Chapter 6

Clinical Application of Fibrinolytic Assays

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Additional information is available at the end of the chapter

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1. Introduction

Haemostasis is a complex balance between thrombus formation and fibrinolysis. Research into bleeding and thrombotic conditions has lead over many years to a detailed knowledge of the role of the components of coagulation, and subsequently many clinical applications have been developed for the testing of platelets, clotting factors and coagulation inhibitors. However, the same cannot be said for the components of fibrinolysis. Fibrinolytic research over the last 30 years has not resulted in the translation of basic science into routine clinical tests of fibrinolytic factors [1, 2], except for D-dimer assays, which are an indirect marker of both thrombosis and fibrinolysis. This has occurred partly due to the difficulties with individual fibrinolytic factor assays, but also due to the inherent limitation of using a single factor assessment to quantify the complex and dynamic process of fibrinolysis.

An ideal assay that could provide an overall or 'global' assessment of haemostasis would take into account the interactions between the proteins of the coagulation and fibrinolytic pathways, blood cellular components and the vessel wall. Such an assay does not currently exist, but refinements of old techniques with updated technology and the development of new global assays have brought improvements in this regard. The ability to assess fibrinolysis is a major benefit of some of these global assays, and has led renewed interest in this field.

Potential clinical applications

There are multiple potential clinical situations in which the ability to detect hypo- or hyperfibrinolysis could in theory be useful. Hypofibrinolysis is a risk factor for thrombosis. Venous thromboembolism (VTE) is a common condition, with an incidence of approximately 1 in 1000 adults [3]. A potential benefit of detecting a fibrinolytic defect would be to identify individuals at higher risk of a first event, which could lead to different management strategies particularly in clinical scenarios such as pregnancy and the peri-operative period where the risk is already

known to be higher. This is relevant because currently only approximately one quarter of patients have a detectable inherited or acquired thrombophilia on testing, and 20% of patients have no cause at all found for their first VTE [3-5]. In addition, recurrent VTE events occur in approximately 20-30% of patients within 5 years [4, 6], and identification of hypofibrinolysis as a risk factor might be useful in informing the duration of anticoagulation. Hypofibrinolysis may also be important in arterial thrombosis such as myocardial infarction, and the ability to detect patients who are at increased risk of infarction or stent occlusion due impaired clot lysis would be an important clinical application [7].

Hyperfibrinolysis can result in an increased bleeding risk during surgery and invasive procedures. As it is associated with increased morbidity and mortality, identifying hyperfibrinolysis is a key aim for fibrinolytic assays [8]. Particularly in major procedures such as liver transplant, cardiac valve repair or revascularisation surgery, challenges to the haemostatic system are complex and continuously evolving, and the ability to rapidly detect hyperfibrinolysis in these patients has lead to an improvement in their care [8-10]. Recently, attention has turned to the need for measuring fibrinolysis in trauma patients since the publication of a large multinational study revealed improvements in survival with the early administration of an anti-fibrinolytic drug [11]. Increased fibrinolysis can also be detected in some patients with inherited bleeding conditions such as haemophilia A, indicating that anti-fibrinolytics may also benefit these patients. Finally, disseminated intravascular coagulation (DIC) is a syndrome with abnormal activation of the both coagulation and fibrinolytic systems. Identifying hyperfibrinolysis in suspected cases could assist in the diagnosis of DIC, which otherwise requires complex scoring systems [12].

In this chapter we review assays of the individual factors of the fibrinolytic system and global tests which provide an overall assessment of the fibrinolysis. In each section we will outline the assay itself and its strengths and weakness, before reviewing the literature regarding its use and current or future clinical applications.

2. Fibrinolytic factors

2.1. Plasmin and α2-plasmin inhibitor

The proteolytic enzyme plasmin is the main mechanism through which intravascular fibrin thrombi are degraded. It circulates as its inactive form, plasminogen, at a plasma concentration of approximately 2 μmol/L and is activated intravascularly by tissue plasminogen activator (tPA) [13, 14]. The localization to intravascular thrombi and formation of the active protease occurs more readily after both plasminogen and tPA are bound to fibrin. Any free plasmin is bound by its primary physiological inhibitor, α2-plasmin inhibitor. This circulates at levels of approximately 1 μmol/L in normal plasma (table 1), and neutralizes plasmin so rapidly that the active enzyme is undetectable in plasma. Assays have therefore been aimed at measuring plasminogen, α2-plasmin inhibitor or the plasmin-α2-plasmin inhibitor (PAP) complex.

Plasminogen activity has been most frequently measured using a functional chromogenic assay, but antigenic assays are also available. In the functional assay, an extrinsic activator,

streptokinase, which acts on free as well as bound plasminogen, is added to a patient's plasma to convert all of the plasminogen to plasmin, which then acts on a chromogenic substrate [14, 15]. Several commercial kits are available. Where low activity levels are found, plasminogen antigen concentrations can be measured by enzyme-linked immunosorbent assays (ELISA) or nephelometry to distinguish between lack of plasminogen (type 1 deficiency) and dysplasminogenaemia (type 2 defects) [15, 16]. PAP complexes have been assayed by ELISA kits [17, 18], and chromogenic assays have also been used to detect unbound α2-plasmin inhibitor activity [19].

	Plasma concentration		Site of Synthesis	Plasma half life (approximate)
	(µg/ml)	(mol)		
Plasminogen	200	2 µmol/L	Liver	1.8 – 2.7 days
Plasmin	Undetectable	-	-	Very Brief
α2-plasmin inhibitor	70	1 µmol/L	Liver	12 hours
tPA	0.005	70 pmol/L	Vascular endothelium	5 minutes
PAI-1	0.02	400 pmol/L	Vascular endothelium, Liver, Adipocytes	2 hours
TAFI	5	75 nmol/L	Liver, Megakaryocytes	10 minutes

Table 1. Synthesis, plasma concentration and half life of fibrinolytic factors [13, 20, 21]

Congenital deficiency and venous thrombosis

The clinical consequences of low plasminogen levels have been studied in patients with congenital deficiency. The prevalence of plasminogen deficiency in the general population has been estimated at 0.3% in a large Scottish cohort of blood donors [22], and 4.3% in a Japanese study [23]. The higher prevalence in the latter population is due the high frequency of a specific plasminogen gene mutation (Ala6012Thr) in East Asian populations causing type 2 deficiency, with similar rates of type 1 disease in each study. Surprisingly, no definite relationship between either type of deficiency and arterial or venous thrombosis has been found [15, 16, 22, 23]. A modestly increased risk suggested by the combined findings of family studies was not statistically significant [15], and has not been confirmed in larger population-based research [22, 23]. Even in severe homozygous plasminogen deficiency there is no excess of thrombotic events; instead the main clinical phenotype is an accumulation of 'ligneous' fibrin-rich, pseudomembranous lesions in the conjunctiva and less commonly in other mucosal membranes [16]. The reason for a lack of thrombotic phenotype is unclear. It may be that residual plasminogen activity in these homozygous patients (the majority display between 4% and 51% activity) is enough to prevent the small vessel thrombosis seen in mouse gene knock-out models where there is no detectable plasminogen [24, 25], or that alternative fibrinolytic proteases released from neutrophils degrade fibrin [26].

Despite the evidence from congenital deficiency states, a small number of studies have investigated plasminogen or α2-plasmin inhibitor levels as a risk factor for venous thrombosis in non-deficient patients. A large case-control study of 770 patients and 743 controls (the MEGA study) found that modest correlations between thrombosis and plasminogen or α2-plasmin inhibitor were lost after adjustment for markers of inflammation and Body Mass Index [27], and the prospective cohort LITE study found no relationship with PAP complexes [18]. A small series of patients with Budd-Chiari syndrome were found to have a slight but statistically significant decrease in plasmin inhibitor levels [28].

Arterial thrombosis

Some large cohort and case-control studies have investigated an association between fibrinolytic proteins and myocardial infarction (MI). High plasminogen levels have surprisingly been consistently associated with a modest elevated risk of myocardial infarction [29-31]. However only one of these studies adjusted for inflammation or smoking [31], which along with elevated cholesterol are potential confounding factors, and the association with MI was lost. An alternative explanation may be that plasmin is known to contribute to the instability of atherosclerotic plaques by activating matrix metalloproteinases [32]. Alpha-2-plasmin inhibitor was also investigated, no association with MI being found in the prospective cohort ECAT study [29], and a slight positive correlation in the retrospective case-control SMILE study [31].

Hyperfibrinolytic states

Raised levels of PAP complexes and decreased levels of α2-plasmin inhibitor have been described in hyperfibrinolytic states, such as acute coagulopathy of trauma [reviewed in 17, 33], disseminated intravascular coagulation (DIC) [34] and the coagulopathy of acute promyelocytic leukaemia [reviewed in 35]. However, no clinical applications have been established, for example as a risk factor of severity.

Rare congenital α2-plasmin inhibitor deficiency has been described [reviewed in 36]. Homozygous deficiency results in a severe bleeding disorder due to increased fibrinolysis with a similar phenotype to congenital haemophilia or Factor XIII deficiency. Conventional haemostasis screening tests are normal, and a functional α2-plasmin inhibitor assay is required to make the diagnosis.

Summary

In summary, research has not resulted in any clinical applications of assays of plasminogen, α2-plasmin inhibitor or PAP complexes in the investigation of fibrinolytic activity despite their central role, perhaps because they circulate at relatively high concentrations and are generally not rate-limiting factors [13]. In rare cases of congenital deficiency of these proteins, functional assays can be used to make the diagnosis.

2.2. Tissue Plasminogen Activator (tPA) and Plasminogen Activator Inhibitor-1 (PAI-1)

Both tPA and PAI-1 have been investigated as markers of fibrinolytic activity. After stimulation, tPA is released locally from endothelial cells and activated platelets, and binds to the

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fibrin surface of the developing thrombus where it catalyses the formation of plasmin. Once bound, it is protected from its principle inhibitor PAI-1, which circulates in plasma and is also secreted by platelets [13, 37]. Free tPA has a plasma half life of approximately 5 minutes due to the action of PAI-1 and simultaneous clearance by the liver [13]. Therefore resting levels of unbound active tPA in plasma are very low and may be measured by ELISAs that detect both the active form and the tPA – PAI-1 complex. PAI-1 levels have been investigated using functional assays and various ELISAs, which utilise a range of monoclonal antibodies with varying sensitivities for unbound PAI-1 and the tPA-PAI-1 complex [38].

There are several issues that complicate the results of these assays. The first is that measuring resting plasma levels of tPA detects mainly inactive complexes with PAI-1, and so raised levels of tPA antigen may indicate inhibited fibrinolysis rather than increased fibrinolytic potential [2]. An inverse relationship between antigen and fibrinolytic activity has even been found [39, 40]. To overcome this, some investigators have measured acute tPA release following stimulation such as prolonged venous occlusion by a tourniquet. However, it is not clear whether this is a better reflection of the physiological situation [7]. In addition, plasma levels of PAI-1 do not reflect its true contribution in inhibiting fibrinolysis as the majority of PAI-1 is released at the site of thrombus by activated platelets [37, 41] and plasma and platelet pools of PAI-1 vary independently of each other [42].

Levels of PAI-1 and tPA are affected by other factors. PAI-1 in particular has many non-fibrinolytic functions, and may be raised in diabetes mellitus and insulin resistance [reviewed in 2]. Both tPA and PAI-1 are acute phase reactants and elevated with raised lipids and pregnancy [29, 31]. Furthermore, there is marked diurnal variation in their levels, being higher in the morning [43].

Arterial and venous thrombosis

The association between tPA and PAI-1 antigen levels with arterial disease and thrombosis has been investigated in multiple large studies with conflicting results, described in two recent reviews [2, 7]. Although some studies have found increased tPA and PAI-1 levels to be associated with an increased risk of arterial disease or recurrent events [reviewed in 44, 45], some major publications including the Framingham study have reported no association [30, 46], and in two studies even a trend to decreased risk was found in subjects with elevated PAI-1 [47, 48]. The use of assays measuring tPA release has not resulted in any clarity, increased levels being associated with major atherothrombotic events in one study [49] and the inverse in another [29]. These confusing results may be partly accounted for by confounding factors demonstrated by studies that have adjusted for a range other risk factors such as diabetes, cholesterol, obesity, and inflammation. In the ECAT, SMILE and Young Finn studies, apparent associations between PAI-1 or tPA and arterial risk were lost when other these other factors were included in the analyses [29, 31, 50].

The data on tPA and PAI-1 as risk factors for venous thrombosis has similarly failed to find any clinically useful associations [reviewed in 1, 2]. Some prospective studies have found no differences in antigen levels between patients who suffered thrombosis and controls [18, 51, 52], whereas a recent large retrospective study has found PAI-1 to be a risk factor for first

venous thrombosis and to be the most important determinant in Clot Lysis Time (CLT), a global test for hypofibrinolysis described later in this chapter [27].

Hyperfibrinolytic states

PAI-1 and tPA levels have been investigated in various hyperfibrinolytic states. PAI-1 has been suggested as a potential therapeutic target in DIC since raised levels correlate with multi-organ failure and outcome [53], whereas in acute traumatic coagulopathy, no association between PAI-1 and severity of injury has been found [17]. In liver cirrhosis, raised tPA levels are proportional to the severity of cirrhosis and risk of variceal bleeding, and may be mediated by a relative deficiency of PAI-1 [discussed in 54].

Summary

Despite a large amount of data on tPA and PAI-1 levels in the literature, no current clinical applications have emerged. The methodological problems and confounding factors discussed above have played a major role in the lack of consistent clinical correlations with disease and outcome.

2.3. Thrombin activatable fibrinolysis inhibitor (TAFI)

TAFI circulates in plasma as a proenzyme and is converted into its active form TAFIa by thrombin or the thrombin-thrombomodulin complex. It then removes specific lysine or arginine residues from partially degraded fibrin, thereby preventing tPA and plasminogen binding which is required for efficient activation of fibrinolysis [13]. It is unstable in plasma, spontaneously degrading to a latent form (TAFIai) with a half life of approximately 10 mins (Table 1).

TAFI can be quantified by ELISA or functional assay. The ELISAs have the advantage of being easy to perform. However there are important differences in the specificities of the antibodies used; they variably measure total antigen, be specific to certain TAFI genotypes, or measure activated TAFI only by being specific for the TAFIa-TAFIai complex [2, 7, 21]. The functional assays measure the ability to cleave residues from small synthetic substrates and have the advantage of measuring all active TAFI, although there may be interference by other carboxypeptidases in plasma. A practical disadvantage is that they are affected by the variable thermal instability of TAFIa, so samples have to be placed on ice immediately and centrifuged at 4^0C, and they also must be collected in tubes containing thrombin and plasmin inhibitors to prevent in-vitro activation [21].

Venous and arterial thrombosis

Raised TAFI levels appear to be consistently associated with venous thromboembolism. In a large case-control study, the Leiden Thrombophilia Study (LETS), TAFI antigen levels above the 90^{th} percentile of controls were found to be associated with an almost two-fold increased risk of first deep vein thrombosis [55], a finding replicated in a smaller case control study using an ELISA specifically designed to be insensitive to different polymorphisms of TAFI [56]. There is some evidence that recurrent venous thromboemboli may be predicted by high TAFI levels; a prospective study of 600 patients with unprovoked venous thrombosis found a two-fold risk

of recurrence if their TAFI levels were above the 75th percentile [57]. Disappointingly, this association could not be confirmed in a follow-up to the LETS study, although this may be because patients with provoked thrombotic events were included [58].

The association between TAFI levels and arterial disease are unclear. Some studies have found a link between high TAFIa (but not total TAFI antigen) and coronary artery disease or myocardial infarction [59, 60], but others have found no association [61, 62] and some have found low total TAFI antigen levels to be associated with increased risk [63, 64].

Other diseases

Associations have been investigated between TAFI and a variety of other disease states such as renal failure, hepatic disease, endocrine disorders, cancer, DIC and pregnancy complications [reviewed in 21] without useful clinical applications arising. One recent study has shown an interesting correlation that suggests the variable bleeding phenotype seen in severe Haemophilia A may be associated with TAFI activation levels [65].

Summary

Once again, the problems with TAFI assays and the lack of consistent significant associations with disease mean that there are no current clinical applications. However the possible association of raised TAFI with recurrent unprovoked venous thrombosis warrants further study.

2.4. Other factors affecting fibrinolysis

Lipoprotein(a) or Lp(a) is a homologue of plasminogen that circulates in plasma and is able to inhibit t-PA mediated plasminogen activation at the fibrin surface [7]. It has been investigated as a risk factor for arterial thrombosis in several prospective studies, and a consistent but weak association has been seen with cardiovascular events in both young and elderly patients [reviewed in 7]. A meta-analysis of prospective studies over the last 40 years, involving approximately 127 000 subjects, found that Lp(a) concentrations were an independent risk factor for both coronary disease and stroke but not clinical outcome. The effect was weak, with a hazard ratio of just 1.13 [66]. Therefore on an individual patient basis, assays of Lp(a) are unlikely to be useful.

Annexin A2 receptors on the endothelial cell surface bind both plasminogen and tPA, and accelerate plasminogen activation 60-fold [13]. Recent publications have shown that autoantibodies to annexin A2 are associated with thrombosis in patients with antiphospholipid syndrome [67]. Another interesting finding has been that autoantibodies to annexin A2 occur in a subset of patients with cerebral sinus thrombosis with or without antiphospholipid antibodies; testing for anti-annexin antibodies may have a role in establishing aetiology in these patients whose thrombosis is otherwise often thought to be idiopathic [68].

2.5. D-dimers

D-dimers are a specific cross-linked fibrin degradation product. Their formation depends on thrombin converting fibrin to fibrinogen and activating factor XIII (FXIIIa), which then cross-

links the D-domains of adjacent fibrin strands prior to cleavage by plasmin. Thus D-dimers can be seen as an indirect marker of both coagulation and fibrinolysis, although low levels can be seen under normal physiological conditions, and raised levels may occur in pregnancy, advancing age and a wide range of inflammatory and malignant conditions as well as thrombosis [reviewed in 69]. For these reasons specific, evaluation of the fibrinolytic system with D-dimer results alone is problematic, and we will only briefly summarise the assays and their established clinical applications below.

D-dimers can be measured using monoclonal antibodies that have variable specificity for an antigen found in the FXIIIa cross-linked fragments of fibrin compared to other fibrin degradation products. Multiple assays have been developed using three techniques: quantitative ELISAs, whole blood agglutination (qualitative), and latex bead agglutination assays that can be performed on routine laboratory coagulation analysers and may be quantitative or qualitative [69]. It is important to note that results are not comparable between different assays, even between those of similar formats. For this reason it is important that clinicians and researchers are aware of the performance characteristics of the individual assay they are using, and the clinical setting for which it has been validated [70]. In addition, many of the commonly used assays may give false positive results due to lipaemia, hyperbilirubinaemia, intravascular haemolysis or high levels of rheumatoid factor [69], and anticoagulation reduces D-dimer levels even within 24 hours of commencement [71].

Venous thromboembolism

D-dimers are typically elevated in acute VTE. However, because they are increased in a variety of other non-thrombotic disorders, raised D-dimers are a sensitive but not specific marker. As mentioned above, D-dimer assays vary; the latex agglutination and ELISA methods are the most sensitive (93-96%) but least specific (43-53%) [72], and consequently a 'negative' or normal range result in these has a high negative predictive value. Multiple studies have shown the D-dimer assay can be successfully combined with clinical probability scoring systems in the diagnosis of suspected lower extremity DVT or PE [reviewed in 69] and their use has been recommended in recent guidelines [73]. In patients with a low or moderate pre-test probability of DVT, a negative D-dimer test of high sensitivity can exclude a DVT without the need for further investigations. Similar strategies can be used in patients with suspected PE [69], but not in upper limb venous thrombosis [73], and the test is less useful in the elderly, hospital inpatients or those with malignancy due to the high prevalence of elevated D-dimers in these groups reducing assay specificity [74].

There is an increased risk of recurrent VTE in patients with evidence of ongoing activation of the coagulation and fibrinolytic systems, as indicated by a raised D-dimer after the cessation of anticoagulant therapy [69, 75]. This may be used to inform treatment decisions regarding the duration of anticoagulation; a major study (PROLONG) randomised patients to restarting anticoagulation or observation if their D-dimer was raised a month after treatment had ceased. The recurrent VTE rate was demonstrated to be significantly less in those who had restarted anticoagulation [76].

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Disseminated Intravascular Coagulation

DIC can be characterised as a syndrome with uncontrolled activation of the both coagulation and fibrinolytic systems, and consequently raised D-dimer levels are a sensitive but non-specific marker [69]. As no single test can establish the diagnosis, D-dimers have been used in scoring systems to establish the likelihood of DIC and monitor the effect of interventions, such as the one developed by the International Society on Thrombosis and Haemostasis (ISTH) [12].

3. Global fibrinolytic assays

The difficulties described above in using individual fibrinolytic markers to measure the fibrinolytic potential of a patient's plasma has lead to increasing interest in global tests that provide an overview of the entire process. Below we discuss thromboelastography, and fibrin generation and lysis assays which have current or potential clinical applications.

3.1. Thromboelastography

Thromboelastography uses viscoelastic changes during coagulation to produce a graphical representation of the fibrin polymerisation process and subsequent fibrinolysis, and as such it has the potential to provide a global evaluation of clot initiation, formation and lysis. First described in 1948 in Germany, two commercial analysers are currently available: the Thromboelastograph or 'TEG' (Haemonetics, MA, USA) and the Rotational Thromboelastogram or 'ROTEM' (Tem International GmBH, Munich, Germany). Both of these are suitable for use as 'point of care' (POC) devices, and this setting is where thromboelastography has developed its main clinical applications.

The principle of the thromboelastogram is as follows [8, 9]; whole blood is added to an incubated cup at 37°C, into which a pin is suspended connected to a detector system. As cup and pin are oscillated relative to each other, the increasing viscosity from the developing fibrin polymerisation affects the magnitude of the movement of the pin, which is converted by a mechanical–electrical transducer to a signal which is displayed as a trace (Figure 1a). As fibrinolysis occurs, the viscosity falls and is also recorded on the trace. As well as the graphical output, values are calculated for various parameters which include: time until initial fibrin formation, clot formation time, rate of polymerisation of fibrin, maximum clot firmness, and clot lysis. Table 2 lists these and the haemostatic variables they are proposed to measure.

Although the TEG and ROTEM are similar in their technologies, there are some important differences. Firstly, in the TEG, the movement is initiated in the cup and a torsion wire monitors the changes in the sample, whereas in the ROTEM, the pin moves on a ball bearing and there is an optical detection system. Some authors claim this modification has led to the ROTEM being more robust in busy clinical settings such as emergency departments and operating theatres [78, 79]. The ROTEM system also has an electronic pipette to help standardise the method. Both systems use proprietary initiators or modifiers of haemostasis, and the TEG can run two assays in series, whereas the ROTEM can run four. The TEG initiates coagulation with

TEG Parameter	ROTEM Parameter	Description	Proposed measured variables
R (Reaction)	**CT**	Clot Time – period to 2mm amplitude	Coagulation factors, platelets, anticoagulants
K	**CFT**	Clot Formation Time – period between 2 – 20mm amplitude	Coagulation factors, fibrinogen, platelets
α - angle	**α - angle**	Rate of clot formation – slope of trace	Coagulation factors, fibrinogen, platelets
MA	**MCF**	Maximum amplitude – maximum clot firmness on trace	Fibrinogen, platelets, fibrinolysis
G	**MCE**	Shear elastic modulus strength or clot elasticity – a representation of clot strength	Platelet function, fibrinogen
LY30, LY60	**LI 30**	Percent decrease in amplitude 30 or 60 minutes after MA is reached	Fibrinolytic factors, fibrinogen
EPL	**-**	Estimated Percentage Lysis - rate of change of amplitude after MA is reached	Fibrinolytic factors, fibrinogen
-	**ML**	Maximum Lysis at the end of ROTEM test (20 - 40 mins)	Fibrinolytic factors, fibrinogen

Table 2. TEG and ROTEM nomenclature for measured parameters [8, 9, 77]

kaolin or a combination of tissue factor and kaolin (± heparinase), and in the ROTEM there are multiple reagent options that may help to distinguish between the causes for abnormal traces, described in table 3 [8, 9]. Relevant to measuring fibrinolysis, one of these (APTEM) contains the fibrinolytic inhibitor aprotinin; correction of an abnormal trace by adding this reagent has been used to suggest the presence of hyperfibrinolysis. It is important to note also that reference ranges vary considerably between the two technologies, and hence results are not directly comparable between the instruments [80].

A major advantage of the thromboelastogram and one that has lead to its principle uses during major surgery, is that it can be used as a bedside measure of global haemostasis, the graphical output appearing as a real-time representation of the patient's clot formation and lysis over a 20-30 minute assay time, avoiding delays inherent in sending samples for laboratory analysis. Furthermore, non-anticoagulated whole blood can be used, and therefore many of the interactions between coagulation, platelets and fibrinolytic factors are preserved. A limitation is that the coagulation is being measured under static (no shear) conditions in a plastic cuvette rather than an endothelialised blood vessel [9], although of course this disadvantage is shared with almost all other *in vitro* assays.

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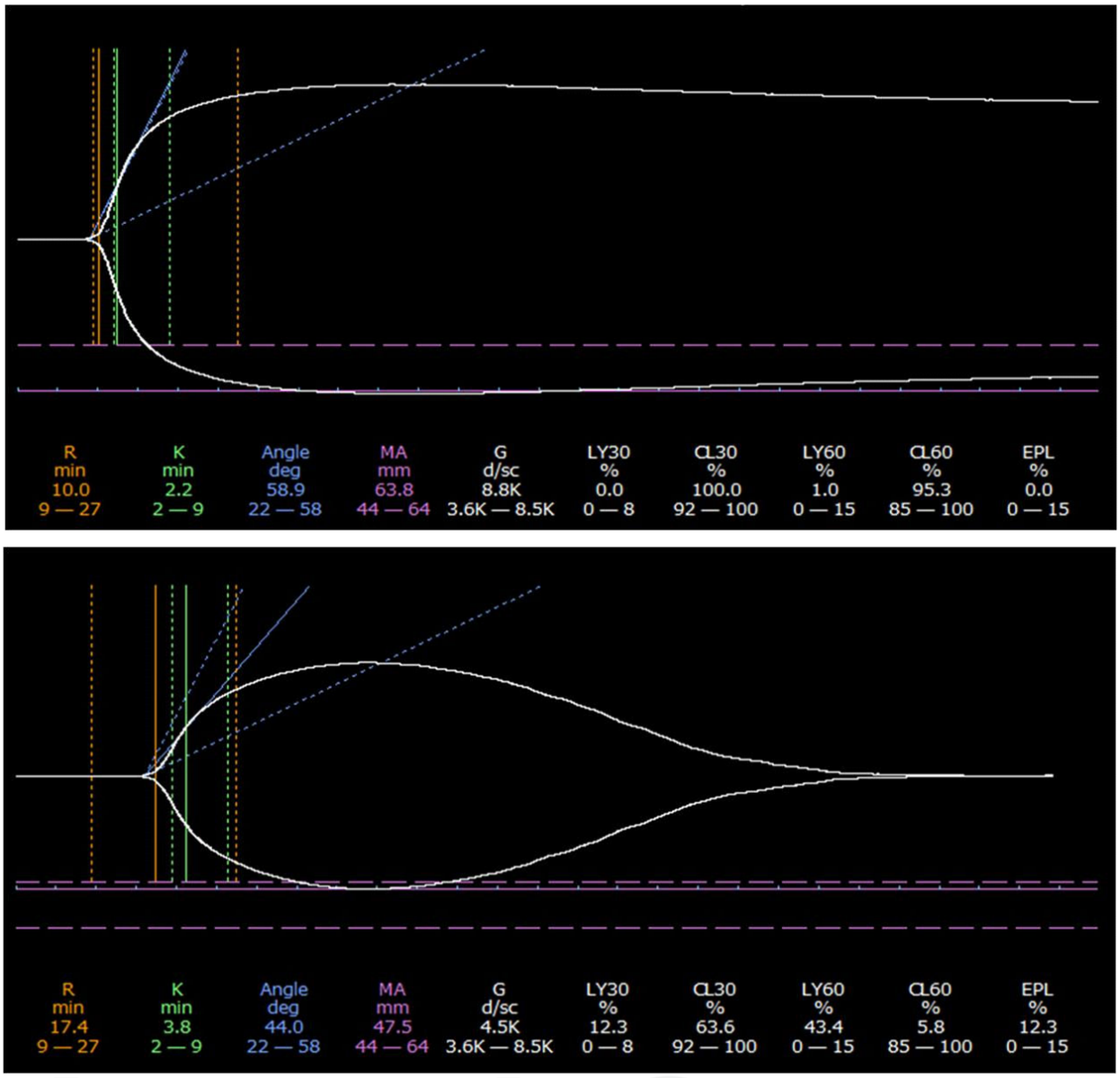

Figure 1. (a) Normal TEG trace. The patient's thromboelastographic trace is shown in white and is produced in real time. The *x-axis* is time; the *y-axis* is millimetres of deviation representing increasing visco-elasticity of the sample as clot forms. Derived parameters are listed below the trace with their proposed normal ranges. R = Clot time; K = Clot formation time; Angle deg = α – angle or Rate of Clot formation; MA = Maximal Amplitude; G = Shear elastic modulus strength; LY30/60 = Percent Lysis at 30/60 minutes; CL30/60 = Inverse of LY30/60 parameter; EPL = Estimated Percentage Lysis; **(b) TEG trace showing hyperfibrinolysis.** The patient's trace in white shows early convergence representing a reduction in visco-elasticity caused by complete fibrinolysis. The key to the derived parameters displayed below the trace is given in Figure 1a above

A concern with thromboelastography has been in the standardisation of methods and reproducibility of results. Pre-analytical variables such as site of blood sampling, time from sampling to analysis, type of reagent and instrument used can alter results significantly [reviewed in 8]. In addition to native whole blood, re-calcified citrate samples may be used when thromboelastography is performed in the haemostasis laboratory, or when a delay

Assay	Reagents	Proposed Use
TEG		
Kaolin	Kaolin	Overall coagulation assessment
Rapid TEG	Kaolin + Tissue Factor	Shorter test time / faster results
Heparinase	Kaolin + Heparinase	Detection of heparin
ROTEM		
In-TEM	Contact activator	Assessment of intrinsic pathway
Ex-TEM	Tissue Factor	Assessment of extrinsic pathway
Hep-TEM	Contact activator + Heparinase	Detection of heparin
Ap-TEM	Tissue Factor + Aprotinin	Detection of fibrinolytic effect when used with EX-TEM
Fib-TEM	Tissue Factor + Platelet antagonist	Qualitative assessment of fibrinogen

Table 3. Selected TEG and ROTEM reagents [9]

between sampling and analysis is expected in a POC setting. However it has been reported that stable results are not produced until after 30 minutes of collection in citrate, which defeats the purpose of the technique as a 'real-time' assessment of haemostasis, and values from citrate samples cannot be correlated to those from non-anticoagulated samples [81].

Operating the TEG or ROTEM in a POC setting means that non-laboratory staff must run these moderately complex tests and hence sufficient numbers need to be properly trained to operate and maintain the equipment to avoid further errors. Quality assessment (QA) must be carried out on a regular basis, the TEG for example requiring quality control (QC) samples to be run each time the machine is switched on, every 8 hours or if the analyser moved [10]. Commercial samples for internal quality control are available and external QA schemes exist, but the results from the latter have been disappointing [82]. Coefficient of variation (CV) results between centres have been reported between 7.6 – 39.9% in the TEG and 3.6 - 83.6% in the ROTEM [82, 83] for coagulation variables on plasma samples, although much better results in both devices can be produced in a single centre using whole blood if the manufacturer's method is followed exactly [9, 84].

No data on the reproducibility on fibrinolytic measures between centres has been published. The effect of fibrinolysis on TEG or ROTEM instruments can be detected using the parameters that are listed in Table 2. However, operators can also use a qualitative difference in the shape of the traces to diagnose hyperfibrinolysis, an example of which is shown in Figure 1b. Although subjective, experienced operators have used this strategy successfully in complex surgery to guide treatment of hyperfibrinolysis as described below, and the effect of giving an anti-fibrinolytic agent *in vivo* or adding aprotinin *in vitro* in the APTEM is clearly demonstrated in the traces of these patients [77, 78, 85-87]. However, the validity and sensitivity of this approach is unclear and in some reviews it has been suggested that only marked hyperfibrinolysis can be identified this way [33, 88]. Some investigators using the TEG have attempted

to standardise the definition of hypofibrinolysis as an 'Estimated Percent Lysis' (EPL) of greater than 15% [88]; this is calculated by the TEG software comparing the area under the curve at the MA amplitude and 30 minutes later. Validation of this definition as a measure of hyperfibrinolysis is lacking, but a recent publication has shown that increasing concentrations of tPA at pathophysiological levels added *in vitro* lead to higher EPL levels in a dose dependent way (but not in the less sensitive RapidTEG) [89]. Also, important clinical correlations with outcome have been found with this EPL threshold (see the studies reviewed below). In the ROTEM there is some data to suggest the maximum lysis measure at 1 hour correlates with tPA, PAPs and PAI-1 [79], and the clot lysis index at 30 mins has 71% sensitivity for hyperfibrinolysis when compared to the less quantitative euglobulin clot lysis test (described in a later section) [78].

In discussing the literature regarding the use of thromboelastography as an assay of fibrinolysis, first the established uses as a POC device in surgery and trauma will be summarised, although detection of hyperfibrinolysis in these situations is only a component of the data utilised. Following this, the research concerning the use of the technique in pregnancy, haemophilia and hypercoagulable states will be discussed.

Hepatic surgery

One of the first clinical applications described was in orthotopic liver transplantation where the TEG can be used to monitor coagulation and guide blood component and anti-fibrinolytic therapy [90]. The haemostatic defect in these patients is complex; they often start with a thrombocytopenia and depletion of clotting factors due to end stage liver disease, but also have low levels of the natural anticoagulant proteins C, S and antithrombin. During surgery they may have a rapidly changing picture due to consumption of factors and dilution. Patients may also develop hyperfibrinolysis caused by tPA build up during the anhepatic stage, followed by a surge of tPA release from the reperfused transplant liver [91, 92]. However, unnecessary use of anti-fibrinolytics should be avoided due to the risk of hepatic artery thrombosis. POC thromboelastography can produce timely information at intervals throughout the surgery on the nature of the defect and requirements for coagulation factor replacement or anti-fibrinolytic therapy, and has been shown to decrease the need for red cell transfusion and plasma products [90, 93-95].

Cardiac surgery

Cardiac surgery requiring cardiopulmonary bypass is a complex balance between anticoagulation with heparin, the effect of anti-platelet agents and the need for haemostasis at the end of surgery [10]. Hyperfibrinolysis may also be seen, particularly post-bypass. There is good evidence from randomised controlled trials and retrospective analysis that the use of thromboelastography-guided algorithms for heparin reversal, blood component and anti-fibrinolytic support reduce the need for blood product transfusion and the rates of surgical re-exploration due to post-operative bleeding [96-100]. For example, a randomised prospective trial of 105 patients undergoing complex cardiac surgery compared TEG-guided transfusion therapy with standard care and found significantly fewer transfusions in the TEG group due to less postoperative requirements [97]. This trial also showed a 75% drop in the number of

patients having FFP infusion and a 50% fall in platelet transfusion. Because the use of TEG and ROTEM has been shown to reduce the potential risk to the patient and to reduce costs [96, 101], their use has been recommended in both US and European guidelines [102, 103].

Trauma

Increased understanding of trauma associated coagulopathy has shown that fibrinolysis is a key component which is associated with increased mortality and can be reliably detected by thromboelastography in the emergency department, combat settings and operating theatres [for recent reviews see 10, 33]. Interest in this area has grown further since the publication of the large multi-centre CRASH-2 study which showed decreased mortality if trauma patients were given tranexamic acid to inhibit fibrinolysis within three hours [11]. However the inclusion criteria were very broad in this study, and POC testing with TEG may be able to target anti-fibrinolytic therapy to those who would benefit most. For example, a recently published prospective study used TEG to evaluate a severely injured cohort of patients for hyperfibrinolysis, defined as an EPL of 15% or more [104]. The incidence was 11% overall, but these patients had 25 times the risk of early mortality. The TEG assay was reported to rapidly identify hyperfibrinolysis within the first hour in all patients, allowing potential early use of anti-fibrinolytics. The results replicate a smaller study using the ROTEM [105]. These developments warrant further prospective randomised trials to demonstrate a reduced morbidity or survival benefit in trauma patients monitored by thromboelastography and treated accordingly.

Haemophilia

Little has been published on the use of thromboelastography in inherited bleeding disorders. However, the TEG has been used to demonstrate hyperfibrinolysis in Haemophilia A, and this was correctable with either recombinant FVIIa or an anti-fibrinolytic or both [86, 106], and the TEG is also able to monitor the effect of rFVIIa used to treat haemophilia patients with acquired inhibitors [107].

Hypercoagulable or hypofibrinolytic states

Thromboelastography has been less successful in demonstrating hypofibrinolysis. This may be because normal subjects only show a minor degree of fibrinolysis in unmodified TEG or ROTEM assays, for example one study showing the normal range of the ROTEM Maximum Lysis at 60 minutes to be 0 – 12% (mean 3%) [84]. Modifications have been suggested to increase the sensitivity to hypofibrinolysis by adding exogenous tPA to the cuvette prior to clot initiation, but there has been little published on the success of this approach; one group described a standard method for measuring tPA induced fibrinolysis using the TEG and subsequently used it to show that children with idiopathic venous thrombosis have significantly reduced fibrinolysis compared to controls [108, 109]. The data concerning the use of thromboelastography in hypercoagulable states has otherwise concentrated on the ability of the technique to show shortened clotting times, rapid fibrin polymerisation or increased clot strength [e.g. 110], and is outside the scope of this chapter.

Pregnancy

Pregnancy is a hypercoagulable state in which many of the component s of the coagulation and fibrinolytic system are altered, but conventional assays fail to provide an overall picture of the patient's haemostatic potential. Thromboelastography has been used in this regard, and has been successful in guiding therapeutic anticoagulation with LMWH and assessing the risk of neuraxial anaesthesia [reviewed in 8, 10]. A minor degree of hypofibrinolysis can be detected by TEG at the end of the third trimester compared to 8 weeks post partum [111], and an algorithm for TEG-guided treatment of post partum haemorrhage has been suggested, similar to those used in trauma [112].

Conclusion

Thromboelastography has an established clinical role in the detection of hyperfibrinolysis in major hepatic and cardiac surgery, and its use in the management of trauma victims is growing. As further awareness and understanding of the assay occurs, further applications in bleeding patients [112], haemophilia and pregnancy are likely [113]. However, an expansion of its role into detection of hypofibrinolysis is unlikely given the insensitivity of current methodologies.

4. Fibrin generation and lysis assays

Attempts had been made to time the dissolution of plasma clots as an overall measure of fibrinolysis even before the development of molecular markers and their immunoassays. However, spontaneous fibrinolysis of plasma clots is an extremely slow process, for example taking as long as 20 hours to achieve just 10% lysis [114]. Laboratory assays were subsequently developed that could measure fibrinolysis over a shorter period, principally the euglobulin clot lysis time (ECLT) [115] and the dilute whole blood clot lysis time (DWBCLT) [116]. However they have not been widely adopted because of several drawbacks. The ECLT measures lysis in only a fraction of plasma precipitated by utilising a low pH and ionic strength; the main determinants are fibrinogen, tPA and plasminogen only, the natural inhibitors being absent. DWBCLT is performed in the absence of calcium and hence excludes the interplay of the coagulation and fibrinolytic systems, and both of these tests were also labour intensive and unsuited to modern clinical laboratories.

4.1. Clot lysis time (CLT)

More recently an automated assay called the clot lysis time (CLT) has been developed to assess the dissolution of a tissue factor induced fibrin clot by exogenous tPA [117]. Citrated platelet-poor plasma (PPP) is added to a microtitre plate at 37^{o}C with tissue factor, calcium, phospholipid to initiate clot formation and exogenous tPA to trigger fibrinolysis. The turbidity at 405nm is measured over time. The CLT is defined as the time from the midpoint of clot formation (between clear and maximum turbidity) to the midpoint of clot lysis (between maximum turbidity and clear). It has a mean (± SD) in normal individuals of 83.8 (± 11.1) minutes [118].

The CLT has several advantages. Firstly, it is relatively simple and easy to run. It can be done on previously frozen samples and is insensitive to method of PPP preparation [118]. It provides an assessment of the overall fibrinolytic capacity of the plasma, being affected by most of the individual factors. Plasminogen, α2-plasmin inhibitor, PAI-1 and TAFI all have been shown to be important variables in the CLT, whilst prothrombin, fibrinogen and factors VII, X and XI have a progressively more minor effect [27]. However, the concentration of the exogenous tPA is above physiological levels, and important interactions with platelets are not accounted for.

The CLT has been poorly standardised between groups, but a recent publication has sought to address this and investigate the biological variation within healthy individuals [118]. It was identified that the assay has a total analytical CV of 13.4%, and there is substantial biological variance over time within normal individuals. Sequential samples are therefore required to establish the true fibrinolytic potential of an individual; the authors suggest a single result may differentiate up to 20% from the true value. These should be done at the same time of day because the assay is affected by the diurnal variation in fibrinolysis that was previously noted in plasma levels of tPA and PAI-1, but no seasonal variation was detected. As it is a turbidometric assay, results may also be affected by marked lipaemia or paraproteinaemia.

Venous thromboembolism

There is definitive evidence that reduced fibrinolytic potential as shown by prolonged CLT is a risk factor for first venous thromboembolism (VTE). The LETS case control study determined the CLTs for 421 patients following their first DVT selected consecutively from anticoagulation clinics and excluded only if aged over 70 or if they had a malignancy [117]. Samples were taken at least 6 months after the diagnosis and at least 3 months after the cessation of anticoagulant and were matched with 469 control samples from patient's partners. A dose-dependent correlation was seen between increased CLT and DVT, with those with CLTs above the 90th percentile having twice the risk of controls, even when corrected for age, sex, clotting factors, antithrombin and TAFI levels. A second smaller study investigated lysis times in a group of 100 patients with a first idiopathic VTE using a similar turbidometric assay [119]. Patients were excluded if they had any known congenital or acquired risk factors for thrombosis or evidence of an underlying inflammatory state; the included VTE patients showed mean clot lysis times that were a 31.9% longer than controls. Findings were replicated in the Multiple Environmental and Genetic Assessment (MEGA) study of 2090 patients with first DVT or PE and 2564 controls [120]. Patients were between 18-70 years and were excluded if death or end-stage disease prevented follow-up samples being taken, whilst the control group was comprised of the patient's partners and random healthy individuals. Again, a dose dependent increase in risk of DVT was seen for increasing CLTs. The authors defined hypofibrinolysis as those with a CLT above the 75th percentile, and these patients had a two-fold risk compared to those in the first quartile. Interestingly, a synergistic effect was seen when CLT was combined with other known risk factors. Immobilisation increased the risk of VTE 4.3-fold in this study, but combined with hypofibrinolysis the risk was over 10 times that of controls. The overall risk with Factor V Leiden heterozygosity increased from 3.5 to 8.1-fold but the combination with prothrombin 20210A mutation did not show the same effect. Most markedly, for women under 50 years on the oral contraceptive, the risk of first VTE went up from 2.6-fold to over 20-times

the risk if they also had hypofibrinolysis. Previously it had been shown that OCP use had either a minor [121] or no effect [122, 123] on prolonging the clot lysis time by itself.

Recurrent VTE

The establishment of hypofibrinolysis defined by CLT as a risk factor for first VTE has led to interest in whether it could predict recurrent events, and therefore be used in clinical decision-making on the duration of anti-coagulation in patients. The LETS population was prospectively studied to see if prolonged CLTs detected after the first DVT predicted recurrent VTE [58]. In a group of 447 patients with a mean follow-up of 7.3 years, no significant association was seen in the 90 patients with recurrence. In a second study, 704 patients with a first unprovoked VTE and no genetic risk factor, malignancy, lupus anticoagulant or other requirement for long-term anti-coagulation, were studied [124]. In the female population (n=378), there was a 3.28-fold risk of recurrence for those with a CLT in the fourth quartile, but no association with recurrence was found in men or the overall population. Whilst these results are disappointing, there may be reasons why some effect was not detected; it should be noted that in the LETS study, patients with temporary risk factors such as immobility were included in the study although they had a low risk of recurrence, and in both studies patients who remained on anti-coagulation for any reason were excluded, including those continuing because they were thought to be high risk for recurrence. In neither of these studies were CLTs combined with other known predictors of recurrence, such as elevated D-dimer or Factor VIII levels, to see if their predictive power could be increased.

Uncommon thrombotic disorders

Some recent interesting research suggests using global methods such as the CLT may provide insight into the pathogenesis of some poorly understood hypercoagulable diseases. For example, CLTs were significantly longer (p=0.001) in 81 patients with retinal vein occlusion compared to a matched control group, even when multivariate analysis adjusted for cardiovascular risk factors [125]. Another study showed CLTs above the 95th percentile gave a 3.4-fold increase risk of Budd-Chiari syndrome [28], and 92 patients with pre-eclampsia were found to have significantly prolonged clot lysis times compared to controls, independent of the presence of antiphospholipid antibodies [126].

Arterial thrombosis

Clot lysis times have also been studied as a risk factor for arterial disease in 3 recent studies. The first used samples from the large case control study population from the Study of Myocardial Infarction Leiden (SMILE) to test CLT in 421 men and 642 controls [127]. Samples in the study group were taken a median 2.6 years after first MI. In men under 50 years the overall risk of MI was 3.2 times (95% CI 1.5-6.7) increased for CLTs in the highest quartile; however, once cardiovascular risk factors were adjusted for the risk was attenuated to just 1.8 (95% CI 0.7-4.8), and no relationship was seen in the over 50s. Another case-control study (from the 'ATTAC' study) examined the association between CLTs and ischaemic stroke, peripheral vascular disease as well as MI in both sexes [123]. Even after adjusting for cardiovascular risk factors there was an approximately 2-fold increase in risk of arterial event associated with CLTs above the 80th percentile (compared to the risk associated with diabetes which was 2.5), and

the association was similar in the 3 categories of disease. Finally, a third case-control group (the RATIO study) found a significant association between CLT and arterial thrombosis in young women aged 18-50 [128]. The risk of MI was increased for those with CLT in the third tertile, but surprisingly the risk of ischaemic stroke was only increased by shortened CLTs, i.e. with hyperfibrinolysis, a finding which is yet to be validated or explained.

Anticoagulants and hypocoagulable states

Given the evidence that some patients with thrombosis have impaired fibrinolysis, one interesting area of investigation concerns the effect of different anticoagulants on fibrinolysis measured by the CLT. Data on their effect could help to interpret CLT studies on patients with VTE without the need to take them off their anticoagulation, which was a methodological problem in the studies on recurrent VTE risk described above. Another possible benefit might be the ability to individualise anticoagulant choices for patients with hypofibrinolysis. Three studies have addressed the effect of anticoagulants. CLTs were measured in an in-vitro study where varying concentrations of anticoagulants including a low molecular weight heparin (LMWH), a selective anti-Xa drug (fondaparinux) and thrombin inhibitors (hirudin and PPACK) [129]. The LMWH and fondaparinux both had a significant effect in shortening CLTs, whereas no effect was seen with the thrombin inhibitors. A second study looked at the in-vivo use of unfractionated heparin (UFH) and LMWH in the treatment of acute PE, and found that fibrinolysis was enhanced significantly in the UFH group versus those on LMWH [130]. A third study showed that fondaparinux shortened CLTs in vitro and in healthy subjects, and this could be partially reversed with rFVIIa [131].

There has been comparatively little research on the utility of CLT in measuring hypocoagulable or hyperfibrinolytic conditions. In haemophilia A, hyperfibrinolysis has been demonstrated by shortened CLTs, and can be abolished by adding FVIII, TAFI or recombinant activated Factor VII (rFVIIa) [132, 133]. Another study used shortened CLTs to demonstrate hyperfibrinolysis in liver cirrhosis [134].

4.2. Overall Haemostatic Potential (OHP)

A limitation of the CLT is that only a single timed variable representing fibrin degradation is produced from the process of fibrin generation and subsequent lysis. A more comprehensive method for investigating this process is provided by the *Overall Haemostatic Potential* assay which was first developed in Sweden by Blombäck and colleagues [135] and modified in subsequent publications [136-138]. The principle of the OHP is the generation of fibrin formation and lysis curves to represent the shifting balance between fibrin generation and lysis using serial spectrophotometric measurements plotted against time. Two other assays with very similar methods have been described and will be also considered in this section. These are the *Clot Formation and Lysis* (CloFAL) assay [139] and the *Coagulation Inhibitor Potential* (CIP) assay [140, 141].

The modified OHP [136, 138] uses a small amount of thrombin to trigger coagulation. Fresh or thawed citrated PPP is added to a microtitre plate well at 37°C containing buffer with calcium chloride and thrombin at a final concentration of 0.03 – 0.04 IU/ml. Light absorbance at 390 or

405nm is recorded every minute for 40-60 mins. As the fibrinogen is gradually converted to fibrin by generated thrombin, the absorbance increases and is recorded at each time point, with the area under the curve (AUC) reflecting the total fibrin generated. The measurements from this well are termed the Overall Coagulation Potential (*OCP*). Each sample is run in parallel with another well containing the same reagents but with added tPA at 330 – 350ng/mL. In this analysis, called the Overall Haemostatic Potential (*OHP*), complete fibrinolysis occurs after initial fibrin generation, recorded by a fall in absorbance over time. The difference between the AUCs of the *OCP* (without tPA) and the *OHP* (with tPA) represents the overall fibrinolytic potential (*OFP*) and is expressed as a percentage of the *OCP*. Examples of these curves are shown in Figures 2a and b. Other derived measures include the *Delay*, which is the time from start of the analysis to onset of fibrin generation and correlates with the APTT, the maximum optical density (*Max OD*) representing the maximal amount of fibrin generated (correlating to plasma fibrinogen levels), and the velocity (*Max Slope*) to describe the rate of fibrin generation [138]. In addition, the OFP may be corrected for variations in *Delay* by standardising the time period over which it is calculated to the 45 minutes starting from the onset of fibrin generation (OFP 45).

Tests are performed in duplicates in a microtitre plate. In a single laboratory the intra-assay CVs were approximately 3.1 – 8.7% and the inter-assay CVs 4.2 – 5.1% for the OCP and OHP values respectively [136]. Experiments in our laboratory have shown that standard precautions may be applied regarding pre-analytical variables; fresh samples are stable if processed within 2 hours or may be frozen and analysed later, and different methods of PPP preparation did not alter assay results either (unpublished data).

The OHP has demonstrated sensitivity to changes in both coagulation and fibrinolytic factors. In vitro experiments using factor deficient plasma have demonstrated significant correlations between OHP parameters and concentrations of factors II, V, VII, VIII, IX and X and XI [142]. These results were replicated in our laboratory, and we also have shown using in-vitro experiments that the OFP parameter is sensitive to varying concentrations of plasminogen, α2-plasmin inhibitor, PAI-1, TAFI and the fibrinolytic inhibitor tranexamic acid [unpublished data and 143]. In vitro spiking experiments have shown fibrinogen concentration outside the normal range also has a negative correlation with fibrinolysis parameters; however, whereas fibrinogen levels can have a marked effect on the CLT assay because of the increase in the clot's peak light absorbance, the nature of the OFP parameter as a ratio of the OCP-OHP:OCP controls for the maximum OD and is a reasonable expression of clot lysis.

The OHP can also be used as a screening assay for heritable thrombophilias; previously unpublished evidence from our laboratory shows sensitivity to antithrombin deficiency with both hypercoagulable and hypofibrinolytic changes (figure 3), the latter defect likely related to increased TAFIa levels. The *Coagulation Inhibitor Potential* (CIP) assay is a newer modification of the OHP specifically designed for thrombophilia screening which uses heparin pentasaccharide to potentiate antithrombin and the snake venom Protac to activate Protein C [140, 141]. It has shown excellent sensitivity of 100% and reasonable specificity of 70-80% in two small series of patients with FV Leiden, or Protein C, S or antithrombin deficiency [140, 144].

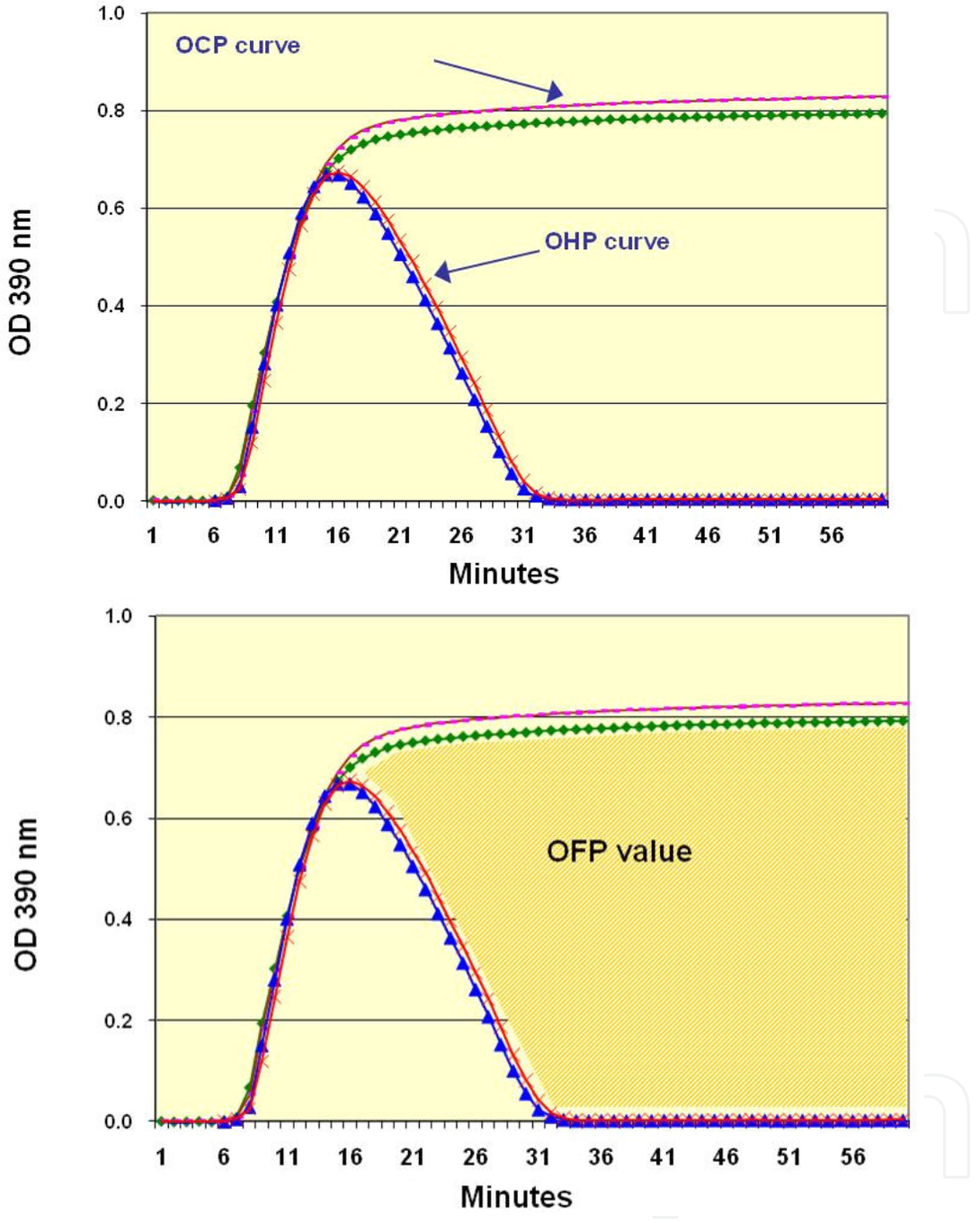

Figure 2. (a) Examples of Overall Coagulation Potential (OCP) and Overall Haemostatic Potential (OHP) curves. **(b)** The Overall Fibrinolytic Potential (OFP) is the difference of the area under the curves of the Overall Coagulation Potential (OCP) and Overall Haemostatic Potential (OHP)

Whilst there are clear advantages of the OHP as an assay for fibrinolysis, such as its simplicity, inexpensiveness and reproducibility, there have been several criticisms of the method [145]. The first is that thrombin is used to initiate coagulation. In earlier versions of the assay, higher thrombin concentrations were used and there was evidence of direct fibrinogen activation

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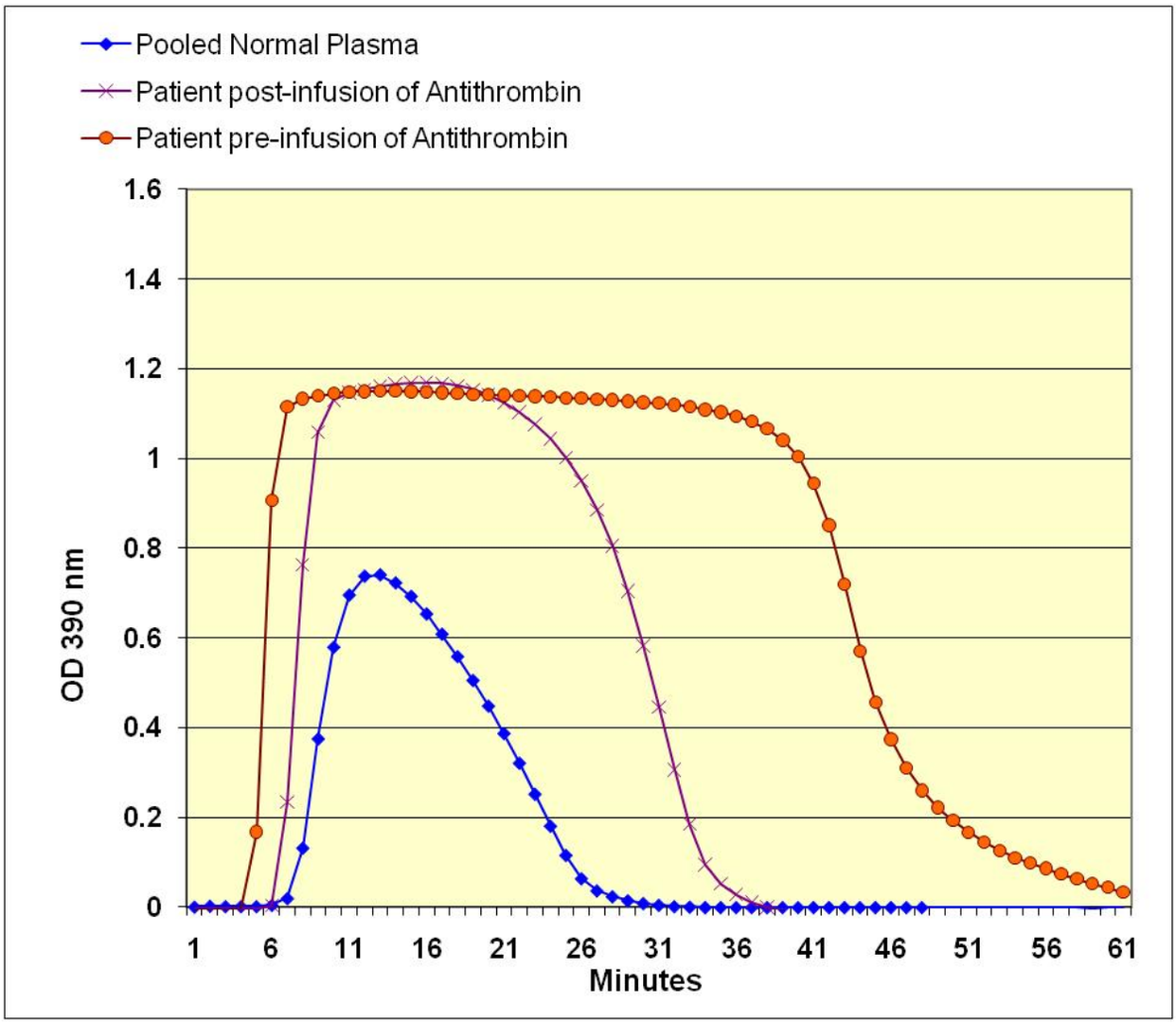

Figure 3. Overall Haemostatic Potential (OHP) curves of a patient with congenital antithrombin deficiency before and after antithrombin concentrate infusion compared to a pooled normal plasma control. The pre-infusion OHP curves show decreased *Delay*, increased *Max Slope* and *Max OD*, and impaired fibrinolysis. *Delay*, *Max Slope* and fibrinolysis are partially corrected by antithrombin infusion.

occurring from the exogenous thrombin. However the very low concentrations used in the modified version produce no detectable clot in thrombin deficient plasma, suggesting the exogenous thrombin is only enough to trigger coagulation via a feedback reaction and not directly converting fibrinogen [145]. Even so, a further refinement of the method has been published which replaces the thrombin with a low concentration of tissue factor and phospholipid to more accurately mirror in-vivo coagulation and this method has also been shown to be sensitive to changes in coagulation factor and PAI-1 concentrations [137]. The *CloFAL* assay has also been independently developed with a very similar method to the OHP using tissue factor as an initiator, and a different algorithm to evaluate fibrinolysis [139].

A second criticism of the OHP is that plasminogen activation depends almost exclusively on the exogenous tPA, a modification of the method which is essential to produce measurable

fibrinolysis within a reasonable time frame but which makes the assay insensitive to changes in endogenous plasminogen activators [145]. Thirdly, the effects of the cellular components are excluded by the use of PPP, unlike in the TEG for example. Finally, the OHP and related assays currently lack standardisation and are not available commercially, and variations in type of initiator, concentrations of reagents and derived parameters making results between groups difficult to compare. As yet, no inter-laboratory CVs have been published. OFP results are also likely to show a diurnal variation due to their sensitivity to higher PAI-1 levels in the morning, and this has been demonstrated in patients with obstructive sleep apnoea [146].

Arterial thrombosis

Impaired fibrinolysis demonstrated by the OHP assay has been found in patients with coronary heart disease. A large retrospective study compared 800 patients three months after their first MI with 1123 normal controls from the local population [147]. Fibrinolytic parameters below the 10th percentile of the control group's values conferred an odds ratio of first MI of 1.66 (95% CI 1.22-2.27) after correction for cardiovascular risk factors in multivariate analysis. In another study, serial samples from patients admitted to hospital with acute coronary syndrome showed decreased fibrinolysis that improved on treatment with LMWH [148]. Patients with stable coronary artery disease also have reduced fibrinolysis; 56 patients with angiography proven coronary atherosclerosis had significantly reduced OFP values compared to controls [149]. The lowest tertile of OFP results conferred an odds ratio of 16.1 for coronary artery disease compared to the highest tertile. This major increase in risk was partly accounted for by higher PAI-1 levels although the global OFP measure remained an independent risk factor when adjusted for this.

The OHP has also demonstrated hypofibrinolysis in patients with cerebrovascular events. In one study, samples were taken from a group of 44 young patients a median of 5 years after they had experienced an episode of acute cerebral ischaemia and compared to healthy age matched controls. Little difference in the incidence of traditional thrombophilic measures was seen between the groups, but increased OCP parameters and decreased OFP results were demonstrated [150]. Another study examined TAFI levels and the OHP in patients at the time of acute ischaemic stroke and 60 days later [151]. Patients had impaired global fibrinolysis compared to controls which was partly, but not fully, explained by raised TAFI levels.

Venous thrombosis

Currently there is less published evidence regarding the use of the OHP to detect hypofibrinolysis in patients with venous thrombosis, although the assay does show promise in this area. The Swedish group first published data on a group of 88 women who had had a pregnancy related VTE, with the majority having evidence of activated protein C resistance with or without the Factor V Leiden mutation [152]. Blood samples were taken for OHP analysis from the patients 8 months to 13 years after the last VTE and compared to samples from a control group of healthy women. They were able to demonstrate a significant persisting hypercoagulability and hypofibrinolysis in the group which was most marked in those who had Factor V Leiden. Subsequently another publication studied 161 patients referred to haematology clinic with clinically defined hypercoagulable states [138]. The study group was heterogenous

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and included patients with VTE (n=73), arterial thrombi, antiphospholipid antibodies or lupus anticoagulant, and pregnancy complications. Despite this, significant differences in fibrin generation and lysis were found in the study population compared to a control group of blood donors, giving the OHP assay an estimated sensitivity of 96% for detection of clinically defined hypercoagulable states. The results of this study complement the excellent sensitivity the OHP has demonstrated in detecting inherited thrombophilic states that was described previously [140], and data showing that the OHP can discriminate factor XII deficient patients with a prothrombotic phenotype [153].

These promising results merit further prospective study to investigate whether the OHP assay can predict first or recurrent VTE. It may be particularly useful in identifying hypercoagulability in patients with otherwise unprovoked idiopathic VTE; in our laboratory we have noted impaired fibrinolysis in this group (unpublished), and similar results have been published using the CloFAL assay in paediatric VTE [154].

Pregnancy

The OHP assay demonstrates both significantly increased fibrin generation and decreased hypofibrinolysis in normal pregnancy [J Curnow et al, personal communication, 136]. In high risk pregnancy, these changes have also been described [155] and unpublished data from our laboratory shows a significant worsening in these parameters compared to patients with uncomplicated pregnancy, which may indicate a role for the OHP in prospective studies of thrombosis risk.

Anticoagulation monitoring

The OHP shows potential applications in the monitoring of overall coagulation and fibrinolytic changes with anticoagulation. As described above, improvement of fibrinolysis was seen in acute coronary syndrome patients once LMWH was started [148], and similar improvement may be seen with warfarin and the new oral anticoagulants (unpublished data). However, the response appears to be variable even when conventional assays show anticoagulation to be within the therapeutic window; for example, in a study of pregnant women with previous thromboembolism, dalteparin prophylaxis was shown to improve hypercoagulable OHP variables in the majority, but some patients were seen to remain hypercoagulable even with therapeutic anti-Xa results [155]. Further study is required to investigate if this translates to an increased thrombosis risk and whether changing treatment based on OHP results can result in improved patient care.

Hypocoagulable states

As mentioned previously, the OHP is sensitive to factor deficiencies in vitro. Results from Haemophilia A patients indicate the OHP may even be a better predictor of clinical phenotype than APTT levels or FVIII assays [145], and in patients with inhibitors, the OHP may be used to monitor the fibrin generation response to recombinant FVIIa treatment [143]. Studies evaluating the OHP's ability to monitor FVIII dosing are ongoing [145].

Summary

Fibrin generation and lysis assays show sensitivity to both hypo- and hyperfibrinolytic states, unlike the thromboelastography. Although they have not entered clinical practice currently, they show great promise in identifying global changes in coagulation and fibrinolytic tendency. In this regard, the OHP has an advantage over the CLT because both aspects of haemostasis are derived. Clearly, further study is required, but the OHP and related assays have the potential to better characterise global coagulation responses than conventional assays, possibly leading to individualised treatment for patients with thrombotic and bleeding conditions.

5. Conclusions

Currently, fibrinolysis is rarely measured in clinical practice. This has been mainly due to the failure of assays of the specific factors to show consistent relationships with disease. In part, this failure has been related to methodological flaws in the assays, for example in the variable specificities of the antibodies used to detect TAFI or PAI-1. However, the principle problem is that the validity of one-off sampling of individual factors as a representation of the complex fibrinolytic pathway is questionable, especially when the sampling has occurred at a time distant to the pathological event being investigated.

D-dimer assays have been one notable success in clinical practice where they have established applications in the diagnosis of VTE and DIC. However, as a non-specific marker of thrombosis and fibrinolysis, they provide little information of the fibrinolytic potential of an individual. Global assays have the ability to improve clinical fibrinolytic testing in this regard. Thromboelastography is increasingly establishing itself as a tool to detect hyperfibrinolysis, particular in point-of-care settings such as cardiothoracic and liver transplant surgery. Fibrin generation and lysis assays such as the CLT and OHP have the disadvantage of requiring platelet poor plasma, but in contrast to thromboelastography, appear to be sensitive to both hyper- and hypofibrinolysis. Results in studies of arterial and venous thrombosis have been encouraging, but further research needs to be undertaken to find out whether the data has clinical applications, for example in predicting an individual patient's risk of recurrent thrombosis. In the meantime, the development of new whole blood tests is ongoing [156, 157], with the hope of achieving a better simulation of in-vivo fibrinolytic conditions.

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