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Assays to quantify fibrinolysis: strengths and limitations. Communication from the ISTH SSC Subcommittee on Fibrinolysis

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Abstract

Fibrinolysis is a series of enzymatic reactions that degrade insoluble fibrin. Plasminogen activators convert the zymogen plasminogen to the active serine protease plasmin, which cleaves and solubilizes crosslinked fibrin clots into fibrin degradation products (FDPs). The quantity and quality of fibrinolytic enzymes, their respective inhibitors, and clot structure determine overall fibrinolysis. The quantity of protein can be measured by antigen-based assays, and both quantity and quality can be assessed using functional assays. Furthermore, variations of commonly used assays have been reported, which are tailored to address the role(s) of specific fibrinolytic factors and cellular elements (e.g. platelets, neutrophils, and red blood cells).

Although the concentration and/or activity of a protein can be quantified, how these individual components contribute to the overall fibrinolysis outcome can be challenging to determine. This

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difficulty is due to temporal changes within and around the thrombi during the clot breakdown, particularly the fibrin matrix structure and composition. Furthermore, terms such as “fibrinolytic activity/potential”, “plasminogen activation” and “plasmin activity” are often used interchangeably despite having different definitions. The purpose of this review is to 1) summarize the assays measuring fibrinolysis activity and potential, 2) facilitate interpretation of data generated by these assays, and 3) summarize strengths and limitations of these assays.

Keywords

fibrinolysis; plasminogen; enzyme-linked immunosorbent assay; enzyme assays; plasminogen activators

1. INTRODUCTION

Fibrinolysis is a series of enzymatic reactions that degrade insoluble fibrin. Plasminogen activators convert the zymogen plasminogen to the active serine protease plasmin, which cleaves and solubilizes crosslinked fibrin clots into fibrin degradation products (FDPs)¹. The overall fibrinolysis is dependent on the quantity and quality of the various fibrinolytic enzymes such as tissue-type plasminogen activator (tPA) and plasmin, their respective inhibitors plasminogen activator inhibitor type 1 (PAI-1) and alpha-2-antiplasmin (α_2 AP), and clot structure. Fibrinolysis is further regulated by thrombin-activatable fibrinolysis inhibitor (TAFI; proCPU; proCPB2)^{2,3}, which functions by dampening plasminogen activation by t-PA.

In a clinical setting, increasing the rate of fibrinolysis is used to reverse thrombotic occlusion (e.g., recombinant tPA for acute treatment of stroke, myocardial infarction, or pulmonary embolism), whereas decreasing the fibrinolytic rate is used to reduce bleeding (e.g. tranexamic acid [TXA] used to prevent hemorrhage in trauma and the post-partum setting)⁴. Ongoing monitoring of fibrinolysis has been considered an emerging science for the use of managing bleeding during surgical procedures (e.g. cardiac surgery) as well as preventing venous thromboembolism in hospitalized patients. Furthermore, fibrinolysis assessment has been proposed for subsets of critically-ill patients at risk for bleeding and thrombotic complications such as those at risk for peri-partum complications, trauma, malignancy, sepsis, and COVID-19⁵ and those undergoing extracorporeal membrane oxygenation and liver transplantation⁶. Global point-of-care testing to identify an individual's fibrinolytic potential is not widely implemented. Instead, laboratory analyses often rely on select measurements of biomarkers in a plasma sample, most notably D-dimers and fibrinogen.

In contrast, basic mechanistic investigation of fibrinolysis is based on quantitation of the various fibrinolytic factors. Total concentrations are measured via antigen-based assays, while several specific functional tests are available to determine their activity. Despite the array of assays available, it remains challenging to attribute individual fibrinolytic factor contribution to the overall fibrinolytic outcome due to the dynamic nature of the environment surrounding the clot. In addition, functional tests such as clot lysis assays are often performed using an agonist to exogenously trigger clot formation and lysis, which can lead to maximal activation of the fibrinolytic processes. The consequence is quantitation of

an artificially generated fibrinolytic activity in a plasma sample rather than measuring its endogenous fibrinolytic potential. As such, it is important to understand both the meaning and limitation of each of these assays when interpreting the results.

The purpose of this review is to 1) summarize assays measuring fibrinolysis activity and potential, 2) facilitate interpretation of data generated by these assays, and 3) summarize strengths and limitations of these assays.

2. AVAILABILITY OF PLASMINOGEN ACTIVATORS

Plasminogen is synthesized in hepatocytes and circulates in blood at ~180 µg/mL. tPA or urokinase-type plasminogen activator (uPA) cleaves plasminogen to plasmin – the enzyme that digests fibrin. However, unlike uPA, tPA binds fibrin with high affinity, which improves its ability to activate plasminogen by 3-orders of magnitude by forming a plasminogen-tPA-fibrin complex⁷. Therefore, the presence of fibrin to act as a cofactor for promoting tPA-mediated plasminogen activation largely dictates the overall fibrinolytic activity. Plasminogen is typically quantified using commercially available ELISAs. However, the total antigen levels do not indicate whether plasminogen is activatable or how much was activated. As such, in clinical laboratory settings, plasminogen quantitation is routinely performed via functional assays, which quantify levels of functional plasminogen. In addition, the cleavage of plasminogen to plasmin can be visualized by the shift in its molecular weight (from ~92 kDa to ~85 kDa) by western blotting, this method is not as reliable for quantitation. The following sections describe methods for quantifying plasminogen activation.

2.1. tPA total concentration and enzymatic activity

tPA is a 68 kDa glycoprotein, consisting of 530 amino acid residues and containing 7–13% carbohydrates. Under physiological conditions, the total concentration of tPA in plasma is ~1–10 ng/mL^{8–11}. Upon occlusive thrombosis, endothelial granule-stored tPA can be rapidly released to increase the plasma tPA concentration^{12,13}. tPA is also released from endothelial cells in response to shear stress¹⁴. tPA readily activates plasminogen in a fibrin-dependent manner. Active tPA has a short half-life (~2.4 minutes)¹¹. tPA in a citrated plasma sample can be quantified by: 1) total tPA antigen, which includes tPA complexed with PAI-1, and 2) tPA activity. Total tPA antigen is often measured using commercially available ELISAs, which detects both free and bound (inhibited) forms of tPA. tPA activity can be measured using a tPA-specific chromogenic/fluorogenic substrate^{11,15}. Acidification of blood samples immediately after collection preserves tPA activity¹⁶.

Retinoic acid, sodium butyrate and PAI-1 enhance tPA gene expression in several endothelial beds and hepatocytes^{8,17,18}. Other factors that lead to fluctuations in circulating levels of tPA include the circadian rhythm, mechanisms that regulate exocytosis, vigorous exercise, excitation of the sympathetic nervous system, venous tourniquet, hypoperfusion, and vessel occlusion,^{19–21} although the mechanism mediating these changes is unclear²². In addition, the ability of secreted PAI-1 to bind to endothelial cell-bound tPA and readily form the tPA-PAI-1 complex²³ may also contribute to the fluctuating levels of circulating tPA. The tPA-PAI-1 complex also has a short half-life of ~5 minutes via

hepatic clearance¹¹. As such, measurable elevation of tPA-PAI-1 complex level directly implies a decrease in free tPA concentration and activity, and consequently a decreased potential to initiate fibrinolysis on vascular endothelium. The association between elevated levels of the tPA-PAI-1 complex and fibrinolysis hindrance is further corroborated by reports that show increased levels of plasma tPA, which mostly exists as tPA-PAI-1 complex, is a risk factor for cardiovascular diseases^{24 25}. In particular, individuals who have chronic cardiometabolic conditions, such as obesity, diabetes, or dyslipidemia, have higher circulating tPA concentrations^{8,12,26}. Although tPA production by hepatocytes is increased in obesity, this increase is overcompensated by a larger increase in PAI-1, resulting in a net decrease of tPA activity in plasma^{8,13}. In addition, plasma from obese individuals has shown higher concentrations of total tPA (free and complexed with PAI-1) despite lower concentrations of PAI-1-free tPA and tPA activity relative to normal individuals⁸, suggesting a likelihood of higher levels of tPA-PAI-1 complex presence. Therefore, quantitation of various forms of PAI-1 and tPA in circulation is necessary to fully understand the state of fibrinolysis and its potential in an individual²⁷.

2.2. uPA concentration and activity

uPA circulates in plasma at a concentration of 5–10 ng/mL and is also present in urine and the extracellular matrix. uPA is synthesized by several cell types, including epithelium and monocytes/macrophages. Despite its presence in plasma, it is unknown whether circulating uPA promotes endogenous fibrinolysis. Unlike tPA, uPA activates plasminogen in a fibrin-independent manner despite a homologous Kringle domain^{7,28}.

Until the late 80s, it was believed that unlike single-chain tPA, single-chain uPA (scuPA) did not possess catalytic activity, since the reaction rate of scuPA with specific inhibitors and synthetic uPA substrates was low ($\leq 1\%$)²⁹. However, recent data show scuPA has catalytic activity³⁰. scuPA has plasminogen-activator activity³¹ and can form an SDS-stable complex with PAI-1³². The activity of uPA is typically determined by the rate of hydrolysis of amide (p-nitroanilide Glu-Gly-Arg, S-2444) or ether (Z-Lys-pNP) substrates due to the high catalytic efficiency of their hydrolysis³³. However, the presence of tranexamic acid (TXA) – a lysine analog that competitively blocks both plasminogen and plasmin binding to fibrin – may promote uPA activity³⁴. scuPA can directly activate plasminogen without conversion into uPA³⁵. However, scuPA is 250-fold less potent in generating plasmin than two-chain uPA³¹. Similar to tPA, uPA activity towards plasminogen activation can also be quantified by using either the lysis of standard fibrin clots³⁶, fibrin plates³⁷, and casein³⁸, or the plasmin-specific substrate in solution to determine the rate of activation³⁹. Also similar to tPA, uPA is rapidly inhibited by PAI-1⁴⁰.

3. THE INHIBITORS OF FIBRINOLYSIS

3.1. Plasminogen activator inhibitor-1

PAI-1 is synthesized mainly in the liver, adipose tissue, vessel wall, and megakaryocytes and its gene expression is readily modified by many factors under physiologic- and pathologic- conditions^{41–44}. The normal plasma concentration of PAI-1 is 17–300 ng/mL^{8,45–47}. The presence of LDL or free fatty acid can increase hepatic expression of

the gene encoding PAI-1 and therefore, increase plasma levels of PAI-1 protein⁴⁸. This mechanism is corroborated by strong associations between lipid profile and plasma PAI-1 concentration. Active PAI-1 spontaneously undergoes a large structural change to become an inactive 'latent' form, leading to a short circulating half-life of active PAI-1 (~2 hours)⁴⁹. Total PAI-1 concentration can be measured by commercially available ELISA kits detecting PAI-1 antigen. The active PAI-1 levels can be inferred by loss of tPA activity¹¹. The tPA-free PAI-1 can be quantified by ELISA⁵⁰.

3.2. α_2 -antiplasmin

α_2 AP is a member of a serine protease inhibitor superfamily with a unique C-terminal bait peptide. α_2 AP is a 70 kDa protein that circulates at 0.7–1 μ M⁵¹, which is about half the concentration of circulating, intact Glu-plasminogen. Although α_2 AP is highly specific for plasmin, there are two functional forms: plasmin-binding and non-plasmin-binding, and these are present at a ratio of 65:35, respectively⁵². During clot formation, α_2 AP becomes crosslinked to fibrinogen and fibrin by activated factor XIII. Based on activity measurements, ~1 nM α_2 AP is incorporated into ~1 μ M of fibrinogen.⁵³ Crosslinking of α_2 AP to fibrin protects fibrin from premature lysis.⁵⁴

α_2 AP inhibits plasmin in two stages. The first stage involves binding between the lysine residues within the C-terminal region of α_2 AP and the lysine-binding kringle domain 1 of plasmin⁵⁵. The second stage involves plasmin-mediated cleavage of Met355-Arg354 bond, which triggers a conformational change in α_2 AP, thus halting the completion of the proteolytic process and forming a stable, covalently bound intermediate plasmin- α_2 -antiplasmin (PAP) complex^{51,56}. The circulating half-lives of α_2 AP and the PAP complex are 2.6 days and 0.5 days, respectively.

Chromogenic methods have been developed to determine functional levels of α_2 AP (*i.e.* plasmin-binding form)⁵⁷. Typically, exogenous plasmin is added to a plasma sample, and residual plasmin activity is quantified spectrophotometrically to infer the available inhibitory capacity of endogenous α_2 AP^{58–60}. Plasma also contains other plasmin inhibitors (*e.g.*, α_2 -macroglobulin [α_2 M], C1-protease inhibitor, antithrombin III, and α_1 -antitrypsin) which can lead to over-estimation of functional α_2 AP levels. To minimize the influence of α_2 M, several adjustments have been implemented: 1) the chromogenic reaction time can be shortened to the first 10 seconds due to differences in the relative ability of α_2 AP (fast) and α_2 M (slower) to inhibit plasmin, and 2) addition of methylamine to the reaction reduces α_2 M activity⁵⁷. In addition, chromogenic methods can also be sensitive to the presence of pharmacologic agents (*e.g.* heparin, ϵ -aminocaproic acid, TXA, etc.). Alternatively, by targeting the specific epitope of α_2 AP, immunochemical assays can bypass complications imposed by other endogenous inhibitors. Unlike the functional assay, however, the immunochemical assays indiscriminately quantify total α_2 AP antigen (*i.e.* plasmin-binding- and non-plasmin-binding-forms, PAP complex). Several ELISAs have been developed that specifically target total and truncated α_2 AP isoforms^{61–63}, as well as PAP complexes⁶⁴.

3.3. Thrombin-activatable fibrinolysis inhibitor

TAFI also regulates fibrinolysis⁶⁵. TAFI is activated by proteolytic cleavage by thrombin, plasmin, or the thrombin-thrombomodulin (TM) complex, which removes the activation peptide and liberates the carboxypeptidase catalytic domain (TAFIa)³. Contrary to its name, TAFIa functions as more of a fibrinolysis attenuator rather than a true inhibitor. TAFIa exerts antifibrinolytic function by removing C-terminal lysine and arginine residues on fibrin and fibrin degradation products (FDPs)⁶⁶, which leads to 1) decreased cofactor function of fibrin in the activation of plasminogen by tPA, and 2) increased dissociation of plasmin from fibrin (i.e., decreased protection of plasmin) that promotes its rapid inhibition by α_2 AP⁶⁷. Margins of avascular injury sites where thrombin is generated proximal to TM could be the primary site where this mechanism functions. While studies using TAFI knockout mice suggest that TAFI plays a minor role *in vivo*⁶⁸, findings from a recent murine model of venous thromboembolism suggests TAFI promotes venous thrombus stability and reduces embolization⁶⁹. In addition, observations of a patient with a bleeding tendency and a mutation in *THBD* (encoding thrombomodulin) that would prevent TAFI activation⁷⁰, as well as on the bleeding phenotype caused by loss of TAFI in animal models of hemarthropathy underscore the potential physiologic importance of TAFI⁷¹.

Levels of TAFI and its truncated forms (e.g., TAFIa and inactivated TAFIa) in plasma can be quantified using commercially available antigen-based methods. However, these values are insufficient to quantify functional activity of TAFIa present in plasma. Direct quantitation of TAFIa in plasma is ideal. However, these measurements are challenging for three major reasons: 1) unlike TAFI, TAFIa is intrinsically unstable at physiologic conditions where its half-life is around 7 minutes⁷², 2) probing for carboxypeptidase activity of TAFIa in plasma using a small synthetic substrate may be overwhelmed by the large presence of constitutively active carboxypeptidase N, and 3) there are only two proprietary functional assays available to specifically measure TAFIa activity in plasma, neither of which are commercially available^{73,74}. As such, functional studies such as clot lysis times have been implemented to infer TAFI activation potential. Since there are no known physiologic inhibitors of TAFIa, inhibitors such as potato tuber carboxypeptidase inhibitor and guanidinoethyl-mercaptosuccinic acid (GEMSA) are often used in *in vitro* assays⁷⁵. Differences in functional output such as clot lysis time that arise upon inclusion of these inhibitors are then used to infer the activation potential of TAFI in these *in vitro* systems. However, this still does not translate to active TAFIa levels that are present in the sample. In addition, TAFI activation potential measurement is further compounded by the presence of soluble TM (sTM) at varying levels in plasma⁷⁶, which may lead to differential kinetics of TAFI activation *ex vivo*.

3.4. Soluble thrombomodulin (sTM)

TM is a transmembrane protein primarily located on vascular endothelial cells. sTM encompasses the extracellular portion of TM and is shed from the endothelial cell surface via proteolytic cleavage by neutrophil proteases and metalloproteases that can be released under proinflammatory conditions⁷⁷. In plasma, sTM is present as a heterogeneous collection of fragments⁷⁸. It has been reported that the low concentrations of sTM natively

present in plasma are sufficient to exert an antifibrinolytic effect, and even the addition of 1 ng/mL of rabbit TM is sufficient to extend lysis time^{76,79}

Currently, sTM is measured as antigen concentrations using immunoassay kits, most commonly in the ELISA format. However, these assays are neither standardized nor harmonized, and do not use established reference materials for calibration, which makes comparison between studies difficult. Indeed, a 10-fold difference was observed with the same samples in one comparison of 3 different ELISA kits⁸⁰. As sTM in human plasma is present as multiple different fragments, the nature of the antibodies used may affect between-assay and between-sample variability. Plasma concentrations of sTM are generally low in healthy individuals, with one study reporting concentrations of 3.1 ± 1.3 ng/mL in 157 healthy volunteers⁸¹. However, in acute conditions such as trauma, disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS), myocardial infarction, and COVID-19, the levels of sTM can be increased several-fold^{69,81–84}. Indeed, studies in severely-ill COVID-19 and sepsis patients reveal elevated sTM concentrations sufficient to potentially exert an antifibrinolytic effect^{69,84,85}. sTM antigen is therefore an important marker of endothelial cell damage and more generally, a proinflammatory state. With respect to COVID-19, elevated sTM is predictive of mortality^{69,84,86,87}. The contribution of fibrinolysis impairment to these associations remains to be evaluated.

4. BIOMARKERS OF PLASMINOGEN ACTIVATION SYSTEM

4.1. D-dimers and fibrin(ogen) degradation products (FDPs)

FDPs, including D-dimer are generated from plasmin-mediated proteolysis of fibrinogen and fibrin. In particular, D-dimer is the smallest fragment that is only generated when crosslinked fibrin is digested⁸⁸. As such, the presence of D-dimers more accurately represents both coagulation (i.e. generation of fibrin clots) and fibrinolysis (i.e. degradation of fibrin clots). In addition, long half-life of D-dimers in circulating plasma (~16 h)⁸⁹ obfuscates accurate interpretation of single time-point measurement. Thus, sequential measurements are required to confirm active fibrinolysis.

D-dimer measurements are used as a biomarker for coagulation and incorporated into diagnostic and predictive algorithms. In particular, a normal (or negative) D-dimer has a high negative predictive value for acute deep vein thrombosis⁹⁰ and pulmonary embolism⁹¹. As a result, D-dimers are routinely measured when coagulation dysfunction is suspected. More recently, elevated D-dimers have been detected in patients with COVID-19 and associated with disease severity and death in some^{92–94} though not all⁶⁹ studies. D-dimer can be measured using commercially available kits. However, rapid implementation of these assays has led to large variability and inconsistency in their sensitivity (e.g. cut-off limits), specificity (FDPs vs D-dimers vs fibrinogen), and the reported units (D-dimer units vs. fibrinogen equivalent units)⁹⁵. Consequently, harmonizing data from these non-standardized methods of reporting D-dimer can be problematic in large meta-analysis-driven cohort studies. Although the use of a gold-standard reference of D-dimer has been proposed, generation of this standard has proven challenging due to its instability⁹⁶.

4.2. Plasmin- α_2 -antiplasmin (PAP) complex

α_2 AP is the main inhibitor of plasmin. Upon binding, plasmin is rapidly inhibited by α_2 AP, producing a covalent complex (PAP). Due to its stability, PAP is considered a good biomarker for the activation of the fibrinolytic system *in vivo*. Quantitation of the PAP complexes in blood or plasma often relies on a highly specific ELISA⁹⁷. Similar to D-dimers, PAP complex has a long half-life (~12 h)⁸⁹, which makes accurate interpretation based on single time-point measurement challenging.

There are conflicting data on the value of measuring PAP complexes. The Cardiovascular Health Study of 5,201 healthy men and women ages more than 65 years showed that PAP levels predicted future acute myocardial infarction (AMI) (OR: 3.1)⁹⁸. However, presence of PAP complex in plasma is weakly associated with an increased risk of VTE in healthy patients⁹⁷. Other studies reported significantly higher PAP levels in patients undergoing diverse clinical conditions in which fibrinolysis is activated, including sepsis, AMI, malignancy, diabetes, pregnancy, and thrombolytic therapy⁶⁴. Similar to D-dimers, many factors apart from fibrinolysis activation (e.g. age, body-mass index, and triglyceride levels) can influence PAP levels, which may explain the reported discrepancies between studies evaluating clinical associations.

5. ASSESSMENT OF FIBRINOLYTIC ACTIVITY AND POTENTIAL

For decades, clot lysis assays that are monitored via turbidity have been used as a standard method to quantify overall fibrinolytic potential of a sample. Furthermore, variations of this global and simplistic assay have been developed to address the role(s) of specific fibrinolytic factors as well as cellular elements (e.g. platelets, neutrophils, and red blood cells). We identify functional assays that can be utilized to assess overall fibrinolytic potential.

5.1. Euglobulin clot lysis time

The euglobulin clot lysis time (ECLT) is an established method to quantify fibrinolytic potential. The euglobulin fraction of plasma is an isoelectric precipitate of an acidified condition that lacks most of the α_2 AP and α_2 M but retains tPA, PAI-1, plasminogen, and fibrinogen⁹⁹. The ECLT is good for estimating endogenous tPA activity, which is determined by the balance of plasma concentrations between tPA and PAI-1^{100–102}. ECLT has been used to identify rapid and abrupt increases in circulating levels of tPA in plasma in various settings including post-vigorous exercise¹⁰³, venous occlusion¹⁰⁴, and a hyper-fibrinolytic state after trauma¹⁰⁵. A modified ECLT in which the fraction is supplemented with a physiologic concentration of calcium ion (Ca^{++} -ECLT) has also been developed. The inclusion of calcium ions leads to shortened ECLT¹⁰⁶, in a manner that can be reversed by activated protein C. As such, enhanced thrombin generation and the resulting PAI-1 neutralization by generated thrombin are thought to be the main drivers of shortened ECLT due to calcium presence¹⁰⁷. Thus, the difference between ECLT and Ca^{++} -ECLT simply represents PAI-1-dependent inhibitory activity of fibrinolysis in plasma. Significantly shortened ECLT and the lack of the Ca^{++} -dependent shortening of ECLT were the clues in identifying two confirmed PAI-1 gene-deficient patients having life-threatening

bleeding^{108,109}. ECLT and Ca^{++} -ECLT are also useful tools to detect a state of fibrinolysis-shutdown caused by a sudden increase in plasma levels of PAI-1.

5.2. Plasminogen activator-induced plasma clot lysis time

The plasminogen activator-induced plasma clot lysis times (PA-CLT) is often reported to assess and quantify the overall fibrinolytic property of a sample^{53,75,76,110}. The PA-CLT is performed by simultaneous addition of agonists to initiate clotting (e.g., thrombin, tissue factor, Ca^{++}) and fibrinolysis (e.g., tPA or uPA) to citrated plasma. Alternatively, the clots may be generated first and then overlaid with tPA or uPA to mimic the clinical setting in which tPA is infused to degrade existing ischemic thrombi (thrombolysis)¹¹¹. The simplicity of the reaction system and minimal sample processing make the PA-CLT an ideal method to investigate the susceptibility of plasma clots to fibrinolysis, and repeatedly shown to associate with risk of thrombosis clinically^{112–115}. Moreover, when used in concert with selective inhibitors to target specific coagulation factors, the PA-CLT has revealed biological pathways that modulate the fibrinolytic response. For example, this method was used as the functional assay to identify the presence of TAFI during its purification and in characterizing the role of TAFI in attenuating fibrinolysis⁷⁵. The TAFIa-specific inhibitor potato tuber carboxypeptidase inhibitor has been used frequently in these studies^{110,116,117}. In addition, the impact of TM on fibrinolysis has been defined by selective inhibition of sTM using antibodies⁷⁶. The PA-CLT is also sensitive to inhibitory molecules that target specific factors such as PAI-1^{118–120} and $\alpha_2\text{AP}$ ^{121–123}, which can be included to infer the respective roles of PAI-1 and $\alpha_2\text{AP}$ in overall fibrinolysis.

5.3. Plasmin generation assay

Inherent in the ability of exogenous tPA to induce clot lysis is its ability to convert plasminogen to plasmin. Historically, there have been fewer assays that directly measure the kinetics of this process as it occurs in plasma. Studies that have approached this gap have largely based these assays on the existing thrombin generation assay (TGA). The TGA enables us to assess thrombin generation measurements, providing a precise vocabulary to define abnormalities in this process. Leveraging this advantage, plasmin generation assays (PGAs) developed by several groups share some common elements^{124–128}. In general, procoagulant activity in the PGA is initiated by the addition of exogenous tissue factor to recalcified plasma from humans or from mice, and fibrinolytic activity is triggered by the addition of exogenous tPA. Plasmin generation is detected via cleavage of a fluorogenic substrate, and parameters are defined from the accrual of fluorescence or via a mathematical derivative of this fluorescence curve. Subtle differences between these assays include the concentrations of tissue factor and tPA used, and use or not of diluted plasma. Some variations of these assays detect thrombin and plasmin simultaneously^{124,125}, whereas others perform these measurements separately but in parallel^{126,128}.

Collectively, these studies using the PGAs have shown these assays are specific to plasmin and sensitive to $\alpha_2\text{AP}$, whereby activity measured represents free-plasmin in the system. The PGA is also sensitive to the ability of fibrin to serve as a cofactor for plasmin generation; mice with a deficiency in fibrinogen¹²⁹ or that express fibrinogen that cannot be converted to fibrin¹³⁰ show reduced plasmin generation¹²⁸. Fibrin(ogen) sensitivity of the PGA has

also been demonstrated in human plasma with abnormal levels of clotting factors that result in altered fibrin formation^{124–127}. Given the contribution of fibrin cofactor activity to plasmin generation, the PGA is also sensitive to TXA. The PGA shows less variability than ROTEM to pharmacologically-relevant concentrations of TXA¹³¹. The PGA is also sensitive to other antifibrinolytic agents, including *e*-aminocaproic acid^{124,125}, giving these assays have several potential clinical applications.

Although both tPA and uPA circulate at low levels in plasma, the PGA is not sensitive to endogenous concentrations of these proteins¹²⁸. This insensitivity differs notably from the ECLT assay^{102,104,132}. Given the higher concentrations of tPA needed to trigger measurable plasmin generation, the PGA is also not sensitive to plasma concentrations of PAI-1. However, the plasmin generation response to the addition of exogenous tPA is dose-dependent. Moreover, the PGA is sensitive to low levels of circulating sTM that facilitate thrombin-mediated activation of TAFI. In a mouse model of diet-induced obesity, increased circulating sTM produced a TM- and TAFI-dependent prolongation in the time to peak plasmin generation¹²⁸. Notably, the effects of sTM on plasmin generation are below levels required for measurable TM-mediated suppression of procoagulant activity (thrombin generation). Collectively, a growing body of work suggests the PGAs, particularly when used in concert with thrombin generation and turbidity assays, yield a multi-dimensional impression of the integrated effects of procoagulant and fibrinolytic activities in health and disease.

5.4. Whole blood assessment of fibrinolysis

Distinct and dynamic phenotypes of fibrinolysis, with direct clinical consequences, are well documented following trauma¹³³ and appear to be relevant to COVID-19. Adverse clot formation has been observed in COVID-19 patients who are both hypercoagulable and hypofibrinolytic⁵. The regional distribution of specialized endothelial cells contributing to the fibrinolytic system underscores the importance of localization of this process¹³⁴. The release of tPA from precapillary arteriole and post-venular endothelial cells allows for a rapid response to prevent low-flow occlusion of small vessels. The importance of continuous clearing of fibrin deposition is observed after trauma with extensive thrombin generation stimulated by tissue injury. This homeostatic process is enhanced to maintain microvasculature patency distal to the injury site^{135,136}.

The plasma-based assays discussed above omit the contribution of cellular components, including platelets, red blood cells, and leukocytes, all of which can influence fibrinolysis^{137–139}. Thus, whole blood assessment may provide further insights into the global fibrinolytic properties in a rapid manner due to minimal sample handling required. Several whole blood assays, such as the thrombolysis plate assay and global fibrinolytic capacity have recently been described, but these are not currently available for clinical use^{89,132}. At this time, viscoelastic hemostatic assays such as thrombelastography (TEG) and rotational thromboelastometry (ROTEM) appear to provide the best point-of-care measures of fibrinolysis within 60 minutes. The TEG thresholds between 3% and 7.5% LY30 (percent degradation of clot 30 minutes after reaching maximum amplitude) has been associated with an increased risk for massive transfusion and mortality^{140–142}. Identifying

the hyperfibrinolytic patient has been relatively straightforward with these assays, although the optimal activator and the optimal percent lysis threshold remain to be defined. Whole blood-based assays can be sensitive to components such as S100A10 (or the p362/p112 complex)¹⁴³, which effectively binds tPA and plasminogen in a lysine-dependent manner through its C-terminal lysine residue.

Evaluating the other end of the spectrum of the fibrinolytic system, impaired or inhibited fibrinolysis, is a comparably understudied and active area of research¹⁴⁴. Endogenous hypofibrinolysis is attributed to chronically elevated PAI-1, impaired endothelial tPA generation/release, or cleaved plasminogen. Reduction of fibrinolytic potential *ex vivo* can be observed as an event in response to acute hyperfibrinolysis in trauma and liver transplantation. The addition of tPA¹⁴⁵ and plasmin challenge¹⁴⁶, TEG assays may enhance the calibration, but these are not currently FDA approved in the USA. Furthermore, diagnostic potential and application of viscoelastic assays in clinical setting requires further validation, and as such there are conflicting reports that describe what viscoelastic tests detect and measure. For example, the study by Raza et al. stratified patient populations by PAP complex levels, along with having hyperfibrinolysis identified by ROTEM¹⁴⁷. As discussed above interpreting the results of the viscoelastic tests is confounded by the PAP complex also has a long half-life that exceeds 12 hours in healthy individuals⁸⁹.

6. Conclusion

There are many commercially available antigen- and function-based assays to quantify various steps within the fibrinolytic cascade. Table 1 provides a summary of various assays that are currently available and are used. For antigen-based assays, it is important to know whether the assay is specific for one form of a protein, or whether it is sensitive to a number of species including enzyme-inhibitor complexes. Similarly, for functional assays, it is important to understand the conditions of the assay being performed (e.g. thrombin or tissue-factor initiated clot formation versus no agonist), which directly reflects their interpretation.

Fibrinolysis assessment in clinical settings is state-of-the-art translational science, with its implementation into practice remaining a major area of interest and ongoing debate. Particularly, there is a need for ongoing monitoring of fibrinolytic state to improve surgery outcomes and disease progression. As such, it is important to choose appropriate assays for the scientific questions being asked while ensuring to not over-interpret their findings.

Addendum

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Table 1:

Table of various assays of fibrinolysis.

Target	Method	Use
Overall Fibrinolysis		
Euglobulin (ECLT)	Kinetic	Endogenous tPA activity. Identify elevated PAI-1.
Clot Lysis (PA-CLT)	Kinetic	Overall fibrinolytic potential. Activator could be in the clot or overlay on existing clots (with exogenous tPA).
Viscoelastic	Kinetic	Whole blood analysis to determine coagulopathy; hypo/hyperfibrinolysis.
Plasminogen	ELISA	Total plasminogen in plasma.
	Western Blot	Full length plasminogen has molecular weight ~ 92 kDa, whereas active plasmin is ~ 85 kDa; not quantitative.
	ELISA	Total tPA level (free tPA + tPA/PAI-1 complex).
tPA	ELISA	PAI1-free tPA level (free tPA without PAI-1-bound, but the tPA is not necessarily functional)
	Kinetic	tPA activity chromogenic/fluoregenic assay (measures functional tPA).
uPA	ELISA	Total uPA (scuPA and tcuPA)
	Kinetic	uPA activity chromogenic assay; TXA presence may obfuscate the assay
PAI-1	ELISA	Total PAI-1 level (active and latent forms, tPA-PAI-1 complex).
	Kinetic	Measures available functional PAI-1 levels.
tPA:PAI-1 complex	ELISA	Specifically measures the tPA-PAI-1 complex.
α_2 AP	ELISA	Total α_2 AP (plasmin-binding/non-binding, plasmin-antiplasmin complex).
	Kinetic	Infer functional levels by quantifying residual plasmin activity upon inhibition.
Plasmin: α_2 AP complex (PAP)	ELISA	Specifically measures PAP complex and not individual components.
Plasmin Generation	Kinetic	Quantify rate of active plasmin being generated over time. Clinically used to quantify functional plasminogen levels.
TAFI	ELISA	Measures TAFI and its products.
TAFIa	ELISA	Measures TAFIa and inactivated TAFIa (TAFIai).
	Kinetic	Specifically measures functional TAFIa levels; not commercially available.
sTM	ELISA	Total soluble TM levels in sample.
	Kinetic	Characterize its cofactor activity towards protein C and TAFI activation.
D-dimers/FDPs	ELISA	Clinically used to determine ongoing thrombosis/fibrinolysis in patients; lacks sensitivity and standardization to compare globally.