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20TH ANNIVERSARY REVIEW ARTICLE

The fibrinolysis renaissance

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Abstract

Fibrinolysis is the system primarily responsible for removal of fibrin deposits and blood clots in the vasculature. The terminal enzyme in the pathway, plasmin, is formed from its circulating precursor, plasminogen. Fibrin is by far the most legendary substrate, but plasmin is notoriously prolific and is known to cleave many other proteins and participate in the activation of other proteolytic systems. Fibrinolysis is often overshadowed by the coagulation system and viewed as a simplistic poorer relation. However, the primordial plasminogen activators evolved alongside the complement system, approximately 70 million years before coagulation saw the light of day. It is highly likely that the plasminogen activation system evolved with its roots in primordial immunity. Almost all immune cells harbor at least one of a dozen plasminogen receptors that allow plasmin formation on the cell surface that in turn modulates immune cell behavior. Similarly, numerous pathogens express their own plasminogen activators or contain surface proteins that provide binding sites for host plasminogen. The fibrinolytic system has been harnessed for clinical medicine for many decades with the development of thrombolytic drugs and antifibrinolytic agents. Our refined understanding and appreciation of the fibrinolytic system and its alliance with infection and immunity and beyond are paving the way for new developments and interest in novel therapeutics and applications. One must ponder as to whether the nomenclature of the system hampered our understanding, by focusing on fibrin, rather than the complex myriad of interactions and substrates of the plasminogen activation system.

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KEYWORDS
α_2-antiplasmin, fibrinolysis, PAI-1, plasminogen activators, plasminogen, tranexamic acid

1 | INTRODUCTION

The enzymes that comprise the fibrinolytic system belong to a large superfamily of serine proteases and share significant ancestral homology with those of the coagulation pathway, and the complement pathway [1]. In fact, phylogenetic studies have revealed that the zymogen plasminogen and the primordial plasminogen activators (PAs) evolved alongside the complement system, approximately 70 million years before coagulation first appeared in cartilaginous fish [2]. Hence, the primary role of plasminogen, and its active form plasmin, did not involve fibrinolysis. Instead, it is suggested that the primordial function of plasmin(ogen) lies in infection and immunity [3], although it is plausible that plasmin formed in protochordates had a more generalized role in protein turnover. By the time the coagulation system evolved, the primordial plasminogen activating system was able to readily adapt to include fibrin, the terminal point in coagulation, among its various substrates.

Nowadays, fibrinolysis is viewed, very simplistically, as the proteolytic process by which fibrin is degraded to allow clearance of a blood clot from the circulation (Figure 1). The central enzyme, plasmin, is formed by the cleavage of circulating zymogen, plasminogen. The enzymes primarily responsible for plasminogen activation are tissue-type and urokinase-type plasminogen activators (tPA and uPA, respectively). Plasmin is a potent proteolytic enzyme with broad substrate specificity and must be tightly controlled within the vasculature to mitigate deleterious damage. The classic inhibitors of fibrinolysis are serine protease inhibitors (SERPINs) that rapidly trap their target protease in a stable 1:1 complex. The complex is subsequently cleared via the liver by low density lipoprotein-receptor-like protein-dependent mechanisms. Plasminogen activator inhibitor-1 (PAI-1) is the SERPIN charged with curtailing the activity of tPA and uPA [4,5], while alpha$_2$-antiplasmin (α_2AP) is the dominant inhibitor of plasmin. The various checkpoints to limit proteolytic activity extend beyond these inhibitors to other regulatory proteins which serve to modulate binding and function of PAs and plasmin to fibrin or other surfaces. Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) is a carboxypeptidase which exerts its antifibrinolytic function by cleaving C-terminal lysine residues from fibrin, thereby preventing docking of plasminogen. As a result, the cofactor function of fibrin in tPA-mediated plasmin generation is attenuated [6].

This 20th anniversary article of the *Journal of Thrombosis and Haemostasis* details the key features of fibrinolysis driven by its initial discovery as the enzymatic system for proteolytic removal of fibrin. These early observations paved the way for new and exciting discoveries. These include expanding our insights into connections between this primordial system and its initial partner, inflammation and immunity, and other pathways, including coagulation and complement. Unearthing the role of fibrinolysis in various pathways has exposed that fibrin is by no means the only target of cascade, and that surfaces including cells and misfolded proteins, are crucial in mediating and augmenting plasminogen activation. Thrombolytic therapy was initially developed in the 1950s with streptokinase, but with key developments in recombinant DNA technology, tPA was approved for clinical use in the late 1980s. We are now in an era when the limelight has once again shifted to various components of fibrinolysis and how these can be manipulated in clinical medicine. Many of these are unrelated to fibrin removal.

2 | AGE OF DISCOVERY

Blood clots were first reported to spontaneously dissolve in 1838 (Denis P.S, “Sang de L'homme; Figure 2). Subsequent work led to suggestions that fibrin had to be altered in to a “globulin family” member, which was reliant on the present of salt. Others argued that these changes could not take place until “putrefaction” occurred. In 1893, a French chemist, Albert Dastre, reported the phenomenon “Fibrinolyse dans le sang,” and so the term “fibrinolysis” was coined. Dastre was attempting to isolate fibrin from clotted blood and reported that the longer the blood samples were left standing, the less fibrin was recovered. He stated the following:

> “C'est cette dispartion de la fibrine que je nomme *Fibrinolyse*” [7].

In 1903, another group identified that fibrinolytic activity in plasma could be increased in the presence of chloroform, but that activity was subsequently reduced when plasma was added back to the sample (Delezenne and Pozerski, 1903) [8], thus indicating that plasma also contained an inhibitor to fibrinolysis.

The quantum leap occurred in 1933 with a publication by Tillett and Garner, detailing the discovery of a fibrinolytic agent produced by hemolytic streptococci [9]. These authors named this entity as “fibrinolysin.” It was later revealed by Haskell Milstone in 1940 that fibrinolysin could not destroy fibrin alone but did so when added to plasma [10]. Hence, it became apparent that a “plasma zymogen” existed that was initially referred to as “pro-fibrinolysis” but later became known as “plasminogen” by Christensen and MacCloud in 1945 [11] who stated:

> “*The inactive enzyme as it occurs in serum and plasma may be designated as ‘plasminogen’ to indicate its source, the plasma, and also to indicate that it is in an inactive precursor state.*”

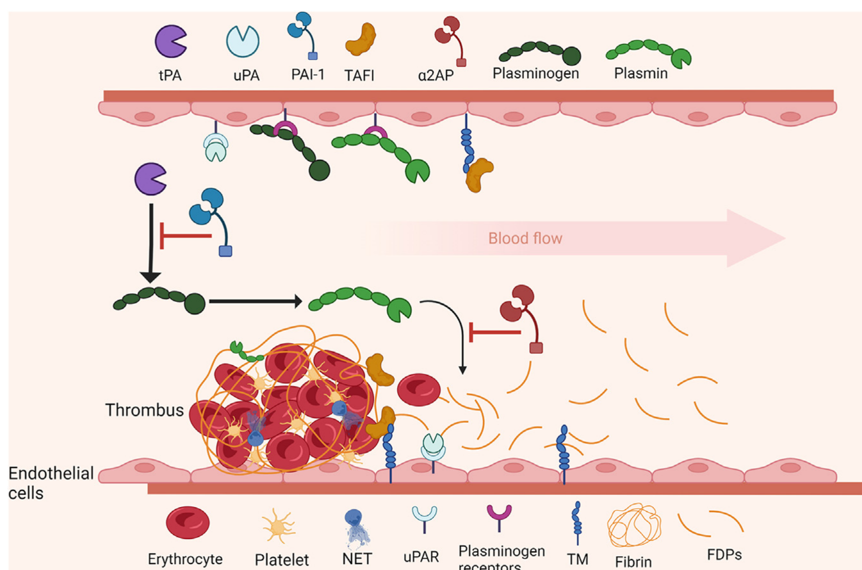

FIGURE 1 Fibrinolytic dissolution of thrombi. Fibrinolysis is initiated by plasminogen activators that convert circulating plasminogen to its active form plasmin, thus promoting degradation of fibrin. Soluble fibrin degradation products can be cleared from the circulation. Fibrinolysis is regulated at the level of plasminogen activation, via plasminogen activator inhibitor-1 or by direct inhibition of plasmin by α2-antiplasmin. Thrombin activable fibrinolysis inhibitor impedes fibrinolysis by removing C-terminal residues from fibrin, these lysine residues are vital for plasminogen binding to fibrin. Various cellular receptors, including those for plasminogen and urokinase protease activated receptor, localize fibrinolytic factors within the vasculature to augment surface-mediated plasminogen activation.

The following year, MacFarlane and Pillings in their article published in *Lancet* [12], further extended the nomenclature to include the inhibitor described ~40 years earlier (above), where they stated:

> "*It has long been recognised that normal plasma or serum contains an antiproteolytic factor (Delezene and Pozerski 1903)* [12] *associated with the albumin fraction……This factor is probably concerned with the absence of fibrinolysis in normal blood, and may be called, for convenience,* "*antiplasmin.*"

Fibrinolysin was subsequently renamed as "streptokinase" to indicate its source (streptococci) and the presumption that the reaction required energy. Two years later, Astrup and Permin published a landmark article in *Nature*, "Fibrinolysis in the Animal Organism" [13] where they found that "profibrinolysin" (ie, plasminogen) could be transformed into its active form by "an activator contained in animal tissue cells." These authors initially referred to this entity as "fibrinokinase" or "cyto-fibrinokinase." What they had discovered, in fact, was tissue-type plasminogen activator (tPA). The "tissue" in tPA reflects the fact that the protein is derived from "tissue." A second fibrinolytic entity discovered in human urine in 1952 by Sobel et al. [14] was subsequently referred to as urokinase (uPA).

Proteases require exquisite regulation to deter adverse downstream effects. The first reports of a PA inhibitor to attenuate fibrinolytic activity came later in 1963, when Brakman and Astrup identified a fibrinolytic inhibitor in pregnant women that did not directly inhibit plasmin, instead it seemed specific for urokinase [15]. This inhibitor was later classified as plasminogen activator inhibitor 2 (PAI-2). Additional reports of a specific PA inhibitor in plasma followed [16,17]. However, there was uncertainty as to whether specific PA inhibitors had any necessary function [15], as the abundance of circulating α_2-AP, was considered sufficient for regulation of the fibrinolytic system [18–20].

Further evidence for PA inhibitors was revealed in 1982, when it was discovered that direct addition of tPA to plasma significantly attenuated its functional activity [21,22]. Subsequently, synthesis and release of a highly stable PA inhibitor, of M_r 55,000, was described from bovine aortic endothelial cells [23]. The same inhibitor was later identified in platelets, which accounts for a significant proportion of circulating PA inhibitor [24]. Subsequently, advancement in laboratory techniques permitted differentiation between PA and plasmin inhibitors, purification of PAI-1 and PAI-2 [25,26], development of specific antisera to PAI-1 and to cloning of the PAI-1 [27,28] and PAI-2 [29] cDNAs.

Like other SERPINs, PAI-1 forms a 1:1 enzyme-inhibitor complex with its target proteases, rendering them enzymatically inactive. However, PAI-1 is very unstable with a half-life of 1 hour [30], whereas conversion to its thermodynamically stable latent form prolongs this to approximately 2 to 4 hours [31]. The levels of PAI-1 in healthy individuals are highly variable (~1-40 ng/mL) and while the concentration is generally in excess over its target protease tPA, it is at relatively low concentrations compared with other SERPINs. The extracellular matrix protein, vitronectin, acts as a stabilizing factor for PAI-1 [32], and augments its half-life [33].

α_2AP, the principal inhibitor of plasmin *in vivo* [34], has a unique attribute, as its extensive C-terminal lysine-rich tail initially docks to the kringles of plasmin, forming a non-covalent complex [35]. Subsequent cleavage of the reactive center loop by plasmin permits the formation of a covalent 1:1 plasmin-antiplasmin (PAP) complex. The main plasma pool of α_2AP is secreted by hepatocytes [36] and circulates at 70 μg/mL, some 1000-fold higher than PAI-1 [37]. Despite being the dominant fibrinolytic SERPIN in plasma, its concentration is surprisingly lower than the zymogen concentration of its target enzyme, plasminogen (2 μM, ~180 μg/mL) [38]. α_2AP is covalently cross-linked to fibrin through the action of the transglutaminase, activated factor XIIIa, thereby localizing its mechanism and action and augmenting potency [39,40]. The cross-linking of α_2AP into a

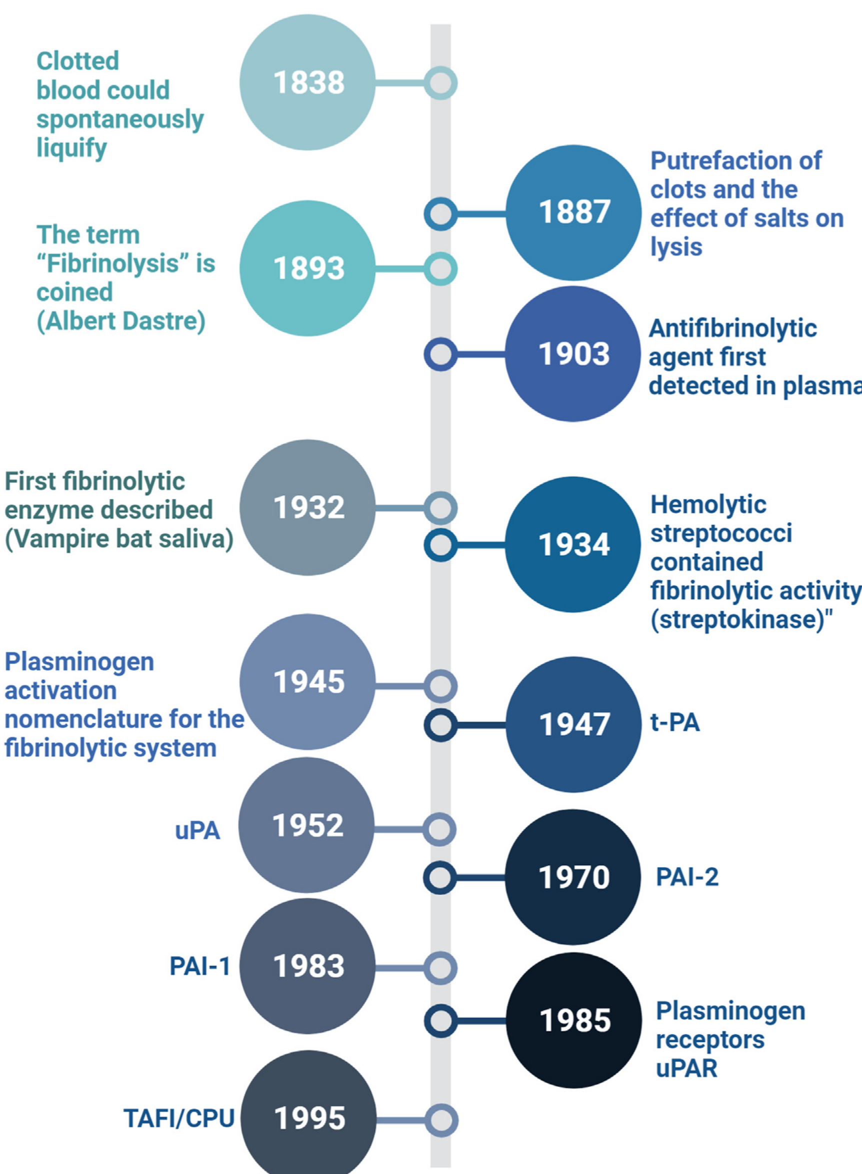

FIGURE 2 Discovery timeline of the fibrinolytic system. Major events propelling the identification of the system and subsequently individual factors involved in plasminogen activation and its regulation.

thrombus is dependent on shear stress [41] or compaction of clots [42].

Strangely, while PAI-2 is highly expressed in multiple cells, including monocytes, granulocytes, trophoblasts, epithelial, and endothelial cells [43–45], only a small percentage of PAI-2 is secreted into the circulation. This is due to the fact that PAI-2 lacks a signal peptide for release [46], and therefore its function is assumed to be mostly intracellular and differs from the extracellular form, in terms of glycosylation [47]. Elevated PAI-2 is observed in pregnancy and is related to increased cellular expression by the trophoblastic epithelium of the placenta [48]. Nonetheless, this SERPIN has the capacity to inhibit many extracellular proteases, despite reduced efficacy toward tPA and uPA compared with PAI-1 [49–51]. Monocyte-derived PAI-2 has the capacity to inhibit uPA- but not tPA-mediated lysis and can cross-link directly to fibrin [45]. Intriguingly, ablation of PAI-2 results in a 12-fold increase in uPA activity thereby augmenting thrombus resolution [52]. An added layer of complexity is that depletion of PAI-2 results in a concomitant decrease in PAI-1 [52], therefore making it challenging to dissect the individual effects of these inhibitors [51].

The *CPB2* gene product [53] was discovered independently by various groups in the early to mid-1990s giving rise to a discord in nomenclature. In 1989, Hendriks et al. [54] described a labile enzyme that interfered with their assay for carboxypeptidase N, later referred to as carboxypeptidase U, with "U" symbolizing unstable [55]. Within a few years, the *CPB2* gene product accumulated other pseudonyms, including plasma carboxypeptidase B [56] and carboxypeptidase R [57]. The regulatory role of this pro-carboxypeptidase in fibrinolysis was not appreciated until 1995, when Bajzar et al. [58] described the purification and characterization of a carboxypeptidase that was activated by thrombin. They termed this novel fibrinolytic inhibitor "thrombin-activatable fibrinolysis inhibitor" (TAFI). TAFI activation by thrombin was later found to be accelerated in excess of 1000-fold by

thrombomodulin [59] but alternatively can be activated by plasmin [60]. The activated carboxypeptidase, TAFIa (which was identical to the earlier descriptions of CPU), removes C-terminal lysine residues from partially-degraded fibrin, thereby reducing the availability of binding sites for plasminogen [61] and attenuating its cofactor capacity in tPA-mediated plasmin generation.

3 | THE CELLULAR REVOLUTION

The crucial role of fibrin in amplifying tPA-mediated activation of plasminogen and protection of plasmin has been appreciated for decades [62]. However, the same general characteristics apply to other surfaces including various circulatory cells, endothelial cells, and other misfolded protein aggregates, such as amyloid, that are prevalent on dead cells [63–65]. Plasminogen binding to immune-derived cells, including, monocytes, neutrophils, and platelets was first documented in 1985 [66], and there have been a plethora of proteins reported as binding partners, including include Plg-R_{KT}, α-enolase, S100A10 (functioning with annexin A2), actin, cytokeratin 8, and integrins $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ [67]. The binding of plasminogen to these receptors tends to be low affinity, but high capacity, with some cell-surface proteins only found on cells undergoing apoptosis. The majority of these proteins/receptors does not express a putative C-terminal lysine to dock plasminogen but are assumed to undergo a post-translational modification that permits interaction. Plg-R_{KT} is a true membrane protein and is synthesized with a C-terminal Lys residue. Interestingly, tPA shares many of these cellular binding partners, including annexin II and Plg-R_{KT} [67]. Receptors for tPA have been characterized in less detail [68,69], but these may be the same or similar molecules. The extent of competition between activator and substrate for these various binding partners is not well defined; however, given the sizable difference in the circulating concentration, one would assume that plasminogen binding was dominant in most scenarios.

In marked contrast, the counterpart uPA, and the zymogen form single-chain uPA (scuPA), bind with high affinity to a well-characterized receptor, uPAR (CD87) [70], where they are afforded some protection from PAI-1 inactivation [71]. uPAR is a glycosylphosphatidyl inositol-anchored protein but elicits signaling [72] via various intracellular proteins. Other proteins also bind uPAR, including vitronectin and integrins in complex with caveolin [73]. The uPA/PAI-1 complex is then internalized, and uPAR is recycled to the surface via a process that involves lipoprotein-receptor-like protein [74,75]. uPAR has clear roles in migration and metastasis but its role in proteolytic removal of fibrin and misfolded protein aggregates is not well defined. Recently, it has been documented that factor (F) XII binding to uPAR regulates neutrophil migration, providing additional links between fibrinolysis and the contact system [76]. The role of uPAR appears to be in protection of active uPA from inhibition, as there is no significant augmentation of plasminogen activation. Direct interaction of a uPA variant with the cell membrane affords a similar enhancement of uPA-mediated plasminogen activation to that observed with uPAR [77]. In contrast, the power of uPAR appears to lie in stimulating activity of the zymogen scuPA [78,79]. Intriguingly, co-localization and reciprocal activation of scuPA and plasminogen occur on platelets [80], which do not express uPAR, indicating that there are additional binding partners for these proteins yet to be discovered. Studies on extracellular vesicles also convey the distinct differences between tPA and uPA, demonstrating that binding of plasminogen and (sc)uPA do not have to be on the same cells or surface to facilitate fibrinolysis, while the same phenomenon is not observed with tPA [81]. These observations highlight fundamental differences in the interactions of PAs with binding partners which plays a role in downstream plasminogen activation, which may provide clues as to their function in (patho)physiological processes.

Platelets are directly involved in clot stability and lysis. On the antifibrinolytic side, there is the physical barrier to lysis that results from clot retraction, added to which platelets have a pool of FXIII [82] that stabilizes fibrin and participates in the clot retraction process [83]. Further, platelets harbor the 3 main inhibitors of fibrinolysis, PAI-1, α_2AP, and TAFI [84]. These platelet-derived pools result from synthesis and packaging of the inhibitors at the megakaryocyte stage and/or endocytosis of the inhibitors from plasma. Indeed, while platelets are devoid of a nucleus, they are known to synthesize various proteins from mRNA captured at the megakaryocyte stage, one of the reported proteins is PAI-1 [85]. The traditional view was that PAI-1 was released into the platelet secretome, but a considerable proportion of active PAI-1 is retained on the activated platelet membrane [86], perhaps to mediate surface-based reactions. In terms of activity, platelet PAI-1 is less active than plasma PAI-1, but these cytoplasts deliver around 50% of the total circulating active PAI-1. The platelet pools of α_2AP and TAFI are not as substantial, accounting for <1% of the total blood pool [87,88]. The interaction of platelets and fibrin is regulated by the integrin αIIbβ3 and is key to the process of clot retraction. Intriguingly, an elegant study revealed that the processes of clot retraction and fibrinolysis are mechanistically coupled *in vivo* to modulate thrombus size [89]. This role of the fibrinolytic pathway may relate to the fibrinolytic pathways primordial function of eliminating protein aggregates, as the crucial role of platelets as innate immune cells has now emerged [90].

On the profibrinolytic side, activated platelets exhibit endogenous plasmin activity [91] and surface-bound plasmin, formed from local plasminogen, afford protection from inhibition by α_2AP. Plg-R_{KT} has now been identified on platelets and accounts for around 40% of binding of plasminogen to the activated platelet membrane [92], while the remainder is bound via fibrin-dependent mechanisms. Separate studies have indicated that plasminogen binding to thrombin-activated platelets is entirely fibrin(ogen) dependent [93] and that accumulation is attenuated by plasma TAFI [93,94]. Plasminogen and uPA bound to different cellular surfaces and microvesicles are capable of stimulating profibrinolytic activity [81], a feature that is not associated with tPA-mediated activation of plasminogen. Binding of soluble Glu-plasminogen to cell surfaces enhances its activation [67] induces a conformational change in the zymogen that is distinct from Lys-plasminogen [95]. These studies and others highlight the importance of the cell membrane in supplying fibrinolytic proteins and

catalyzing plasminogen activation, particularly in the context of (sc) uPA.

Vascular endothelial cells (VECs) primarily bind plasminogen and tPA through Annexin A2-S100A10 [67]. Single-chain tPA is found within the secretory granules that are released via constitutive and regulated mechanisms [96]. Localization of these factors on VECs facilitates local plasminogen activation [97]. However, this is counterbalanced by the fact that PAI-1 is also secreted in abundance by VECs and can reportedly dissociate tPA from the VEC surface [96]. PAI-1 is an acute phase protein that is up-regulated in response to several stimuli, including thrombin, proinflammatory cytokines, insulin, angiotensin, and lipopolysaccharide [98] and as such this dynamic pool of PAI-1 is considered to govern the fibrinolytic potential of VECs. The adhesive glycoprotein thrombomodulin is highly expressed on VECs and is crucial in the anticoagulant capacity of the endothelium but also participates indirectly in fibrinolysis by promoting thrombin-mediated activation of TAFI [59].

Studies on human thrombi reveal that the inhibitors of fibrinolysis, especially PAI-1, accumulate in great excess over proteases [99–101], providing a potential explanation as to why established thrombi are often resistant to lysis. On the other hand, subsequent studies provided compelling evidence that the basis for resistance to lysis was not related to PAI-1, as neutralizing antibodies to PAI-1 incorporated into the clot and surrounding plasma did not potentiate clot lysis [102]. This implies that blood clots harbor other inhibitory agents that attenuate tPA-mediated lysis. Prime suspects are the SERPINs, protease nexin 1 (PN-1) which is phylogenetically the closest relative to PAI-1, and the dominant inhibitor of the contact/complement pathways, C1-inhibitor [103]. As discussed earlier, there are several lines of evidence to suggest a significant contribution of uPA in thrombus clearance, with monocyte-bound uPA significantly reducing thrombus size in a model of venous thrombosis [104]. In this context, the activity of uPA will be preserved by binding to uPAR rendering it resistant to inactivation by PAI-1 and potentially other inhibitors.

4 | THERAPEUTICS: THE CLASSICAL ERA

4.1 | Thrombolytics

Given the importance of the fibrinolytic system in clot removal, there was significant interest in exploiting the system clinically to enhance patient outcomes following thrombotic events. The clinical use of streptokinase preceded both tPA and uPA which only became feasible in the 1980s, due to significant advances in recombinant protein technology. While initially licensed for myocardial infarction, thrombolysis became standard of care for acute ischemic stroke (AIS) in the 1990s [105]. The preferential use of tPA over uPA relates to the avid binding of the former PA to partially degraded fibrin, which augments localized plasminogen activation [106]. On the other hand, and as mentioned above, uPA also plays a physiological role in clot removal. Others have argued that physiological fibrinolysis requires both tPA *and* uPA where these 2 PAs act in a complementary manner [107]. In this scenario, tPA was proposed to initiate lysine-dependent lysis and uPA facilitates subsequent lysis and, in fact, becomes the dominant lytic. This role for uPA is also supported by gene-knock out studies in mice [108,109]. Interestingly, a phase II trial for AIS, outlined in 2022, tested dual thrombolytic therapy with low-dose tPA followed by infusion of a mutant pro-uPA (DUMAS study) [110]. The outcome of this trial is not known at the time of writing this article. Nonetheless, despite its limited window of efficacy, tPA remains the only drug licensed for AIS. Thrombolysis is now frequently used in combination with thrombectomy and is shown to improve long-term functional outcomes and enhance patient mortality [111].

Current thrombolytic agents are compromised by poor efficacy and an unfavorable safety profile, arising from off-target effects leading to hemorrhagic complications and tissue damage [112–114]. These detrimental qualities are attributed to systemic activation of plasminogen that promotes fibrinogenolysis, initiation of other enzyme cascades (for example matrix metalloproteinases), a plethora of effects within the central nervous system [115] and stimulation of immune and inflammatory pathways via plasminogen receptors. In addition, unrestrained proteolytic activity of plasmin, can result in degradation of proteins and molecules that function in tissue repair and wound healing, including fibronectin and vascular endothelial growth factor [116–119].

The clinical use of tPA is complicated by the fact that this PA has a half-life of only around 5 minutes. From a patient or clinical perspective this is highly inconvenient, as it requires an initial bolus followed by continuous 1-hour drug infusion. The advent of third generation thrombolytic agents circumvented this issue by engineering tPA variants with significantly extended plasma half-life [120], several of which have been developed and used clinically [121]. Tenecteplase, the most successful of these variants, shares 98.8% amino acid identity with native tPA, but is endowed with an enhanced plasma half-life (~30 min), reduced ability for plasminogen activation to be potentiated by native fibrinogen and increased resistance to inactivation by PAI-1 [122]. Whether tenecteplase can also initiate other pathways know to be triggered by tPA is currently not entirely clear. A recently completed large scale phase III trial comparing tenecteplase with tPA (alteplase) reported tenecteplase to be "non-inferior" to tPA [123]. However, given the practical advantages of tenecteplase (bolus administration), and its efficacy in conditions in which PAI-1 is elevated [124,125], it is likely to become the lytic of choice.

Even with the advent of tenecteplase and the added practicality of its use, thrombolytic efficacy, and overall improvement to patient outcome has not advanced in 30 years. The field is now desperate for more efficacious agents, with novel lytics, adjunctive treatments, and enhanced delivery methods under consideration. One unique approach includes coupling of thrombolytic agents to magnetic colloidal microwheels beads and using magnets to transport the lytic agent to the clot [126,127]. Other delivery platforms for lytic agents including various formulations of nanoparticles and nanocarriers [128] and/or use of microbubbles are emerging. The emerging technology makes clever use of ultrasound [129], shear-activated release [130], or

direct targeting of thrombolytic agents to activated platelets [131]. Indeed, a recent elegant article illustrates the feasibility of direct delivery of plasmin into the clot using thrombin-responsive nanoparticles [132]. These innovative approaches are driving a revolution in future thrombolytics that will hopefully mitigate off-target effects and complications associated with current standard of care.

Studies have now revealed that neutrophil extracellular traps hamper plasminogen activation and interfere with clot degradation [133]. This provides another plausible explanation of the lack of thrombolytic efficacy in various conditions, including AIS, in which advances in thrombectomy have revealed these thrombi to be rich in neutrophil extracellular traps and other cellular factors [134]. An innovative approach to address the influence of DNA has been the use of Dornase, [135,136], a drug licensed for cystic fibrosis that degrades DNA, thereby augmenting tPA-mediated lysis *ex vivo*. Interestingly, a clinical trial is underway evaluating Dornase (DNase-1) as an adjunctive treatment with tenecteplase-mediated thrombolysis in patients with AIS ("Improving Early Reperfusion With Adjuvant Dornase Alfa in Large Vessel Ischemic Stroke" [EXTEND-IA DNase]; NCT05203224). The outcome of these studies is eagerly awaited.

A similar approach has recently been suggested in which attention is focused on degradation of the platelet–von Willebrand factor (VWF) component of the clot [137]. The designed compound "microlyze" targets destruction of the platelet–VWF complex and as an adjunct to tPA augmented dissolution of clots reperfusion in preclinical models of AIS while mitigating bleeding outcomes [138]. Another targeted molecule includes single-chain antibodies to the platelet integrin $\alpha_{IIb}\beta_3$ fused to the protease domain of uPA that have shown promise in mouse ischemic stroke model [139].

4.2 | The other side of the coin: antifibrinolytic agents

While conventional fibrinolysis focus on clot removal, by far the most common clinical intervention of the fibrinolytic system has been to block it, rather than to enhance it. Antifibrinolytic agents were developed in the early 1960s by the Okamotos in Japan [140] to reduce bleeding in women with postpartum hemorrhage. Tranexamic acid (TXA) is the most widely used antifibrinolytic agent and listed by the World Health Organization as an essential medicine. It is used to control bleeding in a variety of scenarios with little risk of thromboembolic complications. A series of mega trials further supports the use of TXA in trauma and in traumatic brain injury [141,142], postpartum hemorrhage [143], and in cardiac surgery [144]. Interestingly, a recent study evaluating the effect of prehospital TXA on functional outcome revealed no benefit of TXA (assessed at 6 months after injury), although mortality was reduced [145].

While the aforementioned studies focus on the hemostatic effects of TXA, the wider appreciation of the role of plasmin in other systems raised the question as to whether TXA might have beneficial consequences outside of its more conventional use to curb bleeding. For example, plasmin has been shown to be important in the production of melanin, and TXA has been shown to reduce melanin production. This has led to the widespread use of TXA for skin whitening purposes and also in patients with melasma [146].

Plasmin has been shown to play a role in promoting immunosuppression and inflammation [147,148] raising the idea that TXA may interfere with the immunosuppressive and proinflammatory roles of plasmin thereby augmenting immunity. In support of this concept TXA administration reduced postoperative infection rates in non-diabetic patients undergoing cardiac surgery [149]. Others have reported that TXA has the capacity to reduce complement activation in some trauma patients [150,151]. The potential of TXA to modulate immunity has prompted a clinical trial to assess the ability of TXA to reduced infection rates in patients undergoing gastrointestinal surgery (NCT04192435). This trial is designed to test the idea that TXA might be used as a non-antibiotic adjunct for infection control and open the doors to repurposing of this drug in a variety of conditions in which the immune system is overactivated.

The plasminogen activating system is linked to promoting permeability of the blood brain barrier [116] and also to the removal of misfolded/aggregated proteins [152] and amyloid [153], as detailed earlier. Interestingly, these processes are also blocked by TXA, illustrating the importance of lysine binding in these contexts and also the utility of TXA in various settings [154]. The mere presence of misfolded proteins and amyloid stimulates the plasminogen activating system, akin to fibrin, linking back to its primordial role within the immune system to foster removal of foreign surfaces. It is plausible that amyloid-mediated plasminogen activation could increase blood brain barrier permeability and promote intracerebral hemorrhage in patients with cerebral amyloid angiopathy (CAA) [155]. This has therefore led to the notion that TXA might attenuate this process by blocking amyloid-driven plasminogen activation and reduce bleeding risk in patients with CAA.

5 | "THE REBIRTH": TARGETING OTHER COMPONENTS OF THE FIBRINOLYTIC PATHWAY

Given the direct contribution of the plasminogen system to many (patho)physiological processes, there has been considerable interest in targeting many of the proteins involved. A number of clinically approved drugs indirectly reduce plasma PAI-1. These include insulin sensitizing agents for management of type 2 diabetes, such as metformin, and angiotensin-converting enzyme inhibitors (used to treat hypertension) [156]. However, these drugs have only been examined in experimental models [157–160], and there is currently limited information from human studies. Various small molecules, peptides, monoclonal antibodies, and antibody fragments have all been used to modulate PAI-1 activity [161–166]. Drugs targeting PAI-1 in the translational phase have produced promising results [167–169]. The small molecule Tiplaxtinin, (PAI-039), prevents venous thrombosis,

angiotensin II-induced atherosclerosis and obesity in a ferric chloride-induced vascular injury model in rats [167]. A unique nanobody to PAI-1 that selectively stabilizes the active form of PAI-1 has potential as a diagnostic or analytical tool [161,169]. Other drugs that elicit pharmacologic inhibition of PAI-1 have reached phase 1 and 2 clinical trials. A small molecule inhibitor, TM5614, is currently being trialed in a single-center, randomized controlled trial for high-risk patients hospitalized with severe COVID-19 and requiring oxygen [170]. Another PAI-1 inhibitor, ACT001, is currently in phase 1 clinical trials for treatment of glioblastoma, the most aggressive primary malignant brain tumor in adults [171,172]. Other PAI-1 antagonists have shown preclinical efficacy [173] and are currently being explored as a therapeutic for fibrosis and fibroproliferative disorders (MDI Therapeutics).

Neutralization of endogenous α_2AP is an alternative approach to augment plasmin activity and drive fibrinolysis. Inhibition of α_2AP promotes spontaneous thrombolysis and enhanced tPA-mediated thrombolysis in an *in vivo* model of pulmonary embolism [174]. A reduction in circulating α_2AP activity in plasma was noted in the study. Considering that reduced plasma activity of α_2AP during thrombolytic therapy is associated with better recanalization of the occluded vessels [175], the decrease in the plasma activity of α_2AP may promote more efficacious thrombolysis. Alternatively, prevention of TAFI activation and direct inhibition of TAFIa are 2 potential pharmacologic strategies for profibrinolytic drugs. Several small molecule inhibitors against TAFIa have been patented by major pharmaceutical companies, however, few have made it beyond the preclinical stage [176]. A potent neutralizing diabody to PAI-1 and TAFIa rapidly enhances clot breakdown in translational models of AIS [168]. Simultaneous inhibition of PAI-1 and TAFIa may improve current thrombolytic therapy; for example, via co-administration with tPA, thereby permitting a lower dose of the thrombolytic to augment its safety profile [168,177].

6 | REMARKS AND REVELATIONS

The fibrinolytic process has been exploited in clinical medicine for decades, either by boosting plasmin levels to remove dangerous clots, or to block the process to preserve clots and mitigate bleeding. These are the conventional uses of the system where the term “Fibrinolysis” is appropriate. However, it is now evident that the plasminogen activating system extends well beyond clotting and bleeding. These areas, including immunity, inflammation, amyloid clearance, and complement, are not entirely “new,” as they have been documented in the literature for decades. However, these non-canonical effects of the plasminogen activating system are hitting the limelight and are now appreciated by the wider community outside of the defined field of plasminogen activation and fibrinolysis. This is propelled by novel insights at the fundamental level on regulation of this cascade and its role in directing various (patho)physiological processes. Furthermore, translational approaches of these ideas have seen the advent of large clinical trials, which are now underway, or planned to explore new opportunities to modulate and target this system for clinical benefits in various conditions from those pertaining to hemostasis and thrombosis, through to immune regulation and importantly development of diseases, such as Alzheimer’s and vascular dementia and CAA. The term “fibrinolysis,” which refers to only one of the substrates of plasmin, does not technically apply to some of these new indications. Taking this into consideration one might deliberate as to whether the term fibrinolysis has indeed hampered research and development in the field. Perhaps the more general term “plasminogen activation” should be adopted to accurately reflect the diverse biological functions of this key primordial pathway.

We welcome the opportunity to present this review on the history and renaissance of “fibrinolysis” for the 20th anniversary edition of *Journal of Thrombosis and Haemostasis*. It is evident that this system is not as simplistic as many have considered, and we look forward to seeing how this landscape changes over the next 20 years.

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AUTHOR CONTRIBUTIONS

N.J.M. and R.L.M. conceived, wrote, and edited the article.

DECLARATION OF COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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