# THE SERPIN FAMILY IN THE CARDIOVASCULAR SYSTEM

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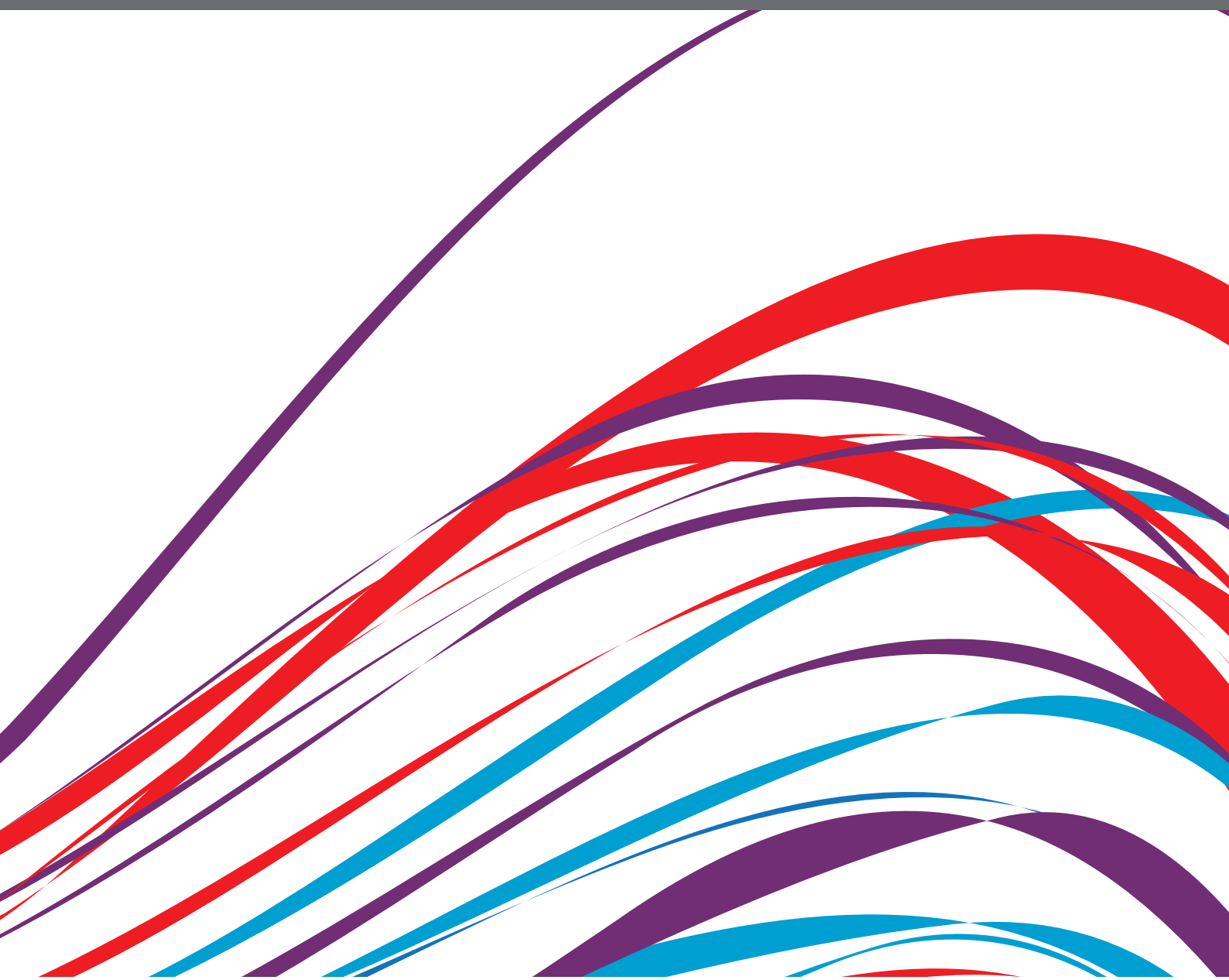

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# THE SERPIN FAMILY IN THE CARDIOVASCULAR SYSTEM

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EDITORIAL
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# Editorial: The Serpin Family in the Cardiovascular System

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**Keywords: serpins, thrombosis, fibrinolysis, inflammation, therapy**

## Editorial on the Research Topic

## Editorial: The Serpin Family in the Cardiovascular System

In this special Research Topic, five original research articles and eight reviews are published under the title of ≪ Serpins in the cardiovascular system ≫. This unique Research Topic on serpins (SERine Protease INhibitors) provides an overview of the recent advances on the role of anticoagulant and antifibrinolytic serpins, as well as viral serpins or non-inhibitory serpins, in the cardiovascular system. There is a wealth of knowledge to be discovered in these intricate regulators, the serpins, that represent up to 2–10% of circulating blood proteins and control many physiologic processes (1–4) (**Figure 1**).

A mini review by Shu et al. shares the recent resolution of structural studies of angiotensinogen, the unique non-inhibitory serpin evoked in this Research Topic. The authors describe the crystallographic studies explaining the interaction of human angiotensinogen with human renin and hence the mechanism of angiotensin release.

The two antifibrinolytic serpins, Plasminogen activator inhibitor type-1 (PAI-1) and Alpha2-Antiplasmin (α2-AP), were thoroughly described in this topic. PAI-1 was described in two complementary reviews. One was written by Sillen and Declerck who provide a precise review of its structure/function and describe diverse approaches for the development of PAI-1 inhibitors. The other study, written by Morrow et al., details the role of PAI-1 in a variety of pathophysiological conditions, in particular in thrombo-inflammation and in the metabolic syndrome. An overview of α2-AP in thrombosis and cardiovascular diseases was reported by Singh et al. The authors describe how targeting this fast-reacting inhibitor of plasmin may provide potential therapeutic opportunities. Protease nexin-1 (PN-1), another antifibrinolytic serpin described in this Research Topic, was detailed by Madjene et al. In contrast to the two previous serpins depicted above, PN-1 displays both anticoagulant and antifibrinolytic properties, which makes this serpin special. The authors summarized the many functions attributed to this enigmatic serpin in health and cardiovascular diseases.

The potential use of serpins as therapeutic targets for bleeding or thrombotic disorders was reviewed by Bianchini et al. who summarize the drawbacks and advantages of targeting specific serpins involved in the finely tuned balance between procoagulant and anticoagulant systems.

The use of serpins for therapeutic is a recent growing area of clinical investigation. Such an idea of therapeutic serpins was assessed by Maas and De Maat who describe the different approaches of engineering serpins to optimize their potential therapeutic relevance. A complementary review on serpin roles in fibrinolysis and inflammation and their potential therapeutic applications was written by Yaron et al. It provides an overview of a highly potent class of serpin virus-derived biologics. One such serpin, Serp-1, is a 55 kDa secreted myxomavirus serpin that, like PN-1, can

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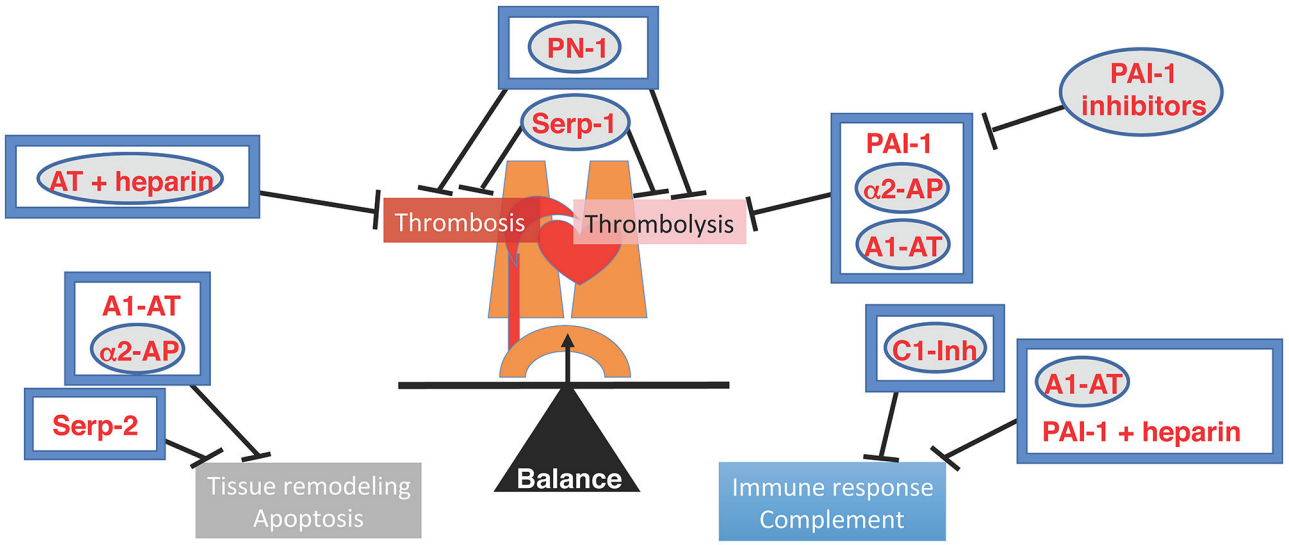


**FIGURE 1 |** Illustration of the Cardiovascular system and the balancing of Serine proteases and SERPIN interactions. Native regulatory pathways are in boxes. Therapeutic serpins are in circles. Serpins reviewed in this special issue have a gray backdrop. AT, Antithrombin; A1-AT, alpha1-antitrypsin; $\alpha 2$ AP, alpha2-antiplasmin; C1-Inh, C1-inhibitor; PAI-1, plasminogen activator type-1; PN-1, protease nexin-1.

bind and inhibit proteases in both the thrombotic and thrombolytic cascades targeting sites of serine protease activation (1, 5–7) Guo et al. This serpin has been tested in a wide range of animal models of inflammatory disorders, and it also appears successful in a small phase-2 clinical trial in unstable coronary patients receiving stent implants. This work, together with mammalian serpins already used in clinic for genetic disorders such as C1-inhibitor (C1-inh) (8) and α1-antitrypsin (A1-AT), provides a basis for using serpins to target dysfunctional protease pathways. The authors discuss the potential use of serpins for treating COVID-19 since a non-negligible number of serine proteases such as thrombin, urokinase-type plasminogen activator (uPA) and complement, the main targets of serpins, are critically involved in SARS-Cov-2 infection. Indeed, this viral serpin, Serp-1, as well as antithrombin (AT), A1-AT, and C1-inh are being discussed and investigated as potential therapeutics for use in acute respiratory distress syndrome (ARDS), cytokine storm, and microthrombotic coagulopathy in COVID-19 (8–10). As serpins are inhibitors, these naturally evolved regulatory inhibitors of proteases have outstanding potential as therapeutics in severe viral infections complicated by aggressive inflammatory and coagulopathic disorders where treatment remains very limited.

In this topic, we have also three excellent examples of how genetic variants of serpins help to understand the impact of serine protease inhibitors in cardiovascular diseases. One of these variants, named the AT variant p.Leu131Phe (AT Budapest 3), is classified among the type II Heparin-binding site (HBS) deficiencies as it does not severely reduce the secretion to the plasma but impairs the activation of AT by heparin (11). In this topic, Natae et al. have evaluated this variant in the Hungarian Roma population, where the p.Leu131Phe variant has a founder effect and reaches a high frequency (1%), aiming to find genetic-environmental interactions that might explain the increased susceptibility to venous thrombosis of this population. This study finds five positive and significant genetic and environmental interactions that play a role in venous thrombosis and should be considered for the assessment of thrombotic disease susceptibility. This study was fully complemented by the manuscript of Bereczky et al. who have done a brilliant archaeogenetic analysis that revealed the age and evolution of the p.Leu131Phe variant, a 400-year-old founder mutation. The bonus of this study was the analysis of the role of this and other type II HBS variants on the risk of arterial thrombosis. Interestingly, the study of Bereczky et al., found consistent evidence to consider AT type II HBS deficiencies as a risk factor not only for venous but also for arterial thromboembolism, especially in selected populations as young patients without advanced atherosclerosis, data supported by other studies (12, 13). This finding encourages the diagnosis of these genetic variants, particularly in populations where they could be frequent (e.g., in Hungary, Spain, and Finland), and would be useful for the management of carriers, as an early diagnosis might warned carriers to avoid any modifiable cardiovascular risk factors. Finally, the study of Hamada et al. is another example of the therapeutic expectations of "a la carte" recombinant serpins with new functions as mentioned by Maas and De Maat. Here, the authors explore A1-AT, an elastase inhibitor whose deficiency is involved in pulmonary emphysema, to create a strong and specific FXIa inhibitor that might be used as a new anticoagulant. By using elegant methods, Hamada et al. have engineered a recombinant A1-AT variant AAT-RC-2 as a specific FXIa inhibitor whose potential clinical benefit must be evaluated in further studies.

We believe that this original topic on serpins will allow readers to appreciate the extraordinary contribution of numerous proteins of this superfamily in the cardiovascular system and show how attractive targets they could be for drug discovery.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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# Targeting PAI-1 in Cardiovascular Disease: Structural Insights Into PAI-1 Functionality and Inhibition

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Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) superfamily with antiprotease activity, is the main physiological inhibitor of tissue-type (tPA) and urokinase-type (uPA) plasminogen activators (PAs). Apart from being crucially involved in fibrinolysis and wound healing, PAI-1 plays a pivotal role in various acute and chronic pathophysiological processes, including cardiovascular disease, tissue fibrosis, cancer, and age-related diseases. In the prospect of treating the broad range of PAI-1-related pathologies, many efforts have been devoted to developing PAI-1 inhibitors. The use of these inhibitors, including low molecular weight molecules, peptides, antibodies, and antibody fragments, in various animal disease models has provided ample evidence of their beneficial effect *in vivo* and moved forward some of these inhibitors in clinical trials. However, none of these inhibitors is currently approved for therapeutic use in humans, mainly due to selectivity and toxicity issues. Furthermore, the conformational plasticity of PAI-1, which is unique among serpins, poses a real challenge in the identification and development of PAI-1 inhibitors. This review will provide an overview of the structural insights into PAI-1 functionality and modulation thereof and will highlight diverse approaches to inhibit PAI-1 activity.

**Keywords: plasminogen activator inhibitor 1 (PAI-1), PAI-1 inhibitors, serpin (serine proteinase inhibitor), fibrinolyisis, cardiovascular disease**

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## INTRODUCTION

Hemostasis is an essential physiological process that preserves the integrity of the vascular system and secures sufficient blood flow throughout the circulatory system. The balance between clot formation (coagulation) and clot dissolution (fibrinolysis) is very tightly regulated in a spatiotemporal manner and requires a dynamic interplay with other systems involved, such as the vascular system and platelets (1). Briefly, upon vascular injury, a sequence of cellular and molecular events is triggered that can be characterized by three distinct but overlapping phases of initiation, amplification, and propagation (coagulation) (2, 3). The end result of the coagulation cascade is the conversion of fibrinogen, a soluble plasma protein, into an insoluble fibrin meshwork that constitutes blood clots. To limit the coagulatory response to the site of injury and prevent vascular occlusion, the prothrombotic response is balanced by the fibrinolytic system. Fibrinolysis revolves around the enzymatic activation of plasminogen into the key fibrinolytic enzyme plasmin through tissue-type (tPA) and urokinase-type (uPA) plasminogen activators (PAs) (4). Tissue-type PA is produced by vascular endothelial cells and released in response to thrombin and venous occlusion.

It is primarily involved in the activation of plasminogen that is required for fibrin dissolution in the circulation (5, 6). In contrast, uPA is expressed by a variety of cells, including renal epithelial cells, inflammatory cells, and cancer cells. It is considered more important in pericellular proteolysis during tissue remodeling and cell migration through the activation of cell-bound plasminogen (7, 8). Plasminogen activator inhibitor-1, a member of the serpin superfamily, is a key component of the plasminogen/plasmin system as it is the primary inhibitor of tPA and uPA.

## SYNTHESIS, DISTRIBUTION, AND BIOCHEMICAL PROPERTIES OF PAI-1

PAI-1 was first detected almost four decades ago as an inhibitor of the fibrinolytic system associated with cultured bovine endothelial cells (9). Not much later, several research groups demonstrated its presence in human plasma (10–12), as well as various other cell types throughout the body, including the spleen, liver, kidney, lung, and adipocytes, albeit at different concentrations and with variable functional activities (13, 14). Furthermore, PAI-1 expression and release are strongly regulated by various factors, including growth factors (e.g., transforming growth factor-β, epidermal growth factor), inflammatory cytokines (e.g., tumor necrosis factor-α and interleukin-1β), hormones (e.g., insulin, glucocorticoid, and angiotensin II), glucose, and endotoxin of Gram-negative bacteria (15, 16). In the blood, PAI-1 occurs in two distinct pools, free in plasma or retained in platelets (17). Plasma PAI-1 circulates mainly in the active conformation at relatively low levels (5–50 ng/mL) (17) showing a large interpersonal variability caused by factors including race/ethnicity (18), gender (19), and body composition (20). In contrast, platelet PAI-1 serves as the main blood pool of PAI-1 with concentrations up to ∼300 ng/mL (17). Initially, several studies showed that platelet-derived PAI-1 is less active compared to plasma PAI-1, considered being only 2–5% functionally active (21, 22). However, the pre-analytical methods used in these studies, such as sonication or freeze-thawing, may have reduced the activity of platelet-derived PAI-1 since more recent studies were able to demonstrate a substantially higher activity for PAI-1 (23, 24). Even though platelets do not contain a nucleus, they retain the ability for *de novo* PAI-1 synthesis through translationally active PAI-1 messenger RNA, of which the synthesis rate is importantly increased by platelet activation (23). As a result, at least 50% of platelet-derived PAI-1 was shown to be in the biologically active form and capable of forming an irreversible PAI-1/tPA complex. Importantly, platelet-derived PAI-1 has a substantial role in conferring thrombolysis resistance to the clot through local accumulation caused by its release from activated platelets and subsequent partial retention of functional PAI-1 on the platelet membrane (24–26).

The 12.3 kb human PAI-1 gene (*SERPINE1*) was mapped to chromosome 7 (7q21.3-q22) and contains nine exons and eight introns (27, 28). The exons encode for a 23 amino acid long signal peptide and the 379 amino acid long mature PAI-1 protein (29). Additionally, a mature form comprising 381 amino acids, including two extra amino-terminal (N-terminal) residues, has been identified and is most likely the result of cleavage at an alternative cleavage site for signal peptidases (30). Native PAI-1 is a 45-kDa single-chain glycoprotein that lacks cysteines. Based on the amino acid sequence, three potential sites for N-linked glycosylation were identified of which Asn209 and Asn165 display a heterogeneous tissue-type specific glycosylation pattern while Asn329 is not utilized *in vivo* (31, 32). Even though glycosylation often has a critical role in determining protein structure, function, and stability for many proteins, glycosylation of PAI-1 is not a prerequisite for its ability to inactivate PAs or to interact with its cofactor vitronectin (33). In contrast, several studies demonstrated that glycosylation can have a tremendous effect on the neutralizing activity of PAI-1 inhibitors and therefore emphasizes the significance of the source of PAI-1 used in the development of PAI-1 inhibitors (31, 34, 35).

## STRUCTURAL AND FUNCTIONAL PROPERTIES

### PAI-1 Is an Inhibitory Serpin

The serpin superfamily comprises over 1,500 inhibitory and non-inhibitory proteins that are broadly distributed among several species, including humans, animals, viruses, bacteria, and plants (36). Despite their profound structural similarity, serpins are functionally very diverse. Whereas, their biological function often requires inhibition of proteases, some non-inhibitory serpins function as, for example, hormone transporters (37), tumor repressors (38), or molecular chaperones (39). Based on their evolutionary relatedness, eukaryotic serpins have been divided into 16 clades (termed A-P), with clades A-I representing human serpins. PAI-1 is categorized as a clade E serpin and is considered to be the main physiological inhibitor of tPA and uPA. However, other serpins with inhibitory activity toward PAs have been identified and include plasminogen activator inhibitor-2 (clade B), protease nexin I (clade E), and neuroserpin (clade I) (40).

PAI-1 displays the well-conserved structure of serpins (**Figure 1**), characterized by three β-sheets [termed A–C, with strand numbers indicated as s(#)A, s(#)B, and s(#)C] and nine α-helices (termed hA-hI) (42, 43). As the primary inhibitor of PAs, PAI-1 rapidly inactivates both tPA and uPA with second-order rate constants between $10^6$ and $10^7$ $M^{-1}$ $s^{-1}$ following the basic mechanism applied to all serpin/serine proteinase reactions (43, 44). The key to this reaction is that the PA recognizes PAI-1 as a (pseudo)substrate. Therefore, PAI-1 carries a flexible surface-exposed reactive center loop (RCL) of 26 residues long (331-SGTVASSSTAVIVSA*RM*APEEIIMDR-356, designated P16-P10′) that presents a substrate-mimicking peptide sequence (Arg346-Met347, designated as P1-P1′). PAI-1 is synthesized in a metastable active conformation, i.e., with the RCL protruding from the top of the molecule, which is essential for the kinetic trapping of PAs in a thermodynamically favorable complex. Several regions–the hinge region (P15-P9 of the RCL), the breach region (the top of β-sheet A), the shutter domain (the central part

of s3A and s5A and the N-terminal part of hB), the gate region (s3C and s4C), and the flexible joint region (hD, hE, hF, and s1A)–have been shown to be important in controlling and modulating PAI-1 functionality through conformational changes (**Figure 1**).

## Mechanism of Protease Inhibition

The PAI-1/PA reaction is initiated by the formation of a non-covalent 1:1 stoichiometric Michaelis complex (EI) between PAI-1 (inhibitor, I) and the PA (enzyme, E) (**Figure 2**). Initially, PAs bind to PAI-1 through several exosite interactions, defined as secondary interactions between regions outside of the PA active site and the PAI-1 P1-P1′ reactive center (47, 53). The nature of this Michaelis complex is now well-understood from the X-ray structure determination of PAI-1 in complex with active-site mutants of tPA (tPA-S478A) and uPA (uPA-S195A) (47, 53). Through flexible loops on their surface, PAs contact several exosites adjacent to the RCL to facilitate the initial docking step and, in addition to P1 and nearby residues in the RCL, confer proteinase specificity. By forming tight interactions, exosites stabilize the Michaelis complex and lock the PA into a particular orientation to warrant optimal positioning of the P1 residue in the active site of the PA. Furthermore, these additional interactions slow down the dissociation of the PA from its initial docking site, allowing the PA active site serine to attack the P1-P1′ bond to form a tetrahedral intermediate with PAI-1 (54). Successful cleavage of this bond yields the acyl-enzyme intermediate (E∼I) in which the PA is covalently linked to the main chain carbonyl of the P1 residue in PAI-1. Following a branched pathway mechanism, the PAI-1/PA reaction is directed either into the inhibitory or into the substrate pathway.

In the *inhibitory pathway* (**Figure 2**), the formation of the acyl-enzyme intermediate is coupled to a rapid and full insertion of the N-terminal part of the RCL (P16-P1) as strand 4 into the central β-sheet A (s4A) (54). This major conformational change coincides with a 70 Å translocation of the bound PA to the opposite side of the PAI-1 molecule. There, a large part of the PA, including the active site, is deformed by compression against the body of PAI-1. As a result, hydrolysis of the acyl-enzyme intermediate is prevented and the PA remains trapped as a stable PAI-1/PA inhibitory complex (E-I) (49, 55). This mechanism of inhibition was demonstrated by the crystallographic structure of the $\alpha_1$-antitrypsin/trypsin complex (49), which is in line with the results from studies that investigated serpin exosite distortion by using nuclear magnetic resonance (56, 57) and circular dichroism (58) studies. In this serpin-protease complex (49), trypsin shows a high degree of conformational disorder as compared to its native form, i.e., a loss of structure for ∼37% of the protease. Furthermore, the active site of trypsin is disrupted as Ser195 of the catalytic triad is moved away from its catalytic partners. Several regions in PAI-1 are crucial for the orchestration of loop insertion and are furthermore involved in the energetical coupling of this favorable conformational change to the energy-demanding process of PA distortion (**Figure 1**) (43). Upon cleavage of the P1-P1′ bond, the PA dissociates from its initial docking site on PAI-1 while releasing the distal P′ side of the cleaved RCL from the PA active site cleft. Simultaneously, the breach region at the top and the shutter region near the center of β-sheet A open up to accommodate the RCL as s4A. The hinge region that contains a conserved series of small hydrophobic residues (P15-P9) initially inserts into the breach region and is a prerequisite for rapid loop insertion. Whereas, the surface-exposed RCL only makes a few contacts with the serpin body, it now becomes an integral part of the central β-sheet A. Further insertion of the RCL, however, is obstructed by a steric clash with hF that is located across β-sheet A. Experimental data favor the hypothesis that hF plays an essential role in PA inhibition by (I) being actively displaced until the loop is fully inserted and the PA has passed to the very end of β-sheet A and (II) by temporarily storing the energy derived from loop insertion in order to distort the PA upon return of hF to its original position, ultimately leading to the formation of the irreversible inhibitory complex (59). Through basic residues in hD and hE in the flexible joint region of PAI-1, these PAI-1/PA complexes bind certain receptors of the low-density lipoprotein receptor (LDLR) family, including low-density lipoprotein receptor-related protein-1 (LRP1), leading to endocytosis and degradation of the complex (60, 61).

In the *substrate pathway* (**Figure 2**), the acyl-enzyme intermediate is hydrolyzed prior to PA distortion, resulting in the release of regenerated PA (E) from cleaved RCL-inserted PAI-1 (I*) (48, 62). This substrate behavior has been associated with the pre-existence of a conformational distinct substrate-like subset of PAI-1 (63, 64), or results from a change in the kinetic parameters that define the partitioning between both branches of the PAI-1/PA reaction (65, 66).

## Factors Influencing the PAI-1/PA Reaction

Several factors have been established that determine target proteinase specificity or influence the partitioning between the inhibitory and substrate branch of the PAI-1/PA reaction pathway. The first region to determine target specificity of PAI-1, and serpins in general, consists of residue at the P1 position and the immediately adjacent residues in the RCL. Indeed, by replacing residues P3-P3′ of the PAI-1 RCL with the corresponding residues of another serpin antithrombin III, this PAI-1 mutant acquired thrombin inhibitory properties (67). Interestingly, vitronectin was shown to alter PAI-1 specificity by also enhancing PAI-1 reactivity toward thrombin in a dose-dependent manner (66, 67). Importantly, studies using PAI-1/serpin chimeras, in which the RCL was replaced with that of other serpins, showed that all chimeras were still effective inhibitors of both PAs, and thus strongly suggested a major contribution of regions outside of the RCL to differences in specificity (68). Based on the crystal structure of the PAI-1/PA Michaelis complexes, interactions between several complementary electrostatic surfaces on PAI-1 and the PAs, referred to as exosites, have been identified (47, 53). One particular region of PAs has been shown to make strong and extensive interactions with PAI-1. The positively charged 37-loop of PAs contacts (I) a negatively charged patch comprising residues in s1B, s2B, and the s3B-hG loop, and (II) the P4′ (Glu350) and P2′ (Ala348) residues in the RCL. This 37-loop/exosite interaction has been proven necessary to ensure the rapid and high-affinity association between PAI-1 and PAs in

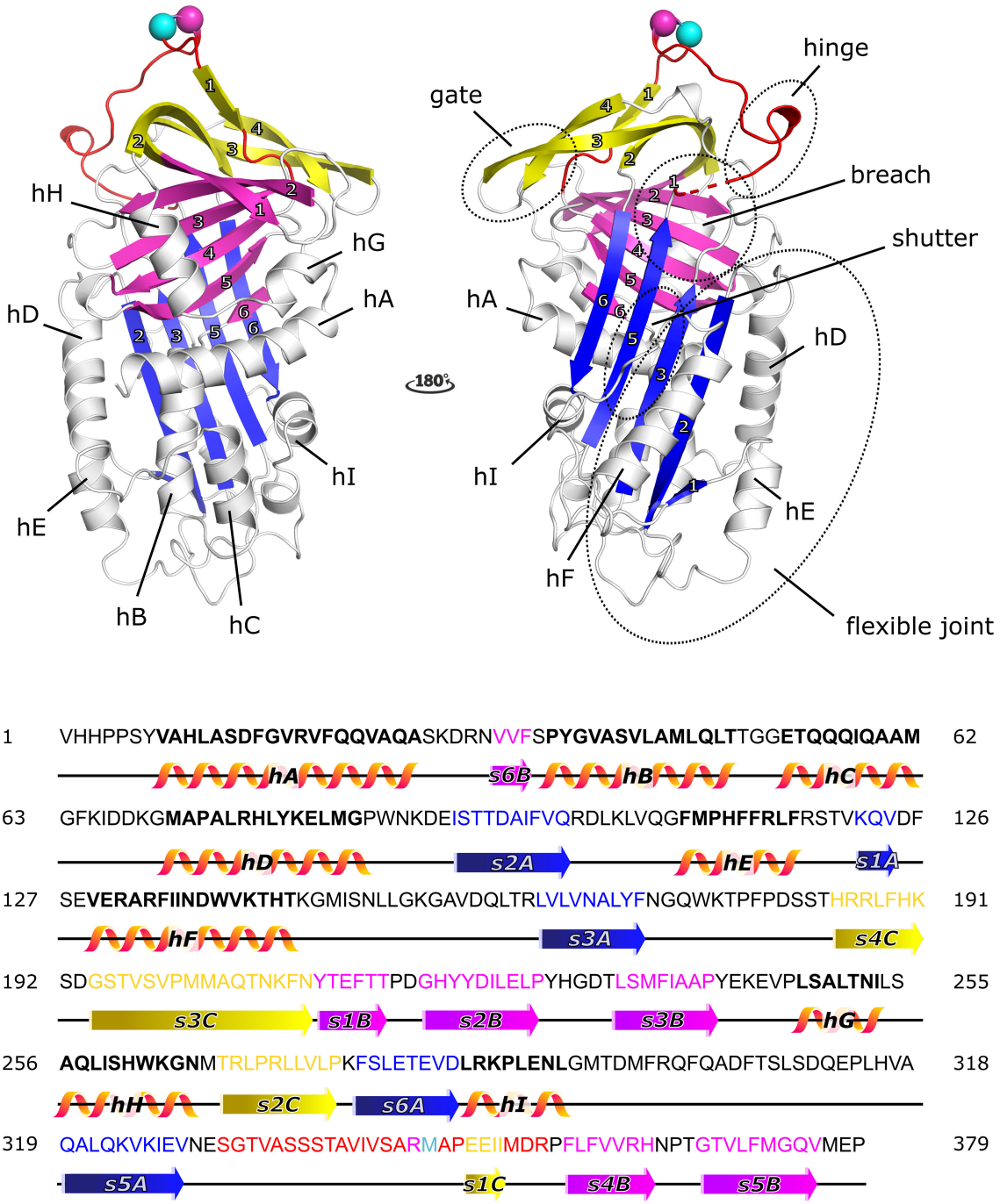


**FIGURE 1 |** Cartoon representation of the crystal structure of the active form of plasminogen activator inhibitor-1 (PAI-1) [PDB ID 6ZRV (41)] and the amino acid sequence of native PAI-1. PAI-1 shows the evolutionarily conserved topology of serpins, consisting of three β-sheets (A–C) and nine α-helices (hA-hI). β-sheet A, B, and C are shown in blue, magenta, and yellow, respectively, with numbers labeling the individual strands. The α-helices are indicated in the figure. The reactive center loop (RCL) of PAI-1 connects strand 5 of β-sheet A (s5A) to strand 4 of β-sheet B (s4B) and comprises strand 1 of β-sheet C (s1C). The RCL is shown in red, with the reactive center Arg346 (P1) and Met347 (P1′) represented by a magenta and cyan sphere, respectively. Other important domains that control and modulate PAI-1 conformational changes (the gate, hinge, breach, shutter, and flexible joint regions) are also indicated. Residues missing in the crystal structure are indicated by a dashed line. The amino acid sequence is presented and secondary structures (α-helices and β-strands) are indicated in the colors corresponding to the cartoon representation.

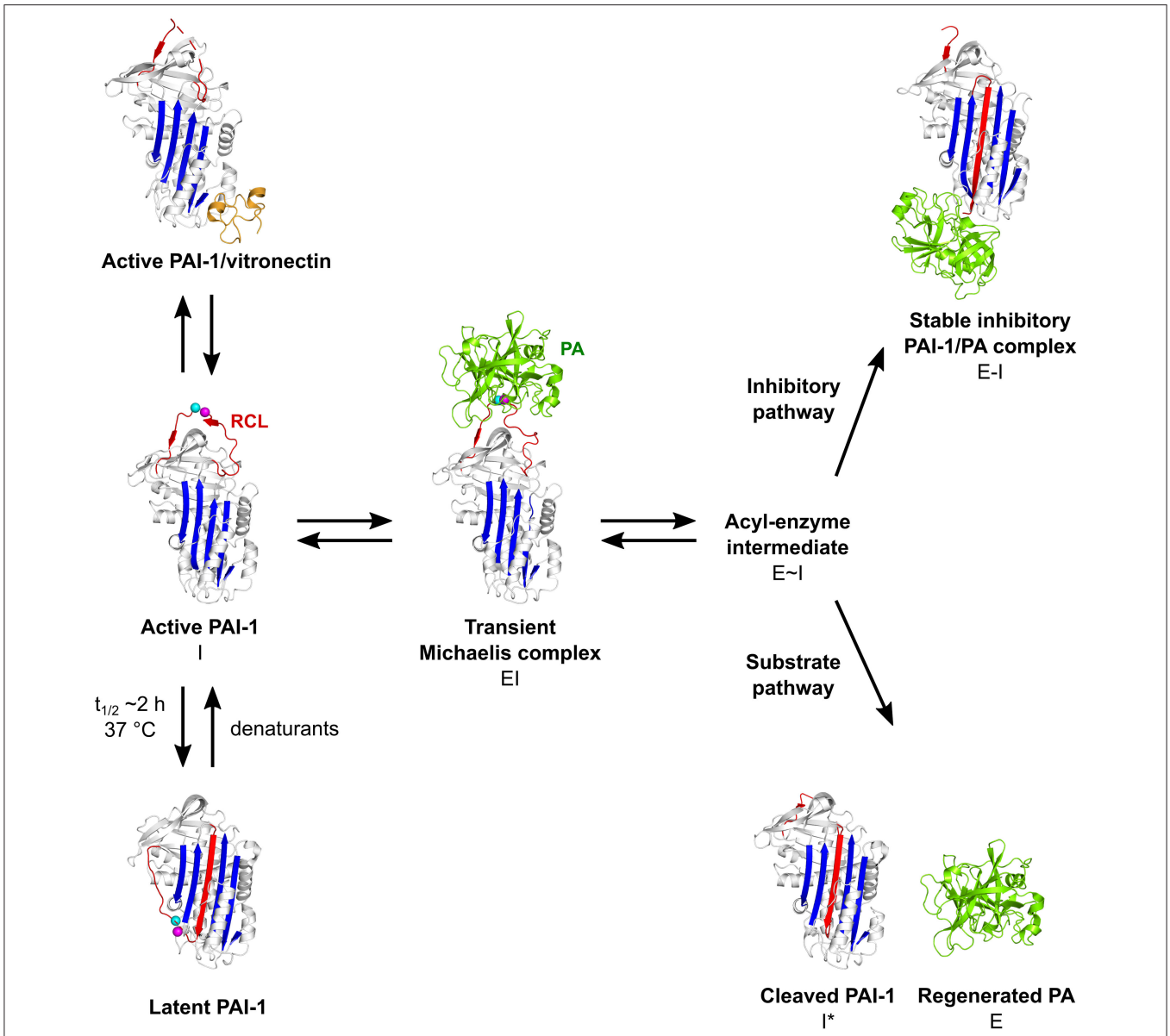


**FIGURE 2 |** Schematic overview of the PAI-1 (I) conformations as well as its interactions with plasminogen activators (PAs, E) and cofactor vitronectin. Following the formation of a non-covalent PAI-1/PA Michaelis complex (EI), the P1-P1′ bond is cleaved to generate an acyl-enzyme intermediate (E~I). From here on, the reaction proceeds through a branched pathway, resulting in either the formation of an irreversible inhibitory complex (E-I) or the generation of cleaved PAI-1 (I*) due to the hydrolysis of the acyl-enzyme intermediate. PAI-1 is shown in white; the central β-sheet A of the PAI-1 molecule in blue; the flexible reactive center loop (RCL) in red, and Arg346 and Met347 (P1-P1′) of the reactive center are indicated by magenta and cyan spheres, respectively. The PA is shown in green. Vitronectin is shown in orange. PDB structures 1DVN (45), 1DB2 (46), 5BRR (47), 3EOX (48), 1EZX (49), 1H4W (50), and 1OC0 (51) were used to generate this figure. Figure adapted from Sillen et al. (52).

studies using 37-loop mutants or antibodies specifically binding to this region on the PA molecule (69–71). Furthermore, the residues in the 37-loop of tPA that are responsible for the direct interaction with PAI-1 are less charged in the 37-loop of uPA, resulting in a stronger exosite interaction of PAI-1 to tPA (72). These stronger exosite interactions result in a twice as large contact area of tPA with PAI-1 when compared to uPA, and provide a rationale for the difference in second-order inhibitory rate constants between the two PAs (47, 53).

As mentioned, the importance of several regions within the PAI-1 molecule for RCL insertion as well as for its interaction with the target PAs has been extensively studied. Ample evidence suggested that the nature of the amino acids and the flexibility of these segments are crucial for PAI-1 functionality and that changes made within these regions result in an altered reaction mechanism. Mutations within the hinge region of the RCL (residues P14-P8) altered the specificity toward tPA or uPA and moreover caused PAI-1 to behave predominantly as a substrate

toward PAs (65, 73, 74). Stable substrate behavior without any detectable inhibitory activity was also conferred upon a PAI-1 deletion mutant lacking hF and the hF-s3A loop (75). Since both regions are involved in RCL insertion, i.e., the hinge region is initially inserted and hF coordinates the final insertion step, mutations in these regions may change the initial conformation of PAI-1 or impair the kinetics of RCL insertion. Therefore, these changes may ultimately result in the hydrolysis of the acyl-enzyme intermediate (76). Apart from these PAI-1 mutants, the behavior of PAI-1 as an inhibitor or as a substrate has also been reported to be influenced by external conditions, such as low temperature and non-ionic detergents, and ligands (77–79).

## Functional Stability of PAI-1

### PAI-1 Spontaneously Converts Into an Unreactive Latent Form

Unlike other serpins, PAI-1 has the unique ability to spontaneously convert into a thermodynamically stable latent form with a half-life of ∼2 h at 37 °C *in vitro*. This active-to-latent transition occurs by slowly self-inserting the N-terminal part of the RCL into the core of the protein, thereby making the P1-P1′ bond inaccessible for PAs (80). Spontaneous latency transition is rather exceptional and is reported in only a few other serpins (81–83). Several lines of evidence indicate that latency transition in PAI-1 is evolutionarily conserved (84, 85). Therefore, it suggests an important role in auto-regulation of PAI-1 activity to reduce the risk of thrombosis due to the otherwise prolonged antifibrinolytic action of PAI-1. Based on the structures of active and latent PAI-1, the dynamical mechanisms involved in the active-to-latent transition were simulated using a computational approach, and could later be supported by experimental evidence (72, 86, 87). In a concerted manner, strand 1 (s1C) is peeled away from β-sheet C and allows the RCL to move around the gate region while it partially inserts up to P11 (Ser336) in the central β-sheet A that opened up at the breach and shutter region to form s4A. To reach this prelatent state, for which experimental evidence indicates that it co-exists with active PAI-1 in solution (88–91), a change in the bend in hA is required. After being held for an extended period in the prelatent form, full insertion of the final P6-P4 residues of the RCL is blocked by steric clashes between the RCL and the hF-s3A loop that overlies β-sheet A, posing a high-energy barrier. Similar to the conformational changes required for PA translocation, the favorable energy that is released upon partial insertion of the RCL is temporarily stored by an outward movement or unfolding of hF to enable full RCL insertion. Finally, hF returns to its native position across β-sheet A and irreversibly locks PAI-1 in its unreactive latent state. Whereas, during the inhibitory reaction with PAs energy can be recovered to distort the active site of the PA, latency transition is an energetically silent process. Therefore, it has been hypothesized that the energy gain from the favorable insertion of the RCL is used to extract s1C from β-sheet C and to position it alongside the PAI-1 molecule (92). Even though this transition is generally considered to be irreversible, limited reactivation by an unknown mechanism may occur *in vivo* (93). *In vitro*, the inhibitory properties can be restored by treating latent PAI-1 with denaturants followed by refolding (94).

### *In vivo* Stabilization

*In vivo*, the active form of PAI-1 is stabilized at least 2-fold by the high-affinity association ($K_D \sim 0.1–1$ nM) with the glycoprotein vitronectin that is abundant in plasma and the extracellular matrix (**Figure 2**) (95–99). The interaction between PAI-1 and vitronectin has been extensively characterized by mutagenesis and competition experiments using monoclonal antibodies, PAI-1/PAI-2 chimeras, and (synthetic) peptides (100–108). Based on these results, the N-terminal somatomedin B (SMB) domain within vitronectin and the flexible joint region, defined by hE, hF, and s1A, within PAI-1 were identified as the primary regions to engage in the interaction. Later, the crystal structure of PAI-1 in complex with the SMB domain of vitronectin (PDB ID 1OC0) provided additional details on the interaction interface, restricting their respective binding sites to the central region of the SMB domain (residues 10–30) and residues in the hE-s2A loop (Arg101), in hE (Pro111 and Phe114), hF (Asp138, Ile135, and Trp139) and s1A (Thr120, Lys122, Gln123, Val124, and Asp125) of PAI-1 (51). Through allosteric modulation of several regions remote from the SMB binding site, vitronectin causes a strong and widespread stabilization of the lower half of the PAI-1 molecule, including hB, hC, hD, hI, and the hI-s5A loop, and induces conformational changes in the RCL without compromising the ability of PAI-1 to associate with PAs (99, 109, 110). By reducing the structural flexibility, binding of vitronectin interferes with the sliding movement that is required to open up the shutter region, and consequently decreases the rate of RCL insertion that ultimately slows down the transition to latent PAI-1. Alternatively, expansion of β-sheet A due to loop insertion during latency transition or during the interaction with PAs results in the dissociation of vitronectin from inactive PAI-1 (95, 111, 112). Apart from the primary high-affinity PAI-1 binding site in the SMB domain, there is experimental evidence for additional PAI-1 binding sites in vitronectin. These sites, comprising a cluster of basic amino acids (residues 348–370 of vitronectin) in the C-terminal region of vitronectin (111–115) as well as the region connecting the SMB domain to the remainder of the vitronectin molecule (residues 111–121 of vitronectin) (116), have been shown to bind PAI-1 with a lower affinity and promote the assembly of higher-order PAI-1/vitronectin complexes (114, 115, 117).

One of the major acute-phase proteins, $\alpha_1$-acid glycoprotein, has also been shown to bind and stabilize the active form of PAI-1. Extensive binding studies allowed to identify a binding region that is distinct from that of vitronectin. This $\alpha_1$-acid glycoprotein binding region resides in the hI-s5A loop, comprising residues Arg300–Asp305 located at the bottom of PAI-1 β-sheet A (118). Even though this interaction occurs at a slower rate and is less stable as compared to the interaction with vitronectin, it might contribute to the PAI-1-mediated effects during inflammation or acute phase reactions (119).

### PAI-1 Mutants With Increased Stability

Since its discovery, a vast amount of PAI-1 variants has been generated by both site-directed and random mutagenesis (120). These mutants have been employed in order to gain insights into the structure/function relationship in PAI-1, to identify

regions that are important for its biological interactions, and to investigate its pleiotropic functions in various pathological processes. Due to its conformational flexibility, structural studies have benefited in particular from the generation of PAI-1 variants of which the functionally active form is stabilized. While single substitutions cause only a moderate stabilization of PAI-1, the combination of multiple mutations often results in a markedly enhanced stability with half-lives up to 450 h (85). Alternatively, the active conformation can also be maintained by introducing Cys-residues to crosslink flexible regions in PAI-1 that are crucially involved in latency transition (121, 122). This way, PAI-1 variants have been generated in which hD of the flexible joint region is connected to the N-terminal part of hA (engineered disulfide bridge between Val8Cys and Ala74Cys), in which s3A and s5A in the shutter region are covalently linked (Gln169Cys–Gly324Cys), in which the N-terminal part of the RCL is connected to the top of s3A in the breach region (Gln174Cys–Gly332Cys), or in which a combination is used. However, the introduction of only one single disulfide bond at the breach region is sufficient to most effectively preclude latency transition (very long half-life PAI-1, $t_{1/2} > 700$ h) without affecting its structure (122).

As mentioned, several crystal structures of PAI-1 in its alternative conformations (active, latent, and cleaved PAI-1) or of PAI-1 in complex with biological ligands have been determined by employing these stabilized active mutants (**Figure 2** and **Table 1**). The first stable mutants to be successfully crystallized in the active conformation were the quadruple mutant PAI-1-N150H-K154T-Q319L-M354I, commonly referred to as PAI-1 14-1B ($t_{1/2} \sim 145$ h) (123, 133) and a variant harboring a fifth mutation, PAI-1-N150H-K154T-Q301P-Q319L-M354I, referred to as PAI-1-stab (46, 134). Later, the structure of active PAI-1-W175F ($t_{1/2} \sim 7$ h) was resolved as well (124). Apart from its prolonged half-life, PAI-1-W175F behaves similarly to wild-type PAI-1 and is therefore a more valid representative of wild-type PAI-1. Comparison of the available PAI-1 14-1B and PAI-1-W175F structures revealed numerous structural differences, with the most prominent one located in the region containing hF and the hF-s3A loop. Three of the mutations in PAI-1 14-1B and PAI-1-stab are clustered in and below the hF-s3A loop (Asn150His, Lys154Thr, and Gln319Leu) and induce a $3_{10}$-like helix covering residues 151–157 that connects hF to the underlying β-sheet A through a hydrogen-bonding network. As a consequence, the energy barrier for hF displacement during the final step in RCL insertion is raised, explaining both the stabilization of the active conformation as well as the increased substrate behavior upon interaction with PAs that is observed for PAI-1 14-1B and PAI-1-stab. In contrast, the stabilization caused by the single amino acid substitution of the conserved tryptophan in PAI-1-W175F appears to be the result of local effects in the breach region that restrict initial loop insertion (124, 135).

## External Conditions Affecting PAI-1 Stability

Apart from being stabilized through interactions with its physiological ligands, several external conditions have been shown to affect the rate of latency transition in PAI-1 *in vitro*. During the search for the optimal purification conditions of recombinant PAI-1, a low temperature (4°C), a low pH (∼5.5), and a high salt concentration (1 M NaCl) contributed to increased PAI-1 stability (98). Since a decrease in pH causes protonation of imidazole groups, it was suggested that one or more histidine residues might be directly responsible for the pH-dependent stability of PAI-1. It was first speculated that $His^{143}$, localized at the top of hF, might be responsible for this effect (136). However, site-directed mutagenesis studies could only demonstrate a direct role for $His^{364}$, situated on the C-terminal end of s4B in close vicinity to hD in the flexible joint region and to the W86-loop (137). The salt stabilization was further investigated based on the observation of an anion-binding site in a crystal structure of PAI-1 14-1B in the active conformation (45). It was suggested that by forming close interactions with partially positive nitrogen residues on each side of the anion-binding site, i.e., with Lys323 and Lys325 in β-sheet A and Ser149 and His150 in the hF-s3A loop, chloride binding increases the energy barrier of the final stage in latency transition. Also, a more pronounced stabilization was correlated with an increased electronegativity of the anion ($F^- \geq Cl^- > Br^- > I^-$), resulting in tighter interactions. Notably, the proposed anion-binding site is located in the hF-s3A loop that is structurally different in PAI-1 14-1B as compared to "wild-type" PAI-1-W175F. Indeed, anion-binding could not be observed within this region in the more recent structure of active PAI-1-W175F (124) and is thus likely to be an artifact resulting from the induced conformational changes in the hF-s3A loop region in PAI-1 14-1B. However, a previously unknown chloride-binding site centered in the gate region could be unambiguously identified. This led to a revised hypothesis of the salt stabilizing effect on PAI-1 inhibitory activity, i.e., delaying latency transition by blocking the gate through bridging of several structural elements located between the s3C/s4C loop and the hG-s3B loop. Also, the preferential stabilization of other halide salts could not be extended to PAI-1-W175F or wild-type PAI-1, which were most dramatically stabilized by sodium chloride yielding half-lives well above 30 h at a 1 M salt concentration (124). Interestingly, two zinc-binding sites could clearly be identified within the same crystal structure of PAI-1-W175F. Since the metals appeared at the interface between two PAI-1 molecules inside the crystal, it remained debatable whether one or both binding sites are physiologically relevant. However, one zinc ion was strongly coordinated by N-terminal His2 and His3 (124). Almost simultaneously it was shown that type I metal ions (calcium, magnesium, and manganese) have modest stabilizing effects on PAI-1 activity, whereas type II metals (cobalt, copper, and nickel) had a more pronounced effect, either destabilizing PAI-1 in the absence of vitronectin or adding up onto the stabilization caused by simultaneous binding of vitronectin (138). Even though competitive binding experiments suggested that these effects were mediated through a single metal-binding site (139), a copper-binding site involving N-terminal His2 and His3 was identified that only accounted for the stabilizing, and not the destabilizing, effect of copper (140). The existence of a second copper-binding site has been further confirmed by the observation that copper facilitates an early step in PAI-1 latency transition by increasing protein dynamics in the flexible joint region and the helices underlying

**TABLE 1 |** List of X-ray crystallographic structures containing human PAI-1 available in the Protein Data Bank (PDB).

| Form | PDB ID | PAI-1 variant | Ligand | Resolution (Å) | References |
|---|---|---|---|---|---|
| Active | 1B3K | PAI-1 14-1B[a] | NA | 2.99 | (123) |
| | 1DB2 | PAI-1-stab[b] | NA | 2.70 | (46) |
| | 1DVM | PAI-1 14-1B | NA | 2.40 | (45) |
| | 3Q02 | PAI-1-W175F | NA | 2.30 | (124) |
| | 3R4L | VLHL-PAI-1[c] | NA | 2.70 | (122) |
| Latent | 1C5G | PAI-1-wt[d] | NA | 2.60 | (125) |
| | 1DVN | PAI-1 14-1B | NA | 2.10 | (45) |
| | 1LJ5 | PAI-1-wt | NA | 1.80 | – |
| | 3Q03 | PAI-1-W175F | NA | 2.64 | (124) |
| Cleaved | 9PAI | PAI-1-A335P | NA | 2.70 | (62) |
| | 3CVM | PAI-1 14-1B | NA | 2.03 | (126) |
| | 3EOX | PAI-1-stab | NA | 2.60 | (48) |
| + Ligand | 1OC0 | PAI-1 14-1B | SMB domain of vitronectin | 2.28 | (51) |
| | 3PB1 | PAI-1 14-1B | Catalytic site mutant of uPA, uPA-S195A | 2.30 | (53) |
| | 5BRR | PAI-1 14-1B | Catalytic site mutant of tPA, tPA-S195A | 3.16 | (47) |
| + Inhibitor | 1A7C | PAI-1-A335E | RCL-derived inhibitory peptide P14-P10 | 1.95 | (127) |
| | 4AQH | Latent PAI-1 14-1B | AZ3976 | 2.40 | (128) |
| | 3UT3 | PAI-1 14-1B | Embelin | 2.42 | (129) |
| | 4IC0 | PAI-1 14-1B | Gallate | 2.32 | (130) |
| | 4G8O | PAI-1 14-1B | CDE-096 | 2.71 | (131) |
| | 4G8R | PAI-1 14-1B | CDE-096 | 2.19 | (131) |
| | 6I8S | PAI-1 14-1B | Fab[e] fragment of MEDI-579 | 2.90 | (132) |
| | 5ZLZ | PAI-1 14-1B | PAItrap 2 | 3.58 | – |
| | 6GWN | PAI-1-W175F | Nanobody Nb42 and Nb64 | 2.03 | (52) |
| | 6GWP | PAI-1-stab | Nanobody Nb42 and Nb64 | 2.28 | (52) |
| | 6GWQ | PAI-1-stab | Nanobody Nb42 | 2.32 | (52) |
| | 6ZRV | PAI-1-W175F | Nanobody Nb93 | 1.88 | (41) |

[a] *PAI-1-N150H-K154T-Q319L-M354I.*
[b] *PAI-1-N150H-K154T-Q301P-Q319L-M354I.*
[c] *Very long half-life PAI-1, PAI-1-Q174C-G332C.*
[d] *Wild-type PAI-1.*
[e] *Antigen-binding fragment.*
*NA, not applicable.*

the shutter region, which could also be observed when copper bound to a mutant lacking His2 and His3 (141). Apart from salts and metals, high concentrations of arginine have been shown to elute subendothelial matrix-bound PAI-1 and to specifically stabilize the PAI-1 active conformation (142, 143). Since arginine residues are clustered in the stretch of basic residues in the C-terminal region of vitronectin, free arginine might contribute to an enhanced PAI-1 stability in a similar way as the arginine-rich C-terminal region of vitronectin.

## (PATHO)PHYSIOLOGICAL ROLES OF PAI-1

As the major physiological inhibitor of plasminogen activators tPA and uPA, PAI-1 plays a regulatory role in the fibrinolytic system by controlling plasmin formation. Not only is the plasminogen activator/plasmin system involved in fibrinolysis, it has also a profound role in multiple physiological processes, including the degradation of extracellular matrix (ECM), tissue remodeling, wound healing, angiogenesis, cell migration, and inflammation (144). Upon uPA-mediated activation of plasminogen, either by two-chain uPA or single-chain uPA bound to the uPA receptor (uPAR), plasmin can degrade several ECM components either directly or indirectly through the activation of matrix metalloproteases (MMPs). Degradation of the ECM may then facilitate cell invasion into the surrounding tissue. Furthermore, by increasing the availability of growth factors, such as vascular endothelial growth factor, fibroblast growth factor, and transforming growth factor-β, the role of plasmin further extends to the control of angiogenesis, cell growth, and cell differentiation. Importantly, independent of its effect on plasmin formation, PAI-1 directly interacts with specific matrix components, including vitronectin, LRP1,

and the uPA/uPAR complex to affect cell migration and intracellular signaling.

The (patho)physiological role of PAI-1 has been extensively studied by comparing the phenotype observed in human PAI-1 deficiency with that of mice engineered to be completely PAI-1 deficient by gene targeting (PAI-$1^{-/-}$ mice). In humans, PAI-1 deficiency is an uncommon disorder that can be caused by mutations in the *SERPINE1* gene leading to the production of non-functional PAI-1 protein (145) or by a complete absence of PAI-1 plasma antigen (146–148). Typically, this disorder is characterized by mild to moderate bleeding in response to injury, trauma, or surgery. In women, PAI-1 deficiency may cause severe blood loss during menstruation and pregnancy-related complications, such as prepartum bleeding, preterm labor, or miscarriage (149–151). PAI-$1^{-/-}$ mice were shown to be viable, fertile, and developed normally (152). Furthermore, disruption of the PAI-1 gene did not appear to impair hemostasis, but was associated with increased resistance to thrombosis and with a milder hyperfibrinolytic state as compared to humans (153). In contrast to PAI-1 deficient mice, several lines of transgenic mice overexpressing native or stabilized PAI-1 of human and murine origin have been established. These lines have been generated to explore the effects of elevated PAI-1 levels on, e.g., the progress of thrombosis (154, 155), pulmonary fibrosis (156), and obesity (157). Furthermore, these transgenic mice often display hair loss and skin abnormalities. Importantly, transgenic mice expressing a reactive site inactive PAI-1 mutant exhibit complete phenotypic rescue, while transgenic mice expressing PAI-1 with reduced affinity for vitronectin manifest all of the phenotypic abnormalities, underscoring the fact that PAI-1 affects physiological processes by acting through multiple pathways (158). In humans, two variations in the promoter region of the PAI-1 gene occur frequently and have been shown to affect PAI-1 levels (159–161). Firstly, the 4G/5G polymorphism refers to a single guanosine insertion/deletion at position 675 upstream of the transcription site (159). It has been suggested that the 4G allele is associated with higher PAI-1 activity since the 5G allele harbors an additional binding site for a transcriptional repressor. Secondly, the G/A polymorphism is characterized by a single nucleotide substitution of guanine for adenine at position 844 upstream of the transcription site, generating a consensus binding sequence for transcription factor Ets-1 which increases the transcription rate (161). Taken together, the association of these PAI-1 gene polymorphisms and/or elevated PAI-1 levels with several pathologies have been extensively studied in both humans and in PAI-1 deficient or transgenic mice.

## PAI-1 in Cardiovascular Disease

Elevated levels of PAI-1 downregulate tPA and uPA activity and create a prothrombotic or hypofibrinolytic state that was suggested to contribute to the pathogenesis of cardiovascular diseases (CVD) (162). As mentioned, several lines of transgenic mice that overexpress PAI-1 have been developed and support a contribution of elevated PAI-1 levels to thrombosis and CVD. The first line of transgenic PAI-1 mice overexpressed native human PAI-1 and was shown to develop transient venous thrombosis in the tail and hind limbs and subcutaneous hemorrhage (154). Later, human PAI-1-stab mice were generated that displayed age-dependent coronary arterial thrombosis and myocardial infarction (155). In contrast, spontaneous thrombosis could not be observed in transgenic mice that overexpress stabilized murine PAI-1 (163). However, it should be noted that (I) the choice of the promoter that drives PAI-1 expression, (II) the nature of the stable variant, and (III) a cross-species difference in PAI-1 function may have contributed to the observed phenotypic differences. In humans, many studies have suggested that PAI-1 gene polymorphisms, possibly leading to higher PAI-1 levels, are an independent risk factor for major adverse cardiovascular events (MACE) including myocardial infarction (MI) (164–167) and ischemic stroke (168), as well as coronary heart disease (CHD) (169), venous thrombosis (170–172), and atherosclerosis (173). However, despite the observed link in these studies, these findings are in contradiction with other available data (174–178). Similarly, independent of the contribution from PAI-1 gene polymorphisms, ample evidence has been provided of a link between elevated PAI-1 levels and MI or stroke (179–181), CHD (182), venous thrombosis (183), and atherosclerosis (184, 185). In a recent systematic review of all studies published between 1991 and 2016 that examined the association of PAI-1 with MACE (defined as death, MI, and stroke) or restenosis (the recurrence of treated coronary artery stenosis), Jung et al. substantiated a link between elevated plasma PAI-1 antigen levels, but not PAI-1 activity levels, and MACE in both incident and secondary event populations (181). MI is most often a result of CHD and is caused by the disruption of an atherosclerotic plaque, thereby exposing a procoagulatory surface of the coronary vessel that gives rise to occlusive thrombus formation (186). Several studies have reported elevated PAI-1 levels in several cell types associated with atherosclerotic plaques in human coronary arteries, including endothelial cells, vascular smooth muscle cells (SMCs), and macrophages (184, 187, 188). In mice, PAI-1 deficiency has been shown to be protective (189) or promoting (190) in the development of atherosclerosis, however, no effect of PAI-1 on atherosclerosis has been observed as well (191). Indeed, overproduction of PAI-1 in a diseased vessel wall may contribute to the progression of atherosclerosis by reducing local plasmin production which is physiologically required for the removal of fibrin, ECM remodeling, and SMC proliferation. However, when the controlling effect of PAI-1 on plasmin formation is abolished, it may contribute to the atherogenic role of plasmin, as plasmin is also involved in lipoprotein modification, macrophage cholesterol accumulation, inflammation, and foam cell formation (192, 193). Furthermore, PAI-1 has been shown to have an ambiguous role in neointima formation (194–198). In this respect, in the same systematic review by Jung et al., low PAI-1 antigen and activity levels were associated with increased restenosis, highlighting the complex role of PAI-1 in vascular remodeling (181). Despite the links provided between PAI-1 and CVD, certain studies could not confirm these associations or the significance was lost after adjustment for other risk factors (199–201). A positive correlation has been demonstrated for plasma PAI-1 levels and known risk factors for developing CVD, including age, sex, obesity, hyperlipidemia, insulin resistance,

and diabetes (162, 181, 202, 203). Age is an important risk factor for most chronic diseases including cardiovascular disease, type 2 diabetes, and metabolic syndrome. Furthermore, PAI-1 levels have been reported to increase with age in various tissues. More recently, PAI-1 has been identified not only as a marker but also as a mediator of cellular senescence associated with aging and aging-related pathologies (204).

# DIVERSE APPROACHES TO INHIBIT PAI-1

As PAI-1 is considered a risk factor in various pathological conditions, many efforts have been devoted to the development of PAI-1 inhibitors, including small molecules, synthetic peptides, RNA aptamers, monoclonal antibodies (mAbs), and antibody derivatives. Whereas, some marketed drugs have been shown to attenuate PAI-1 synthesis or secretion (205), the majority of PAI-1 inhibitors currently in development can influence PAI-1 functionality in at least three possible ways: (I) by directly blocking the initial formation of the Michaelis complex between PAI-1 and PAs, (II) by preventing the formation of the final inhibitory complex, resulting in substrate behavior of PAI-1, or (III) by accelerating the active-to-latent transition of the PAI-1 molecule or an otherwise inert form. Despite extensive *in vitro* and *in vivo* characterization, no PAI-1 inhibitor is currently approved for therapeutic use in humans. This is mainly due to affinity and specificity issues, which are especially observed for small molecules. Furthermore, the structural plasticity of PAI-1 and the possible counteraction of PAI-1 binding partners, such as vitronectin, pose a real challenge to develop PAI-1 inhibitors that retain their capacity to modulate PAI-1 activity *in vivo*. To improve their properties or to guide the rational design of novel PAI-1 inhibitors it is essential to get a deeper understanding of PAI-1 inhibition at the molecular level. In addition to the several crystal structures of PAI-1 in active, latent, or cleaved conformation (45, 48, 124), a handful of structures containing PAI-1 in complex with inhibitory peptides, small molecules, and antibody fragments have been described (**Table 1**). Furthermore, by using a broad range of biophysical and biochemical methods, including competitive binding experiments, mutagenesis, and computational docking, the presumptive binding regions of the majority of PAI-1 inhibitors have been mapped and can be related to the mechanisms by which they interfere with PAI-1 functionality.

## Synthetic Peptides

### RCL-Mimicking Peptides

Insertion of the RCL into the central β-sheet A is a crucial step in the inhibitory mechanism of serpins in order to translocate and irreversibly trap the target proteinase. In this regard, synthetic peptides derived from the RCL-sequences of antithrombin III and $\alpha_1$-antitrypsin were shown to convert the respective serpins from an inhibitor to a substrate. By binding between s3A and s5A in β-sheet A and thus becoming s4A, these peptides prevent endogenous RCL insertion upon interaction with the target proteinase, resulting in cleavage of the serpin and release of regenerated proteinase (206). Taking a similar approach, several peptides that mimic different fragments of the RCL of PAI-1, such as P14-P1 (207), P14-P10 (127), P14-P9 (208), P14-P7 (136), and P8-P3 (208), were designed and evaluated for their PAI-1 modulating properties. The first peptide, corresponding to residues P14-P1 of the RCL, was shown to rapidly inhibit PAI-1 activity by accelerating the conversion to a non-reactive PAI-1 form and effectively enhanced *in vitro* fibrinolysis in both platelet-poor and platelet-rich clots (207). However, when PAI-1 was bound to its biological cofactor vitronectin, the PAI-1 neutralizing effects of this peptide were considerably reduced. A comparable mechanism was observed for the P8-P3 peptide that mimics the C-terminal part of the RCL that inserts at the bottom of the β-sheet A in latent PAI-1. Unlike peptides P14-P1 and P8-P3, peptides P14-P7, P14-P10, or P14-P9 that correspond to the N-terminal part of the RCL converted PAI-1 from an inhibitor to a substrate for tPA (127, 136, 208). By showing that binding of P14-P7 and the formation of latent PAI-1 are competitive events, the first evidence was provided for a binding site in the central β-sheet A cleft (136). The high-resolution crystal structure of PAI-1 mutant PAI-1-Ala335Glu in complex with two P14-P10 peptides (PDB ID 1A7C) further confirmed this presumption (127). The structure revealed that both peptide molecules bound inside the cleft between s3A and s5A, with the first molecule occupying the same space as RCL residues P14-P10 in latent and cleaved PAI-1, and the second one occupying the same space as residues P6-P2 in cleaved PAI-1. Since the different effects of RCL-derived peptides on the outcome of the PAI-1/PA reaction, i.e., conversion to either inert or substrate PAI-1, did not seem compatible with one common binding position inside the β-sheet A cleft, it was finally suggested that peptides mimicking the C-terminal part of the RCL (P8-P3) act by accelerating the irreversible transition to an inert form of PAI-1, whereas peptides that mimic the N-terminal part of the RCL (P14-P9) primarily induce PAI-1 substrate behavior (208).

### Other Peptides

A few other peptides have been isolated from a phage-display peptide library including paionin-1 (209), which does not impair PAI-1 activity, and paionin-4 (210), which accelerates the active-to-latent conversion of PAI-1. *In silico* docking of paionin-1 into the crystal structure of PAI-1 suggested a binding site in the flexible joint region that was supported by site-directed mutagenesis and competitive binding of other molecules targeting the same region, such as XR-5118 and bis-ANS. As paionin-1 is able to prevent binding of the PAI-1/uPA complex to LRP1, paionin-1 or other compounds binding in the same region may be of benefit in cases where disruption of the signaling function of uPA/uPAR/LRP1 is desired. Paionin-4 presumably binds PAI-1 at the loop between hD and s2A and is suggested to induce a conformational change that facilitates loop insertion.

## RNA Aptamers

RNA-aptamers are 8–15 kDa single-stranded nucleic acid ligands that tend to bind to highly positive regions on proteins and block protein-protein interactions. In this respect, a few RNA aptamers have been developed in order to interfere with the interactions between PAI-1 and its binding partners. WT-15 and

SM-20 are able to disrupt the functional interaction between vitronectin and PAI-1 without compromising the PA-inhibitory function of PAI-1 (211). Expression of these aptamers in human breast cancer cells decreased cell migration and invasion and additionally decreased PAI-1 and uPA levels while increasing the stable PAI-1/uPA complex (212). Other aptamers, R10-4 and R10-2, were able to interfere with the formation of the stable PAI-1/tPA complex but not with the PAI-1/uPA complex, suggesting a binding site not directly involved in PAI-1/PA interactions, without disrupting the PAI-1/vitronectin interaction (213).

## Small Molecules

Since the mid-90s, several low molecular weight (LMW) inhibitors possessing a large structural diversity have been described and grouped based on their chemical composition [extensively reviewed by Fortenberry (214) and Rouch et al. (215)]. Lead molecules have been discovered using various approaches, including through isolation from bacterial biomass or a library of natural products, by high-throughput screening (HTS) of synthetic libraries, and by structure-based virtual screening. Subsequently, many of these compounds have been modified based on structure-activity relationship (SAR) studies in order to improve their selectivity and specificity and their inhibitory and physicochemical properties. Even though only a few structures of PAI-1 complexed with small molecules were determined, they provided evidence for a common compound-binding pocket within the flexible joint area of PAI-1 (**Figure 3**).

The first published crystal structure of a PAI-1/inhibitor complex involved compound AZ3976 (PDB ID 4AQH) (**Figure 3C**) (128). AZ3976 was identified by HTS of the AstraZeneca compound collection and shown to inhibit PAI-1 activity in *in vitro* chromogenic and clot lysis assays. Titration of PAI-1 with AZ3976 revealed that the compound was only bound to 30% of the total PAI-1 present, corresponding to the non-reactive subpopulation (latent or cleaved) in the preparation of active PAI-1. This was confirmed by affinity data, showing a high binding affinity toward latent PAI-1 whereas no binding was observed toward the active form. Based on the structure of latent PAI-1 complexed with AZ3976, a deep ligand-binding pocket within the flexible joint region was identified with the entry located between hD and s2A. Importantly, this binding site appeared to be more accessible in latent PAI-1, however, tight binding of AZ3976 requires small conformational changes. Since AZ3976 has been shown to accelerate the active to latent transition of PAI-1, it was therefore suggested to bind to a latent-like prelatent form from which latent PAI-1 is then generated more rapidly.

Not much later, a second crystal structure of PAI-1 containing a small molecule inhibitor, embelin, was published (PDB ID 3UT3) (**Figure 3F**) (129). Embelin was identified as a PAI-1 antagonist by screening a library of natural products. Structural and site-directed mutagenesis results have shown that embelin binds to a small and charged groove aligned by hD, hF, s2A, and the hE-s1A loop in active PAI-1 (129), located adjacent to the larger and deeper pocket in (pre)latent PAI-1 that can be occupied by AZ3976 (128). It was proposed that embelin fixes s2A to the neighboring hD and hE, and thereby interferes with the sliding movement of s2A and s3A into the flexible joint region in order to accommodate RCL insertion. Indeed, consistent with this theory, embelin has been shown to act by a two-step mechanism including a fast reversible step of inducing PAI-1 substrate behavior, followed by a slow irreversible induction of an inactive form. Despite the ability of AZ3976 and embelin to inhibit PAI-1 activity *in vitro*, the presence of vitronectin abolished the capacity of both compounds to bind and modulate PAI-1 activity. Since the binding sites for AZ9376 and embelin partially overlap with the binding site for vitronectin in active PAI-1, the protective effects of vitronectin might be caused by sterically blocking their binding sites or, in the case of AZ3976, by preventing the formation of the prelatent structural state to which AZ3976 preferably binds. Importantly, the binding pocket within the flexible joint region, which has been observed in the crystal structures of PAI-1 complexed with AZ3976 and embelin, is consistent with the binding sites for other small molecules that were determined mainly through competitive binding studies, mutagenesis studies, and molecular modeling. Several negatively [AR-H029953XX (216), ANS, Bis-ANS] and positively [XR5118 (217, 218)] charged amphipathic inhibitors have been shown to bind overlapping but non-identical binding sites within this hydrophobic pocket, resulting in variable induced molecular changes in PAI-1 and in a differential susceptibility to vitronectin-bound PAI-1 (219). First, it was demonstrated that both groups inhibit PAI-1 via a two-step mechanism, involving a rapid reversible conversion into a PAI-1 form exhibiting substrate behavior, followed by a slower irreversible conversion into a non-reactive form. However, a different study showed that both AR-H029953XX and XR5118 induce a direct conversion of PAI-1 into a non-reactive form, possibly due to the differences in compound concentrations that were used to conduct experiments. A concentration-dependent effect could also be observed for tiplaxtinin (PAI-039), which induces PAI-1 substrate behavior at lower concentrations and converts PAI-1 to a non-reactive form at high concentrations (88). Interestingly, several studies reported that PAI-1 polymerization could be induced by negatively charged organochemical inhibitors following conversion to a non-reactive form (219–221).

Apart from this common compound-binding pocket, a third crystal structure of the cocrystallized PAI-1/CDE-096 complex (PDB ID 4G8O) elucidated the binding mode of CDE-096 which is, in contrast to the aforementioned compounds, active against both free and vitronectin-bound PAI-1 (**Figure 3D**) (131). CDE-096 was synthesized based on a SAR study of a high-affinity polyphenolic PAI-1 inhibitor (222). Structural studies, substantiated with site-directed mutagenesis results, revealed a binding site at the interface composed of residues from the s3A/s4C loop, β-sheets B and C, and hH, referred to as the sB/sC pocket. Using a combination of biochemical experiments, a mechanism of action was proposed that involves reversible allosteric modulation of RCL conformation to block initial PAI-1/PA Michaelis complex formation. Furthermore, although CDE-096 and vitronectin reduce PAI-1's affinity for one another, their binding is not strictly mutually exclusive, suggesting allosteric modulation through reciprocal communication between the

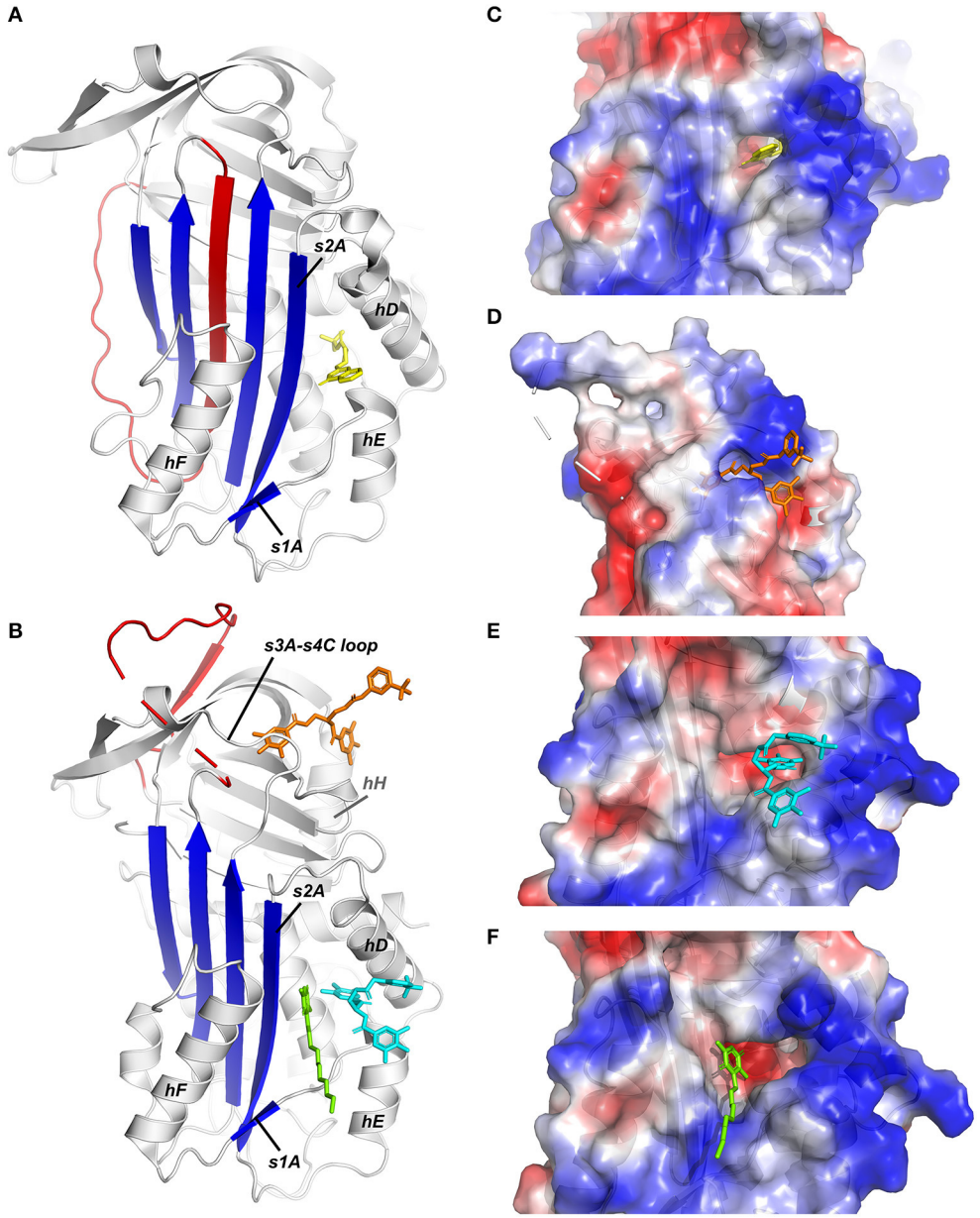


**FIGURE 3 |** X-ray crystallographic structures of small molecule inhibitors bound to PAI-1. **(A)** Structure of latent PAI-1 in complex with AZ3976 (128). **(B)** Superimposition of the structures of active PAI-1 in complex with embelin (129) and CDE-096 (131). PAI-1 is colored white with the central β-sheet A colored blue and the RCL colored red. Secondary structure elements involved in binding to the compounds are indicated. AZ3976 is colored yellow, and embelin is colored green. CDE-096 in the structures obtained by cocrystallization or crystal soaking is colored orange or cyan, respectively. **(C)** AZ3976 bound to a deep pocket aligned by hD and s2A in latent PAI-1 [PDB ID 4AQH (128)]. **(D)** CDE-096 bound to active PAI-1 obtained by cocrystallization [PDB ID 4G8O (131)]. **(E)** Detail of the structure of CDE-096 bound to active PAI-1 obtained by crystal soaking [PDB ID 4G8R (131)]. **(F)** Detail of the structure of embelin bound a groove aligned by hD, hF, s2A, and the hE-s1A loop in active PAI-1 [PDB ID 3UT3 (129)].

high-affinity compound- and vitronectin-binding sites. In crystal soaking studies that require high concentrations of CDE-096 to be incubated with preformed PAI-1 14-1B crystals, a second possible binding site was observed and shows overlap with the binding site for AZ3976 in latent PAI-1 (PDB ID 4G8R) (**Figure 3E**). However, based on the mutagenesis results and the capacity of CDE-096 to bind both active and latent PAI-1 with similar affinity, it was argued to be an artifact induced by the high concentrations used for crystal soaking.

Another class of small molecules was discovered by virtual screening of a library of commercially available chemicals. To address the lack of efficacy when translated into *in vivo* settings often encountered in high-throughput screening of large compound libraries, two filters were applied representing (I) the

general drug-likeliness based on clinically used drug molecules, and (II) the specific lead-likeliness based on the RCL peptide P14-P10 as well as reference inhibitors that bind in the region of the vitronectin binding site in PAI-1 (219, 223). Next, docking simulations for the remaining compounds focused on the cleft in β-sheet A that is occupied by the RCL following loop insertion. TM5007, the most effective compound, exhibited high specificity for the PAI-1/PA system and was furthermore effective in *in vivo* models of thrombosis and fibrosis (223). SAR studies on TM5007 resulted in the selection of TM5275 with an improved inhibitory profile and better oral bioavailability (224). A similar docking simulation suggested that TM5275 binds within the cleft between the strands of β-sheet A, albeit at a different position compared to its precursor TM5007. Whereas, TM5007 docked into the space occupied by P8-P3 in the latent form, TM5275 docked at the P14-P9 position. The differences in their presumed binding sites within the cleft also seemed to correlate with their mechanisms of action, i.e., by either preventing PAI-1/PA complex formation (TM5007) or inducing substrate behavior of PAI-1 (TM5275) (223, 224). Further structure-optimization by substituting the lipophilic moiety and varying the acyl-type linker length led to the discovery of smaller derivatives, including TM5441 and TM5484 (225). Although these compounds have originally been designed to bind the central β-sheet A cleft, there is no experimental evidence that confirms their binding site or their mechanism of action.

Even though many small molecules have been shown to be potent PAI-1 inhibitors *in vitro* or *in vivo*, several other factors, such as the lack of information regarding their exact inhibitory mechanism and/or binding area in PAI-1, the inability to modulate the activity of vitronectin-bound PAI-1, and the possibility to induce PAI-1 polymerization may partially hamper the future rational design of novel effective small molecules.

## Antibodies and Antibody Derivatives

Conventional antibodies are Y-shaped heterotetrameric glycoproteins (150 kDa) composed of two light and two heavy chains that are linked together by multiple disulfide bonds (**Figure 4A**) (228). Each light chain comprises one variable ($V_L$) and one constant domain ($C_L$), whereas each heavy chain comprises one variable domain ($V_H$) and three constant domains ($C_H1$, $C_H2$, and $C_H3$). The Ig unit can be divided into three functional components, namely two identical antigen-binding fragments (Fabs) and one crystallizable fragment (Fc). In each Fab fragment, the variable fragment (Fv) is responsible for the recognition of and high-affinity binding to a specific antigen and is composed of the variable domains of both chains ($V_L$ and $V_H$). The amino acid residues of the V-regions that are in direct contact with the antigen are referred to as the paratope, whereas the binding site for the antibody on the surface of the antigen is referred to as the epitope. By connecting the $V_H$ and $V_L$ domain of a conventional antibody through a flexible polypeptide linker consisting of serines and glycines, a ∼25 kDa single-chain variable fragment (scFv) can be created that usually retains the antigen-binding capacity of the parental mAb (**Figure 4B**). Subsequently, two scFvs can be combined in order to generate a ∼55 kDa diabody that can either target the same epitope on another molecule of the same antigen (bivalent), another epitope on the same antigen (biparatopic), or another antigen (bispecific) (**Figure 4B**).

Later it was discovered that, besides conventional antibodies, the sera of camelids (such as camels and llamas) (229) as well as cartilaginous fish (such as nurse sharks) (230) naturally contain functional heavy-chain-only antibodies (HCAbs) (231). In contrast to conventional antibodies, camel HCAbs (∼90 kDa) are devoid of the light chains and the heavy chain $C_H1$ domains that normally serve to anchor the light chains (**Figure 4A**). The $V_HH$ domain can be isolated from the HCAb and be produced as such at a large scale in bacterial expression systems, ultimately yielding a recombinant single-domain antibody referred to as nanobody (Nb) (**Figure 4B**). In addition to having high binding affinities toward antigens in the nano- or even picomolar range, Nbs express a favorable stable behavior in harsh conditions, including high temperatures, high protein concentrations, high pressure, and the presence of detergents or denaturants (232, 233). Furthermore, resistance to pepsin or chymotrypsin can be conferred upon nanobodies by introducing an additional disulfide bond, suggesting the possibility of oral administration (234). Furthermore, their small size makes them good candidates when it is required to penetrate dense tissues in order to bind hard to reach targets or to deliver functional molecules to the cytoplasm. As they can easily be linked, nanobodies can serve as "building blocks" to construct multispecific, multivalent, or multiparatopic molecules.

### Antibody-Based PAI-1 Inhibitors

Due to the efforts of many research groups, a large panel of mAbs that interfere with PAI-1 activity is readily available. More recently, nanobody libraries have been constructed as well (35). In contrast to inhibitory peptides and small molecules, the epitopes of antibody-based PAI-1 inhibitors have been mapped to different regions of the PAI-1 molecule (**Figure 5**). Antibodies have been shown to affect PAI-1 functionality at distinct levels during the PAI-1/PA reaction, i.e., by preventing Michaelis complex formation, by inducing substrate behavior of PAI-1, or by accelerating the conversion to latent PAI-1 (243).

Only recently, the first crystal structure of PAI-1 complexed with an inhibitory antibody fragment belonging to the first class was reported (**Table 1**) (132). This structure containing the Fab fragment of neutralizing antibody MEDI-579 revealed that its epitope is concentrated around the C-terminal region of the RCL (residues Ala345–Glu350 or P2–P4′) and the neighboring exosites for the 37-loop (Tyr210, Glu212, Tyr220, and Tyr241) and 60-loop (Leu269, Pro270, and Arg271) of PAs (**Figure 5A**) (132). As a consequence, by simultaneously interfering with exosite interactions and shielding the P1-P1′ reactive center, MEDI-579 prevents the initial interaction between PAI-1 and PAs. A similar mechanism has been described for nanobody VHH-s-a93 (Nb93) that binds an epitope slightly overlapping with that for MEDI-579 (41). The X-ray crystallographic structure of the PAI-1/Nb93 complex revealed that Nb93 binds PAI-1 in a PA-like manner through interactions with the almost full-length RCL and adjacent exosites for PAs other than that for their 37-loop (**Table 1** and **Figure 5D**) (41). In addition,

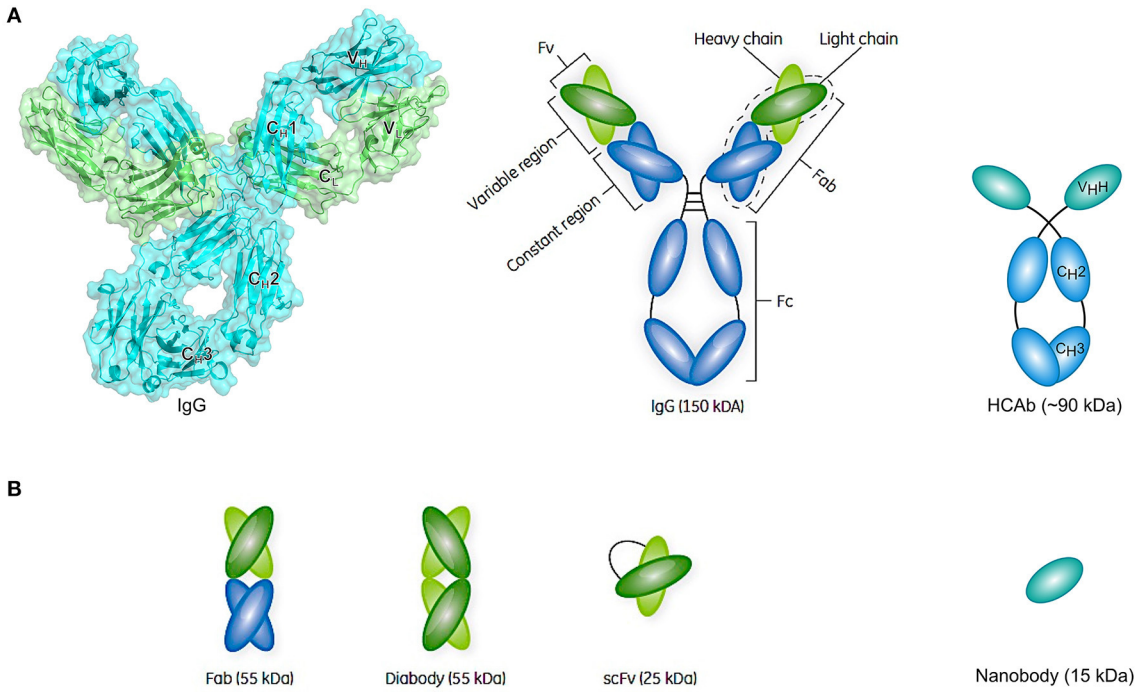


**FIGURE 4 |** Schematic representation of an antibody structure and different antibody fragment formats. **(A)** The left panel shows the cartoon and biological surface representation of the full-size crystal structure of Pembrolizumab, a human anti-PD1 immunoglobulin G4 (IgG4) antibody [PDB ID 5DK3 (226)]. A conventional antibody is a Y-shaped heterotetrameric glycoprotein consisting of two identical heavy (cyan) and two identical light chains (green). The heavy chain comprises one variable domain ($V_H$) and three constant domains ($C_H1$, $C_H2$, and $C_H3$), whereas the light chain comprises one variable ($V_L$) and one constant ($C_L$) domain. The panel on the right shows a simplified schematic representation of a conventional antibody and a heavy-chain-only antibody (HCAb). Each arm of the conventional antibody represents the antigen-binding fragment (Fab) that comprises the constant region ($C_H1$ and $C_L$ domains) and the variable fragment (Fv) containing $V_L$ and $V_H$. The stem of the antibody comprises two copies of the $C_H2$-$C_H3$ domains that form the crystallizable fragment (Fc). The HCAb comprises two heavy chains, each combining one variable $V_HH$ domain, referred to as nanobody, and two constant domains (CH2 and CH3). **(B)** Schematic representation of a selection of antibody fragments, including Fab, diabody, single-chain variable fragment (scFv), and nanobody. Figure adapted from Rodrigo et al. (227).

Nb93 was shown to selectively bind and stabilize the active conformation of PAI-1 by anchoring the RCL to the top of the PAI-1 molecule. Notably, similar binding sites including the RCL of PAI-1 and/or neighboring exosites for PAs have been described for other mAbs (**Figure 5B**), including ESPI-12 (between residues 342–349), MAI-12 (between residues 320–379), MA-42A2F6 (Lys243 and Glu350), MA-56A7C10 (Glu242, Lys243, Glu244, Glu350, Asp355, and Arg356), and MA-44E4 (His185, His186, and Arg187), suggesting that they directly interfere with Michaelis complex formation as well (132, 235, 244, 245). The epitopes of two other nanobodies, VHH-s-a27 (Nb27) and VHH-2g-42 (Nb42), could not be deduced by mutational studies (35). However, it was hypothesized that they might interfere with the PAI-1/PA reaction by directly preventing PAI-1/PA complex formation as well. Indeed, using a hybrid approach employing structural and biochemical methods, Nb42 was shown to destabilize the initial Michaelis complex by binding to the exosite region for the 37-loop of PAs (**Table 1** and **Figure 5B**) (52). Interestingly, MA-124K1 that inhibits rat PAI-1 was found to bind the exosite region for the 37-loop of PAs (Glu212 and Glu220) and thereby inhibits PAI-1 activity while simultaneously enhancing the binding of PAI-1 to vitronectin (236).

The second class of mAbs, referred to as "switching antibodies," redirect the inhibitory PAI-1/PA reaction toward the substrate branch. Within the category of substrate-inducing antibodies, two different subclasses have been identified. Even though both subclasses ultimately increase the relative fraction of cleaved PAI-1, each class acts through a distinct mechanism by binding different epitope regions localized in the lower half of the PAI-1 molecule (**Figure 5B**). Several mAbs, including MA-33H1F7 (Glu130, Arg131, and Lys154), MA-55F4C12 (Glu128, Val129, Glu130, Arg131, and Lys154), and Mab2 (Arg131, Arg133, Phe134, Asn137, Asp138, Leu152, and Lys154) were shown to have overlapping epitopes located in hF and the loop connecting hF with s3A of the central PAI-1 β-sheet (237, 238). These mAbs have been shown to slow down the rate of cleaved RCL insertion, possibly by restricting the structural rearrangements within this region during RCL insertion. A different epitope was identified for switching antibody MA-8H9D4 that binds the loop between hI and s5A at the bottom of the PAI-1 molecule (Arg300, Gln303, and Asp305) and possibly residues in hC (Glu53) and hI (Arg287, Glu297, and Asp297) (239). A similar epitope, i.e., comprising residues in hB, hC, and the hI-s5A loop, was identified for nanobody

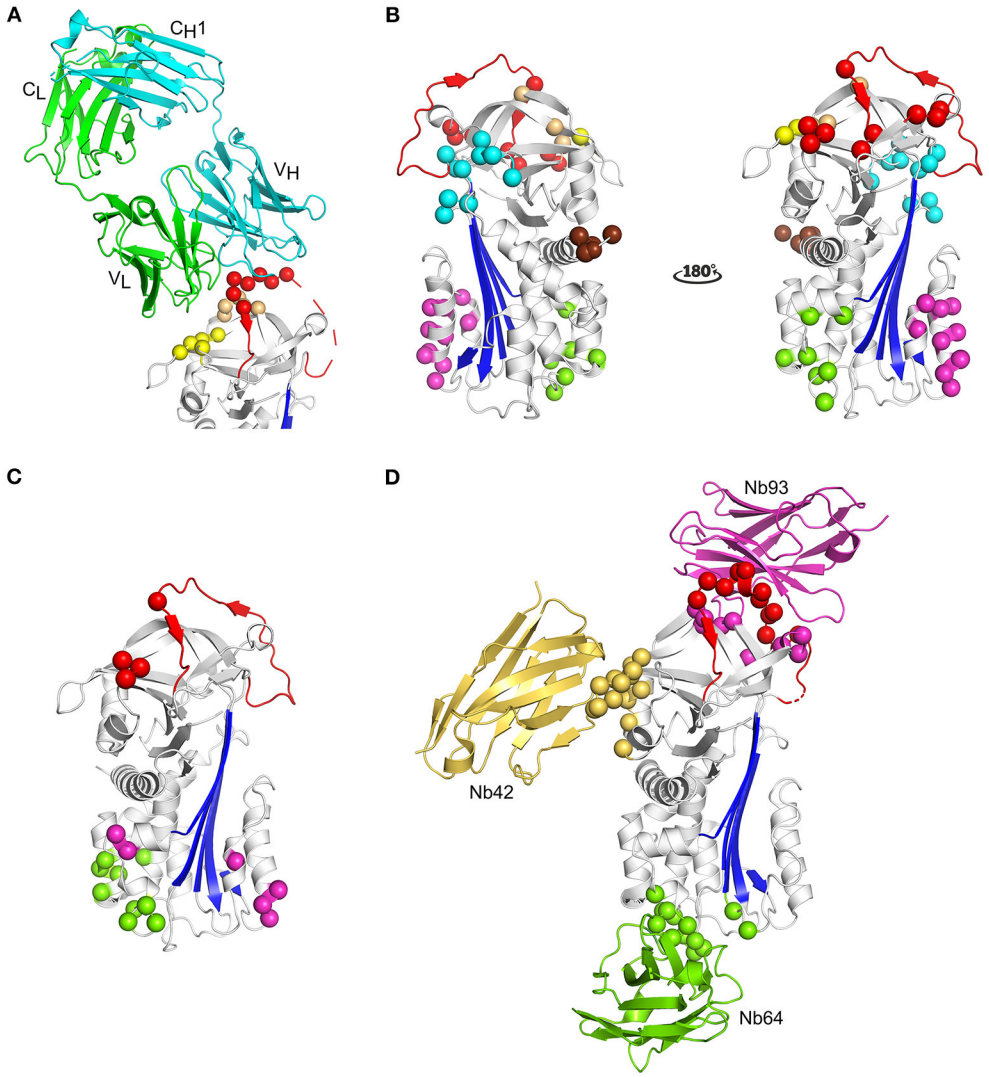


**FIGURE 5 |** Localization of different epitopes in the structure of active PAI-1. **(A)** Cartoon representation of the crystal structure of PAI-1 in complex with the Fab fragment of MEDI-579 (PDB ID 6I8S) (132). The heavy and the light chain of the Fab fragment are colored cyan and green, respectively. The constant and variable heavy ($C_H1$ and $V_H$) and light ($C_L$ and $V_L$) domains are indicated in the figure. Residues closer than 4 Å to MEDI-579 are indicated as spheres. Residues located in the RCL are colored red, residues residing in the exosite regions for the 37-loop and 60-loop of PAs are colored yellow and orange, respectively. **(B)** Localization of different epitopes of monoclonal antibodies (mAbs) as determined by mutagenesis studies. The epitopes of mAbs that prevent the interaction between PAI-1 and PAs are indicated as red (MA-42A2F6, MA-56A7C10, and MA-44E4) (235) and yellow (MA-124K1) (236) spheres. The epitopes of switching mAbs that bind to hF or the hF-s3A loop (MA-33H1F7, MA-55F4C12, and Mab2) (237, 238) are indicated as magenta spheres. The epitope of switching mAb MA-8H9D4 (239) that binds to the hI-s5A loop is indicated as green spheres. The epitopes of latency-inducing antibody MA-33B8 (240, 241), MA-H4B3 (90), and MA-159M12 (242) are indicated as cyan, orange, and brown spheres, respectively. **(C)** Localization of different epitopes of nanobodies as determined by mutagenesis studies (35). The epitope of substrate-inducing nanobody Nb98 is indicated by green and magenta spheres, whereas only the magenta spheres indicate the epitope of Nb64. The epitope of Nb93 that interferes with PAI-1/PA complex formation is indicated as red spheres. **(D)** Cartoon representation of the superimposed crystal structures of PAI-1 in complex with Nb42 [PDB ID 6GWN, 6GWP, and 6GWQ (52)], Nb64 [PDB ID 6GWN and 6GWP (52)], and Nb93 [PDB ID 6ZRV (41)]. Nb42, Nb64, and Nb93 are colored yellow, green, and magenta, respectively. Residues closer than 4 Å to Nb42 are indicated as yellow spheres. Residues closer than 4 Å to Nb64 are indicated as green spheres. Residues closer than 4 Å to Nb93 are indicated as red and magenta spheres. Red spheres represent residues located in the RCL of PAI-1, whereas magenta spheres represent residues located in the exosite binding regions for PAs.

VHH-2w-64 (Nb64). The crystallographic structures of the PAI-1/Nb64 complex later confirmed the crucial involvement of the latter loop (**Table 1**) (52). Based on structures of other serpin/serine proteinase complexes, the binding site of Nb64 in all probability overlaps with the position of the PA in the final inhibitory complex (**Figure 1**, **Figure 5D**). In contrast to the first subclass binding hF, MA-8H9D4 (246), and Nb64 (52) neither affected the formation of the initial PAI-1/PA complex, nor the kinetics of RCL insertion for the PAI-1/tPA reaction. It was therefore suggested that MA-8H9D4 and Nb64

interfere with the final step of inhibitory complex formation by hindering the PA to come close enough to the PAI-1 surface for PA distortion. Furthermore, strong functional additivity has been observed for the MA-33H1F7/MA-8H9D4 and MA-55F4C12/MA-8H9D4 antibody pairs which demonstrates that these mAbs bind structurally distinct epitopes and affect different steps of the PAI-1/PA reaction (246). Importantly, the effects of Mab2, MA-8H9D4, and Nb64 are potentiated by simultaneous binding of vitronectin to the opposite side of hF in PAI-1, which further increases the rigidity within this region (99). Another substrate-inducing nanobody VHH-s-a98 (Nb98) was suggested to bind a cavity aligned by hB and hC (Gln47, Glu53, and Gln55-Gln56-Gln57), the hF-s3A loop (Glu128-Val129-Glu130-Arg131 and Lys154), hI (Glu291 and Asn292), and the hI-s5A loop (Gln303 and Asp305). Since this region harbors binding sites for both subclasses of switching Abs (MA-33H1F7 and MA-55F4C12 vs. MA-8H9D4 and Nb64), the exact mechanism by which Nb98 induces substrate behavior remains unclear.

The third class of mAbs, including MA-35A5, MA-33B8, M5, and MA-H4B3, have the ability to accelerate the active to latent transition of PAI-1 and bind epitopes that are spread more across the PAI-1 surface (**Figure 5B**). The major determinants of the MA-33B8 epitope were simultaneously reported by two research groups, and are comprised in the same region that covers the turn connecting hD with s2A (Asn87, Lys88, and Asp89), the top of s3A (Gln174 and Lys176), the loop connecting s2B with s3B (His229, Gly230, and Thr232), and the loop connecting s5A with the RCL in the breach region (Asn329 and Ser331) (240, 241). Interestingly, this epitope is relatively less accessible in the active form of PAI-1 and undergoes a structural rearrangement to become more compact in the loop-inserted forms of PAI-1. Furthermore, since the putative epitope contains residues located on both sides of the RCL insertion site, i.e., on s5A and s3A, and MA-33B8 promotes loop insertion, the binding must occur to a prelatent form of PAI-1 in which the RCL is already partially inserted. Additional evidence for a prelatent form that can be accelerated into latency transition has been provided by the binding site and inhibitory mechanisms of M-5 and MA-H4B3. The dominant epitope residue for M-5 was mapped to Asp181 located directly below the RCL at the loop connecting s3A with s4C (247). Since this residue was shown to be more accessible in the latent form, it was hypothesized that M-5 displaces and forces insertion of the RCL into the central β-sheet A upon binding. The epitope of MA-H4B3 includes residues on s1B, s2B, and s3B (Tyr210, Glu212, and Tyr241) as well as s2C (Arg271) and thus partially overlaps with the epitope for MEDI-579 that prevents Michaelis complex formation (90). However, since the epitope of MA-H4B3 was shown to be occluded by s1C in active PAI-1, it was suggested that the epitope only becomes available upon s1C detachment during latency transition. Thus, by binding to a prelatent form that exists in equilibrium with active PAI-1, MA-H4B3 accelerates the rate of RCL insertion, resulting in an enhanced latency transition. Another mAb, MA-159M12, binds to the N-terminal part of hA (Pro2, Leu3, Pro4, and Glu5) and accelerates the active to latent transition in rat PAI-1 (242). However, MA-31C9 that targets a similar region in human PAI-1 (His3, Ser6, Tyr7, and His10) has been shown to be non-inhibitory. Furthermore, introduction of the epitope of MA-159M12 in human PAI-1 only caused MA-159M12 to bind PAI-1 with low affinity. This observation emphasized that two mAbs generated toward the same region in different orthologs can display very divergent functional effects, either caused by subtle structural differences between human and rat PAI-1 or by subtle differences in the binding mode of these mAbs (35, 235–238, 248).

Alternatively, single-chain variable fragments have been derived from several of the aforementioned mAbs (249–251). Since crystallization attempts with these scFvs and their full-size mAbs remained unsuccessful thus far, a mutagenesis approach was used to identify epitope (235, 239) and paratope (251, 252) residues involved in the interaction between PAI-1 and the scFvs. Subsequently, this information was used to drive protein-protein docking in order to predict the structures of the respective PAI-1/scFv complexes (253). Notably, due to the *in vitro* and *in vivo* potency and cross-reactivity toward rodent PAI-1, one scFv (scFv-33H1F7) was developed into a bispecific diabody format together with scFv-TCK26D6 that inhibits the antifibrinolytic enzyme thrombin activatable fibrinolysis inhibitor (TAFI) (254). Further *in vivo* evaluation and comparison with the standard thrombolytic therapy (tPA) showed that the simultaneous administration of MA-33H1F7 and MA-TCK26D6 or the use of diabody Db-TCK26D6x33H1F7 holds a great promise in both prophylaxis and treatment of thrombotic disease (255, 256).

## CONCLUSIONS

Over the last four decades, the role of PAI-1 in various pathophysiological processes including cardiovascular disease has been extensively studied (257, 258). As the main physiological inhibitor of PAs, PAI-1 exerts an antifibrinolytic activity mainly by attenuating the plasmin-mediated fibrin degradation and thereby contributes to the pathogenesis of thrombotic cardiovascular diseases. Apart from being a regulator of the plasminogen activation system, PAI-1 has a pleiotropic biological function stemming from its ability to interact with ligands, such as the extracellular matrix protein vitronectin and cellular low-density lipoprotein receptors including LRP1. As a consequence, PAI-1 is also involved in the (patho)physiological processes associated with tissue remodeling, cell migration, and inflammation. Even though the precise role of PAI-1 in these diverse pathological processes is not always fully understood, elevated levels of PAI-1 have been shown to be related to the incidence, severity, and prognosis of various diseases. Therefore, significant clinical interest has been tied to PAI-1 as a putative drug target for the treatment of PAI-1-related pathologies. A very diverse collection of PAI-1 inhibitors has already been developed, including peptides, RNA aptamers, small organochemical molecules, antibodies, and antibody fragments. Even though several antagonists have been extensively characterized *in vitro* and *in vivo*, no PAI-1 inhibitors were approved to date for therapeutic use in humans. However, it should be noted that a few PAI-1 antagonists are currently proceeding through clinical trials, underscoring the

persistent clinical interest in safe and efficient modulators of PAI-1 activity. The growing number of available structures from PAI-1 in complex with biological ligands and inhibitors may provide access to useful information for guiding the development of the continuously growing segment of PAI-1 antagonists.

## AUTHOR CONTRIBUTIONS

MS and PJD contributed to the conception and the design of the manuscript. MS did the literature research, wrote the first draft of the review, and generated the figures and tables. PJD reviewed the manuscript and provided the critical feedback. All authors contributed to manuscript revision, read, and approved the submitted version.

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# Alpha2-Antiplasmin: The Devil You Don't Know in Cerebrovascular and Cardiovascular Disease

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Alpha2-antiplasmin (α2AP), the fast-reacting, serine protease inhibitor (serpin) of plasmin, was originally thought to play a key role in protection against uncontrolled, plasmin-mediated proteolysis of coagulation factors and other molecules. However, studies of humans and mice with genetic deficiency of α2AP have expanded our understanding of this serpin, particularly in disease states. Epidemiology studies have shown an association between high α2AP levels and increased risk or poor outcome in cardiovascular diseases. Mechanistic studies in disease models indicate that α2AP stops the body's own fibrinolytic system from dissolving pathologic thrombi that cause venous thrombosis, pulmonary embolism, arterial thrombosis, and ischemic stroke. In addition, α2AP fosters the development of microvascular thrombosis and enhances matrix metalloproteinase-9 expression. Through these mechanisms and others, α2AP contributes to brain injury, hemorrhage and swelling in experimental ischemic stroke. Recent studies also show that α2AP is required for the development of stasis thrombosis by inhibiting the early activation of effective fibrinolysis. In this review, we will discuss the key role played by α2AP in controlling thrombosis and fibrinolysis and, we will consider its potential value as a therapeutic target in cardiovascular diseases and ischemic stroke.

**Keywords: alpha2-antiplasmin, ischemic stroke, pulmonary embolism, deep vein thrombosis, fibrinolysis, thrombosis, plasmin**

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## ALPHA2-ANTIPLASMIN (α2AP) IS THE SERPIN THAT KILLS PLASMIN

α2AP (also known as α2-plasmin inhibitor, antiplasmin, serpinf2, plasmin inhibitor), is an ultrafast covalent inhibitor of plasmin (1–3) and, is a crucial member of the serine protease inhibitor (serpin) family. α2AP was first described by three different investigators as the fast-acting inhibitor of plasmin (4–6), who named it differently as α2-plasmin inhibitor (5), antiplasmin (6) and primary plasmin inhibitor (4, 7). α2AP is present in the blood at nearly half the concentration (∼1 μM) of its target enzyme precursor, plasminogen (∼2 μM) (6, 8). Structurally, α2AP is a unique serpin (**Figure 1**) with a 12 amino acid N-terminus, a central serpin domain and a C-terminal tail that is ∼55-residues long (10–12). Mechanistically, the C-terminal lysine residues of α2AP initially bind non-covalently to the kringle domains of plasmin to form a 1:1 stoichiometric complex (13). Plasmin then cleaves the reactive center loop of α2AP at $Arg^{376}$-$Met^{377}$ bond and forms an inactive, covalent complex (1–3, 14). However, mutations in the α2AP molecule or monoclonal antibodies against α2AP can change the plasmin-α2AP interaction to an enzyme-substrate reaction (an alternate mechanism of serpin interaction) where active plasmin leaves the complex after cleaving α2AP (15).

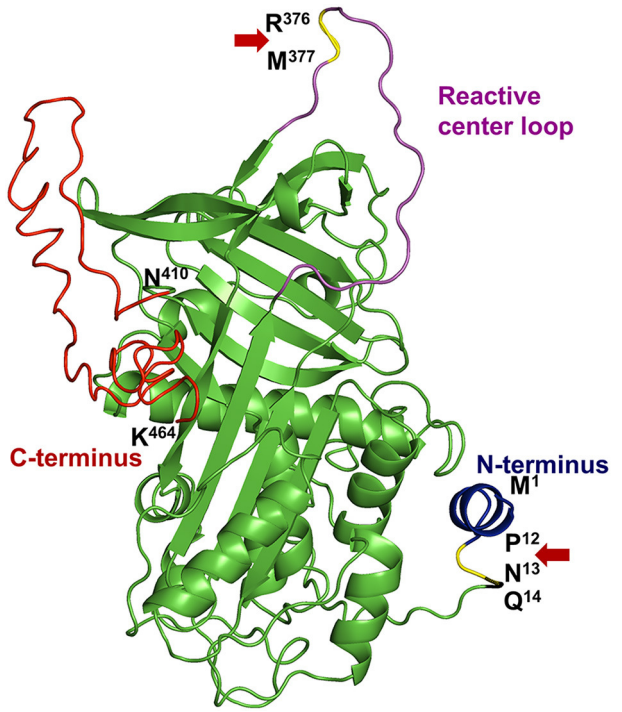


**FIGURE 1 |** Structural elements of α2AP. The protein structure of human α2AP was generated by homology modeling using an online I-TASSER server (9) based on the crystal structure of mouse α2AP (PDB: 2R9Y) (10). α2AP has a central serpin domain and, N- & C-terminal extensions with specific functions. The N-terminus (blue) of α2AP is cleaved in the circulation by APCE at the $Pro^{12}$-$Asn^{13}$ site ($P^{12}$-$N^{13}$, yellow highlighted and Red arrow) to form $Asn^{13}$-α2AP which represents 70% of blood α2AP levels. $Gln^{14}$ ($Q^{14}$) residue of α2AP is cross-linked to fibrin by transglutaminase enzyme, activated factor XIII. The reactive center loop (magenta) protrudes out from the central serpin domain (green) of α2AP. Plasmin cleaves at $Arg^{376}$-$Met^{377}$ bond ($R^{376}$-$M^{377}$, yellow highlighted, and red arrow) in the reactive center loop to form the inactive complex. A ∼55 residue long C-terminus (red) ($Asn^{410}$-$Lys^{464}$; $N^{410}$-$K^{464}$) of α2AP is important for initial binding with plasmin due to the presence of multiple lysine residues.

## α2AP EXPRESSION

α2AP is primarily synthesized by hepatocytes in the liver and released into the blood (16, 17). After synthesis, α2AP is enzymatically modified in the circulation at both the N- and C-terminus, which affects its fibrin-crosslinking and plasmin(ogen) binding capabilities respectively (18). Lower levels of α2AP are also detected in the human kidney, blood platelets, the gastrointestinal tract, muscles, lungs, placenta, and brain (cerebral cortex, hippocampus, and cerebellum) (https://www.proteinatlas.org/ENSG00000167711-SERPINF2/tissue) (19–21). α2AP is present among diverse species from mammals to birds and fish and there is significant protein sequence homology among various species such as humans, mice, bovine, etc. (11, 22–26). Human and mouse α2AP have similar kinetic constants for inhibition of autologous and heterologous plasmin *in vitro* (27). Administration of physiologic concentrations of human α2AP to α2AP-deficient ($\alpha2AP^{-/-}$) mice restores fibrinolytic inhibition and thrombosis to approximately normal levels (28). Since mouse and human α2AP have similar properties and cross-species reactivity, $\alpha2AP^{-/-}$ mice have provided an excellent translational model to examine the function of α2AP.

## α2AP AND CONTROL OF FIBRINOLYSIS

α2AP is covalently cross-linked to fibrin in the thrombus by activated factor XIII, a transglutaminase (29–31) which is a major source of the resistance of *in vitro* plasma clots to plasmin-mediated fibrinolysis (32–35). Once released into plasma, $Met^1$-α2AP is clipped by α2AP cleaving-enzyme (APCE) at the N-terminus to generate the truncated $Asn^{13}$-α2AP (**Figure 1**), which is incorporated into the fibrin network 13 times faster than uncleaved $Met^1$-α2AP (36). Plasmin activity is partially protected from α2AP inhibition when its lysine binding sites are engaged with fibrin in the clot (37–39) or on the surface of a cell, such as endothelial cells (40). The relative contribution of activated factor XIII-mediated fibrin-fibrin cross-linking (41) vs. fibrin-α2AP crosslinking to thrombus resistance has been debated (35). Most of the studies suggest that fibrin-α2AP cross-linking is the major determinant of fibrinolytic resistance of the thrombus (34, 42–44). Under *in vivo* conditions, activated factor XIII also may contribute to the dynamics of thrombosis through secondary interactions such as red blood cell retention (45, 46), or cross-linking of other fibrinolytic inhibitors such as plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI). *In vitro*, fibrinolysis assays showed that α2AP works synergistically with other major fibrinolytic inhibitors including TAFI or PAI-1 (47). *In vivo*, TAFI-deficient mice have variable effects in different pulmonary embolism models (48, 49). In contrast, $\alpha2AP^{-/-}$ mice showed greater fibrinolytic dissolution of *ex vivo* pulmonary thrombi as compared to PAI-1 deficient mice (50), suggesting that α2AP is the dominant contributor to thrombus resistance against fibrinolysis (50).

## ROLE OF α2AP IN ANIMAL MODELS OF CARDIOVASCULAR AND CEREBROVASCULAR DISEASES

### Role of α2AP in Ischemic Stroke

Human ischemic stroke is primarily caused by thrombotic arterial occlusion of a middle cerebral artery which interrupts the supply of blood, oxygen and nutrients, leading to ischemia, inflammation, breakdown of the blood-brain barrier and neuronal cell death (51). Higher blood levels of α2AP are associated with an increased risk of human ischemic stroke and may contribute to the failure of recombinant-tissue plasminogen activator (r-tPA) therapy for reperfusion in stroke patients (52, 53).

Experimental studies show that α2AP regulates fibrinolysis during ischemic stroke and has deleterious effects that worsen brain injury by enhancing thrombo-inflammatory mechanisms (54). In a mouse model of thromboembolic occlusion of the middle cerebral artery, Houng et al. (55) showed that increased

blood levels of α2AP reduced thrombus dissolution after treatment with r-tPA. Increased blood levels of α2AP also worsened brain infarction and brain swelling (55) (**Figure 2**). In contrast, α2AP inactivation (α2AP-I) enhanced r-tPA-mediated thrombus dissolution, in addition to reducing cerebral infarction, brain swelling and brain hemorrhage (55) (**Figure 2**). Treatment with the α2AP-I led to reduced TUNEL-staining, decreased caspase-3 expression and diminished breakdown of the blood-brain barrier. Subsequent studies showed that α2AP had dose-dependent deleterious effects in ischemic stroke in mice: it reduced thrombus dissolution and worsened cerebral infarction, brain swelling, and blood-brain barrier breakdown (56) (**Figure 2**). Increasing blood levels of α2AP enhanced ischemic brain injury, in part through a matrix-metalloproteinase-9 (MMP-9)-dependent mechanism (67). In contrast, both α2AP deficiency ($\alpha2AP^{-/-}$) or α2AP-I reduced brain infarction, hemorrhage and brain swelling (54–56). Also, α2AP deficiency or α2AP-I reduced microvascular thrombosis and MMP-9 expression (54, 56). Importantly, α2AP-I significantly enhanced acute (24 h) and longer-term (1 week) survival by comparison to r-tPA therapy and controls (54–56). Also, α2AP-I significantly improved neurobehavioral outcomes (54–56).

The effects of α2AP on ischemic stroke have also been studied in ischemic stroke, induced by mechanical arterial ligation or occlusion. Nagai et al. found that $\alpha2AP^{-/-}$ mice had reduced ischemic brain injury in comparison to control mice with normal α2AP levels after permanent ligation of the middle cerebral artery (57) (**Figure 2**). Similarly, inhibition of α2AP activity by intravenous infusion of plasmin/microplasmin or a monoclonal antibody significantly reduced focal ischemic brain injury after middle cerebral artery ligation in mice and hamsters (58). In ischemic stroke caused by middle cerebral artery photothrombotic occlusion, Suzuki et al. (68) found that doses of microplasmin that were equally effective for reducing ischemic cerebral infarction to r-tPA, caused less intracerebral bleeding and reduced tail bleeding time. However, higher doses of microplasmin that fully depleted circulating α2AP increased intracerebral bleeding (68, 69). It is interesting to note that α2AP deficiency or inhibition improved stroke outcomes in stroke models caused by an occluding thrombus and by mechanical ligation, suggesting the role of α2AP in stroke may extend beyond its role in thrombus dissolution.

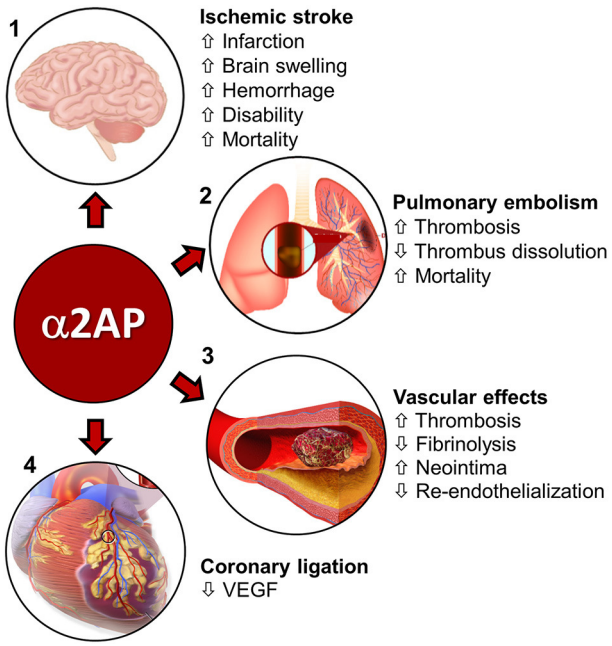


**FIGURE 2 |** Role of α2AP in cardiovascular diseases. **(1)** Ischemic stroke—α2AP increases cerebral infarction, brain swelling, hemorrhage, and, disability and mortality during ischemic stroke in mice (55–59). Image source-(https://www.injurymap.com/free-human-anatomy-illustrations). **(2)** Pulmonary embolism-α2AP decreases thrombus dissolution and, increases thrombosis in the lungs and mortality during pulmonary embolism in mice (28, 60–62). Image source—(http://apsfa.org/pulmonary-embolism/). (http://www.nhlbi.nih.gov/health/dci/Diseases/pe/pe_what.html). **(3)** In arterial (carotid artery injury) and venous (IVC, jugular vein) injury/thrombosis models, α2AP increases thrombosis and decreases fibrinolysis (63, 64). α2AP is also associated with increased neointima formation and reduced endothelialization over time (65). Image source—(https://commons.wikimedia.org/wiki/File:Blausen_0088_BloodClot.png). **(4)** Coronary artery ligation—α2AP decreases VEGF levels in mice, which affect pulmonary vascular permeability (66). Image source—modified from (https://commons.wikimedia.org/wiki/File:Blausen_0463_HeartAttack.png).

## Role of α2AP in Deep Vein Thrombosis

Since its discovery, the effects of α2AP were considered to be restricted to inhibiting the dissolution of formed thrombi; it was not thought to have a role in regulating thrombus formation or thrombosis (70). However, new data show that α2AP regulates thrombus initiation and thrombus development, and is required for the occurrence of stasis induced deep vein thrombosis in mice (64) (**Figure 2**). In mice with normal levels of α2AP, thrombosis induces plasmin generation (64), however thrombosis proceeds because the plasmin generated, is insufficient to overcome the anti-fibrinolytic effects of α2AP. In contrast, in α2AP deficiency, plasmin-driven fibrinolysis prevents the initiation and establishment of thrombosis. Indeed, in venous thrombosis induced by stasis (no blood flow), or by stenosis (reduced flow), $\alpha2AP^{-/-}$ mice do not develop thrombosis even after hours to weeks (64). The requirement for α2AP to enable the development of thrombosis appears to be mediated through its inhibition of plasmin because another plasmin inhibitor, ε-aminocaproic acid (64) will restore thrombus formation in the absence of α2AP. How α2AP affects other key components of venous thrombosis such as neutrophils, monocytes or coagulation system components needs further investigation.

In a jugular vein thrombosis model (endothelial injury) in mice, α2AP deficiency caused delayed occlusion and early reperfusion in comparison to wild type controls (**Figure 2**) (63). An α2AP-I alone or in combination with r-tPA increased the dissolution of human plasma thrombi in a jugular vein thrombosis model in rabbits (71). The combination of r-tPA with the α2AP antibody did not increase fibrinogen degradation (71)

suggesting that α2AP-I may enhance the specificity of fibrinolysis by plasminogen activators.

## α2AP in Pulmonary Embolism

Pulmonary embolism is caused when the thrombi formed in the deep veins of the legs or other sites detach from the vascular wall and travel to the lungs to cause serious, life-threatening complications (72). Therapy with r-tPA is limited to high-risk pulmonary embolism patients because clinical trials have shown r-tPA can cause serious or fatal bleeding (72). *In vivo* studies in mice and other animals have shown that thrombus dissolution can be achieved by α2AP-I with increased efficacy without increased bleeding. In a pulmonary embolism model in ferrets, α2AP-I by a monoclonal antibody increased experimental thrombus dissolution by r-tPA without increased fibrinogen degradation (60). Similarly, $\alpha2AP^{-/-}$ mice showed enhanced dissolution of pulmonary emboli made from $\alpha2AP^{+/+}$ or $\alpha2AP^{-/-}$ mouse plasma (61) (**Figure 2**), but in two different bleeding tests, $\alpha2AP^{-/-}$ mice did not show enhanced bleeding when compared to $\alpha2AP^{+/+}$ mice (61). Other hemostatic parameters including plasminogen, PAI-1 levels, hematocrit and fibrinogen levels were comparable in $\alpha2AP^{-/-}$ and $\alpha2AP^{+/+}$ mice (61). The role of α2AP was also studied in a pulmonary thrombosis model induced by photochemical irradiation of Rose Bengal in the jugular vein in $\alpha2AP^{-/-}$ mice (73) (**Figure 2**). In this model, α2AP deficiency was associated with decreased deposition of endogenous fibrin in pulmonary vessels and increased survival in comparison to wild type controls ($\alpha2AP^{+/+}$) (73). There were no differences in the bleeding time in $\alpha2AP^{-/-}$ mice treated by r-tPA (73). Inhibition of the crosslinking of α2AP to fibrin by activated factor XIII markedly enhanced fibrinolysis in experimental pulmonary thromboembolism (44). Finally, the comparative effects of an α2AP-I and r-tPA were examined in a humanized model of pulmonary embolism in mice. The α2AP-I alone showed comparable efficacy to high dose r-tPA in thrombus dissolution (28) (**Figure 2**). Treatment with r-tPA increased fibrinogen consumption and prolonged bleeding times but α2AP-I did not cause these effects. Combination treatment with very low dose r-tPA and α2AP-I was more effective at dissolving thrombi than a much higher dose of r-tPA alone, but the combination did not cause increased fibrinogen degradation and/or prolonged bleeding time (28) (**Figure 2**).

## α2AP in Arterial Injury and Thrombosis

The role of α2AP in arterial thrombosis was investigated by Matsuno et al. (63) by inducing endothelial injury of the murine carotid artery (**Figure 2**). α2AP deficiency did not change the time for thrombotic occlusion of the carotid artery but it significantly accelerated spontaneous reperfusion indicating that α2AP played a major role in arterial fibrinolysis (63). In studies of femoral arterial injury induced by electric current, α2AP did not appear to affect smooth muscle cell migration and neointima formation 2–3 weeks after injury (74). However, in carotid artery injury induced by Rose Bengal photo-irradiation, there was increased re-endothelialization and reduced neointima formation in $\alpha2AP^{-/-}$ mice in comparison with $\alpha2AP^{+/+}$ mice (65) (**Figure 2**). The increased re-endothelialization was attributed to the increased plasmin-mediated generation of vascular endothelial growth factor in $\alpha2AP^{-/-}$ mice (**Figure 2**). Finally, Ang II and N(omega)-nitro-L-arginine methyl ester (L-NAME)-induced vascular remodeling (perivascular fibrosis) was significantly decreased in $\alpha2AP^{-/-}$ mice compared with wild-type mice (75).

## α2AP in Coronary Artery Ligation

Coronary thrombosis is the primary cause of human myocardial infarction (76) but a reproducible model of coronary thrombosis has not been established in mice (77), which limits the translational relevance of experimental studies. Nevertheless, in a left coronary artery permanent ligation model in mice, α2AP deficiency was associated with increased plasmin-mediated vascular endothelial growth factor release, which enhanced pulmonary vascular permeability (**Figure 2**). Unfortunately, the effects of α2AP deficiency on thrombosis or fibrinolysis could not be assessed in this model. The role of α2AP in an ischemia-reperfusion model of myocardial infarction has not been studied.

## α2AP in Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is a rare but severe thrombotic disorder causing microvascular thrombosis in various organs and low platelet counts (78). In ADAMTS deficiency-induced experimental TTP in mice, there are increased levels of von Willebrand factor in the blood and microthrombi. However, increased plasmin activity by α2AP deficiency in mice causes increased proteolysis of von Willebrand factor and resolves the signs of disease (79).

# NON-FIBRINOLYTIC EFFECTS OF α2AP

α2AP may have non-fibrinolytic effects under different pathophysiological conditions. α2AP deficiency in mice decreases fibrosis in different models of fibrotic diseases (80, 81). Cancer is one of the major risk factors for deep vein thrombosis and α2AP enables deep vein thrombosis (64); however, it also restricts lymphatic remodeling and metastasis in a mouse model of cancer (82). In the brain, α2AP is expressed mainly by hippocampal neurons and is required for dendrite growth through p38 microtubule-associated protein kinase pathways in mice (83, 84). α2AP deficiency has been associated with impairment in motor function, cognitive function, anxiety, and depression-like behavior in mice (85). In a mouse model of Alzheimer's disease, chronic depletion of blood α2AP by antisense oligonucleotide treatment increased the activation of macrophage/microglial cells and increased fibrillar plaque, though it did not alter total plaque deposition (86). α2AP deficiency accelerates wound healing, perhaps through an increase in the release of vascular endothelial growth factor (87). Inhibitors of mouse α2AP increase liver repair after injury when compared to controls (88). α2AP deficiency also decreases arteriosclerosis after vascular injury (65).

## DEFICIENCY OF α2AP IN HUMANS—CONGENITAL AND ACQUIRED

Congenital deficiency of α2AP (Miyasato disease) in humans is very rare and has been associated with a phenotype of delayed traumatic or spontaneous rebleeding, usually in the form of hematomas or hemarthroses (89–91). Spontaneous cerebral bleeding has not been reported as an issue in humans (89–91). Bleeding in α2AP deficiency is usually controlled by standard measures or with tranexamic acid or ε-aminocaproic acid, which block plasmin-mediated fibrinolysis (91). Indeed, α2AP-deficient patients have successfully undergone heart surgery with these agents. Homozygous genetic deficiency has been described in a 62-year-old patient (92), indicating that the life-long absence of α2AP can be tolerated. Heterozygous individuals normally do not show bleeding phenotype unless there is a trauma or surgery; and sporadic reports of α2AP heterozygous deficiency in patients as old as 83 years are reported (93).

Congenital deficiency can be either quantitative with reduced protein levels or qualitative with reduced protein function but both are difficult to detect as routine coagulation tests and other hemostatic parameters are normal in patients. Quantitative deficiency of α2AP with reduced protein levels may be caused by a point mutation (α2AP-Paris Trousseau, 15% levels, and α2AP $Val^{384}$-Met, ∼50% levels), or a deletion (α2AP-Okinawa, <1%) or a frameshift mutation (α2AP-Nara, <1% level) (91). A qualitative or functional deficiency of human α2AP (Enschede) is due to an insertional mutation in the reactive center loop of α2AP (an additional alanine), which causes it to behave like a substrate of plasmin instead of an inhibitor (94, 95).

Acquired deficiency of α2AP may be caused by thrombolytic agents (e.g., plasminogen activators, plasmin, and microplasmin) or disease conditions such as severe liver disease and acute leukemia (54). Increased levels of α2AP are associated prospectively with an elevated risk of myocardial infarction (96) and ischemic stroke (52). In Alzheimer's disease patients, α2AP expression increases in the brain tissue and is associated with amyloid β plaques (20). During early studies of plasminogen activators, levels of α2AP were noted to fall before or synchronously with fibrinogen levels, which was a harbinger of clinical bleeding complications (97, 98). Indeed, α2AP supplementation was considered as an adjuvant to r-tPA therapy to prevent bleeding complications (99). However, more recent studies show that α2AP is the dominant inhibitor of physiologic fibrinolysis and that elevated levels of α2AP may be harmful in cardiovascular and cerebrovascular diseases (1, 26).

## THERAPEUTIC STRATEGIES TARGETING α2AP

Thrombosis is the leading cause of cardiovascular and cerebrovascular deaths (100). There have been two primary strategies for treating thrombotic diseases: anticoagulation to prevent thrombus formation or expansion, and fibrinolytics to dissolve existing thrombi. Anticoagulation therapy is widely used to prevent thrombosis in patients with myocardial infarction, ischemic stroke, deep vein thrombosis and pulmonary embolism (51, 72, 101). The value of anticoagulation is limited by bleeding and by the fact that it does not dissolve existing thrombi. Fibrinolytic (thrombolytic) therapy triggers the dissolution of existing thrombi. Plasminogen activators such as r-tPA, tenecteplase, and streptokinase are the most widely used fibrinolytic agents. The value of plasminogen activator therapy is limited by bleeding and other toxicities, which restrict therapy to a small subset of those who might benefit from thrombus dissolution for treatment of ischemic stroke, myocardial infarction, pulmonary embolism, etc. (51, 72) Experimental studies suggest that targeting α2AP is a novel paradigm for preventing thrombosis and dissolution of thrombi without compromising safety. Several strategies have been described including specific monoclonal antibodies, peptides, and microplasmin to neutralize the activity of α2AP.

### Monoclonal Antibodies Inhibiting α2AP Activity

Reed et al. (62, 71, 102) and Sakata et al. (103) reported the use of monoclonal antibodies to inhibit human α2AP activity to enhance thrombus dissolution. Mouse monoclonal antibodies caused spontaneous or r-tPA-mediated human clot dissolution (33, 62, 102, 103). A mouse monoclonal antibody inhibitor of α2AP synergistically increased fibrinolysis by r-tPA and other types of plasminogen activators increasing the potency of these agents by 20–80-fold (62). Despite increases in fibrinolysis, equipotent combinations of α2AP-I with very low dose plasminogen activators caused less fibrinogen breakdown than the plasminogen activator alone. As noted earlier, α2AP-I has been shown to enhance fibrinolysis in several different animal models of venous thrombosis, pulmonary embolism and ischemic stroke (28, 44, 55, 56, 59, 60, 62, 64, 71). More recently, in a humanized model of pulmonary embolism in mice, an α2AP-I (TS23, a monoclonal antibody that inactivates human α2AP), enhanced the dissolution of pulmonary emboli with a potency similar to higher dose r-tPA (3 mg/kg), though unlike r-tPA, this α2AP-I did not increase arterial or venous bleeding (28). The α2AP-I, TS23 prevented thrombus formation during venous stasis in mice (64). This α2AP-I has been tested in Phase I trials in humans (NCT03001544) and Phase II trials are planned.

### Microplasmin/Plasmin

Microplasmin is a truncated version of plasmin that contains only the catalytic domain (104). Microplasmin is a non-specific enzyme that is inhibited by α2-macroglobulin and by α2AP. Infusions of microplasmin will induce secondary depletion of α2AP, which were thought to be important for its function. A single bolus of plasmin/microplasmin in mice significantly reduced focal ischemic injury in mice (58). Microplasmin also reduced ischemic brain injury and neurological function in a rat middle cerebral artery thrombosis model (105) and improved behavioral outcomes in an embolic stroke model in rabbit (106). A Phase 1 trial in humans showed that α2AP inhibition by microplasmin induced a dose (0.1–5 mg/kg) related inhibition effect on α2AP activity in healthy volunteers (107). In a double-blind randomized phase II trial in stroke patients, 1–4 mg/kg

microplasmin neutralized blood α2AP by up to 80% and was well-tolerated, however, no effect on reperfusion or clinic outcome was observed possibly due to the small sample size (108). The development of microplasmin as a cardiovascular therapeutic was discontinued and, is used now for the clinical treatment of human retinal disease (109).

Infusion of plasmin will also deplete α2AP and this was used as an experimental treatment for thrombotic injury in mice (58). Marder et al. (110) used a similar strategy with a different hypothesis namely, that catheter-directed localized delivery of plasmin will increase the thrombus dissolution and then the released plasmin will be neutralized by α2AP in circulation so that plasmin will not have any side effects (111). Plasmin (4 mg/kg) dissolved thrombi in abdominal aorta thrombosis and did not increase bleeding (112). Plasmin of up to a dose of 8 mg/kg completely neutralized 60% of α2AP activity but also caused fibrinogen, factor VIII depletion, as well as increased bleeding (113). Safety trials for plasmin in patients with acute lower extremity arterial or bypass graft occlusion showed enhanced thrombus dissolution with bleeding events in <20%(114). Phase I/II of human plasmin in acute ischemic stroke patients showed that human plasmin was tolerable for plasmin dose up to 80 mg within 9 h of stroke, but recanalization was achieved in a limited number of patients (25%) (115). There have been no new reports of clinical development of plasmin.

### Inhibitors of APCE

APCE is a 97 kDa, prolyl-specific protease in plasma that cleaves $Met^1$-α2AP at $Pro^{12}$-$Asn^{13}$ to generate $Asn^{13}$-α2AP, which is cross-linked to fibrin 13 times faster than $Met^1$-α2AP (36). APCE shares a strong amino acid sequence homology to fibroblast activation protein, an integral transmembrane protein and may represent its soluble isoform or a derivative (36). It was proposed that the specific inhibitors of APCE can reduce the amount of α2AP crosslinking to fibrin and thus enhance fibrinolysis. Chemically modified peptide inhibitors of APCE increased fibrinolysis in plasma clot lysis assays (116, 117).

### α2AP Mimicking Peptides

Synthetic peptide mimicking α2AP regions have been tested as a competitive inhibitor of α2AP to interfere in factor XIII-mediated cross-linking, plasminogen binding and activation to achieve enhanced fibrinolysis or clot lysis *in vitro* (118–121). The effects of these peptides or inhibitors have been studied during clot formation, but not on preformed plasma clots or *in vivo* thrombi in experimental models.

## SUMMARY

Several different approaches have been taken to investigate the therapeutic potential of interfering with α2AP function to prevent thrombosis and dissolve existing thrombi. Systemic use of microplasmin has been limited by off-target effects and its use is currently limited to the treatment of retinal disease. Plasmin administration required catheter delivery by expert teams and only achieved limited thrombotic dissolution and recanalization in ischemic stroke. The development of α2AP mimicking peptides and APCE inhibitors appears uncertain as there are no reports of clinical trials. Monoclonal antibody approaches have been extensively evaluated in experimental models; they have shown high specificity, potency and the fewest off-target effects and are in development for Phase II trials in thrombotic diseases.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Since its original description by three different laboratories in 1976, our understanding of α2AP and its role in cardiovascular has evolved significantly. Epidemiologic and observational studies suggest that α2AP contributes significantly to the risk of thrombotic events in cardiovascular and cerebrovascular diseases. Numerous *in vitro* and *in vivo* studies, including studies in genetically-deficient mice and humans indicate that α2AP regulates endogenous and pharmacologic fibrinolysis. In addition, α2AP has been implicated in experimental models of wound healing, fibrosis, neuronal function, liver repair, and Alzheimer's disease. Disease-relevant models of thrombosis have shown that blocking α2AP function significantly enhances thrombus dissolution and improves outcomes, without causing bleeding. Taken together, these data suggest that therapeutically targeting α2AP has promise for both treatment and prevention of acute thrombotic cardiovascular and cerebrovascular diseases.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

SSa edited the manuscript. SSi and GR wrote, edited, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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# Serpins in Hemostasis as Therapeutic Targets for Bleeding or Thrombotic Disorders

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Bleeding and thrombotic disorders result from imbalances in coagulation or fibrinolysis, respectively. Inhibitors from the serine protease inhibitor (serpin) family have a key role in regulating these physiological events, and thus stand out as potential therapeutic targets for modulating fibrin clot formation or dismantling. Here, we review the diversity of serpin-targeting strategies in the area of hemostasis, and detail the suggested use of modified serpins and serpin inhibitors (ranging from small-molecule drugs to antibodies) to treat or prevent bleeding or thrombosis.

**Keywords: serpin (serine proteinase inhibitor), antithrombin (AT), protein Z-dependent protease inhibitor (ZPI), plasminogen activator inhibitor 1 (PAI-1), therapy, protease nexin I (PN-1)**

## INTRODUCTION

Coagulation (the formation of a solid fibrin clot at the site of vessel injury, to stop bleeding) and fibrinolysis (the disaggregation of a clot, to prevent the obstruction of blood flow) are interconnected pathways that both help to maintain the hemostatic balance. Coagulation is initiated at site of injury by exposure of tissue factor (TF), which forms a complex with circulating factor VIIa (FVIIa) and thus activates FIX and FX. In turn, FXa catalyzes the conversion of prothrombin into thrombin—the last enzyme in the coagulation pathway. Thrombin amplifies its own generation by activating cofactors FVIII and FV, which bind to their cognate enzymes (FIXa and FXa, respectively) - thereby considerably increasing their reactivity. Furthermore, thrombin promotes platelet aggregation and fibrinogen proteolysis – leading to the formation of a firm thrombus composed of polymerized fibrin and platelet aggregates (1). Fibrinolysis is triggered once the clot is formed, since fibrin acts as an essential cofactor for plasminogen activation and plasmin-catalyzed fibrin degradation (2). Since coagulation and fibrinolysis proceed by amplifying cascades of enzymatic reactions, these pathways must be finely tuned to prevent excessive bleeding or thrombosis. This fine tuning is performed by (i) cofactors that potentiate or modulate enzyme reactivity, and (ii) protease inhibitors (**Figure 1**).

Coagulation and fibrinolysis are predominantly regulated by protease inhibitors from the serpin superfamily (3). Antithrombin (AT, *SERPINC1*) is one of the most potent physiological anticoagulants by targeting most of the procoagulant enzymes (especially FXa and thrombin).

Other serpins are also involved in regulation of the coagulation cascade, such as heparin cofactor II (*SERPIND1*), protein Z-dependent protease inhibitor (ZPI, *SERPINA10*), protease nexin I (PN-1, *SERPINE2*), and protein C inhibitor (*SERPINA5*) (4–8). The fibrinolysis process is also regulated by serpins. Plasminogen activator inhibitor 1 (PAI-1, *SERPINE1*) inhibits tissue plasminogen activator (tPA) and α2-antiplasmin (*SERPINF2*) inhibits plasmin (9). Members of the serpin family are characterized by a common core structure consisting of 3 β-sheet, 7–9 α-helices, and a variably exposed reactive center loop (RCL). In their native conformation, serpins are in a thermodynamically metastable state and tend to adopt a more stable conformation through insertion of the RCL as a supplementary strand into β-sheet A. This conformational transition occurs either spontaneously (the latent conformation) or upon proteolytic cleavage in the RCL (the cleaved conformation) and serves as the mechanistic basis of protease inhibition by serpins (10). Hence, the serpin-protease reaction follows a branched pathway suicide substrate inhibition mechanism, involving recognition of the RCL as a substrate to form a reversible Michaelis complex. The P1-P1′ scissile bond [according to the nomenclature of Schechter and Berger (11)] is then cleaved, giving an acyl-enzyme intermediate in which the RCL's P1 residue is covalently bound to the protease's catalytic triad. At this point, the two competing branches of the pathway are either (i) hydrolysis of the acyl-enzyme intermediate to yield a cleaved serpin and free proteinase, or (ii) inhibition of the target protease *via* a well-described "mousetrap" mechanism (12). Indeed, upon cleavage, the serpin undergoes a rapid conformational change in which the RCL's N-terminal side inserts into β-sheet A, dragging the protease toward the opposite side of the serpin. Hence, this process traps the protease in an inactive covalent complex with the serpin. Even though a serpin's specificity is mainly determined by the RCL's sequence and its complementarity with the cognate protease's catalytic groove, it also involves exosite interactions between the two protein partners (13). Furthermore, serpin reactivity can be modulated by cofactors that boost the inhibition rate through an allosteric and/or template-based mechanism (14, 15).

Due to the pivotal role of serpins in the regulation of hemostasis and their particular mechanism of action, these proteins have been considered as promising therapeutic targets in the treatment of bleeding or thrombotic disorders (16, 17). Here, we aim at illustrating, through different examples, the broad range of novel strategies used to modulate the activity of serpins involved in coagulation and fibrinolysis. This review focuses principally on basic and preclinical research that might open up novel perspectives and meet unmet needs in the therapeutic management of hemostatic dysfunction.

## PART I: MODIFIED ANTITHROMBIN AS A REVERSAL AGENT FOR HEPARIN DERIVATIVES

Any discussion of serpins as therapeutic targets must necessarily cover the therapeutic use of heparins (18). Historically, heparin constituted the first class of drug that acted by modulating the reactivity of anticoagulant serpins. Thanks to knowledge of how heparin derivatives bind to antithrombin, how antithrombin is conformationally activated and how procoagulant enzymes are inhibited, the clinical development of heparin derivatives has continued—notably with the release of low-molecular-weight heparin and fondaparinux (the minimal pentasaccharide

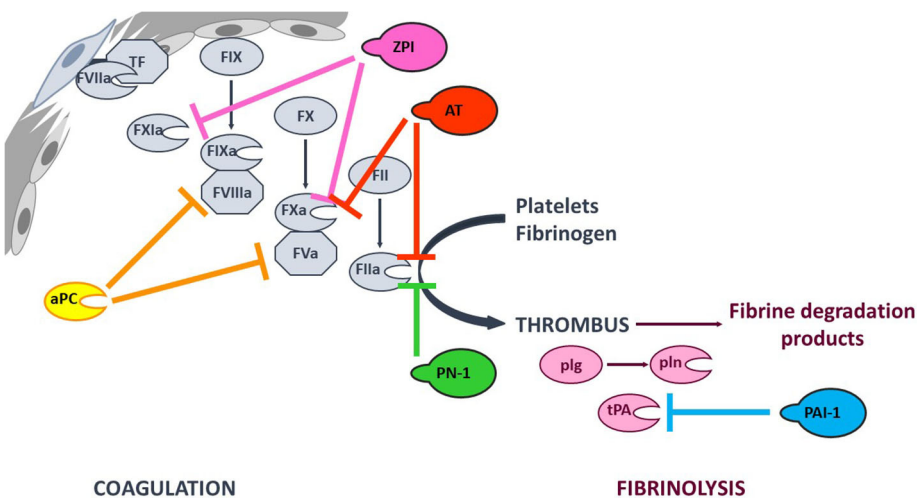


**FIGURE 1 |** Simplified overview of the coagulation and fibrinolysis processes. Coagulation is initiated at site of injury by TF-FVIIa complex that activates FIX and FX. FXa catalyzes thrombin (FIIa) formation, that amplifies its own generation by activating FXI and cofactors FVIII and FV. Thrombin also promotes platelet aggregation and fibrinogen into fibrin conversion to form a stable thrombus. Fibrinolysis is triggered once the clot is formed by tPA-induced plasminogen (plg) activation. The resulting plasmin (pln) enzyme is then responsible of fibrin degradation. The targets of the principal anticoagulant and antifibrinolytic serpins, as well as that of anticoagulant aPC are indicated with color capped arrows.

sequence required to potentiate AT anticoagulant activity) (19–21). When fondaparinux was approved for clinical use, it presented several advantages over unfractionated and low-molecular-weight heparins. It was safer because of its synthetic origin and its favorable pharmacokinetic profile, and it was at least as effective as heparins in several indications (22). However, fondaparinux's main shortcoming is the lack of an effective reversal agent to neutralize its anticoagulant activity (23). Protamine is the only agent approved for heparin-derivative reversal but it is ineffective for fondaparinux and can lead to adverse events (24, 25). Faced with the unmet need for a safe reversal agent for all heparin derivatives, our group engineered a recombinant inactive AT (riAT) that was devoid of anticoagulant activity but retained the ability to bind tightly to heparin. This was achieved by inserting a proline between arginine 393 and serine 394 residues within the RCL and by replacing asparagine 135 with a glutamine residue. The combination of these two mutations dramatically reduced the rate of FXa and thrombin inhibition (by about a 1000-fold) and significant enhanced (by about 3-fold) the AT's affinity for fondaparinux. This riAT was subsequently shown to act as specific, efficacious antidote to heparin derivatives by competing with native AT in plasma (**Figure 2**) (26). In *ex vivo* studies, riAT dose-dependently reversed anticoagulant activity of fondaparinux, unfractionated heparin, and low-molecular-weight heparin, and was found to be as effective as protamine in neutralizing heparin in a thrombin generation assay. Furthermore, the riAT did not exhibit any apparent prothrombotic activity in the latter assay (27). In *in vivo* models, the intravenous administration of riAT to mice treated with high-dose fondaparinux considerably reduced the fondaparinux plasma concentration (as assessed in a chromogenic assay) (26). Lastly, riAT has also been studied for its ability to neutralize the anticoagulant effects of heparin after a cardiopulmonary bypass (CPB) procedure. The results obtained in a rat model of CPB showed that (in contrast to protamine) riAT was not associated with adverse events (such as hemodynamic instability and histamine release) but was as potent as protamine in reversing heparin's anticoagulant activity. These properties suggested that riAT could safely replace protamine in a post-CPB anticoagulant/reversal protocol (28). Although riAT is promising, its immunogenicity and safety must be evaluated before it can be considered for therapeutic use in humans.

# PART II: SERPIN-TARGETING STRATEGIES IN THE TREATMENT OF HEMOPHILIA

Hemophilia is a bleeding disorder that results from a deficiency in FVIII (hemophilia A) or FIX (hemophilia B). Replacement therapy *via* the intravenous infusion of the missing factor is the current standard of care for hemophilia, although it requires frequent administrations and can induce the development of alloantibodies (inhibitors). The limitations of standard treatments have prompted a search for other therapeutic options that correct the bleeding phenotype by downregulating natural anticoagulants and thereby rebalancing hemostasis. This type of approach would be suitable for patients with hemophilia A or B and with or without inhibitors. In this respect, the inhibition of anticoagulant serpins (notably AT) and the use of modified serpins that promote a procoagulant response appear to be promising strategies (**Figure 1**) (17, 29).

## Inhibition of at in the Treatment of Hemophilia

The most advanced strategy in this field is currently in late-stage clinical development. It consists in silencing AT expression using a small interfering RNA (siRNA, fitusiran). The results of a Phase I clinical trial demonstrated a reduction in the AT level of more than 75% from baseline, together with enhanced thrombin generation in patients receiving the siRNA. In a Phase II trial, a reduction in the AT level of ∼80% was observed; this resulted to a fall in the median annualized bleeding rate to 1 in all patients, 48% of whom were bleed-free and 67% of whom did not experience any spontaneous bleeding episodes. Although fitusiran has shown promise, this siRNA might inherently limited by its mechanism of action, which induces inertia of action upon treatment initiation and discontinuation (30, 31). Accordingly, Barbon et al. developed an alternative strategy by engineering a bivalent single-domain antibody (sd-Ab) against AT, which binds to AT and abrogates its anticoagulant activity. The bivalent sd-Ab successfully restored thrombin generation in plasma and reduced blood loss in a FVIII-deficient mouse model of tail vein transection. Furthermore, long-term efficiency and safety of this bivalent sd-Ab in hemophilic mice have been demonstrated following gene transfer with an adeno-associated virus vector. Several weeks after vector administration, the sd-Ab was present

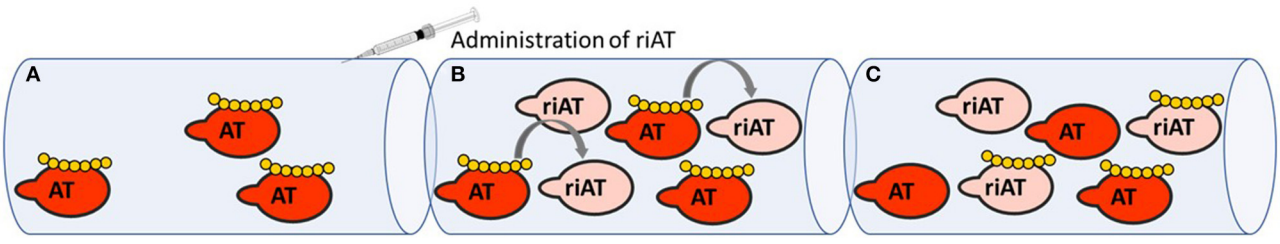


**FIGURE 2 |** Mechanism of action of riAT as reversal agent for heparin derivatives anticoagulant activity. **(A)** In situation of bleeding risk associated with the use of heparin derivatives (yellow), **(B)** administration of riAT (light pink) could displace heparin derivatives from endogenous AT (red) to riAT by a competition mechanism. **(C)** Hence, heparins are found trapped in an inactive complex with riAT, reducing thereby their apparent anticoagulant activity.

in the plasma of treated mice and retained its ability to rescue the bleeding phenotype. No signs of a prothrombotic state or an immune response were evidenced. The strength of this approach lies in the sd-Ab's direct mechanism of action and the short half-life of unbound sd-Ab- warranting its consideration as an alternative strategy in the management of hemophilia (32).

## Use of a Modified Serpin Targeting Activated Protein C in the Treatment of Hemophilia

Another strategy involves targeting activated protein C (aPC). In association with its cofactor protein S, aPC catalyzes the rapid degradation of the cofactors FVa and FVIIIa and thereby shuts down prothrombinase and tenase complex activity (33). Thus, inhibition of aPC might prolong thrombin propagation and restore the hemostatic balance in patients with hemophilia. The challenge was thus to design a modified serpin that potently inhibits aPC but cross-reacts minimally with procoagulant enzymes. After a structural analysis revealed that the catalytic groove of aPC is more permissive to bulky and basic residues than that of thrombin, Polderdijk et al. built a modified serpin on the scaffold of the Pittsburgh variant of a1-antitrypsin, in which the native P2-P1′ sequence Pro-Arg-Ser was replaced by the Lys-Arg-Lys sequence. This modified serpin failed to inhibit thrombin and conserved its strong inhibition of aPC, despite residual inhibitory activity against FXa and FXIa. Importantly, the modified serpin dose-dependently promoted thrombin generation in the presence of thrombomodulin in a thrombin generation assay and restored defective procoagulant activity in FIX-deficient mouse models of bleeding and thrombosis (34). The safety and efficacy of this modified serpin is currently being evaluated in a Phase I/II clinical trial in healthy volunteers and in patients with severe hemophilia (35).

## Protease Nexin-1 and Protein Z-Dependent Protease Inhibitor as Alternative Targets in the Treatment of Hemophilia

Although inhibition of AT or aPC appears to a promising strategy for the treatment of hemophilia, other anticoagulant serpins, such as PN-1 and ZPI have also been investigated in this respect. PN-1 is stored within α-granules in platelets and is released from activated platelets during the clotting process, where it acts as a potent inhibitor of thrombin and FXIa (7). ZPI is a plasma protein that inhibits two key procoagulant enzymes, FXa and FXIa. Whereas, FXIa is rapidly inhibited by free ZPI, FXa inhibition requires the presence of protein Z as a cofactor for ZPI (6). The anticoagulant properties of PN-1 and PZ/ZPI system have been evidenced in a mouse model of $FeCl_3$-induced thrombosis, in which deletion of the gene coding for PN-1, ZPI, or PZ significantly increased vessel occlusion (7, 36). The relevance of counterbalancing the bleeding phenotype in hemophilia by blocking the PN-1 or PZ/ZPI system was highlighted by work on double-knockout mouse models. When combined with FVIII deficiency, PN-1 gene deletion tended to limit tail bleeding after vein or artery transection (37). Similarly, PZ or ZPI gene deletion in FVIII-deficient mice attenuated bleeding in a tail vein rebleeding model (38). It was also shown that polyclonal antibodies against PN-1 or PZ (to neutralize ZPI-mediated FXa inhibition) were able to promote thrombin generation in plasma from patients with hemophilia (37, 38). These findings have prompted the search for PN-1 and ZPI inhibitors with potential therapeutic value. A modified ZPI bearing two alanine mutations (the first in the RCL's P1 position, precluding protease inhibition, and the second in the PZ binding site, enhancing the affinity for PZ by about 20-fold) have been designed to serve as a decoy for PZ. By competing with native ZPI for PZ, the modified ZPI was found to antagonize PZ/ZPI anticoagulant activity and promote thrombin generation in plasma from patients with hemophilia (39). With a similar objective, bivalent sd-Abs directed against PN-1 have also been described recently. These sd-Abs specifically bind to and inhibit PN-1, so that they successfully neutralize PN-1-induced elongation of the clotting time in normal or FVIII-deficient plasma (40). However, it remains to be determined whether the procoagulant response produced by neutralization of PN-1 or ZPI is sufficient to prevent bleeding, since to date, none of these strategies has proved its efficacy *in vivo*. Nevertheless, these targets might offer safer alternatives with regard to the risk of thrombotic adverse events; this emerged as a potential issue when a Phase II clinical trial of fitusiran had to be temporarily interrupted because of a case of fatal thrombosis (31). Although AT and PC deficiencies are undoubtedly associated with an increased risk of thrombosis, this link is much less conspicuous with ZPI and PN-1 deficiencies. In contrast to AT or PC, whose gene deletion results in perinatal or embryonic lethality, ZPI or PN-1 knockout mice are viable (7, 36, 41, 42). In addition, while clinical studies clearly evidence an increased risk of thrombosis in patients with AT or PC deficiency, they lead to conflicting results regarding the role of ZPI and fail to establish a convincing association between ZPI deficiency and thrombophilia (43, 44).

# PART III: TARGETING PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 IN THE TREATMENT OF THROMBOSIS

The serpin PAI-1 is also involved in hemostasis because it is the main regulator of fibrinolysis. PAI-1's antifibrinolytic properties (due to the inhibition of tPA) have prompted a search for PAI-1 inhibitors of value in the treatment of thrombotic disorders. In the vasculature, tPA is considered to be the main promoter of fibrinolysis; it binds to fibrin and converts plasminogen into plasmin, which in turn catalyzes fibrin proteolysis (45). Along with the use of anticoagulant drugs to downregulate the procoagulant pathway, treatment of thrombosis may require the use of thrombolytic therapy to restore blood flow in occluded vessels. To this end, tPA infusion has been successfully used as a thrombolytic agent in the management of acute myocardial infarction, ischemic stroke, and pulmonary embolism. However, it is also associated with life-threatening adverse bleeding events (46). Given that PAI-1 deficiency is characterized only by mild-to-moderate bleeding without spontaneous bleeding episodes

(whereas high plasma levels of PAI-1 have been linked to the development of thrombosis), PAI-1 inhibition might safely promote thrombolysis in the treatment of vascular thrombosis (45). Several research groups have attempted to develop small-molecule PAI-1 inhibitors (47, 48). The most intensively studied PAI-1 inhibitor to date is tiplaxtinin (also referred to as tiplasinin or PAI-039). This compound blocks the formation of the covalent complex between PAI-1 and its target proteases, and thus converts PAI-1 into a substrate. Thanks to docking studies and mutagenesis experiments, researchers have determined that the tiplaxtinin binding site is in the helix D/E region—close to PAI-1's vitronectin binding site. The results of *in vivo* studies in rats and dogs have shown that tiplaxtinin is a highly potent accelerator of thrombus re-permeabilization after acute carotid injury (49). Other molecules with anti-PAI-1 activity have been described; for example, our group identified annonacinone (a natural product found in plants of the Annonaceae family) via high-throughput screening. Like tiplaxtinin, annonacinone binds to an hydrophobic pocket between the β-sheet stand 2A and the α-helixes D and E (close to PAI-1's vitronectin binding site) and inhibits PAI-1/tPA complex formation by enhancing PAI-1's behavior as a substrate. Annonacinone is more potent than tiplaxtinin in inhibiting PAI-1 *in vitro*, with $IC_{50}$ values of 9 and 28 μM, respectively, in a chromogenic assay. Annonacinone promotes fibrinolysis in plasma (as assessed by thromboelastography) and potentiates tPA-induced thrombolysis in a murine model of $FeCl_3$-induced venule occlusion (50). However, the compound's highly challenging chemical synthesis and lack of efficacy against vitronectin-bound PAI-1 have not warranted the clinical development of annonacinone. Another family of chemical compounds as PAI-1 inhibitors was developed through virtual screening and structure-activity relationship studies. In particular, the molecule TM5275 which specifically inhibits PAI-1, presents the advantage to be orally bioavailable. Docking simulations suggested that TM5275 binds to strand 4 of the PAI-1's β-sheet A. Like tiplaxtinin and annonacinone, TM5275 inhibits PAI-1 activity *in vitro* by favoring its substrate behavior. Orally administered TM5275 exhibits antithrombotic activity in rodent and non-human primate models of thrombosis and does not significantly lengthen the bleeding time (51). However, in spite of these promising results, no clinical development of TM5275 has been reported yet. Finally, although several studies have suggested that small molecule PAI-1 inhibitors could provide benefits in the treatment of thrombotic events, whether used alone or in combination with tPA, none of them have entered clinical development as thrombolytic agents. Because in plasma, PAI-1 is predominantly bound to vitronectin that stabilizes its active conformation, one possible explanation is that most of these molecules inhibit PAI-1 less effectively when the latter is bound to vitronectin because of overlapping binding sites between inhibitors and vitronectin (52). In addition, PAI-1 is involved in multiple other functions than fibrinolysis, and PAI-1 inhibitors may exhibit toxicity or adverse drug reaction. Hence, specific regulation of PAI-1 antifibrinolytic activity is an intensively pursued but as-yet unattained objective.

## CONCLUSION

The studies presented in this review highlight the great interest in serpin-targeting strategies for future therapeutic application in the field of hemostasis. These strategies cover a broad range of approaches to regulate serpins, these include (i) silencing of serpin synthesis, (ii) inhibition of serpin activity by chemical compounds or sd-Abs, and (iii) bioengineering of modified serpin to refine its function or to serve as inactive bait for cofactors. Although the principal aim of hemostasis is to prevent exsanguination after injury, it also participates in the process of wound healing (53). Hence evaluation of the long-term effects of the serpin-targeting strategies on healing beyond their direct impact on coagulation and fibrinolysis will require special attention. The ongoing clinical trials for fitusiran and modified serpin as aPC inhibitor will provide valuable information on efficacy and safety of serpin-targeting strategies for the treatment of hemophilia and will surely motivate further basic and preclinical researches to continuously improve therapeutic management of bleeding and thrombotic disorders.

## AUTHOR CONTRIBUTIONS

EB performed literature searches and wrote the manuscript. CA, MR, MV, and DB reviewed and edited the review. All authors contributed to the article and approved the submitted version.

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# Age and Origin of the Founder Antithrombin Budapest 3 (p.Leu131Phe) Mutation; Its High Prevalence in the Roma Population and Its Association With Cardiovascular Diseases

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**Background:** Antithrombin (AT) is one of the most important regulator of hemostasis. AT Budapest 3 (ATBp3) is a prevalent type II heparin-binding site (IIHBS) deficiency due to founder effect. Thrombosis is a complex disease including arterial (ATE) and venous thrombotic events (VTE) and the Roma population, the largest ethnic minority in Europe has increased susceptibility to these diseases partly due to their unfavorable genetic load. We aimed to calculate the age and origin of ATBp3 and to explore whether the frequency of it is higher in the Roma population as compared with the general population from the corresponding geographical area. We investigated the association of ATBp3 with thrombotic events in well-defined patients' populations in order to refine the recommendation when testing for ATBp3 is useful.

**Methods and Results:** Prevalence of ATBp3, investigated in large samples ($n = 1,000$ and 1,185 for general Hungarian and Roma populations, respectively) was considerably high, almost 3%, among Roma and the founder effect was confirmed in their samples, while it was absent in the Hungarian general population. Age of ATBp3—as calculated by analysis of 8 short tandem repeat sequences surrounding *SERPINC1*—was dated back to XVII Century, when Roma migration in Central and Eastern Europe occurred. In our IIHBS cohort ($n = 230$), VTE was registered in almost all ATBp3 homozygotes (93%) and in 44% of heterozygotes. ATE occurred with lower frequency in ATBp3 (around 6%); it was rather associated with AT Basel (44%). All patients with ATE were young at the time of diagnosis. Upon investigating consecutive young (<40 years) patients with ATE ($n = 92$) and VTE ($n = 110$), the presence of ATBp3 was remarkable.

**Conclusions:** ATBp3, a 400-year-old founder mutation is prevalent in Roma population and its Roma origin can reasonably be assumed. By the demonstration of the presence

of ATBp3 in ATE patients, we draw the attention to consider type IIHBS AT deficiency in the background of not only VTE but also ATE, especially in selected populations as young patients without advanced atherosclerosis. We recommend including the investigation of ATBp3 as part of thrombosis risk assessment and stratification in Roma individuals.

**Keywords: antithrombin deficiency, antithrombin Budapest 3, founder effect, Roma population, cardiovascular disease, thrombosis**

## INTRODUCTION

Thrombosis is a common complex disease including arterial thrombotic diseases as myocardial infarction (MI), ischemic stroke (IS), or peripheral arterial occlusive disease and venous thrombotic events (VTE), which are the major contributors of morbidity and mortality in all developed countries (1). Antithrombin (AT) is a single-chain glycoprotein belonging to the SERPIN family, and it is one of the most important regulators of hemostasis (2). The mature protein, which is composed of 432 amino acids, has two glycoforms in the circulation, the major α- (90–95%) and a β-glycoform (<10%), the latter of which lacks *N*-glycosylation on Asn135 (Asn167 according to HGVS nomenclature, http://www.HGVS.org/varnomen) (3). Major targets of AT are serine-protease coagulation factors, and its effect is the most pronounced to thrombin and activated FX (FXa). Heparin, low molecular weight heparin, pentasaccharides, and heparan sulfate proteoglycan molecules are able to increase its inhibitory effect by more than 500-fold (4, 5). Heparin-binding site (HBS) of AT encoded by exon 2 of *SERPINC1* (the gene encoding AT) is responsible for the interaction between heparins and AT (2). Mutations in this region lead to functional AT deficiency, namely, type IIHBS with decreased heparin-AT interaction. Most frequent type IIHBS mutations are AT Basel (p.Pro73Leu), AT Padua (p.Arg79His), and AT Budapest 3 (p.Leu131Phe) (6–8). Heterozygous AT-deficient patients have a high thrombotic risk in general (9). The different AT deficiency subtypes, however, do not have the same clinical phenotype, and based on the results of some clinical studies, type IIHBS deficiency seems to exert a lower thrombotic risk (10–12). Additionally, the situation is even more complicated, since type IIHBS homozygotes present the most severe thrombotic symptoms among all AT-deficient patients (13). The first-line laboratory assay in the diagnosis of AT deficiency is a functional amidolytic test in which the inhibitory effect of AT on active FXa or thrombin is investigated in the presence of heparin (14, 15). This assay shows reduced AT activity in all AT deficiency types, including type II variants. Since progressive AT activity (performed by functional assay in the absence of heparin) is normal only in type IIHBS deficiency, this second-line test serves as an excellent tool for differentiating these patients from other AT-deficient individuals (16).

While thrombophilia testing, including AT deficiency, has its role in VTE, testing for thrombophilia in arterial thrombotic events (ATE) is rather controversial (17–20). Most of the guidelines are against inherited thrombophilia testing in ATE (21, 22). In unselected population, this approach has rationale. However, in a recent study, examining patients with MI with non-obstructive coronary arteries (MINOCA) thrombophilia was found in almost one-fourth of patients (23). According to a more recent concept of atherothrombosis, ATE and VTE have more in common than it was supposed earlier (24, 25). Based on the presence of the same structural elements of *ex vivo* arterial and venous thrombi and based on the findings of clinical studies in which dual pathway inhibition was beneficial, it is reasonable to suppose that certain risk factors of VTE may play a role also in arterial diseases (26–29).

Population-specific differences may also contribute to modify the general view of thrombophilia investigation in selected samples. Young individuals with ATE have different risk factor profile upon comparison with older patients in several clinical studies (30). The atherosclerotic burden is less remarkable in this group and not only the risk profile but also the clinical presentation may be different from older people (31). It seems that smoking has a more pronounced impact in the development of MI in the young, and among its several adverse effects, hypercoagulability seems to be the most important (32, 33). The presence of certain genetic determinants can also influence clinical decision making in certain populations. As an example, the prevalence of Factor V Leiden mutation (FVL, rs6025) shows high variability worldwide, and it is frequent in certain populations, while it is missing from others (34, 35). Beside AT Cambridge II, AT Basel and AT Budapest 3 (ATBp3) are the most frequent and well-known founder mutations in AT deficiency, which may increase the otherwise low prevalence of AT deficiency in the affected populations (6, 8). The founder effect of ATBp3 in the Hungarian population was confirmed previously by haplotype analysis, by investigating rs3138521, rs5877, rs5878, rs2227596, rs941989, rs1799876, rs677, and rs2227612 and short tandem repeat (STR) markers, namely *SERPINC1*-Alu5 and Alu8, D1S196, and D1S218 (8). In all ATBp3 individuals, the pathogenic "T" allele was associated with the same haplotype, while the normal "C" allele was associated with different haplotypes both in ATBp3 heterozygotes and in control subjects. ATBp3 homozygous patients shared one distinct Alu5 and Alu8 repeat number variations $(ATT)_6$ and $(ATT)_{15}$, respectively. The STR marker closer to *SERPINC1* (D1S218) showed bi-allelic distribution in ATBp3 homozygotes carrying $(AC)_{24}$ and $(AC)_{25}$ and D1S196 was variable. The age and origin of this mutation however have not been investigated, as yet.

The Roma ethnic group as the largest and most widespread minority group in Europe is one of the major subjects of ethnicity-based studies (36). The Roma population has significantly worse cardiovascular health as compared with the

general population in the same country, where they settled down (37). Roma people have a higher cardiovascular morbidity rate, they may be younger at the time of first thrombotic episodes and their risk factor profile is much worse as compared with the majority population (38–40). Among modifiable risk factors, smoking, dyslipidemia, and obesity were found significantly more frequent in Roma people (40, 41). According to a number of papers, the genetic load of Roma population to atherosclerosis and thrombosis were significantly higher comparing it with the general reference population, and among thrombophilia factors, the FVL allele was more frequent (i.e., 11.12% in the Roma vs. 4.29% in the general population) in most recent studies (42, 43). Several other susceptibility allele frequencies were also significantly higher in the Roma population among SNPs investigated in the same cross-sectional study (43). These results suggest that the combination of high genetic susceptibility for thrombotic diseases with the presence of an unfavorable environmental risk factor profile makes this ethnic population extremely sensitive for these diseases. It was, however not investigated, if the founder ATBp3 mutation, which—as opposed to the previously mentioned SNPs each with small effect size—represents a severe thrombotic risk, may be frequent in the Roma population.

The aims of the present study were to explore the frequency of the founder ATBp3 mutation in the Roma general population and compared data with the general reference sample from the corresponding geographical area. We aimed to calculate the age and origin of the founder ATBp3 in the general population and to test our hypothesis of its Roma origin. We aimed to investigate the association of type IIHBS mutations, including the prevalent ATBp3, with VTE and ATE in well-defined populations to see whether it is relevant to give a recommendation for more extensive testing for AT deficiency in certain populations.

# PATIENTS AND METHODS

## Study Population

### Reference Groups for Genetic Epidemiology Studies

A large number of individuals ($n = 1{,}000$, median age 55, range 29–102 years, male/female 46.6/53.4%) representing the general Hungarian population were recruited in the framework of the Hungarian General Practitioners' Morbidity Sentinel Stations Program (44). Another group ($n = 1{,}185$, median age 40 years, range 18–87, male/female 41.0/59.0%) representing the Roma general population living in segregated colonies in Northeast Hungary was also recruited. The details of the sampling methodology and data collected are described elsewhere (41, 45). These two samples were considered as reference samples.

For confirmatory analysis in a separate study (46), healthy Hungarian individuals ($n = 450$, median age 34 years, range 18–68, male/female 38.6/61.4%) free from any cardiovascular disease and considered a healthy reference group were recruited. Except for moderate hypertension (HT) in their case histories (blood pressure between 145/90 and 165/95 mmHg), all chronic diseases were considered exclusion criteria. Roma individuals ($n = 402$, median age 44 years, range 18–77, male/female 26.6/73.4%) from the corresponding geographical area as the general Roma population were also recruited.

### Patients' Groups Recruited for the Investigation of the Association Between Type IIHBS AT Deficiency and Thrombotic Diseases

To investigate the association of AT IIHBS deficiency and thrombotic diseases, different patients' groups were collected. First, $n = 243$ non-related genetically confirmed type IIHBS AT-deficient patients (index patients) diagnosed at our center between January 2007 and August 2020 and their affected family members (total $n = 328$), were involved. Inclusion criteria were low AT levels measured by anti-FXa heparin cofactor AT activity assay (hc-anti-FXa) and the confirmed type IIHBS mutation in the genetic test.

A second group including young adults with ST-elevation MI in their case histories below the age of 40 years was collected ($n = 119$, median age 36 years, male/female 79/21%) in order to investigate the association of hemostasis alterations with MI in this young age group, where the prevalence of occlusive arterial diseases was low. The diagnosis of MI based on the current guideline for universal definition of MI based on the biomarker detection plus the presence of clinical symptoms or ECG changes characteristics for myocardial ischemia or identification of coronary thrombus (47). Age-matched clinical control (CC) individuals ($n = 101$, median age 36 years, male/female 59/41%) were also recruited, who had undergone coronary angiography, but no coronary artery disease has been revealed and no MI was recorded in their case histories. Indication of coronary angiography for them was the clinical suspicion for stable angina, as they had at least one positive non-invasive test for assessment of myocardial ischemia. Detailed definitions of MI and CC patients' inclusion and diagnostic criteria and the circumstances of enrolling are described elsewhere (33).

Finally, a group consisting of consecutive, non-related patients with VTE in their case histories below the age of 40 was investigated ($n = 110$, median age 31 years, male/female 52/48%). Thrombosis was diagnosed and categorized into spontaneous and provoked according to guidance of the International Society of Thrombosis and Haemostasis (48). Clinical and laboratory data were collected as previously described (13). Patient clinical, laboratory, and genetic data were recorded in a database for further evaluation. As data collection by ethnic status is not allowed in health care services, we were not able to collect information about the ethnicity of the patients.

### Ethical Approval

All enrolled individuals were informed about the study according to the study protocol and gave written informed consent. Ethical approval for the study was obtained from Committee of the Hungarian Scientific Council on Health (3166/2012/HER and NKFP/1/0003/2005; 8907-O/2011-EKU, 61327-2017/EKU). The study was approved by the Ethical Committee of University of Debrecen, Hungary (reference No. 2462-2006), and all performed procedures were in accordance with the 1964 Helsinki declaration and its later amendments.

## Laboratory Methods

Fasting blood samples were collected into 0.109 mol/L citrate vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ, USA) at least 3 months after the acute thrombotic episode or coronarography (if relevant) and stored at −80°C until use. Native blood samples were also collected and stored at −80°C. For diagnosing AT deficiency, hc-anti-FXa and progressive (p-anti-FXa) AT activity were measured (Labexpert Antithrombin H+P, Labexpert Ltd, Debrecen, Hungary, reference intervals 80–120 and 82–118%, respectively) on a Siemens BCS-XP coagulometer. AT antigen was measured by immunonephelometry (Siemens, N Antiserum to Human Antithrombin III, Marburg, Germany; reference interval 0.19–0.31 g/L). Protein C activity and free protein S antigen were measured by commercially available assays from Siemens (PC chromogenic and Innovance free PS antigen). Fibrinogen was measured by the Clauss method using Labexpert reagent. Measurement of other plasma or serum parameters was executed by routine methods using Roche reagents and instruments (Roche Diagnostics GmbH, Mannheim, Germany). DNA was isolated from the buffy coat of citrated blood samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) in case of patients' samples. DNA was isolated using the MagNA Pure LC system (Roche Diagnostics) with a MagNA Pure LC DNA Isolation Kit-Large Volume according to the manufacturer's instructions in case of the reference samples.

Sanger sequencing searching for *SERPINC1* mutations in the exons, the flanking intronic regions and in the promoter was executed by ABI3130 Genetic Analyzer and Sequencing Analysis 5.4 software (Thermo Fisher Scientific, Carlsbad, CA, USA) in AT-deficient group and in case of MI, CC, and VTE samples upon abnormal hc-anti-FXa AT activity results according to protocols described earlier (8). ATBp3 mutation, FVL, and the prothrombin 20210G>A polymorphisms were determined according to protocols developed in our laboratory on Roche LightCycler 480 instrument by using real-time PCR and melting curve analysis in all groups.

For the determination of the age of ATBp3, genomic DNA from 36 unrelated index subjects with ATBp3 mutation and their family members out of the large AT-deficient group were haplotyped for alleles at eight STRs; seven dinucleotide and one tetranucleotide repeats; heterozygosity >0.7, namely D1S212 (4.92 ΔcM), D1S2659 (3.27 ΔcM), D1S218 (0.73 ΔcM), D1S2790 (1 ΔcM), D1S1165 (2.43 ΔcM), D1S2815 (2.53 ΔcM), D1S196 (7.35 ΔcM), and D1S460 (54.8 ΔcM) flanking the disease locus. Distances of the markers from ATBp3 are given in the brackets (**Figure 1**). The fragments containing the STRs were amplified by PCR, and the amplicons were tested for length polymorphism by capillary electrophoresis on an ABI3130 Genetic Analyzer (Thermo Fisher Scientific). Analysis of STR sequences was implemented by the GeneMapper v4.1 software (Life Technologies). Two hundred individuals out of the $n$ = 1,000 Hungarian reference population as control subjects and $n$ = 94 individuals out of the $n$ = 1,185 Roma reference samples were also tested for these STR markers.

## Statistical Analysis and Computations

Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to examine the normality of data distribution. Results of continuous variables were expressed as median and interquartile range. Differences between groups were analyzed by Student's $t$-test and ANOVA when normally distributed or by Mann-Whitney test and Kruskal-Wallis test in the case of non-normal distribution. Significance values were adjusted by the Bonferroni correction for multiple comparisons. Differences in category frequencies were evaluated by $\chi^2$-test. A $p$-value of 0.05 or less was considered to indicate statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 23.0), Chicago IL, USA.

The age of an allele is the duration of time elapsed since it was created by a mutation (49). Age estimates can be made by different methods considering variations at marker loci and allele frequency. Linkage disequilibrium (LD) index values ($\delta$) were calculated according to Bengtsson and Thomson (50). Genetic distances [recombination fraction ($\theta$)] were obtained from physical distances (Genome Reference Consortium Human Build 38) between STRs and *SERPINC1* by applying a conversion factor of 1.17 cM/Mb derived from the Marshfield map (51). The age of ATBp3 [in generations ($g$)] was initially estimated by two moment methods. The first one is based on the algorithm of Risch et al., where $g = \log\delta/log(1-\theta)$ (52). The second method generates a Markov transition matrix ($K$), which gives the probability that, in a single generation, any one haplotype will be transformed into any other one. $K$ is calculated as the weighted sum of matrices corresponding to the recombination ($R$), mutation ($M$), and no event occurring ($I$): $K = \theta R + \mu M + (1-\theta-\mu)I$ (53). Under this model, it was possible to correct the decay of LD over generations for the mutation rate ($\mu$) at marker loci (under the assumption of $\mu = 2.1 \times 10^{-3}$ for D1S1165 and $\mu = 5.6 \times 10^{-4}$ for dinucleotide-repeat STRs) (54). The number of generations that have passed since the foundation event was estimated by multiplying the state vector by $K$ iteratively until the observed proportion of ancestral haplotypes on mutation-bearing chromosomes were reached. The number of times that $K$ had been multiplied yielded the estimate of the age ($g$).

Besides simple parametric age estimators, we reanalyzed LD data in a Bayesian perspective using the Markov chain Monte Carlo method (55), implemented in the DMLE+ program, version 2.3 (available at dmle.org), and a second likelihood method (56), which estimates the distance (in generations) from probands sharing a common haplotype to the most recent common ancestor (MRCA) by the means of the ESTIAGE software. By DMLE+, the posterior probability distribution ($P$) of the ATBp3 (c.391C>T) mutation's age was inferred assuming a proportion of mutation-bearing chromosomes in our sample ($f$ = 0.012) that conservatively represents the expected allele frequency in the Hungarian population and allows obtaining good convergence of $P$. The population growth parameter ($r$ = 0.079) was calculated by the equation $T1 = T0 \times eg \times r$, in which T1 is the size of the actual population (9.8 million of inhabitants), T0 is the estimated size of the population at the time of the founder event (first half of the XVII century; 3.5

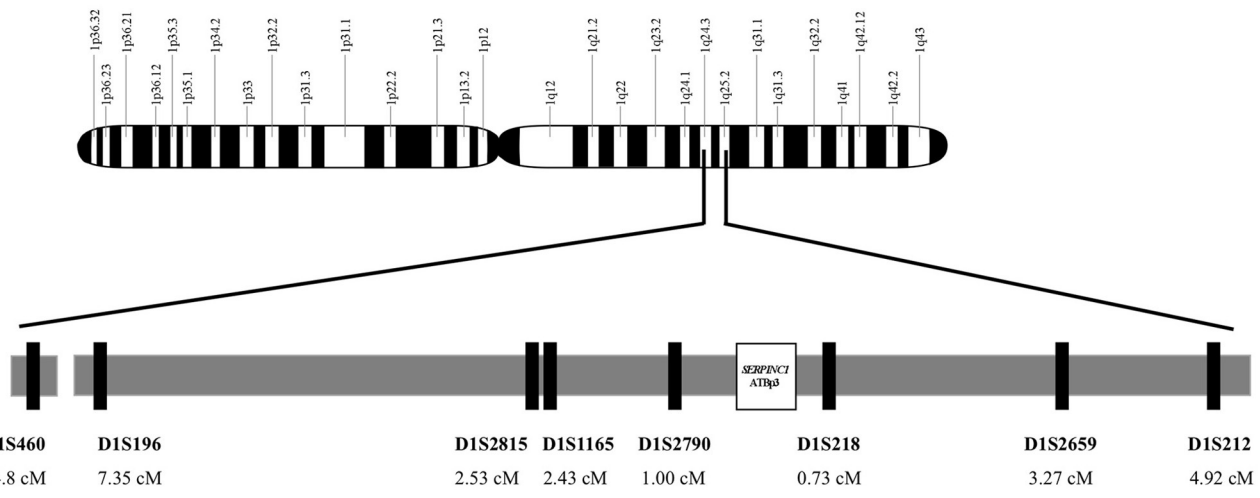


**FIGURE 1 |** Genomic map of chromosome 1. Localization and position of *SERPINC1* gene and the analyzed short tandem repeat markers on region 1q24-25. Short tandem repeat markers are D1S460 $(CA)_n$, D1S196 $(AC)_n$, D1S2815 $(CA)_n$, D1S1165$(CTTT)_n$, and D1S2790 $(CA)_n$ locate proximal to *SERPINC1* and D1S218 $(AC)_n$, D1S2659 $(CA)_n$, and D1S212$(CA)_n$ locate distal to *SERPINC1*.

millions) and $g$ is the number of generations passed since the founder event that had been estimated by the moment methods (57). The stepwise mutation model for STRs (mean $\mu = 10^{-3}$) was adopted to keep in consideration microsatellite instability while running ESTIAGE.

# RESULTS

## Results of Genetic Epidemiology Studies

### Age and Origin of the Type IIHBS Antithrombin Budapest 3 Mutation

As the founder effect of ATBp3 mutation was confirmed previously, we aimed to investigate the age and origin of the most recent common ancestor (MRCA) of the ATBp3 (c.391C>T) and to provide a plausible historical and demographic scenario in which the founder effect could have started. By investigating the $n = 36$ unrelated ATBp3 mutants and their family members ($n = 70$, altogether $n = 106$) a fully conserved ancestral haplotype: D1S212: 20; D1S2659: 11; D1S218: 24/25; D1S2790: 20; D1S1165: 13; D1S2815: 18; D1S196: 12; D1S460: 7 (where numbers after the markers represent the most frequent repeat number variations) was identified in 14 independent chromosomes (five independent chromosomes if only the index patients were taken into consideration, **Supplementary Table 1**). In addition, related haplotypes, likely derived from the ancestral one by either recombination or mutation at the flanking markers, were found in the remaining chromosomes. We analyzed the frequencies of repeat alleles associated with ATBp3 and compared them with the corresponding allele frequencies in 200 unrelated control Hungarian subjects recruited from the general Hungarian population who did not carry ATBp3 mutation. The most frequent haplotype in the Hungarian population for the investigated markers were as follows: D1S212: 20; D1S2659: 15; D1S218: 25; D1S2790: 21; D1S1165: 11; D1S2815: 18; D1S196: 12; D1S460: 7. As it is shown by LD index values obtained for the STR markers, the correlation between D1S218, D1S2790, D1S1165, and D1S2815 markers with ATBp3 allele was higher than in the case of markers D1S2659, D1S196, D1S212, and D1S460 (**Table 1**). The mean ± SD overall age estimate for the *SERPINC1* c.391C>T mutation, based on the LD data for the eight STRs, was 11.5 ± 5.47 $g$ according to the first moment method (**Table 1**) and it was 11.8 ± 7.1 $g$ according to the iterative procedure of the second one. The Markov chain Monte Carlo method provided similar estimation of 13 $g$ (95% credible set (CS): 3–42 $g$), and the age estimation was 14 $g$ (95% CS: 5–37 $g$) by the second likelihood method.

Assuming an average of 25 years per generation and that the average birth year of the mutation carriers investigated is 1990, the present results suggest MRCA bearing the c.391C>T mutation back to middle of the XVII century. This dating points to the origin of the founder effect at a very turbulent period of the history of Hungary with settlement of different populations including Roma tribes.

### Prevalence of Antithrombin Budapest 3 Mutation in the General Hungarian and Roma Population

As the age estimation of ATBp3 rose up the hypothesis of its Roma origin, we investigated the prevalence of this mutation in a large group of Roma individuals ($n = 1{,}185$) representing the Roma general population living in Northeast Hungary. The carrier frequency was 2.80% in this group, which was a considerably high as compared with our group representing the general Hungarian population ($n = 1{,}000$), where no ATBp3 carriers were found. To confirm our findings, second sets of samples were collected independently from the first recruitment. $N = 402$ Roma individuals and $n = 450$ apparently healthy individuals from the corresponding geographical area together with demographic and clinical data were involved. While, again,

**TABLE 1 |** Estimation of the age of the c.391C>T mutation in the *SERPINC1* gene by different methods.

| Marker | Distance | Repeat number variations | LD | MRCA age | | Founder event of ATBp3 (date) | |
|---|---|---|---|---|---|---|---|
| | ΔcM | | δ | *g* | *y* | Date | 95% CS |
| D1S212 | 4.92 | 20 | 0.326 | 11.5 ± 5.47 | 287 ± 137 | 1,703 ± 137 | NA |
| D1S2659 | 3.27 | 11 | 0.628 | | | | |
| D1S218 | 0.73 | 24/25 | 0.945 | | | | |
| D1S2790 | 1.00 | 20 | 0.899 | | | | |
| D1S1165 | 2.43 | 13 | 0.741 | | | | |
| D1S2815 | 2.53 | 18 | 0.731 | | | | |
| D1S196 | 7.35 | 12 | 0.470 | | | | |
| D1S460 | 54.8 | 7 | 0.086 | | | | |
| All haplotypes | | DMLE+ | | 13 | 325 | 1,665 | 940–1,915 |
| All haplotypes | | ESTIAGE | | 14 | 350 | 1,640 | 1,065–1,865 |

*Distance (ΔcM, centiMorgan) is the genetic distance between the marker and the SERPINC1 locus on chromosome 1. Repeat number variations represent the most frequent repeat numbers in homozygous ATBp3 probands. Historical date of the founder event that has likely increased the frequency of the c.391C>T mutation in the Hungarian population, based on the MRCA age and a mean date of birth for mutation's carriers equal to 1990.*

*NA, not applicable; CS, credible set (Bayesian analog of confidence interval in classical frequentist statistics); LD, linkage disequilibrium between the allele and the SERPINC1 c.391C>T mutation; MRCA, most recent common ancestor of the c.391C>T-mutation-bearing chromosomes, whose age is expressed in generations (g) and years (y), assuming a mean value of 25 y/g; DMLE+, software implementing the Markov chain Monte Carlo algorithm to allow Bayesian estimation of the MRCA age; ESTIAGE, software implementing a likelihood-based method of inferring the MRCA age from multilocus haplotypes.*

no ATBp3 was detected within the general Hungarian group, the carrier frequency of ATBp3 was 2.74% in the Roma group. In this latter group, 10 heterozygotes (age range 24–60 years) and one homozygote (age 28 years) were registered (**Table 2**). None of these patients suffered VTE, as yet, and two of them had MI in their case histories. However, some of them were very young at the time of data collection and did not present any cardiovascular risk factors. Notably, almost all Roma ATBp3 individuals had a BMI greater than 25 kg/m$^2$.

To confirm the founder effect in the Roma population the same STR markers were analyzed in $n = 44$ ATBp3 carriers and in $n = 50$ non-carriers selected from the two Roma cohorts. The most frequent repeat number variations matched with the previously found distinctive repeat numbers (marked in gray in the upper right corner of **Figure 2**) except for markers D1S1165 and D1S212. As all but one Roma ATBp3 mutation carriers investigated for STR markers were heterozygous for the ATBp3 mutation, they also have a normal "C" allele, which might be associated with various repeat numbers. The most frequent haplotype in the Roma general population for the investigated markers were as follows: D1S212: 16; D1S2659: 15; D1S218: 25; D1S2790: 22; D1S1165: 12; D1S2815: 18; D1S196: 12; D1S460: 10. Other types of AT deficiency were not revealed within our Hungarian and Roma reference groups.

## Results of the Investigations on the Association Between Type IIHBS AT Deficiency and Thrombotic Diseases

### Prevalence of Thrombosis Among Type IIHBS AT-Deficient Patients

Within our large cohort of AT-deficient patients ($n = 435$), the ratio of type IIHBS AT deficiency was extremely high; it was 75.4% ($n = 328$ type IIHBS AT-deficient patients, including $n = 19$ AT Basel, $n = 30$ AT Padua and $n = 279$ ATBp3 individuals). Most of them were index patients, only $n = 85$ individuals were found during family screening. As detailed clinical information was eligible in the case of $n = 230$ type IIHBS AT-deficient patients from the point of view of cardiovascular events, we took only them into consideration for further analysis. Current median age of our type IIHBS cohort was 34 years; however, ATBp3 homozygous patients were significantly younger due to their early diagnosis of AT deficiency because of early thrombotic episodes (**Table 3**). Accordingly, their age at the first thrombotic episode was also significantly lower as compared with others. There were more relatives registered in ATBp3 heterozygous group than in other groups, due to some positive cases found by family screening of ATBp3 homozygotes. The prevalence of thrombotic events, as expected, was significantly higher in the group of ATBp3 homozygotes as compared with any other groups. VT events (VTE) were registered in almost all patients with ATBp3 homozygosity (93%), while the frequency of VTE was below 50% in other groups. The frequency of provoked thrombosis (thrombosis during pregnancy or childbirth, trauma, surgery, oral anticoncipient use within 1 month of the thrombotic episode) was rather low in our study group; $n = 2$ patients in the AT Padua group, $n = 2$ patients in ATBp3 homozygotes group, and $n = 11$ among ATBp3 heterozygotes. There was only one patient with unprovoked VTE among AT Basel patients. VTE was recurrent in one-third of the patients in average, and there were no significant differences among the different groups. The highest number for recurrent VTE was 7, registered in an ATBp3 homozygous patient. Three to six episodes of VTE were registered in other ATBp3 homozygotes and in some ATBp3 heterozygotes, while a maximum of two VTE episodes were described in AT Padua. Isolated PE was found in eight ATBp3 homozygotes, in three ATBp3 heterozygotes, and in three AT Padua patients, and one ATBp3 homozygous patient suffered

**TABLE 2 |** Demographic and clinical data of Roma carriers of antithrombin Budapest 3 collected from the confirmatory study.

| ID | Gender | Age (year) | ATBp3 genotype | ATE or VTE in case history | Presence of CV risk factors |
|---|---|---|---|---|---|
| 1 | Male | 24 | Heterozygote | No | Obesity |
| 2 | Male | 27 | Heterozygote | No | No |
| 3 | Female | 28 | Homozygote | No | No |
| 4 | Female | 35 | Heterozygote | No | Obesity |
| 5 | Female | 42 | Heterozygote | No | Obesity, HLP |
| 6 | Female | 53 | Heterozygote | Yes (MI) | Obesity, DM, HT |
| 7 | Female | 54 | Heterozygote | No | No |
| 8 | Male | 56 | Heterozygote | No | Obesity, HT, HLP |
| 9 | Female | 57 | Heterozygote | No | DM, HT |
| 10 | Female | 58 | Heterozygote | Yea (MI) | Obesity, HT, HLP |
| 11 | Female | 60 | Heterozygote | No | Obesity, DM, HT |

*The term CV (cardiovascular) risk factors cover hypertension (HT), diabetes mellitus (DM), hyperlipidemia (HLP), smoking, and obesity (BMI above 25 kg/m$^2$).*

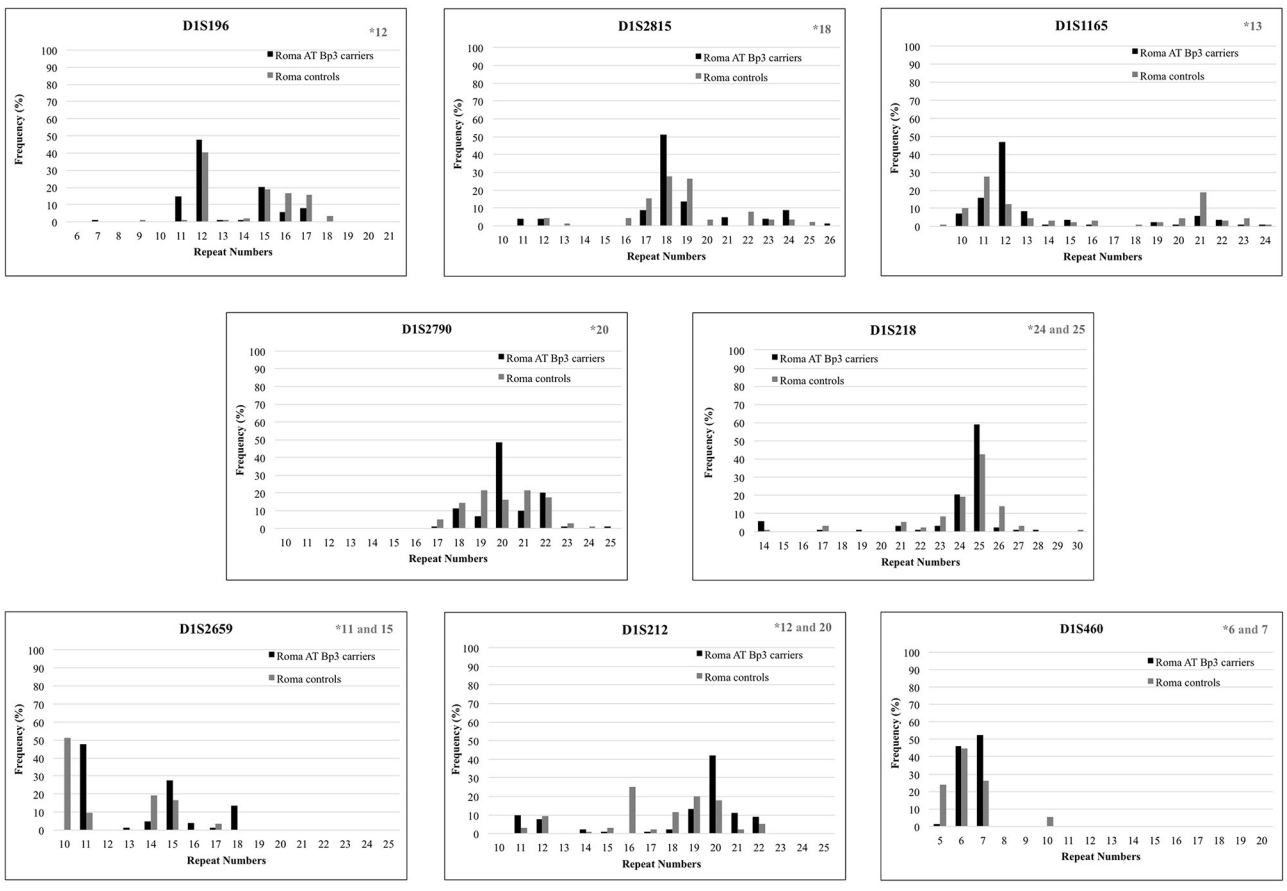


**FIGURE 2 |** Repeat number variations of eight short tandem repeat markers in Roma ATBp3 carriers and in Roma controls. The most frequent repeat number variations detected at ATBp3 homozygous patients previously are marked in gray in the upper right corner of each graph.

from three episodes of PE. We investigated whether carriership of FVL influences the clinical phenotype in ATBp3 patients. In ATBp3 homozygotes, only FVL heterozygous patients ($n$ = 4) were registered. Clinical phenotype of ATBp3 homozygosity seemed severe enough not to be influenced by the presence or absence of a heterozygous FVL as clinical presentation of thrombosis in terms of the number of thrombotic episodes and the age at first symptoms were similar in patients with FVL and in wild type patients. In the group of ATBp3 heterozygotes, five patients were registered as FVL homozygotes. Among them, only

**TABLE 3 |** Clinical and laboratory characteristics of the type IIHBS antithrombin-deficient cohort.

| | All ($n = 230$) | AT Basel ($n = 9$) | AT Padua ($n = 26$) | AT Budapest3 HoZ ($n = 44$) | AT Budapest3 HeZ ($n = 151$) | *p*-value |
|---|---|---|---|---|---|---|
| Current age years[a] (median; min–max); IQR | 34 (2–76); 26 | 51 (28–65); 23 | 42 (18–70); 23 | 24 (2–69); 21 | 34 (2–74); 26 | <0.001 |
| Male/female (*n*) | 93/137 | 3/6 | 6/20 | 22/22 | 62/89 | 0.162 |
| Index pts/relatives[b] (*n*) | 162/68 | 7/2 | 22/4 | 36/8 | 97/54 | 0.041 |
| Any thrombotic event Y/N[c] (*n*) | 131/99 | 5/4 | 12/14 | 41/3 | 73/78 | <0.001 |
| Age at first thrombotic event years[d] (median; min–max); IQR | 26 (0–68); 24 | 42 (15–51); 34 | 50 (22–66); 17 | 15 (0–48); 16 | 33 (0–68); 19 | <0.001 |
| VT %[e] | 52 | 11 | 38 | 93 | 44 | <0.001 |
| Recurrent VT % within VT patients | 35 | 0 | 29 | 38 | 34 | 0.831 |
| ATE %[f] | 8 | 44 | 8 | 5 | 6.6 | <0.001 |
| Ratio of FVL carriers HeZ/HoZ % | 21/2 | 0 | 24/0 | 9/0 | 25/3 | 0.261 |
| Ratio of FII 20210A carriers HeZ % | 3.9 | 0 | 7.6 | 5.9 | 2.9 | 0.738 |
| Antithrombin activity %[f] (median; min–max); IQR | 56 (9–78); 11 | 57 (44–74); 11 | 57.5 (40–74); 12 | 17 (9–59); 10 | 57 (37–78); 8 | <0.001 |
| Antithrombin progressive activity %[g] (median; min–max); IQR | 85 (56–126); 15 | 100 (73–117); 17 | 105 (73–126); 23 | 73 (56–100); 11 | 85 (60–114); 10 | <0.001 |
| Antithrombin antigen g/L[g] (median; min-max); IQR | 0.25 (0.13–0.35); 0.05 | 0.3 (0.25–0.32); 0.03 | 0.3 (0.24–0.35); 0.04 | 0.2 (0.13–0.28); 0.03 | 0.25 (0.17–0.31); 0.04 | <0.001 |

*Isolated PE was diagnosed in 14 cases (n = 3 AT Padua, n = 3 ATBp3 HoZ, n = 8 ATBp3 HeZ).*

*IQR, interquartile range; HeZ, heterozygote; HoZ, homozygote.*

[a] *Current age of ATBp3 homozygotes is significantly lower than current age of other groups ($p < 0.001$ as compared with AT Basel and Padua and $p = 0.006$ as compared with ATBp3 HeZ); current age of ATBp3 heterozygotes is significantly lower than current age of AT Padua ($p = 0.014$).*

[b] *Ratio of index patients vs. relatives was lower in ATBp3 HeZ than in other groups (ATBp3 HoZ vs. ATBp3 HeZ $p = 0.028$, AT Padua vs. ATBp3 HeZ $p = 0.044$, AT Basel vs. ATBp3 HeZ $p = 0.496$).*

[c] *Prevalence of any thrombotic events was the highest in ATBp3 homozygous group (AT Basel vs. ATBp3 HoZ $p = 0.012$, AT Padua vs. ATBp3 HoZ $p < 0.001$, ATBp3 HeZ vs ATBp3 HoZ $p < 0.001$).*

[d] *Age at first thrombotic event of ATBp3 homozygotes is significantly lower than that of other groups ($p < 0.001$ as compared with ATBp3 HeZ and AT Padua and $p = 0.005$ as compared with AT Basel). Age at first thrombotic event of ATBp3 heterozygotes is significantly lower than that of AT Padua ($p = 0.002$).*

[e] *Prevalence of VT events was the highest in ATBp3 homozygous group (AT Basel vs. ATBp3 HoZ $p < 0.001$, AT Padua vs. ATBp3 HoZ $p < 0.001$, ATBp3 HeZ vs ATBp3 HoZ $p < 0.001$).*

[f] *Antithrombin heparin cofactor and progressive activity and AT antigen of ATBp3 homozygotes are significantly lower than AT activity and antigen of other groups ($p < 0.001$ in all comparisons).*

[g] *Progressive antithrombin activity and AT antigen of ATBp3 heterozygotes are significantly lower than AT activity and antigen of AT Basel and Padua ($p = 0.004$ and $p < 0.001$ for AT progressive activity, respectively, and $p < 0.001$ for AT antigen for both).*

two patients had thrombotic episodes in their case histories and they were relatively old, one of them was 47 years old at the time of his VT, the other patient had one episode of PE at the age of 65 years. Others had no thrombosis, so far; however, they are still young, below the age of 20 years. If FVL heterozygotes and wild-type patients were compared within the group of ATBp3 heterozygotes, no significant differences were found in terms of the frequency of patients with thrombotic episodes (around 50%) and types of thrombosis (frequency of VT 44 and 45% for wild type and heterozygotes, respectively, PE 6% in both groups, ATE 10 and 6% for wild type and heterozygotes, respectively); however, heterozygotes were a bit younger at the time of the first symptoms. (Median age of wild-type and FVL heterozygous patients at first thrombosis was 34 and 27 years, respectively, $p = 0.05$). FVL heterozygotes presented recurrent thrombosis more often than wild-type patients (frequency of recurrence for wild type and heterozygotes were 16 and 40%, respectively, $p = 0.04$). To conclude these findings, carriership of FVL in ATBp3 heterozygous subjects may shift the age at the first thrombotic episode to a lower range and it may have an impact on the severity (if it is characterized by recurrence).

The number of ATE was much less than that of VTE; however, it was not negligible. It was remarkable that AT Basel, patients showed a higher frequency of ATE, as compared with others; four patients out of the 9 suffered from ATE without having VTE in their case histories and in two of them the ATE was recurrent (**Table 4**). They were all young at their first ATE. A young female patient with ATBp3 homozygous mutation suffered ischemic stroke at the age of 40 years and formerly she had three episodes of VTE, the first was registered at the age of 15. Another young female with two VTE in her case history suffered from ischemic stroke at the age of 27 years without reporting any risk factors for cardiovascular diseases. It is interesting that both patients have hypoplasia of the vena cava as diagnosed by computer tomography. Both patients were put on life-long anticoagulant treatment after their second thrombotic episodes by warfarin. Among ATBp3 heterozygotes, $n = 10$ patients were registered with ATE. Half of them had VTE in

**TABLE 4 |** Major clinical characteristics of type IIHBS antithrombin deficient patients with arterial thrombotic disease.

| Patient ID | AT deficiency type | Gender | VT No. (age at first event in years) | ATE (age at event) | Presence of other thrombophilia | Presence of CV risk factors | Other clinical conditions |
|---|---|---|---|---|---|---|---|
| 1 | AT Basel | Male | 0 | MI (15) | 0 | HLP | 0 |
| 2 | AT Basel | Female | 0 | MI (50) | 0 | ND | 0 |
| 3 | AT Basel | Male | 0 | MI (42), stroke (45) | 0 | 0 | 0 |
| 4 | AT Basel | Female | 0 | Stroke (49) | 0 | 0 | 0 |
| 5 | AT Padua | Male | 0 | Stroke (43) | 0 | ND | 0 |
| 6 | AT Padua | Female | 0 | Stroke (40) | 0 | 0 | 0 |
| 7 | AT Bp3 HoZ | Female | 3 (15) | Stroke (40) | 0 | 0 | v. cava inferior hypoplasia |
| 8 | AT Bp3 HoZ | Female | 2 (17) | Stroke (27) | 0 | 0 | v. cava inferior hypoplasia |
| 9 | AT Bp3 HeZ | Female | 1 (29) | Stroke (39) | 0 | 0 | 0 |
| 10 | AT Bp3 HeZ | Male | 0 | Stroke (14) | 0 | HLP, Hcy | 0 |
| 11 | AT Bp3 HeZ | Female | 1 (23) | Stroke (23) | 0 | 0 | 0 |
| 12 | AT Bp3 HeZ | Male | 2 (27) | Stroke (19), MI (24) | FVL HeZ | Smoking | 0 |
| 13 | AT Bp3 HeZ | Male | 0 | Stroke (2), +2 stroke | 0 | 0 | ASD and VSD |
| 14 | AT Bp3 HeZ | Male | 0 | MI (47) | FVL HeZ | Smoking, HLP | 0 |
| 15 | AT Bp3 HeZ | Female | 1 (47) | MI (38) | 0 | HT, obesity | Hypothyreosis |
| 16 | AT Bp3 HeZ | Female | 1 (37) | MI (48) | 0 | HT | Aorta stenosis |
| 17 | AT Bp3 HeZ | Female | 0 | Stroke (18) | FVL HeZ | 0 | 0 |
| 18 | AT Bp3 HeZ | Female | 0 | MI (38) | 0 | Smoking | 0 |

*The term CV risk factors cover hypertension (HT), diabetes mellitus, hyperlipidemia (HLP), hyperhomocysteinemia (Hcy), smoking and obesity (BMI above 25 kg/m$^2$). No., number; HeZ, heterozygote; HoZ, homozygote; ASD, atrial septal defect; VSD, ventricular septal defect; ND, no data.*

their case histories and two of them suffered from more than one episode of ATE. The youngest patient is a currently 11-year-old boy who suffered his first episode of ischemic stroke at the age of 2 followed by two additional episodes until now. His case is however different from others, since—although there was no embolic source confirmed in the background—due to the presence of atrial and ventricular septal defects, we cannot exclude the venous origin of vascular occlusion. The eldest patient with ATE was still relatively young; she was 48 years old at the time of MI.

## Prevalence of Type IIHBS Mutations in Young Patients With Myocardial Infarction

As ATE was registered in our type IIHBS AT-deficient cohort, even in ATBp3 heterozygotes and patients with these thrombotic symptoms were relatively young at the onset of the disease, we investigated the prevalence of type IIHBS AT deficiency among young individuals with MI. As the prevalence of MI is low below 40 years of age, we could recruit $n = 119$ individuals with MI below the age of 40 in their case histories and $n = 101$ age-matched individuals without MI (clinical controls, CC). Recruitment lasted for 5 years. Only those individuals in both groups, who underwent cardiac catheterization upon admission, were included. After informed consent $n = 92$ MI and $n = 74$ CC patients were eligible for the study (**Table 5**). Concerning classical thrombophilia risk factors, almost 10% of MI patients were carriers of the FVL; however, based on the allele frequency data of the general population in Hungary—this is not surprising and it was not different from the CC group. Prevalence data of FII20210A carriers were also similar in the two groups. While no protein C or protein S deficiencies were found among these patients, there were three young MI patients with type IIHBS AT deficiency in our study group. Two of them were heterozygous carriers of the ATBp3 mutation, while one patient was a heterozygous carrier of the AT Basel mutation; all patients were males. There were no AT-deficient individuals in the CC group. Among the three patients with type IIHBS mutations in the MI group, the young patient with AT Basel suffered from MI (occlusion of LAD) at the age of 16. Two years after the coronary stent implantation, he had ischemic stroke (a. cerebri media). He was put on anticoagulant therapy (first with warfarin, then with apixaban $2 \times 5$ mg) combined with aspirin (100 mg/day). At the age of 26, while on dual pathway inhibition, he suffered inferior STEMI with right coronary artery (RCA) occlusion and PCI was performed. He has no other known thrombophilia and he is also wild type for FVL and FII20210. He has no classical cardiovascular risk factors, except for an elevated Lp(a) above 800 mg/L. The two patients with ATBp3 suffered from MI at the age of 39 and 40. The first patient had inferior STEMI with RCA occlusion; the second patient had STEMI with LAD occlusion. Both patients underwent coronary stent implantation. Due to in-stent restenosis, the second patient had re-coronarography and drug-eluting stent implantation. Both patients were put on

**TABLE 5 |** Clinical and laboratory characteristics of the young MI and venous thrombosis populations and their healthy controls.

| | HC ($n = 215$) | CC ($n = 74$) | MI ($n = 92$) | VTE ($n = 110$) | *p*-value (MI vs. VTE) | *p*-value (MI vs. CC) | *p*-value (VTE vs. HC) |
|---|---|---|---|---|---|---|---|
| Age years (median; min–max); IQR | 27 (18–40); 12 | 35 (20–40); 6 | 36 (14–40); 4 | 30.5 (17–40); 11 | <0.001 | 0.129 | 0.076 |
| Male/female (*n*) | 87/128 | 47/27 | 70/22 | 57/53 | 0.001 | 0.089 | 0.059 |
| Diabetes mellitus (%) | 0 | 12.0 | 14.0 | 4.0 | 0.010 | 0.819 | 0.015 |
| Active smokers (%) | 25.0 | 21.0 | 60.0 | 20.0 | <0.001 | <0.001 | 0.070 |
| Ex-smokers % | 9.5 | 20.8 | 33.3 | 18.0 | <0.001 | <0.001 | 0.084 |
| ATE+VTE % | 0 | 0 | 4.3 | 1.8 | 0.012 | NA | NA |
| Positive family history ATE or VTE (%) | 45.0 | 60.0 | 74.0 | 62.0 | <0.001 | 0.060 | <0.001 |
| Hypertension (%) | 7.0 | 51.0 | 43.0 | 12.0 | <0.001 | 0.350 | 0.149 |
| Hyperlipidemia (%) | 0 | 39.0 | 86.0 | 0 | NA | <0.001 | NA |
| BMI kg/m$^2$ (median; min–max); IQR | 23.2 (16.3–41.4); 6.39 | 28.0 (19.0–45.0); 10.0 | 28.0 (17.0–46.0); 6.0 | 28.7 (18.9–52.7); 7.32 | 0.221 | 0.586 | <0.001 |
| Type IIHBS AT deficiency % | 0 | 0 | 3.3 | 1.8 | 0.469 | NA | NA |
| FVL carriers HeZ/HoZ % | 8/0 | 9.6/0 | 9.4/0 | 35.5/10.9 | <0.001 | 0.970 | <0.001 |
| FII 20210A carriers HeZ % | 3.3 | 1.4 | 2.3 | 6.3 | 0.304 | 0.652 | 0.251 |
| Antithrombin activity % (median; min–max); IQR | 106 (80–130); 14 | 105 (87–135); 11 | 112 (57–135); 12 | 104 (50–136); 15 | <0.001 | <0.001 | 0.042 |
| AT, PC, and PS deficiency % | 0 | 0 | 3.3 | 5.5* | 0.469 | NA | NA |

*Positive family history was defined as MI, stroke, or non-traumatic lower limb amputation in first-degree relatives.*

*NA, not applicable; IQR, interquartile range; HeZ, heterozygote; HoZ, homozygote.*

*Only PC activity showed normal distribution. Reference intervals for antithrombin activity is 80–120%.*

**There were two patients with genetically confirmed type IIHBS antithrombin deficiency (ATBp3 heterozygotes) and protein C deficiency, one patient with protein S deficiency, and one patient with type I antithrombin deficiency in the venous thrombosis group.*

clopidogrel therapy. None of them suffered from VTE until data analysis. None of them had other thrombophilia; however, they had hyperlipidemia with normal lipid parameters on adequate therapy, and they were active smokers with pack-years of 25 and 20, respectively. The 40-year-old patient was obese, with a BMI of 40, and he had type 2 diabetes mellitus with normal glucose values on therapy.

### Prevalence of Type IIHBS Mutations in Young Patients With Venous Thromboembolic Disease

As a comparison with our young MI patients, we investigated consecutive patients suffering from VTE below the age of 40 ($n = 110$) recruited from our large cohort of 400 VTE patients. In this group, $n = 26$ (24%) patients were registered with provoked thrombosis ($n = 7$ childbirth, $n = 3$ immobility, $n = 11$ oral anticoncipients, $n = 5$ trauma). All other thrombotic episodes were spontaneous. Healthy controls ($n = 215$) below the age of 40 upon recruitment and selected from our healthy reference population (i.e., out of the $n = 450$ healthy Hungarian samples) served as a general reference for all patients' groups (**Table 5**). The representation of females was higher in the VTE group as compared with our MI patients. There were huge differences in the prevalence of classical cardiovascular risk factors between VTE and MI groups, while BMI values did not differ. While 4.3% of MI patients also had VTE in their case histories, only 1.8% of VTE patients registered with arterial events ($p = 0.012$). Among classical thrombophilia risk factors, the allele frequency of FVL was significantly higher in the VTE group (more than 45% of the patients were carriers) and not only heterozygotes but also homozygotes were found. The frequency of FII20210A carriers was only slightly higher in the VT group. Type IIHBS deficiency was registered in two VTE patients (both ATBp3 heterozygotes); moreover, one type I AT deficiency, two PC deficiencies, and one PS deficiency were confirmed by genetic testing. As being exclusion criteria, the healthy reference group was free of diabetes mellitus and hyperlipidemia and the representation of active smokers was comparable with the CC and VTE groups. The median BMI was lower than that of the other groups. Allele frequency values of FVL and FII20210 were similar to that of CC and MI groups and no AT, PC or PS deficiency was explored. The carrier frequency of type IIHBS AT deficiency was also zero.

## DISCUSSION

In this study, we investigated the age and origin of the founder ATBp3 mutation and strengthen the hypothesis of its Roma origin. Both Bayesian estimates were consistent each other and with the MRCA age obtained by the moment methods. All methods suggested that ATBp3 mutation founding was ∼350–400 years ago. The upper limit of the CS age interval was higher in the case of Bayesian methods than the corresponding interval suggested by the mean ± SD value obtained from parametric analysis of LD decay over generations by the two moment methods; this difference might be due to the relatively small sample size that we could collect for analysis;

however, this sample size was the maximum feasible in case of such a rare disease. We observed that homozygous ATBp3 mutation carriers had a bi-allelic distribution of D1S218 marker. This marker shows the least distance from ATBp3. If no recombination is assumed at this point since the mutation occurred, it might happen that the founder event occurred parallel in two alleles carrying two different D1S218 STR variants. It is a more possible explanation of this phenomenon; however, that the mutation is young enough to occur only one recombination event at position D1S218 since the foundation event. Upon confronting the MRCA age with the major population events that occurred at different periods of the Hungarian history, a very turbulent period was recognized. It was characterized by the Fifteen-Year War (1592–1606) then the Thirty-Year War (1618–1648), plus four plague epidemics (1620–1627, 1632–1634, 1643–1645, and 1660–1665), and a variola outbreak (57). These dramatic events brought famine and other calamities to the entire region. In some areas, a massive population decline occurred; however, at the same time, the arrival of Serbs in regions under Ottoman rule and of Romanians, Ruthenians, Vlachs, and Romanies in Royal Hungary, might created population bottleneck, through which a pre-existent or a *de novo SERPINC1* c.391C>T mutation could be introduced into the Hungarian population and became fixed because of strong genetic drift. A rapid population growth afterwards could strengthen a founder effect for c.391C>T. It was mentioned in historical literature that major immigration ways of Roma people to Hungary were from Serbia and from Romania and Transylvania, and during the XVII Century, the Roma population in Hungary had grown significantly due to immigration (58). As no data of official census is available from that century, the exact number of Roma immigrants and population size in XVII Century is not known. Until the end of the XVIII century when the first official census was carried out, however, the frequency of Roma in Hungary reached 1% of total population. In conclusion, the estimated age of the *SERPINC1* c.391C>T mutation, the geographic distribution of families with ATBp3, and the history of the modern Hungarian population are consistent with the hypothesis that the mutation originated (or was originally introduced) and expanded in the later Ottoman period and Royal Hungary. Almost all patients with ATBp3 mutation have been reported so far are of Central Eastern European origin, and this is consistent with the proposed origin of the founder effect (8, 59–62). The considerable carrier frequency (around 3%) in such a rare disease that was found in the Roma general population and the confirmation of the founder effect of the ATBp3 mutation further strengthens the hypothesis that the mutation is of Roma origin, or at least became more prevalent in that population due to high frequency of consanguinity and inbreeding (63). We do not have clear explanation on whether the ATBp3 mutation provided an advantage for carriers; only speculations can be made. Similarly to that of an advantage of being FVL carrier, we may surmise that being heterozygous for ATBp3 mutation prevented high blood loss upon childbirth and injuries in the Roma population with nomadic lifestyle. Since homozygosity exerts an extremely high thrombotic risk, only heterozygosity might have an advantage over wild genotype in this context.

As it was demonstrated earlier, the Roma population is vulnerable from the point of view of cardiovascular diseases (41, 43). They tainted with several environmental and genetic risk factors making them more sensitive to early thrombosis development, including ATE. In addition to obesity and other risk factors, Roma people are often subjected to air pollution living in peripheral, industrial areas (58). Identification of individuals with more severe genetic load is of high importance to offer them adequate preventive and treatment strategies. Since we demonstrated here, that Roma people may be carriers of the type IIHBS ATBp3 mutation with a considerable frequency, the number of ATBp3 AT-deficient individuals among the 700,000–800,000 Roma people living in Hungary according to recent estimations is ∼24,000, which number may be even higher in the Central European region, where the number of Roma inhabitants exceeds 10 million (64). Besides its genetic epidemiological value, the identification of a vulnerable population loaded with the ATBp3 mutation and a documented clinical history will be extremely useful in the search for disease modifying loci within c.391C>T-carrying families. As FVL is also prevalent in the Roma population, the chance of having combined thrombophilia by carrying both FVL and ATBp3 is not negligible. Based on our results it is recommended to include the investigation of ATBp3 AT deficiency as part of thrombosis risk-stratification in Roma individuals.

It is of outmost importance to identify other vulnerable individuals and populations from the point of view of disease control and prevention. Here, we attempted to examine the association of ATBp3 and other type IIHBS mutations with cardiovascular diseases including not only venous but also arterial thrombotic events, as well. The question is interesting from several aspects. It was hypothesized earlier by us and by others that AT deficiency was a rather heterogeneous disease concerning its clinical and laboratory consequences (7, 13, 14). Even, within type IIHBS deficiency, heterogeneity was observed in the clinical symptoms, onset of diseases and risk of recurrent thrombotic events. It is now evident, as supported by cohort studies and case reports, that ATBp3 homozygosity is one of the most severe thrombophilia (9, 10, 13, 65) and is evidently associated with severe VTE. ATBp3 heterozygous state is less severe; however, its association with VTE is also significant. Recently, reports on association of ATBp3 and other type IIHBS AT deficiencies with ATE have been published; however, as hereditary AT belongs to the group of rare diseases, well-designed, large clinical studies with high statistical power cannot be carried out (6, 10, 11, 61). Multicenter studies may overcome this problem; however, they are likely to be subjected to bias due to heterogeneous ethnicity. Until having more robust data (hopefully from the International AT deficiency registry, which has been started most recently under the umbrella of the Scientific and Standardization Committee on Plasma Coagulation Inhibitors of the ISTH), each piece of evidence is helpful for clinicians facing AT-deficient cases. In well-described case reports, most of the AT-deficient patients were young at

the development of their first MI or stroke suggesting that AT deficiency is severe enough to lead to early onset thrombotic complications even at the "arterial side" (66–71). As being a rare disorder, however, only a few publications have been released in which the role of AT deficiency was investigated in ATE, as yet. In observational cohort studies recruiting unselected MI or stroke patients, only a small proportion, if any are expected to carry AT mutations (72–74). Clinically useful conclusions, therefore, are hardly drawn from these studies. Investigation of selected populations may have more rationale and results from these studies may be more relevant for the specific source population from which the sample is selected. As an example to this, AT Cambridge II (p.Ala416Ser according to HGVS nomenclature), which is a relatively frequent mutation in the Spanish population associated with increased risk (OR 5.66; 95% CI 1.53, 20.88) of MI in a large sample ($n$ = 1,224 MI patients and $n$ = 1,649 controls) suggesting AT deficiency as an independent risk factor for MI (75). Another approach for investigating the association of AT deficiency with cardiovascular diseases is to conduct cohort studies in genetically confirmed AT-deficient patients' groups. According to the recent results of such studies, the prevalence of ATE was between 4.8 and 19%, and these events associated more often with type IIHBS AT deficiency than with other types (6, 7, 10, 11).

Since we have one of the largest type IIHBS AT-deficient cohort investigating the prevalence of ATE in different subtypes of type IIHBS AT deficiency, especially with ATBp3, seems feasible. ATE was most prevalent in AT Basel patients and almost all with this genotype have been suffered from at least one ATE episode until the time of data collection without having any VTE. It is to be noted, however, that the sample size of both AT Basel and Padua is much lower than that of ATBp3 in our population to draw a strong conclusion, although our findings are well-correlated with other reports (6, 10). In case of ATBp3, the frequency of ATE was higher in the heterozygote group as compared with homozygotes. The probable explanation for this is the lower age of ATBp3 homozygotes. They were twice younger than heterozygotes upon first hospitalization and suffered severe VTE as first thrombosis. As a consequence of early diagnosis of AT deficiency, they were immediately put on long-term anticoagulation and were warned to avoid any modifiable cardiovascular risk factors. So, according to our experience, ATBp3 homozygotes are rescued from the AT-deficient population before the development of arterial events. ATBp3 heterozygosity is another issue. The ratio of ATE was obviously lower as compared with VTE in our cohort; however, they were all young at the time of MI or stroke development. In eight out of 10 patients, there was no VTE at all in their histories, or at least ATE preceded the venous thrombotic episodes. At least one modifiable cardiovascular risk factor was registered in their cases drawing the attention to the possible combined effect of AT deficiency and environmental risk factors in the early development of ATE. Cardioembolic (or thromboembolic) origin of stroke was excluded in all but one case; patient 13 in **Table 4** had both atrial and ventricular septal defects, which were closed at the age of 2 years; however, he suffered two more episodes of stroke afterwards, before the age of 11.

As ATE developed at a young age in our AT-deficient cohort, we aimed to investigate the effect of type IIHBS AT deficiency on ATE development in selected populations consisted of non-related young MI and VTE patients. The ratio of type IIHBS AT deficiency in MI patients was comparable with that of VTE patients, although two major differences were observed. MI patients had more classical cardiovascular risk factors; especially, the frequency of smoking and hyperlipidemia was very high, while the ratio of additional thrombophilia, especially FVL mutation, as expected, was significantly higher in the VTE group. In conclusion, the presence of type IIHBS AT deficiency is not negligible in young individuals suffering from any types of thrombosis, and searching for AT Basel or ATBp3 in such selected populations, especially, in regions, where these mutations are prevalent, may be beneficial.

Our study has some limitations. As a single-center study, we could not reach large sample size, thus high statistical power in young MI and VTE groups for investigating ATBp3 effect. However, the prevalence of ATE and VTE at a young age is generally low, and establishment of such a strict cut-off value for age (40 years) among inclusion criteria makes recruitment difficult. Moreover, as AT deficiency itself is a rare disease, especially the number of homozygous ATBp3 carriers in the ATE study was small. This limits the impact of our observations. We believe, that by the demonstration of the presence of ATBp3 (and also AT Basel) even in these relatively small groups of ATE (and VTE) patients, we could draw the attention to consider type IIHBS AT deficiency in the background of not only VTE but also ATE, especially in selected populations as young patients without advanced atherosclerosis. Another limitation is the uncertainty of ethnicity in the different patients' groups. As being a sensitive issue, Roma ethnicity is not registered upon hospitalization or patient recruitment except for those epidemiology studies, where directly Roma population is involved. By demonstrating the high prevalence of ATBp3—as compared with its rare disease status—in the Roma general population, we suggest that in regions, where the size of Roma minority is significant, the chance of diagnosing ATBp3 AT deficiency is higher. Collecting data in larger studies will help in clarifying the role of ATBp3 AT deficiency in different types of thrombosis and the risk conferred by the combination of this thrombophilia with other genetic and environmental cardiovascular risk factors in the development of these diseases.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available upon request from the corresponding author (ZB).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee of the Hungarian Scientific Council on Health and Ethical Committee of University of Debrecen, Hungary. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

ZB designed the research, was involved in all aspects of the study, evaluated results, and wrote the manuscript. RG was involved in all aspects of the study, analyzed results, and took part in writing the manuscript. SF was involved in recruiting Roma individuals and in sample collection. MS performed genetic analysis and data evaluation of founder effect. TM recruited patients with venous thrombosis. LB recruited myocardial infarction patients. ZM and ZS performed genetic investigations. RÁ was involved in designing and implementing Roma surveys, data analysis, and manuscript preparation. All authors reviewed the manuscript before submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2020.617711/full#supplementary-material

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# PEGylated Serp-1 Markedly Reduces Pristane-Induced Experimental Diffuse Alveolar Hemorrhage, Altering uPAR Distribution, and Macrophage Invasion

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Diffuse alveolar hemorrhage (DAH) is one of the most serious clinical complications of systemic lupus erythematosus (SLE). The prevalence of DAH is reported to range from 1 to 5%, but while DAH is considered a rare complication there is a reported 50–80% mortality. There is at present no proven effective treatment for DAH and the therapeutics that have been tested have significant side effects. There is a clear necessity to discover new drugs to improve outcomes in DAH. *Ser*ine *p*rotease *in*hibitors, *serpins*, regulate thrombotic and thrombolytic protease cascades. We are investigating a Myxomavirus derived immune modulating serpin, Serp-1, as a new class of immune modulating therapeutics for vasculopathy and lung hemorrhage. Serp-1 has proven efficacy in models of herpes virus-induced arterial inflammation (vasculitis) and lung hemorrhage and has also proved safe in a clinical trial in patients with unstable coronary syndromes and stent implant. Here, we examine Serp-1, both as a native secreted protein expressed by CHO cells and as a polyethylene glycol modified (PEGylated) variant (Serp-1m5), for potential therapy in DAH. DAH was induced by intraperitoneal (IP) injection of pristane in C57BL/6J (B6) mice. Mice were treated with 100 ng/g bodyweight of either Serp-1 as native 55 kDa secreted glycoprotein, or as Serp-1m5, or saline controls after inducing DAH. Treatments were repeated daily for 14 days (6 mice/group). Serp-1 partially and Serp-1m5 significantly reduced pristane-induced DAH when compared with saline as assessed by gross pathology and H&E staining (Serp-1, $p = 0.2172$; Serp-1m5, $p = 0.0252$). Both Serp-1m5 and Serp-1 treatment reduced perivascular inflammation and reduced M1 macrophage (Serp-1, $p = 0.0350$; Serp-1m5, $p = 0.0053$), hemosiderin-laden macrophage (Serp-1, $p = 0.0370$; Serp-1m5, $p = 0.0424$) invasion, and complement C5b/9 staining. Extracellular urokinase-type plasminogen activator

receptor positive (uPAR+) clusters were significantly reduced (Serp-1, $p = 0.0172$; Serp-1m5, $p = 0.0025$). Serp-1m5 also increased intact uPAR+ alveoli in the lung ($p = 0.0091$). In conclusion, Serp-1m5 significantly reduces lung damage and hemorrhage in a pristane model of SLE DAH, providing a new potential therapeutic approach.

**Keywords: lupus, diffuse alveolar hemorrhage, immune modulator, Serp-1, inflammation, recombinant protein therapeutic, vasculitis**

## INTRODUCTION

Systemic lupus erythematosus (SLE), or lupus, is an autoimmune disease characterized by immune cell hyperactivity, production of antibodies against self-antigens, such as double-stranded (ds) DNA, histones, and ribonucleoprotein (RNP). The etiology of SLE is only partially defined and has been linked to abnormal genetic, hormonal, and environmental responses (1–4). The incidence of this disease is 20–70 per 100,000 people, and the incidence in women is 6–10 times that of men. Patients with SLE have a wide range of clinical symptoms, including skin rash, nephritis, non-erosive arthritis, serositis, cardiovascular involvement, hematological, and respiratory disorders (with pulmonary fibrosis and hypertension). In some cases (1) The most serious clinical manifestation of SLE is diffuse alveolar hemorrhage (DAH), with prevalence ranging from 1 to 5%, but causing >50–80% mortality in affected SLE patients (2–4). Lupus DAH is characterized by neutrophilic capillaritis with destruction of alveolar septae and infiltration of hemosiderin-laden macrophages (5–8). The current treatment options for DAH include steroids, cyclophosphamide, rituximab, methotrexate, azathioprine, respiratory support, and *among others*. The efficacy of these treatments is limited and there are many significant side effects, that include hypertension, diabetes, osteoporosis, adrenal suppression, infertility, pulmonary fibrosis, hepatotoxicity, and risk for future malignancies. Therefore, there is an urgent, unmet need for new drugs to improve treatment for DAH.

Pristane (2, 6, 10, and 14 tetramethylpentadecane, TMPD) is an isoprenoid alkane found at high concentration in mineral oil, and in low concentration in vegetables (9). It is also found in the liver of some sharks (10). Intraperitoneal (IP) injections of pristane can induce in mice a wide range of autoantibodies specific to, or associated with, SLE (11, 12), making pristane an accepted method to establish mouse models of SLE. IP injection of pristane in C57BL/6J (B6) mice causes severe alveolar hemorrhage within 2 weeks, manifested by alveolar and perivascular inflammation (capillaritis, small vessel vasculitis), endothelial injury and hemorrhage (7, 13–15). Many previous studies have proven that this model can closely simulate the pathological process of DAH.

Serp-1 is a purified 55 kDa secreted glycoprotein originally derived from MYXV, belonging to the SERPIN superfamily. Our previous research has demonstrated that purified Serp-1 protein treatment is beneficial in a wide range of immune mediated disorders, from arthritis to vasculitis to transplant (16–21). Serp-1 reduces macrophage cell infiltration into transplanted hearts, kidneys and aorta in rodent models, with improved histopathological evidence of acute and chronic rejection (16, 17, 22). In a mouse model of inflammatory vasculitis induced by mouse gamma herpesvirus-68 (MHV-68) infection in interferon gamma receptor deficient mice (IFNγR$^{-/-}$) and also in an aortic transplant model, Serp-1 significantly reduced arterial inflammation and plaque growth. Additionally, Serp-1 treatment reduced lung hemorrhage and consolidation and improved survival in mouse gamma herpesvirus-68 (MHV68) infected mice, a model for inflammatory vasculitis and lethal lung hemorrhage (20, 21). In clinical trials, Serp-1 treatment proved safe and significantly reduced markers for myocardial damage after coronary stent implant in phase I and IIa clinical trials in patients with unstable angina pectoris or non-ST elevation myocardial infarction (NSTEMI), with no significant major adverse reactions (MACE = 0) and no neutralizing antibody detected (23).

Urokinase type plasminogen activator (uPA) binds to the uPA receptor (uPAR). The uPA/uPAR complex sits at the leading, or invading, edge of inflammatory macrophage cells. In addition to a role in thrombolysis, the uPA/uPAR complex also activates plasmin which in turn activates matrix metalloproteinases (MMPs). MMPs break down connective tissue (collagen and elastin), to allow immune cells to infiltrate tissues. The uPA/uPAR complex thus functions both in fibrinolysis and in inflammatory cell activation and invasion, the latter being considered the predominant function. Serp-1 binds and inhibits thrombolytic protease, tissue- and urokinase-type plasminogen activators (tPA and uPA, respectively) as well as thrombotic proteases, thrombin and factor Xa. Serp-1 binds to the uPA/ uPAR complex on the macrophage plasma membrane surface (24, 25). Serp-1 inhibition of macrophage migration is dependent upon uPAR expression *in vitro* in monocytes and *in vivo* in the aortic transplant model. Serp-1 efficacy was previously found to be dependent on uPAR expression in the donor aorta in aortic transplant models in mice (18, 24, 26). Serp-1 treatment is thus projected to either reduce excess thrombolysis, or to rebalance an imbalance in both thrombotic and thrombolytic cascades, and to reduce inflammation in this SLE lung hemorrhage model. In previous studies another member of the Serpin superfamily, Alpha-1-antitrypsin (AAT), has shown anti-inflammatory and immunomodulatory functions, inhibiting the activation and recruitment of inflammatory cells when given for 1 week prior to pristane injection. Human AAT (hAAT) reduced the severity of DAH in B6 mice; hAAT transgenic mice completely prevented DAH induced by pristane (27).

The half-life for Serp-1 in circulating blood was ∼20 min in clinical trial up to 1.36 days in mouse and rabbit models, and

is dependent upon the model examined (23, 28). PEGylation has been demonstrated to improve the half-life and reduce antigenicity in prior work with other proteins (29). In this study, we examined treatment with either PEGylated Serp-1, here termed Serp-1m5, or with the native non-PEGylated secreted Serp-1 in the SLE DAH mouse model for efficacy and compared to Saline control alone. Based on prior studies, we have postulated that Serp-1 will prove effective and safe for the treatment of DAH.

# MATERIALS AND METHODS

## Proteins and Chemicals

Serp-1 (m008.1L; NCBI Gene ID# 932146) was expressed in a Chinese hamster ovary (CHO) cell line (Viron Therapeutics Inc., London, ON, CA). The Serp-1 protein used in this research is GMP-compliant and purified by continuous chromatographic separation. The purity of Serp-1 is >95%, as determined by Coomassie stained SDS-PAGE and reverse-phase HPLC. Serp-1 was endotoxin-free by LAL (limulus amebocyte lysate) assay. Serp-1 was incubated with mPEG-NHS (5 K) (Nanocs Inc., #PG1-SC-5k-1, NY) in PBS buffer (pH 7.8) at 4°C overnight to modify the protein according to standard PEGylation protocols. PEGylated Serp-1 (Serp-1m5) was purified by FPLC using an ÄKTA pure protein purification system with Superdex-200.

Hematoxylin and eosin for H&E staining and trichrome reagents were from Sigma-Aldrich. Information about each antibody used for immunohistochemical staining in this study is provided below when first mentioned.

## Animals

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of Arizona State University under protocol #20-1761R and conform to national and international guidelines for animal care. Eighteen wild-type female C57BL6/J mice aged 6–8 weeks old were treated with pristane. Female mice are reported in prior studies to be preferred for the development of DAH model (13, 27). Each mouse was injected with 0.5 ml of pristane (Sigma-Aldrich) intraperitoneally (IP) at day 0. These mice were then randomly divided into three groups, i.e., saline, Serp-1 or Serp-1m5 treatment groups (6 mice each group, $n = 6$). Six normal mice were also examined, without pristane or Serp-1, and six mice had Serp-1 treatment without pristane. No adverse effects were seen [Toxicity for Serp-1 has been extensively tested and proven to be minimal in preclinical and clinical trials, as previously reported (16–21, 23–26)]. Each mouse was given one IP injection of 100 μL saline or 100 ng/g bodyweight of clinical grade Serp-1 or Serp-1m5 in 100 μL of Saline after pristane induction. The treatments were repeated every day until the 14th day. The mice were euthanized by $CO_2$ asphyxiation on the 15th day and lung tissues were divided; one half was frozen at −80°C for later protein analysis, and one half fixed in 10% neutral-buffered formalin for at least 3 days before processing and paraffin embedding. There were no early deaths or complications in any treatment group.

## Lung Pathological Evaluation

DAH in lung specimens was initially assessed by gross observation of excised lungs, prior to either fixation or freezing. Lung tissues were fixed in 10% neutral-buffered formalin after collection and then processed in a Leica TP1050 tissue processor and embedded in paraffin with a Leica EG1160 embedding station, as previously described (21–26). Tissue blocks were cut into 5 μm sections using a Leica RM2165 microtome.

Sections were stained with hematoxylin and eosin (H&E) and by Masson's trichrome using standard procedures, as previously described (21–27, 30). DAH was classified into three degrees of severity according to the percentage of hemorrhage on H&E stained sections as assessed by a blinded histological analysis of DAH score as follows: (1) No hemorrhage (0%); (2) Partial hemorrhage (25–75%); (3) Complete hemorrhage (75–100%). Prussian blue staining (Electron Microscopy Science company) was performed with standard protocol to analyze the hemorrhage status.

Sections were additionally stained for immunohistochemical analysis (IHC) for CD3 (Abcam, ab6590, 1:100), CD4 (Abcam, ab183685, 1:1,000), Ly6G (Invitrogen, 14-5931-82, 1:100), arginase-1 (Cell Signaling, 93668, 1:200), iNOS (Abcam, ab15323, 1:100), C5b/9 antibody (Abcam, ab 55811), and uPAR (R&D Systems, AF534,1:100). HRP-conjugated secondary antibodies against rabbit or goat IgG were applied at a dilution of 1:500 for 1 h at room temperature. HRP-conjugated secondary antibody given alone without primary antibody was used as negative control for each stain. Antigens were revealed with ImmPACT DAB (Vector Labs, USA), counterstained with Gil's formula #3 Hematoxylin and mounted with Cytoseal XYL.

Slides were examined and images collected as objective-calibrated TIFFs on an Olympus BX51 upright microscope equipped with an Olympus DP74 color CMOS high-resolution camera operated by cellSens Dimensions v1.16. Images (Olympus, Waltham, MA, USA). Images were subsequently analyzed live and processed in ImageJ/FIJI. Positively stained cells were counted per high power field for each group; three high power fields examined per mouse and lung section.

## Lung Tissue Protein Extraction and Analysis

For each mouse, lungs were collected after euthanasia at 15 days post-pristane injection. One lung was fixed in neutral buffered formalin for later histological analysis. The other lung was frozen at −80°C for later biochemical assays. Half of the collected frozen tissue was homogenized as whole lung tissue with a blade homogenizer into 400 μL RIPA with EDTA buffer containing 1× protease inhibitor cocktail (Bimake, Houston, TX, USA, #B14001) and 1 mM phenylmethanesulfonyl fluoride (PMSF) on ice. Homogenized samples were rotated at 4°C for 1 h and centrifuged at 13,000× g for 15 min at 4°C. Supernatant containing total protein was transferred to a new tube for ELISA assays.

Half of the frozen lung tissue was homogenized into 400 μL PBS buffer containing protease inhibitors cocktail, PMSF, and 1 mM EDTA. Homogenized samples were processed by two

cycles freeze-thaw, followed by centrifugation at 13,000× g at 4°C for 15 min. Supernatant without membrane proteins was then collected for cell membrane free soluble uPAR (csuPAR) protein analysis.

## Elisa Assays

uPAR (R&D Systems, DY531) levels in lung tissues were quantified with ELISA kits following manufacturer's instructions. Quantified protein level of lung tissue was normalized to total protein, which was determined with the BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, #23227).

## Mass Spectrometry Analysis of C3 Binding by Serp-1

For Serp-1 interactome analysis, 100 μL of streptavidin magnetic beads (Thermo Scientific, #88816) were washed, resuspended in 1 mL EBC buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, pH 8.0) with 1.0% BSA. Ten microliters of biotin-labeled Serp-1 antibody (AxB7.9-biotin) was added and the mixture rotated at RT (20°C) for 2 h. Beads were washed in EBC, resuspended in 500 μL EBC buffer with 2% BSA and 20 μg Serp-1 added, and incubated with 250 μL plasma at 4°C overnight followed by buffer wash. Total binding proteins were collected by boiling the beads with 40 μL of 6 × SDS reducing dye. SDS-PAGE was performed, and bands cut from gel for interactome analysis by mass spectrometry (MS) at the ASU/Biodesign MS center.

## Flow Cytometry Analysis of Splenocytes

Spleens were isolated from mice and cells dissociated in ice-cold RPMI-1640 containing 20% FBS using a 70 μm cell strainer for immediate Flow cytometry analysis. Red blood cells were lysed using RBC lysis buffer (155 mM $NH_4Cl$, 12 mM $NaHCO_3$, 0.1 mM EDTA) for 10 min at room temperature and pelleted splenocytes were washed with RPMI-1640 containing 20% FBS. Splenocytes were deposited into 96-well round-bottom polystyrene plates ($10^6$ splenocytes per well). Cells were either directly stained as follows or stimulated with Cell Activation Cocktail (Biolegend) in the presence of 1X Brefeldin A (Biolegend) for 90 min prior to staining. Cells were blocked on ice with TruStain FcX anti-mouse CD16/32 Fc receptor blocker (Biolegend) for 10 min and stained with eBioscience Fixable Viability Dye eFluor780 (Thermo Fisher), according to manufacturer's procedure. Surface markers were stained for 30 min at 4°C at manufacturer's recommended dilution in 3% BSA/PBS. Cells were fixed and permeabilized using the eBioscience Foxp3 Transcription Factor staining buffer kit (Thermo Fisher) for 1 h according to manufacturer's procedure. Intracellular markers were stained for 30 min at 4°C at manufacturer's recommended dilution in 3% BSA/PBS. Antibodies used were: CD4-PE/Cy7 (clone RM4-5, Biolegend), CD8-BV480 (clone 53-6.7, BD Biosciences), NK1.1-SB600 (clone PK136, Thermo Fisher), FoxP3-eFluor450 (clone FJK-16s, Thermo Fisher), IFNγ-APC (clone XMG1.2, Biolegend), GATA3-BV711 (clone L50-823, BD Biosciences), RoRγt-BV650 (clone Q31-378, BD Biosciences), CD11c (clone BV480, BD Biosciences), CD11b (clone M1170, BD Biosciences), F4/80(clone BV711, Biolegend), and CD163 (clone PercCP/eF710, Thermo).

Cells were analyzed on an Attune NxT with autosampler (Thermo Fisher) by the ASU Knowledge Enterprise Core Research Flow Cytometry facility and data were processed with FlowJo v10.

## Statistical Analysis

Graphing and statistical analysis were performed using GraphPad Prism v8.4.3 (GraphPad Software, San Diego, CA, USA). Mean values were calculated for each analysis and are presented as mean ± SEM. Differences between groups were compared using analysis of variance (ANOVA), Fishers LSD (least significant difference) secondary analysis and unpaired Student's *T*-test. $P < 0.05$ were considered significant, represented in the figures as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

# RESULTS

## Native Serp-1 and PEGylated Serp-1 (Serp-1m5) Treatment Reduce Pristane-Induced DAH in C57/BL/6 SLE Mouse Model

When each mouse was euthanized by $CO_2$ inhalation, the lungs were immediately collected and imaged by a digital camera, before further processing (**Figure 1A**). All mice survived to 15 days post-pristane injections. According to the severity of the hemorrhage observed on gross pathology specimens, the lungs were divided into four grades: severe, moderate, mild, and no bleeding. As shown in **Figure 1B**, the whole lung pathology specimens demonstrate severe DAH in the saline treatment group, 5/6 severe DAH (5/6, $N = 6$) and 1 moderate DAH (1/6; $N = 6$). The Serp-1 treatment group contained five severe DAH (5/6; $N = 6$) and one non-DAH (1/6; $N = 6$). In the Serp-1m5 treatment group, three cases were severe (3/6; $N = 6$), two cases were mild (2/6; $N = 6$), and one case had no DAH (1/6; $N = 6$) (**Figure 1**; $p = 0.2677$). No hemorrhage was detected in normal healthy mice with or without Serp-1 treatment after euthanasia (Data not shown). Further evaluation of the hemorrhage was based on H&E staining and Prussian blue staining. Four 20 × fields were examined in randomly selected areas on the H&E sections for each mouse and scored according to the degree of bleeding as follows: 0, no hemorrhage; 1, 0–25% hemorrhage; 2, 25–50% hemorrhage; 3, 50–75% hemorrhage; 4, 75–100% hemorrhage. Representative histology images are presented in **Figure 2A**. The average DAH score for each mouse was calculated. Measurements were performed by two independent experimenters blinded to the treatments given to pristane injected mice. On histological examination, the DAH score of the Serp-1 treatment group indicates a trend toward a reduction and the DAH score for the Serp-1m5 treatment group is significantly lower than that of the saline group (**Figure 2B**; Serp-1, $p = 0.2172$; Serp-1m5, $p = 0.0252$). Prussian blue staining was used to detect hemosiderin laden macrophages. Serp-1 and Serp-1m5 treatments both significantly reduced

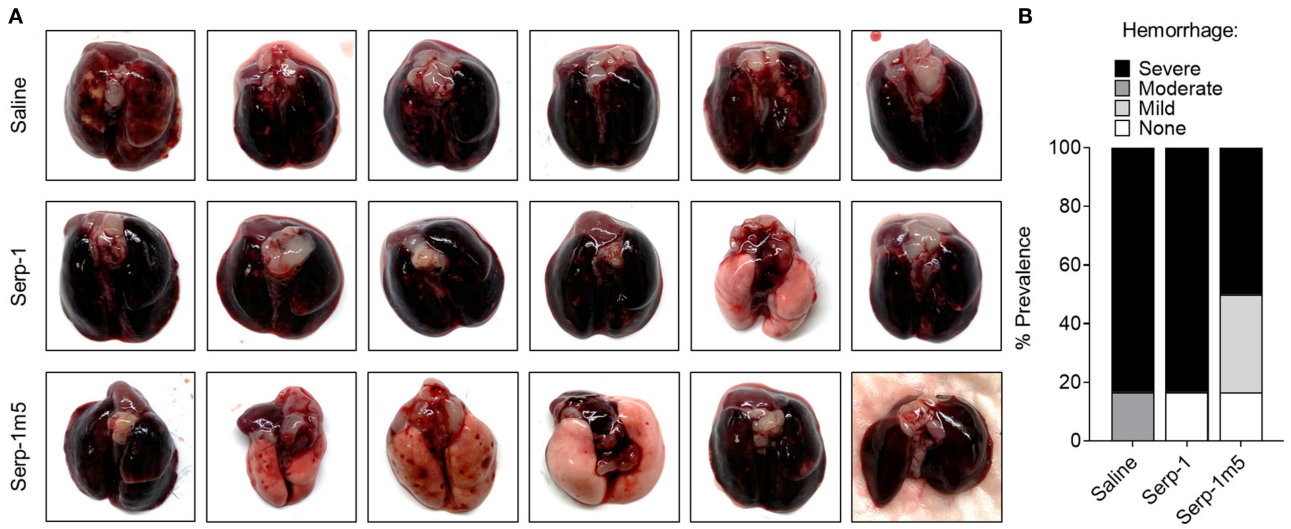


**FIGURE 1 |** Serp-1 and its reengineered derivative Serp-1m5 reduce the prevalence of hemorrhage in pristane induced DAH mouse model. All mice induced by pristane were euthanized by $CO_2$ on the 15th day of treatments with saline (6 mice), Serp-1 (6 mice), or Serp-1m5 (6 mice). Whole lungs from mice in each group were collected **(A)** and examined based on four grades of hemorrhage: severe, moderate, mild, or none **(B)**.

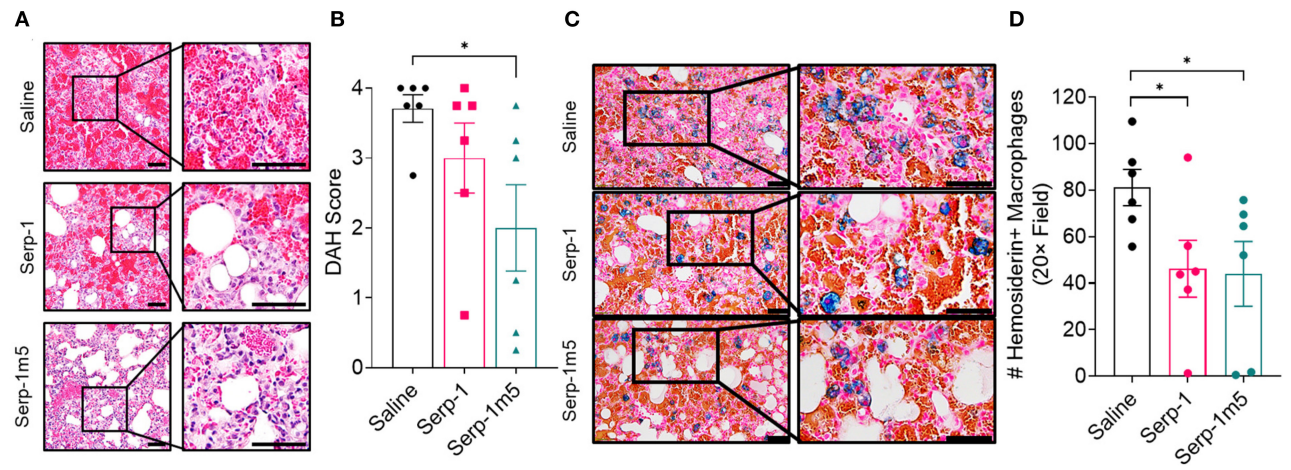


**FIGURE 2 |** Histological analysis of DAH during treatment with saline control ($N = 6$ mice), GMP Serp-1 ($N = 6$ mice), or Serp-1m5 ($N = 6$ mice) proteins given daily. **(A)** Representative micrograph images of H&E stained lung sections at 14 days follow up. **(B)** DAH scores were evaluated by taking four 20× field of each section, and calculating the average score as standardized: 0, no hemorrhage; 1, 0–25% hemorrhage; 2, 25–50% hemorrhage; 3, 50–75% hemorrhage; 4, 75–100% hemorrhage. The DAH score of Serp-1m5 is significantly lower than that of the saline group ($p = 0.0252$). **(C)** Representative micrographs of Prussian blue staining at 14 days for each treatment group. **(D)** Both Serp-1 ($p = 0.0370$) and Serp-1m5 ($p = 0.0424$) significantly reduced detected hemosiderin laden macrophage counts in lungs form the pristane induced DAH model. *$p < 0.05$.

detected hemosiderin laden macrophage cells when they were compared to the saline treatment group (**Figures 2C,D**; Serp-1, $p = 0.0370$; Serp-1m5, $p = 0.0424$). On H&E stained sections, there was a significant reduction in perivascular mononuclear cell infiltrates with Serp-1m5 treatments and a trend for Serp-1 treatment (**Figure 3**) (ANOVA, $P = 0.0104$; Serp-1m5, $p = 0.0026$, Serp-1, $p = 0.1215$). Trichrome staining revealed a trend toward a reduction in collagen or fibrous tissue staining around areas of excess hemorrhage, with both Serp-1m5 and Serp-1 treatments (**Figure 4**).

## Serp-1 and Serp-1m5 Treatment Reduced M1 Macrophages and Neutrophils in Pristane-Induced DAH Model

The DAH in the pristane induced mouse model is reported to be macrophage dependent (13). We characterized the proinflammatory M1 macrophage polarization in the mouse lung tissue sections by IHC staining for iNOS (iNOS+). As shown in **Figure 5**, the lungs of mice treated by Serp-1 and Serp-1m5 treatments had significantly lower numbers of iNOS+

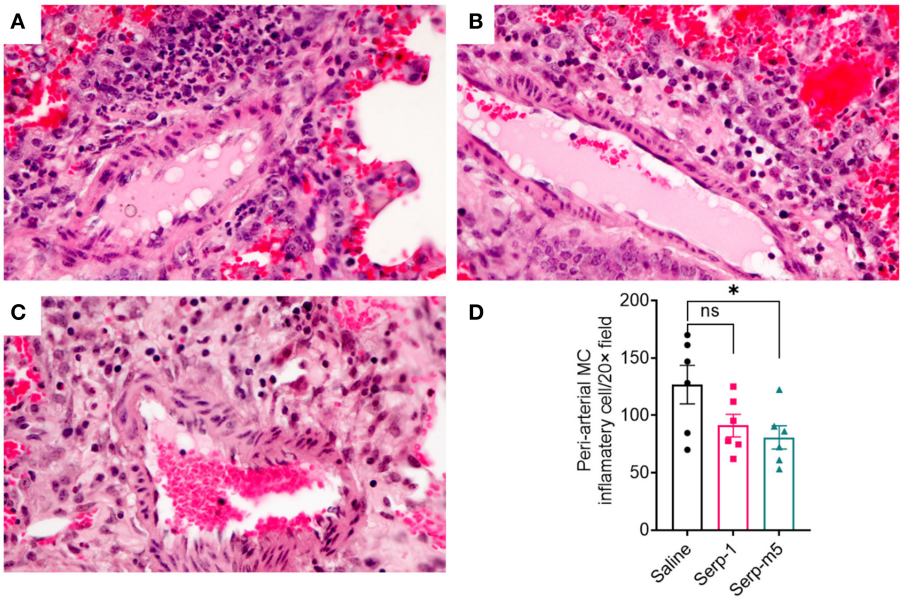


**FIGURE 3 |** Saline treated mice had marked perivascular mononuclear cell infiltrates after pristane induction DAH **(A)**. Serp-1 treatment produced a non significant reduction in perivascular inflammatory cell counts **(B)**. Serp-1m5 treatment significantly reduced perivascular inflammatory cell counts when compared to the saline treated controls **(C)**. Perivascular mononuclear cell counts ± SEM are illustrated in panel **(D)**. *$p < 0.05$.

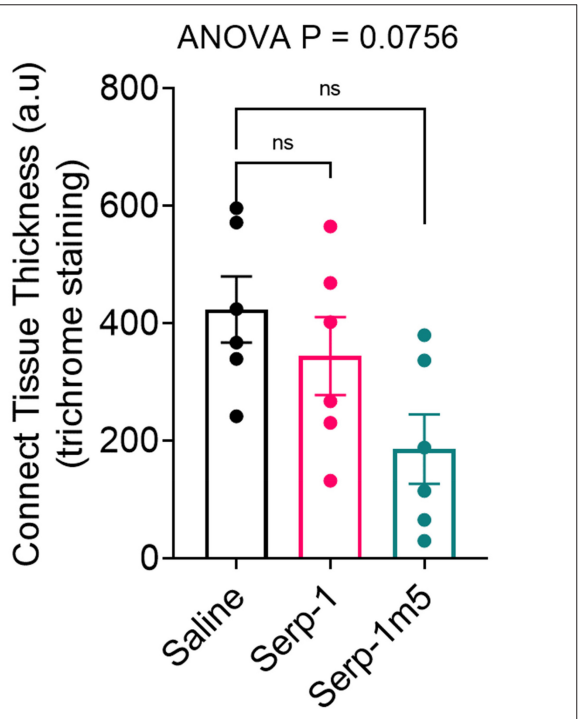


**FIGURE 4 |** Trichrome staining indicated a nonsignificant increase in collagen staining in areas of hemorrhage in the saline treated controls. Serp-1 and Serp-1m5 treatment produced a non-significant decrease in fibrous tissue staining, here illustrated as measured thickness of fibrous tissue.

M1 macrophages than that of saline control (**Figures 5A,B**; Serp-1, $p = 0.0350$; Serp-1m5, $p = 0.0053$). Additionally, lung tissues treated with Serp-1 and Serp-1m5 also have significantly less detected numbers of Ly6G+ neutrophils than the saline treatment group (**Figure 5C**; Serp-1, $p = 0.0371$; Serp-1m5, $p = 0.004$). We also performed IHC staining for arginase-1 (Arg-1) to characterize the anti-inflammatory M2 macrophages (**Figure 5D**), but no statistical differences among these three groups were observed for Arg-1+ M2 macrophage. Arg-1+ M2 macrophage staining detected a nonsignificant trend toward increased numbers. Serp-1 and Serp-1m5 treatment groups have a trend to increased CD3+ T cells (**Figure 5E**) when compared to saline treated mice but this does not achieve significance ($p = 0.3595$). There were no identified changes in different in CD4+ T helper cell staining either (**Figure 5F**; $p = 0.1015$).

## Serp-1m5 Treatment Reduced Macrophage Counts on Flow Cytometry Analysis in Cell Isolates From the Spleen of Mice After 15 Days of Induction With Pristane

In order to examine the potential systemic immune cell responses to Serp-1 and Serp-1m5 treatments on mice after pristane induction of DAH, we examined splenocyte isolates from each mouse at 14 days follow up. We did not observe consistent changes in spleen size among these three groups when we

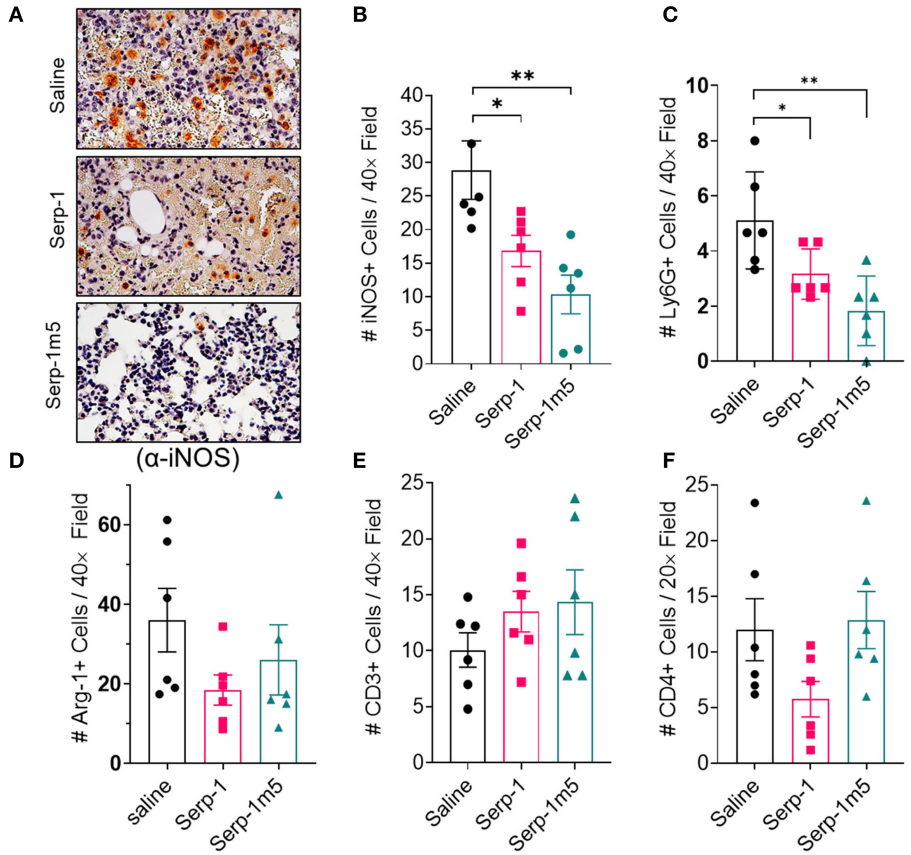


**FIGURE 5 |** DAH Serp-1 and Serp-1m5 treatments reduced M1 macrophage and neutrophil numbers in lung sections after pristane-induced DAH, but did not significantly affect M2 macrophage counts, CD3+ cell, or CD4+ cell infiltration. **(A)** Representative micrographs (40×) of iNOS + IHC staining in lungs at 14 days post-pristine injection. **(B)** Serp-1 and Serp-1m5 group had significantly lower numbers of M1 macrophage counts marked by positive staining for iNOS IHC staining (Serp-1, $p = 0.0350$; Serp-1m5, $p = 0.0053$) when compared to the saline treatment group. **(C)** The Serp-1m5 treated group had significantly lower numbers of Ly6G+ neutrophils (Serp-1, $p = 0.0371$; Serp-1m5, $p = 0.004$). Serp-1 treatment had a strong trend toward reduced neutrophil counts, but did not reach significance. The number of Arg-1 + M2 macrophages **(D)**, CD3+ T cells **(E)**, or CD4+ T cells **(F)** were not statistically different among the three groups, although a non-significant trend toward increased Arg+ cells was seen. $^{*}p < 0.05$, $^{**}p < 0.01$.

collected mouse tissues after 15 days of induction with pristane (17 mice in total–6 with saline, 6 with Serp-1, and 5 with Serp-1m5 treatment; the spleen from one mouse in the Serp-1m5 group was not collected and was not tested by flow cytometry). Flow cytometry analysis of splenocytes demonstrated significantly decreased F4/80+ macrophages (**Figure 6**; $p = 0.0173$) in live splenocytes from Serp-1m5 treated mice. No change in detected CD163+ M2 in F4/80+ macrophages, nor in CD11b or CD11c cells in live splenocytes was seen (**Figure 6B**). A significant increase in CD4+ T cells in live splenocytes ($p = 0.0268$) and the TH1/Th2 ratio ($p = 0.0287$) was detected (**Figure 7**); A significant decrease in Tregs ($p = 0.0285$) and GATA3+CD4+ Th2 cells ($p = 0.0097$) in CD4+ T cells were detected with Serp-1m5 but not the unmodified Serp-1 (**Figure 7**); There was no significant change in the frequency of NK cells, CD8+ T cells, CD11b+cells, CD11c+ cells in living spleen cells, IFNg+CD4+ Th1 cells, Th17 cells in CD4+ T cells, and CD163+F480+M2 macrophages in F4/80+ macrophages among the three groups (**Figures 6**, **7**).

## Serp-1 and Serp-m5 Reduce C5b/9 Complement Positive Cell Counts in DAH Model Lung Sections

Serp-1 pulled down human plasma C3 and vitronectin as determined by mass spectrometry. C5b/9 is a final stage in complement activation, the membrane attack complex. We have demonstrated a significant reduction in cells staining positively in both bronchial (ANOVA $p = 0.0008$) and parenchymal tissues (ANOVA $p = 0.0101$) in lung sections from mice treated. Both Serp-1 and Serp-1m5 produced significant reduction; Serp-1, bronchus $p = 0.0146$; parenchyma $p = 0.0077$ and Serp-1m5, brochus $p = 0.0002$, parenchyma $p = 0.0074$; **Figure 8**).

## Serp-1 and Serp-1m5 Treatment Reduced Cell Membrane Free Soluble uPAR in the Lung Tissue of Pristane-Induced DAH Mouse Model

The uPA-uPAR interaction plays an important role during inflammatory cell invasion and activation. It has been reported

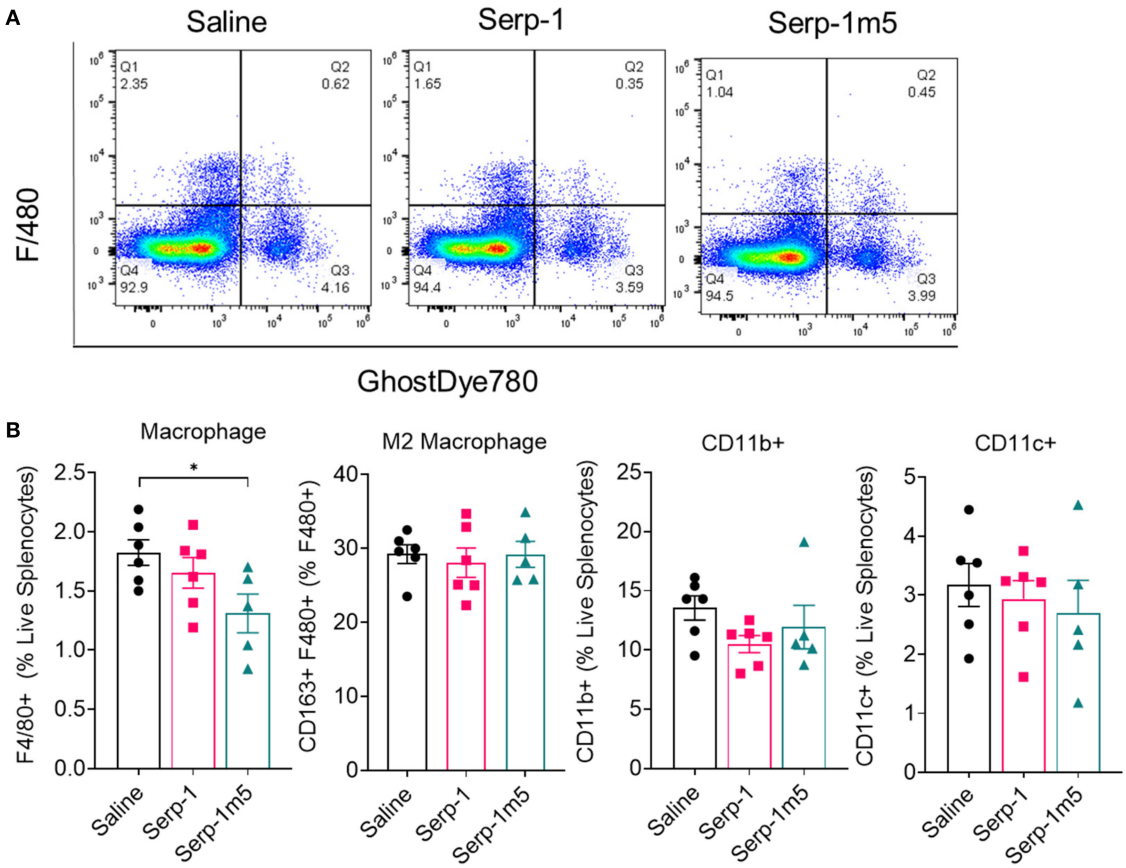


**FIGURE 6 |** Serp-1m5 treatment reduces macrophages in the spleen of mice after 15 days of induction with pristane. **(A)** Representative images of flow cytometry for F4/80+ macrophages in live splenocytes demonstrating reduced F4/80+ cells with Serp-1m5 treatment. **(B)** Flow cytometry of macrophages demosntrated decreased frequency of F4/80+ macrophages ($p = 0.0173$) in live splenocytes in Serp-1m5 treated mice compared with saline treated mice while no significant differences in the frequency of CD11b+cells, CD11c+ cells in living spleen cells and CD163+F480+M2 macrophages in F480+ macrophages among the three groups. *$p < 0.05$.

earlier this year that cell free uPAR, i.e., soluble uPAR (suPAR), is related to organ damage in SLE patients (31). Our research has previously demonstrated that the immune modulation produced by Serp-1 in aortic allografts as well as Serp-1 inhibition of macrophage activation and diapedesis in tissue culture is dependent on the uPAR (25). In prior work, Serp-1 lost its ability to reduce inflammation and to reduce plaque growth in uPAR knock out aortic allograft transplants in mouse models (18). The depletion of uPAR also abolished the function of Serp-1 to promote wound healing (30). We therefor performed IHC staining for uPAR to characterize uPAR expression after pristine induction, with or without serpin treatments. It can be seen from the IHC staining of lung tissue that Serp-1 and Serp-1m5 reduce non-specific uPAR+ clusters (**Figures 9A–C**; Serp-1, $p = 0.0172$; Serp-1m5, $p = 0.0025$), detected as non-cell associated clumps of positive staining in the lungs, when compared to the saline treatment group. In contrast, there was increased detection of intact uPAR+ stained alveoli along the inner rim (**Figures 9A–C**; Serp-1m5 vs. saline, $p = 0.0091$). Control experiments without primary antibody did not detect nonspecific staining. To further quantitatively analyze the dissociation of uPAR, we compared the uPAR extracted from tissue without detergent (PBS only) to the total uPAR extracted with RIPA buffer containing 0.1% SDS. ELISA was performed to determine the concentration of uPAR in each extraction. We set the average ratio of lung tissue cell membrane free uPAR (csuPAR) to total uPAR in each lung treated with saline as one and normalized all the ratios of csuPAR/total uPAR (**Figure 9D**). The ratio of csuPAR to total uPAR of Serp-1 and Serp-1m5 groups was significantly lower than that of the saline treated group (Serp-1, $p = 0.0004$; Serp-1m5, $p = 0.0002$). This data confirmed our observation in the IHC staining to uPAR (**Figures 9A–C**).

## DISCUSSION

The pathogenesis of DAH is reported to be caused by defects in macrophage phagocytic function and reduction in the removal of apoptotic cells. Apoptotic fragments activate autoreactive B cells and T cells, leading to the production of autoantibodies

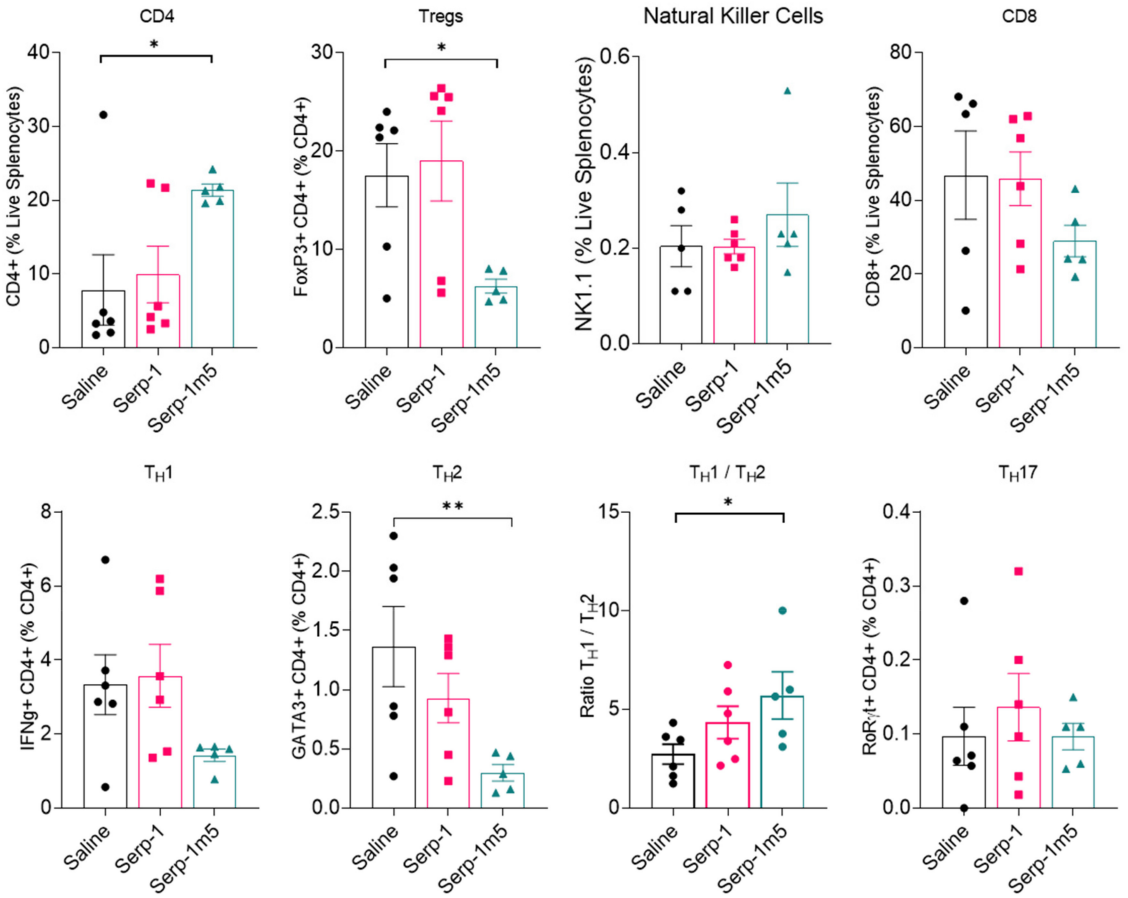


**FIGURE 7 |** Flow cytometry of lymphocytes indicate a decreased frequency of GATA3+CD4+ Th2 cells ($p = 0.0097$), Tregs cells ($p = 0.0285$) in CD4+ T cells and increased frequency of CD4+ T cell in live splenocytes ($p = 0.0268$), ratio of Th1/Th2 ($p = 0.0287$) in Serp-1m5 treated mice compared with saline treated mice while no significant differences in the frequency of NK cells, CD8+ T cells, IFNg+CD4+ Th1 cells, Th17 cells in CD4+ T cells among the three groups. $^*p < 0.05$, $^{**}p < 0.01$.

and the formation of circulating immune complexes (ICs). ICs are then believed to activate the classical complement pathway, thereby causing pulmonary capillary vasculitis, damage to the basement membrane, and capillary leakage with extravasation of red blood cells (RBC) and bleeding into the alveolar cavity. This continuous auto-Ab-mediated enhancement of the complement system also causes complement depletion and reduces the ability of phagocytes to remove dead cell debris, thus initiating a vicious circle (32). Intraperitoneal injection of pristane in B6 mice is an accepted model for lupus that simulates the SLE DAH pathological process (14, 33, 34). After injection, pristane migrates to the lungs, resulting in increased cell death, activation of inflammatory cells such as macrophages, activation of the classical pathway of complement and ICs formation and additionally alveolar hemorrhage similar to human DAH occurs. Studies have demonstrated that this process is macrophage dependent in the mouse DAH model. Neutrophil depletion is however reported to not be protective in prior mouse model studies when using antibody to neutrophil elastase. In contrast treatment with clodronate liposomes (CloLip) to reduce macrophages was able to prevent DAH (13). Cell debris depends on the opsonization of natural IgM, C3, and CR3/CR4 on the surface of macrophage cells to activate downstream inflammatory pathways. $CD11b^{-/-}$ mice are protected against the development of pristane-induced DAH (5), and mice with C3 deficiency and CD18 deficiency are also resistant. Reduction of complement in wild-type mice by cobra venom factor (CVF) can prevent DAH (13), while antibody to suppress neutrophils was not effective.

From prior research, it is understood that macrophages play a decisive role in the SLE DAH pathological process. Our research has demonstrated that the administration of Serp-1 or Serp-1m5 after intraperitoneal injection of pristane can significantly reduce the occurrence and severity of DAH (**Figures 1**, **2**). We performed immunohistochemistry using typical markers of M1(iNOS), M2 (ARG1) macrophages and neutrophils (Ly6G). Quantitation of stained cells demonstrated that Serp-1 and Serp-1m5 treatments significantly reduced M1 macrophage polarization and neutrophil infiltration in the lungs of the DAH mouse model. Flow cytometry analysis of spleen cells was also

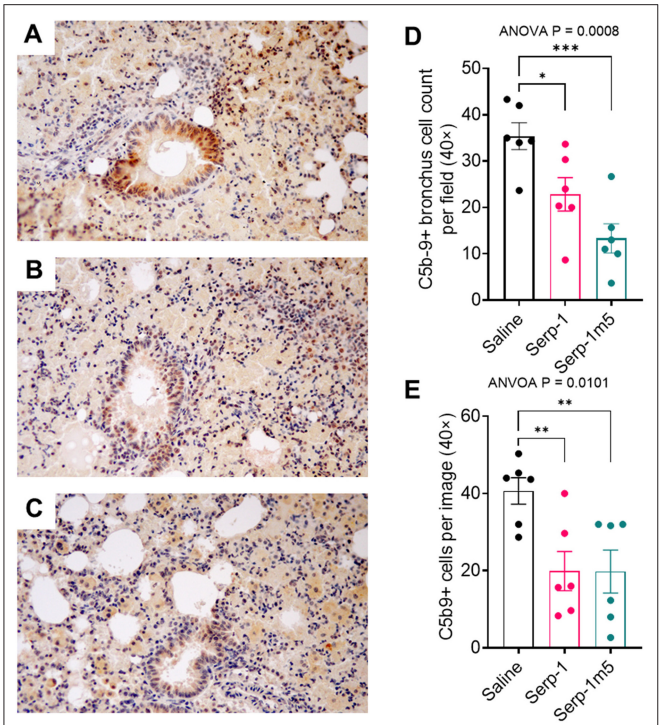


**FIGURE 8 |** C5b/9 staining is markedly increased in the bronchial epithelium and in the surrounding lung parenchyma in saline treated mice with pristane induced DAH **(A)**. Serp-1 **(B)** and Serp-1m5 **(C)** significantly detected reduced bronchial staining. Bar graphs demonstrate significant changes in the mean cell counts ± SEM for cells positively stained for C5b/9 in the bronchial epithelium **(D)** and in the lung parenchyma **(E)**; Bronchus cell counts—$p = 0.0008$ ANOVA; Parenchyma ANOVA—$p = 0.0101$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

consistent with the findings on immunostaining in the lungs. Flow cytometry analysis demonstrated that the macrophage isolates from the spleen of Serp-1m5 treated mice after pristane induction were significantly reduced when compared to saline treated pristane injected control mice. In contrast, the number of M2 macrophages did not change significantly. Therefore, it is deduced that the efficacy of Serp-1 and Serp1m5 treatment is associated with a significant reduction in M1 macrophage. Variable changes in lymphocytes counts on IHC with some significant differences in CD4, Treg, and Th2 counts on flow cytometry were observed and on flow cytometry. These T cell changes were limited to Serp-1m5 treatments. These findings are consistent with previous studies with Serp-1 suggesting a more significant effect on macrophage responses. However, the more pronounced effects of Serp-1m5 both on lung hemorrhage, M1 macrophage and Ly6G counts on IHC and also greater effects of Serp-1m5 on splenocyte analyses might suggested that some of the enhanced activity of the Serp-1m5 protein is due to a larger effect overall on immune cell responses in the DAH model in mice.

M1 macrophages are involved in the pathogenesis of various autoimmune inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, asthma, and SLE (1, 35–39), we have proposed that targeting M1 cells with Serp-1 treatment will provide a potential treatment for autoimmune diseases, suggesting that Serp-1 can inhibit the activation of macrophage M1 to protect SLE patients against the development of DAH. Serp-1 is a proven inhibitor of activated serine proteases, functioning to bind, and inhibit both tPA and uPA as well as thrombin and fXa as noted in the introduction. The thrombotic and thrombolytic cascades also activate immune and inflammatory cell responses and conversely platelets and thrombosis are activated on the surfaces of dysfunctional endothelial cells and/or activated macrophages in the arterial wall. uPA and tPA are plasminogen activators, serine proteases, in the thrombolytic, clot dissolving cascade, and can initiate matrix metalloproteinase activation and connective tissue degradation allowing immune cell invasion. Complement is also serine protease central to immune cell responses. We have posited that Serp-1 inhibits the migration of M1 macrophages to the lung via blockade of the uPA/uPAR complex on the surface of activated inflammatory phagocytes. Activated macrophage expression of uPA and tPA leads to the activation of MMPs allowing cell invasion into damaged tissues by breaking down surrounding connective tissue or the arterial endothelial glycocalyx. It is, however, not known whether Serp-1 may directly promote macrophage polarization to a M2 phenotype. The uPAR is part of a large lipid raft that interacts with many cell surface integrins and low density lipoprotein related protein receptor (LRPR) as well as chemokine receptors. We had previously demonstrated that Serp-1 alters macrophage migration *in vitro* via uPAR and filamin B (an actin binding protein) dependent mechanisms. Our previous studies have demonstrated that Serp-1 can reduce monocyte/ macrophage adhesion and migration across endothelial monolayers *in vitro* and into mouse ascites *in vivo*. Serp-1 applied to monocytes alters the expression of filamin B and CD18, increasing filamin B and decreasing CD-18 expression. These alterations in gene expression are uPAR dependent and application of siRNA to filamin blocked Serp-1 mediated inhibition of monocyte migration *in vitro*. Filamin b and uPAR are co-localized and co-immunoprecipitated with Serp-1 (25). Therefore, we would suggest that Serp-1 exerts its anti-inflammatory effects by modifying uPAR-CD18 and filamin b in monocytes to mediate the decrease of M1 macrophages in lung tissue. We would like to further note that in prior reported studies of the mechanisms of DAH development, DAH was resistant to CD18-deficient mice (5).

Serp-1 treatment may also have the added benefit of reducing activation of fibrinolysis and thus may directly mediate a reduction in bleeding. The uPA/uPAR complex is traditionally considered to act predominantly on cellular activation and immune cell responses rather than as a primary regulator of thrombolysis. tPA, the fibrinolytic serine protease that Serp-1 also inhibits, is considered the central mediator of the regulation of clot lysis in the blood stream. In this study, our immunohistochemical study of uPAR in lung tissues demonstrated that when compared with saline treated DAH

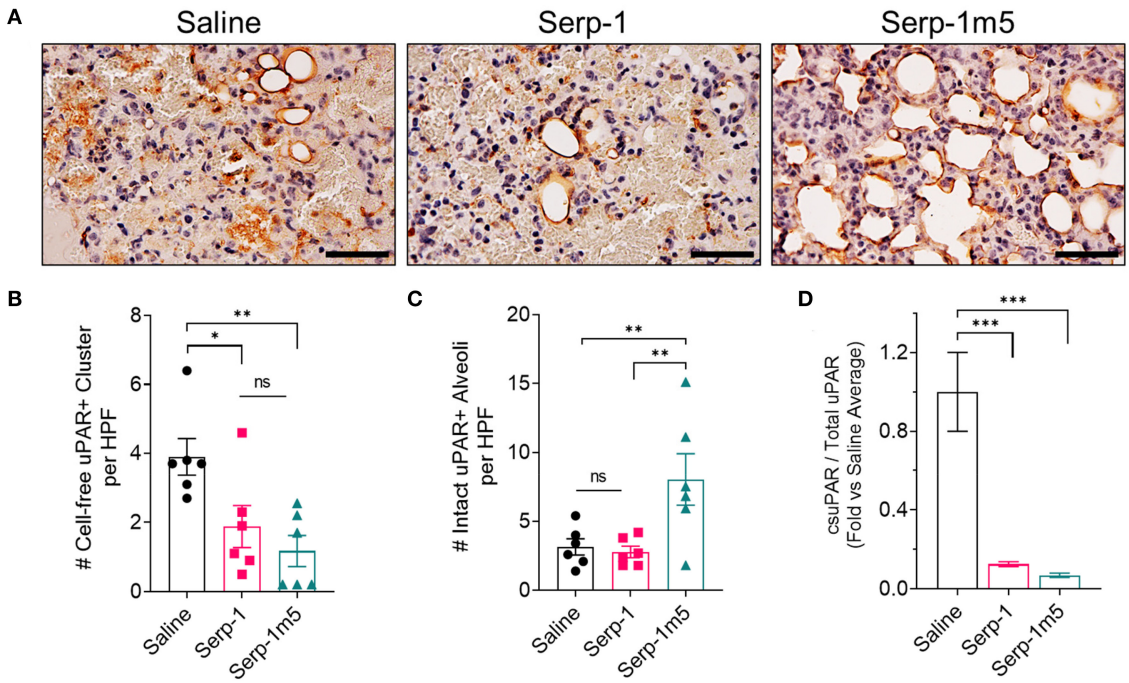


**FIGURE 9 |** Serp-1 and Serp-1m5 treatment reduce soluble uPAR in the pristane-induced DAH model. **(A)** The representative pictures of uPAR IHC staining. **(B)** Serp-1 and Serp-1m5 reduced cell-free uPAR+ clusters in IHC staining when compared to the saline treatment group (Serp-1, $p = 0.0172$; Serp-1m5, $p = 0.0025$). **(C)** Serp-1m5 increased intact uPAR+ stained alveoli along the inner rim when compared to the saline treatment group ($p = 0.0091$; Serp-1m5 vs. saline). **(D)** The ratio of csuPAR to total uPAR of Serp-1 and Serp-1m5 tested by ELISA of lung tissue was significantly lower than that of the saline group (Serp-1, $p = 0.0004$; Serp-1m5, 0.0002). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

mice, the soluble uPAR in the Serp-1 treatment group was significantly reduced, while uPAR on the intact alveolar cells was preserved significantly. This is consistent with the uPAR ELISA analysis of lung tissue that similarly detected Serp-1 reductions in soluble uPAR. uPAR is composed of three homologous domains and is connected to the cell surface through glycosylphosphatidylinositol (GPI) anchors. The three domains of uPAR include the main uPA (40), and the extracellular matrix protein vitronectin binding site (41). uPAR is easily cleaved by several proteases, including physiologically related enzymes such as neutrophil elastase, plasmin and uPA itself (42–44).

Soluble uPAR is currently considered to be a biomarker of inflammation and immune system activation but has not to date been examined nor associated with DAH in SLE patients. Elevated suPAR levels are associated with a variety of inflammatory diseases, such as systemic inflammatory response syndrome (SIRS), cancer, local segmental glomerulosclerosis, cardiovascular disease, type 2 diabetes, asthma, liver failure, COVID, and SLE (31, 45–50). After DAH occurs, the alveolar basement membrane tissue is destroyed by immune complex (IC) deposition and inflammatory cell adhesion, red blood cells then extravasate (leak out of damaged vessels), and abnormal blood coagulation pathways are initiated. Cleavage of uPAR by plasmin and uPA causes the release of soluble uPAR from the cell surface membrane into surrounding tissues, weakening of the anchoring of cells to the extracellular matrix, reducing endothelial cell connections and, at the same time, triggers a series of proteolytic cascade reactions to degrade the components of the extracellular matrix leading to further destruction of alveolar tissue structure and increased bleeding. Therefore, Serp-1 can reduce the cleavage of uPAR, maintain cell adhesion to the extracellular matrix as well as reducing inflammatory cell invasion via inhibiting uPA and plasmin and activation of matrix metalloproteinases. As we have seen in our experiments, soluble uPAR is significantly reduced, and uPAR in intact alveolar tissue cells increased. Meanwhile, Serp-1 reduces the downstream protease cascade by modulating the coagulation and fibrinolysis process, thereby preventing further destruction of lung tissue structure and pulmonary hemorrhage. Activation of complement is also central to immune responses. We have demonstrated Serp-1 binding to C3 in human plasma by MS analysis. We also report here a reduction in C5b/9 IHC complement staining with serpin treatments, which may be secondary to efficacy in reducing uPA/uPAR activity or conversely complement. Prior work would suggest that uPAR is a principal target. However, binding and modulation of a second pathway via C3 may also contribute to the benefits seen with serpin treatments in this lupus DAH model. These findings support a reduction in overall immune cell activation with serpin treatment but do not provide a final proof of mechanism. The studies do suggest a close correlation between

uPAR expression, as well as complement activation, both serine protease pathways, and serpin efficacy in this mouse model of pristane induced SLE DAH.

In summary, Serp-1 and PEGylated Serp-1 (Serp-1m5) effectively reduce the frequency and severity of pristane-induced diffuse pulmonary hemorrhage in mice through multiple factors, while the modified Serp-1 protein has greater efficacy than its wild type does in this experimental DAH model. This research proves that Serp-1 and its derivates are very promising candidates of therapeutics for DAH, which has no proven effective treatment available now. In this direction, the molecular mechanism under the therapeutic effectiveness to DAH and the technologies for the modification will be further studied.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Biodesign Institute, ASU Institutional Animal Care and Use Committee Animal Protocol Review ASU Protocol Number: 20-1761R RFC 1.

## AUTHOR CONTRIBUTIONS

QG, JRY, LZ, and ARL designed the study, LZ and QG developed materials. LZ, QG, and JRY performed the animal studies and histology assays, RB and TO assisted in the protein purification, KB, EA, MB, and LNS performed histology, JW and PF provided scientific feedback and discussion, QG, LZ, JRY, and SL analyzed the data, QG, ARL, and LZ wrote the manuscript. All authors reviewed the manuscript.

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**Conflict of Interest:** The method of Serp-1 modification has been submitted for a provisional patent application with title of "New composition of immunomodulating Serpin, Serp-1" (PCT Application No. 63/017,598). ARL, LZ, QG, JRY, and JW are inventors of this patent. JW is employed by Exalt Therapeutics but has received no funding for his work on this project nor the research presented in this manuscript.

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# Angiotensinogen and the Modulation of Blood Pressure

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The angiotensin peptides that control blood pressure are released from the non-inhibitory plasma serpin, angiotensinogen, on cleavage of its extended N-terminal tail by the specific aspartyl-protease, renin. Angiotensinogen had previously been assumed to be a passive substrate, but we describe here how recent studies reveal an inherent conformational mechanism that is critical to the cleavage and release of the angiotensin peptides and consequently to the control of blood pressure. A series of crystallographic structures of angiotensinogen and its derivative forms, together with its complexes with renin show in molecular detail how the interaction with renin triggers a profound shift of the amino-terminal tail of angiotensinogen with modulation occurring at several levels. The tail of angiotensinogen is restrained by a labile disulfide bond, with changes in its redox status affecting angiotensin release, as demonstrably so in the hypertensive complication of pregnancy, pre-eclampsia. The shift of the tail also enhances the binding of renin through a tail-in-mouth allosteric mechanism. The N-terminus is now seen to insert into a pocket equivalent to the hormone-binding site on other serpins, with helix H of angiotensinogen unwinding to form key interactions with renin. The findings explain the precise species specificity of the interaction with renin and with variant carbohydrate linkages. Overall, the studies provide new insights into the physiological regulation of angiotensin release, with an ability to respond to local tissue and temperature changes, and with the opening of strategies for the development of novel agents for the treatment of hypertension.

**Keywords: serpin, angiotensinogen, renin, tail-in-mouth, allosteric, redox switch, hypertension, pre-eclampsia**

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## INTRODUCTION

Angiotensinogen, a non-inhibitory serpin (1, 2), has a key physiological function as the carrier of the angiotensin peptides that control blood pressure. It acts in this way as a substrate, in what is the first and rate-limiting step in the renin–angiotensinogen system (RAS), with the cleavage of the N-terminal extension of angiotensinogen by the highly-specific aspartyl-protease renin. Cleavage of the N-terminus releases a decapeptide, angiotensin-I, which is then subsequently processed (**Figure 1A**) to give the sub-peptides that influence salt retention and vasoconstriction, and hence, control blood pressure (3, 4). Although angiotensinogen is present in the plasma in relatively high concentration (0.8 μM), its primary function is now believed to occur at a cellular level (5); with its direct role in the control of blood pressure (6) emphasized by the recent demonstration of the hypotensive response to its siRNA suppression (7).

## STRUCTURAL MECHANISM

The role of angiotensinogen in the RAS was for long considered as merely that of an inert substrate. The previous questioning of this passive role (6, 8), suggesting an inherently active role of angiotensinogen in the overall control of the release of angiotensin and, hence, in the modulation of blood pressure, has now been definitively confirmed by our recent structural studies (9, 10). The solving of a series of crystallographic structures at high resolution of human angiotensinogen, together with its physiologically truncated forms and complexes with renin, now provides a video view of the conformational shifts that take place on the interaction of angiotensinogen with renin. The juxtapositioning and complexing of the two molecules requires major shifts in each, not only to reveal the renin-cleavage site in angiotensinogen and position it within the active site of renin but also to give the widespread changes in each that are necessary for their highly specific interlinkage. As highlighted here, this video view of the interaction of the two molecules provides direct insights of medical and biological significance, indicating how the cleavage of angiotensinogen and release of angiotensin-I can be modulated by external factors and providing an explanation for the tight species specificity of the cleavage of angiotensinogen by renin.

## BURIED CLEAVAGE SITE

The crystal structure of angiotensinogen shows that it essentially retains the typical serpin fold, including an exposed, although inert, reactive center loop. The striking difference, however, is seen in the superstructure formed by the 63-residue N-terminal extension containing the angiotensin-I decapeptide. This terminal tail is anchored to the body of angiotensinogen by extensive hydrophobic bonding including two new helices, with the renin-cleavage site at Leu10-Val11 being held in an inaccessible buried site (**Figure 1B**). The advantage of this buried configuration is that it protects the scissile bond in the circulating protein, with the complexity of its conformational exposure and entry into the active site cleft of renin ensuring the precise specificity of the cleavage.

## CONFORMATIONAL SHIFT IN RENIN BINDING AND CLEAVAGE

An enlightenment from the structural findings is that the conformational shift in angiotensinogen on its interaction with renin is seen to not only expose the angiotensin cleavage site but also to involve widespread changes that allow the complementary binding of the two molecules. This tight and extensive interlinkage of renin and angiotensinogen ensures the precise entry of the scissile bond into the active site cleft of renin and explains the proteolytic specificity of the release mechanism. Major conformational shifts take place on the docking of renin, with the angiotensin segment of the N-terminal tail of angiotensinogen being competitively displaced by 10–20 Å from its linkages to the body of the molecule. This is accompanied by a 10-Å displacement of the CD loop of angiotensinogen, which would otherwise sterically block the binding of renin, the two concerted movements being linked by a conserved disulfide bond. The widespread nature of other changes that take place on the binding of the two molecules has been further revealed in the most recent high-resolution structures (10), which show an accompanying rearrangement of helix H of angiotensinogen to allow more extensive bonding between the two molecules.

## SELECTIVITY OF THE RELEASE MECHANISM

The requirement of widespread bonding explains the highly specific interaction of angiotensinogen and renin and emphasizes the tight control exercised over the release of angiotensin. Evidence of this selectivity of release comes from the observed difference in the kinetics of the release of the angiotensin decapeptide from synthetic peptides (11) or from a surrogate serpin carrier (12), as compared to the release from angiotensinogen. This is even more evident in the interspecies selectivity of the interaction with renin, thus human renin will only cleave human angiotensinogen and not that of the mouse or rat (13). The critical factor in this selectivity has now been shown (10) to be due to changes not as expected from other protease studies in the residues surrounding the scissile site, but rather in more peripheral residues involved in the body-to-body interface between the two molecules.

## MODULATION: OXIDATION AND PRE-ECLAMPSIA

The structural findings as well as showing the precision of the cleavage of angiotensinogen by renin also indicated the likelihood that the conformational changes involved could in themselves readily allow a modulation of angiotensin release. With this in mind, attention focused on the disulfide bridge that links the movement of the N-terminus of angiotensinogen and the accompanying shift of the CD loop necessary for the body-to-body binding of renin (9). This conserved S-S bridge, between Cys18 in the N-terminus to Cys138 in the body of angiotensinogen, was known from earlier biochemical studies to have a critical functional role (12), and to be subject to external oxidation (14). Moreover, there were recurrent references in the literature to the occurrence of hypertensive crises at times of oxidative stress (15, 16). Could the oxidation of the disulfide bridge affect the cleavage by renin and the release of angiotensin?

Support for this came with the demonstration (9) that this linking-disulfide existed in humans in a balanced equilibrium between its oxidized (bridged) and reduced (unbridged) states. Blood plasma samples from healthy individuals, regardless of gender or age, showed a remarkably consistent reduced-to-oxidized ratio for the S-S bridge, near 40:60, with the redox poise readily allowing a switch between the two forms. Significantly the switch from the reduced to the oxidized form results in a 4-fold increase in the catalytic release of angiotensin (9). Taken together, these findings strongly imply a modulatory mechanism

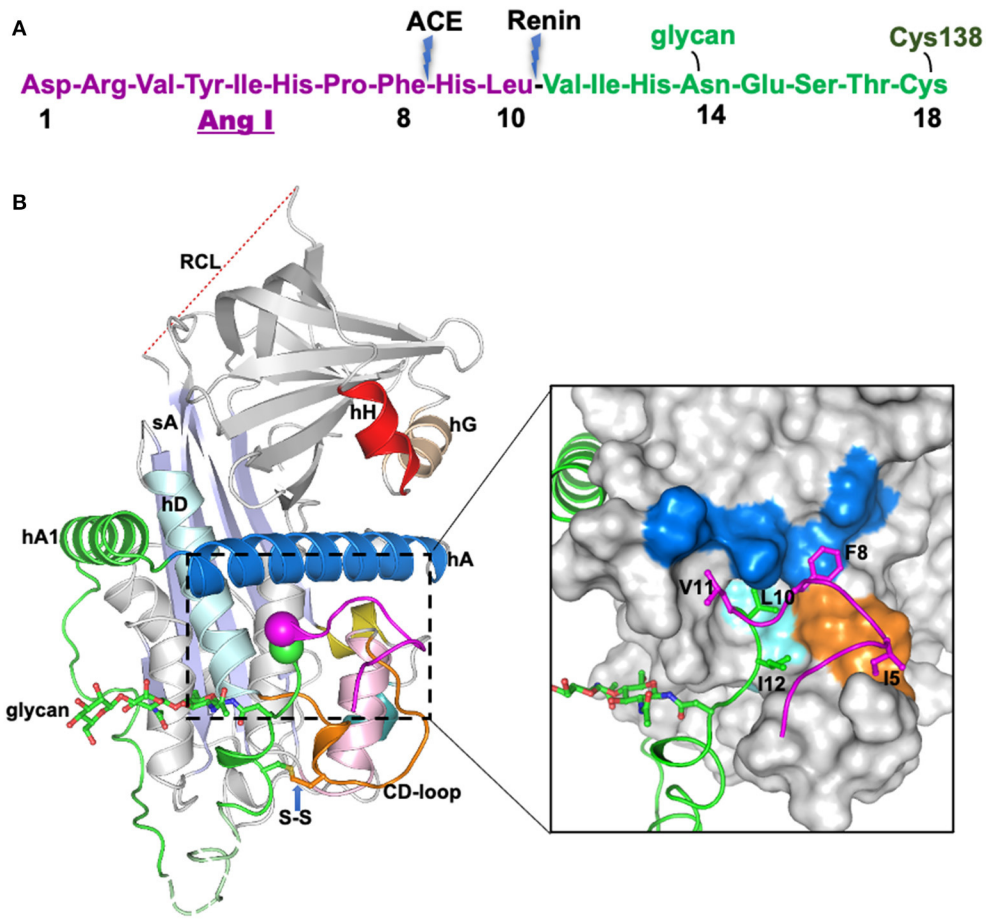


**FIGURE 1 |** The crystal structure of human glycosylated angiotensinogen (9). **(A)** N-terminal tail sequence, indicating renin and ACE (angiotensin converting enzyme) cleavage sites, the glycosylation site and conserved disulfide bond. **(B)** Structure of angiotensinogen shown as a cartoon. Serpin template in gray, helix A in marine, A-sheet light blue, and the disordered reactive center loop (RCL) in red dashes. Angiotensin-I tail in green with the scissile bond shown as magenta and green spheres. Cys18 in the amino tail forms a disulfide bond with Cys138 of the CD-loop (brown). The glycan attached to Asn14 is shown as green sticks. The segment from Glu20-Pro29 (dashed, pale green) is disordered in the structure and modeled for illustration. Surface representation of the main body of AGT, with the extra N-terminus (1-63) shown in cartoon representation. The Angiotensin-I peptide is mainly stabilized by hydrophobic interactions with the main body (colored as yellow surface), and the scissile bond (shown as spheres) is buried in the hydrophobic cavity formed by residues in the CD-loop (V131, P132, and W133), helix A (L68, M72, L76, and F79) and helix D (L142 and V147). The figure was adapted from our previous publication (9) with modifications.

with the deduction being that episodic hypertension could be triggered by the oxidative conversion of angiotensinogen to its more active bridged form. The investigation of this proposal using plasma assays was challenging, as the oxidative switch predictably occurs diffusely, in renal and vascular tissues, rather than in the circulation (5). Confirmation came however, from the more focal oxidative stress that occurs in the placenta and underlies the hypertensive crises of pregnancy: pre-eclampsia. Here, evident changes were demonstrable in maternal plasma samples from pre-eclamptic pregnancies, with a clear increase in the oxidized form as compared to carefully matched normal-pregnancy controls (9). The results in this initial study have now been convincingly supported by subsequent quantitative assays in pre-eclamptics, showing a consistent increase in the proportion of oxidized angiotensinogen (17, 18) coupled with a fall in plasma antioxidants (19). The 4-fold increase in the catalytic efficiency of release of angiotensin by renin from oxidized angiotensinogen may seem small, but evidence that it is a sufficient cause of the resultant hypertension comes from the previous finding of a similar but even smaller increase in activity associated with hypertension, in a family with an angiotensinogen cleavage-site mutation and a history of pre-eclampsia (20).

These findings clearly establish the contribution of redox changes to the regulation of blood pressure, but oxidation is just one factor in the regulation of angiotensin release from angiotensinogen. The less active unbridged form of angiotensinogen, with reduced sulfydryls, is also demonstrably stabilized by nitrosylation (9) in keeping with the known hypotensive action of nitric oxide. Other adjustments of the efficiency of the renin-release of angiotensin were shown (10) to arise from variations of glycan composition, notably so at Asn14

in close proximity to the renin-cleavage site at Leu10. The overall clinical and physiological message is that angiotensinogen is not an inert substrate. It inherently contains complex and responsive adaptations that make it a key initiator in the regulation of blood pressure.

## WHY A SERPIN?

The bonus from the more recent structures of the complex of renin with angiotensinogen (10) is the answer they provide to what has long been an intriguing puzzle. Why has evolution selected the complex serpin framework as the carrier of the angiotensin decapeptide?

The serpins are an ancient protein superfamily, the members of which have evolved over millions of years from their origins as protease inhibitors in early unicellular organisms (21). The survival and now predominance of the serpins in all forms of life is dependent on the efficiency of their inhibitory mechanism, which irreversibly entraps a target protease. The entrapment mechanism involves a profound conformational change, with a large 70-Å movement of the cleaved reactive loop to incorporate it into the main beta sheet of the molecule (22). This transition, from a metastable stressed (S) form to a hyperstable relaxed (R) form, has been conserved in most but not all the later members of the family. In particular, three of the principal serpins in human plasma are carriers of essential hormones: the thyroxine and corticosteroid binding globulins, TBG and CBG, and angiotensinogen. All three carrier serpins have lost their functions as protease inhibitors although TBG and CBG have retained the ability to undergo the S-to-R transition (23). The conformational shift that accompanies the movement of the reactive center loop in TBG and CBG is seen to be directly transmitted to the hormone-binding site in each and, hence, to affect the binding and release of the hormones. Angiotensinogen does not, however, undergo the S-to-R transition in stability (2), but nevertheless, it was confidently expected that, as with TBG and CBG, the release of its hormone would be modulated by the inherent serpin mechanism. Repeated structures of reactive-center cleaved and other forms of angiotensinogen failed however to show any of the conformational shifts expected from the movement of the cleaved loop into the body of the molecule. This was a puzzling disappointment. Based on a primordial form present in lampreys (24), angiotensinogen is believed to have originated as an add-on adaptation of an active protease-inhibitory serpin, so there had been good reason to expect that, as with TBG and CBG, the justification for this adaptation would be an accompanying regulatory advantage.

## TAIL-IN-MOUTH MODULATORY MECHANISM AND THE RELEASE OF ANGIOTENSIN

An unexpected answer came from the later high-resolution structures of the complex of angiotensinogen and renin (10), which showed how angiotensinogen had indeed adapted a subtle feature of the serpin mechanism to allow a fine-tuning of the release of the angiotensin peptide. The first few residues of the amino-tail of angiotensinogen were seen in the complex to extend beyond the active cleft of renin and to insert into the equivalent in angiotensinogen of what in TBG and CBG is the hormone-binding pocket (**Figure 2**). This "tail-in-mouth" action in angiotensinogen requires an unfolding of helix H, which forms one wall of the binding pocket, with the unfolding revealing key sites involved in the bonding that links the bodies of the renin and angiotensinogen. In this way the interaction between the two molecules can be seen to be dependent on widespread conformational changes that allow the complementary binding of the two, with integral to this the movement of the scissile bond of angiotensinogen into the active site of renin and the insertion of the initial residues of the N-terminus into the "helix H" pocket of angiotensinogen. This latter, newly-recognized aspect of the interaction with renin is of critical regulatory significance. The efficiency of cleavage and release of the angiotensin decapeptide, a rate-limiting step in the control of blood pressure, is now seen to be dependent on the completion of the renin-angiotensinogen interface, revealed by the unfolding of the H helix. Conversely the cleavage of the angiotensin decapeptide from the tail will cause a reversion of this complementarity, with an accompanying dissociation of the renin from angiotensinogen and the release of angiotensin-I. Confirmation of the mechanism of this dissociation comes from the observed loss of renin-binding affinity in post-cleavage (des-angiotensin) angiotensinogen (10).

## TEMPERATURE SENSITIVITY OF ANGIOTENSIN RELEASE?

The modulatory potential of this tail-in-mouth mechanism is clear. Any competitive blockage of the serpin "hormone"-pocket of angiotensinogen or decrease in its binding-affinity will hinder the interaction with renin, with a predictable decrease in angiotensin release and, hence, hypotensive consequences. A direct implication follows from recent studies with the thyroxine and corticosteroid binding globulins, TBG and CBG (25–28). These hormone-carrying serpins show how the affinity of the hormone binding-pocket is inherently responsive to changes in temperature, even in their conformationally inactive forms. The temperature sensitivity is much more so however in active TBG and CBG, with the small equilibrated movements of the reactive center loop nudging into and out of the main beta sheet of the molecule directly affecting the flexibility of the binding-pocket. These coupled movements provide a clinically demonstrable "molecular thermocouple" (29, 30), accelerated as temperatures rise above 37°C to give with TBG and CBG a markedly increased release of thyroxine and cortisol in fevers.

The control of blood pressure is multifactorial but the retention in angiotensinogen of this thermally-responsive flexibility of the binding-pocket is likely to contribute to the immediacy of fluctuations in blood pressure observed with variations in temperature. Increased body temperatures, with a lowering of binding affinity, will predictably hinder the interaction with renin and, hence, contribute to a decreased

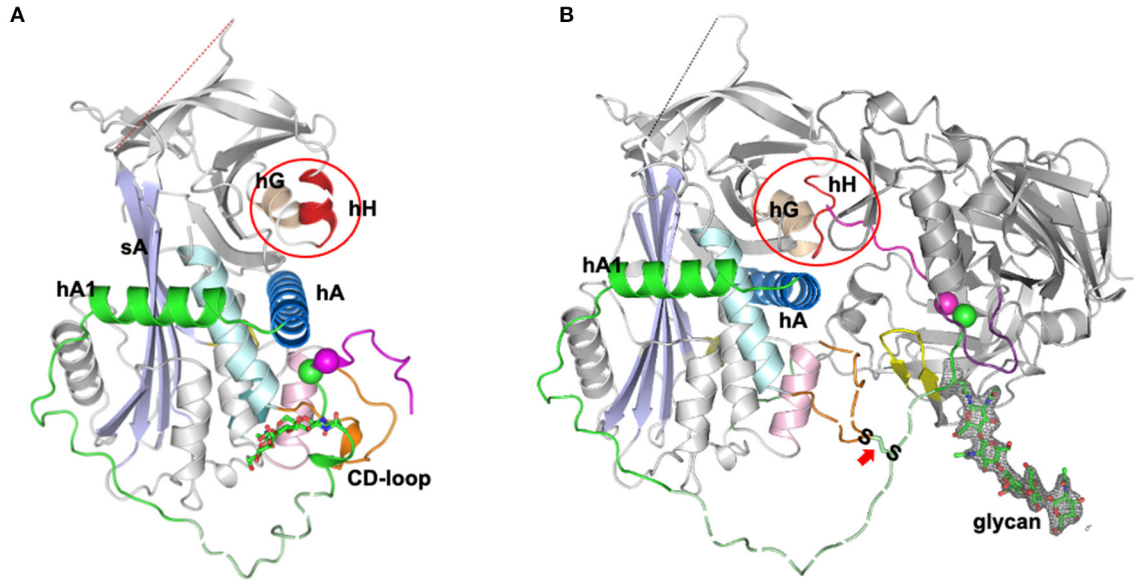


**FIGURE 2 |** Tail-into-mouth shift of N-terminal tail on binding with Renin (10). **(A)** Side view of angiotensinogen showing the rearrangement of the tail (magenta and green) that takes place on the complexing with renin in **(B)**. The scissile bond, Leu10-Phe11 (magenta and green spheres) becomes exposed and the tail is anchored at its terminus by docking into what in other serpins is the hormone-binding pocket, between helix H, the B beta-sheet, and helix A (as circled). Docking of the terminus is accompanied by an unfolding of helix H to reveal sidechains essentially involved in the binding of renin. The S-S bridge between Cys18 of the tail and Cys138 of the displaced CD loop of angiotensinogen is arrowed. Also apparent: the potential influence on cleavage kinetics of glycan variations at Asn14, adjacent to the scissile bond. The clear electron density for the glycan linked to Asn14 is shown in gray mesh and the fragments without clear electron density are shown with dashed lines for illustration. The figure was adapted from our previous publication (10) with modifications.

release of angiotensin, in keeping with the vasodilation and decreases in blood pressure that occur in fevers (31). Such temperature-sensitive changes in affinity are similarly compatible with other fluctuations in blood pressure with ambient temperatures (32); conversely so with a predictable increased affinity at lower temperatures, in keeping with the observed prompt rise in central aortic blood pressure after even short-term exposure to winter cold (33).

## CONCLUSIONS

The new structural understandings of the mechanism of cleavage and release of angiotensin from angiotensinogen have profound medical and physiological implications. Angiotensinogen has long been known as the ultimate source of angiotensin but what is now revealed by the structures of its complexes with renin is angiotensinogen's direct role in regulating the cleavage and release of the peptide and, hence, in the control of blood pressure. This inherent ability to modulate function in response to local tissue changes, as is also so with the plasma carriers of thyroxine and corticosteroids (34), explains why the conformationally flexible serpin framework has been selected as the carrier of angiotensin. The conformational shifts required for the release of angiotensin-I involve not just the exposure of the buried renin-cleavage site but also an accompanying rearrangement of the wider sites required for the body-to-body interface of renin with angiotensinogen. In particular, optimal kinetics for the cleavage and release depends on the precise repositioning of the renin-cleavage bond at Leu10-Val11. This is held in its exposed configuration anchored between the S-S bridge at Cys18 and the N-terminus of the tail bound to the helix H pocket (**Figure 2**).

The realization that each of these anchors can be readily modified by external influences emphasizes the modulatory role of angiotensinogen and opens new prospects for the investigation of the causes and ultimately the treatment of hypertension. The S-S bridge is demonstrably labile *in vivo* and is readily opened and re-formed—reduced and oxidized—by local redox fluctuations. This notably occurs with the placental oxidative stress and consequent hypertension in pre-eclampsia (9). The challenge now is to demonstrate whether such changes, occurring at a wider tissue level, are a contributory cause of hypertension in general. Blocking of the more active oxidized form of angiotensinogen has been shown to occur with nitrosylation—to what extent does this explain the hypotensive action of nitric oxide? More questions follow from the recognition of the tail-into-mouth mechanism that establishes the other anchor of the cleavage site. The vestigial conformational mechanism involved, with associated changes in the affinity of the binding-pocket, has been well studied (27) in other ligand-binding serpins, in antithrombin as well as TBG and CBG. Is the binding of the tail in angiotensinogen similarly sensitive to small changes in temperature? Is the hypotension observed to occur with hypothermia due to a decrease in the affinity of the binding-pocket, and hence in a decrease in angiotensin release? Predictably the binding site in angiotensinogen, as with the other serpins, will be subject to competitive blocking, by drugs and other small molecules, and to modulation by its interaction with tissue receptors.

What can be concluded with confidence is that the recognition of the structurally well-defined helix H binding-pocket in angiotensinogen now provides a basis for the design of new agents to attenuate angiotensin release and thus alleviate hypertension.

## AUTHOR CONTRIBUTIONS

This paper was conceived and written by RWC, RJR, and AZ with input from ZS and JW. ZS and JW prepared illustrations for the paper. All authors contributed to the article and approved the submitted version.

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# Stepwise Reversion of Multiply Mutated Recombinant Antitrypsin Reveals a Selective Inhibitor of Coagulation Factor XIa as Active as the M358R Variant

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Alpha-1 antitrypsin (AAT, also known as alpha-1 proteinase inhibitor or SERPINA1) is the most abundant member of the serpin superfamily found in human plasma. The naturally occurring variant AAT M358R, altered at the P1 position of the critical reactive center loop (RCL), is re-directed away from inhibition of AAT's chief natural target, neutrophil elastase, and toward accelerated inhibition of thrombin (FIIa), kallikrein (Kal), and other proteases such as factor XIa (FXIa). FXIa is an emerging target for the development of antithrombotic agents, since patients with FXI deficiency are protected from thromboembolic disease and do not exhibit a strong bleeding tendency. Previously, we used phage display, bacterial lysate screening, and combinatorial mutagenesis to identify AAT-RC, an engineered AAT M358R with additional changes between RCL positions P7-P3', **C**LE**VE**P**R-**S**TE** [with changes bolded and the P1-P1' (R358-S359) reactive center shown as R-S]. AAT-RC was 279- and 16-fold more selective for FXIa/IIa or FXIa/Kal than AAT M358R; the increased selectivity came at a cost of a 2.3-fold decrease in the rate of FXIa inhibition and a 3.3-fold increase in the stoichiometry of inhibition (SI). Here, we asked which alterations in AAT-RC were most important for the observed increases in selectivity for FXIa inhibition. We back-mutated AAT-RC to AAT-RC-1 (P7-P3' FLE**VE**P**R**S**TE**), AAT-RC-2 (P7-P3' FLEA**E**P**R**S**TE**), and AAT RC-3 (P7-P3' FLEAIP**R**-S**TE**). Proteins were expressed as cleavable, hexahistidine-tagged glutathione sulfotransferase fusion proteins in *E. coli* and purified by proteolytic elution from glutathione agarose, with polishing on nickel chelate agarose. Selectivity for FXIa over Kal of AAT-RC-1, −2, and −3 was 14, 21, and 2.3, respectively. AAT-RC-2 inhibited FXIa 31% more rapidly than AAT M358R, with the same SI, and enhanced selectivity for FXIa over Kal, FXa, FXIIa, activated protein C, and FIIa of 25-, 130-, 420-, 440-, and 470-fold, respectively. Structural modeling of the AAT-RC-2/FXIa encounter complex

suggested that both E (Glu) substitutions at P3 and P3' may promote FXIa binding via hydrogen bonding to K192 in FXIa. AAT-RC-2 is the most selective and active AAT variant reported to date for FXIa inhibition and will be tested in animal models of thrombosis and bleeding.

**Keywords: serpins, inhibitors, blood coagulation factors, antitrypsin, molecular modeling, recombinant proteins**

## INTRODUCTION

Circulating at a concentration of ∼20 μM, $\alpha_1$-antitrypsin (AAT, also known as alpha-1 proteinase inhibitor or SERPIN A1) is the most abundant member of the serpin superfamily found in human plasma (1–3). Although the principal physiological target of AAT is neutrophil elastase (3, 4), inhibitory complexes of AAT and other proteases such as activated coagulation factor XI (FXIa) can be found in human plasma (5, 6). While the rate of FXIa inhibition by native AAT is relatively slow (7), a naturally occurring M358R (AAT Pittsburgh) substitution in its critical reactive center loop (RCL) increases its rate of inhibition > 1,000-fold for FXIa and other clotting-related factors, while similarly reducing its inhibition of neutrophil elastase (8–10). FXI has emerged as an attractive target for the development of antithrombotic agents with improved therapeutic profiles (11), because reductions in FXI or FXIa levels correlate with lower thrombotic risk without provoking hemorrhage in mice (12–14) and humans (15–20). We hypothesized that mutating additional residues in the RCL of AAT M358R would yield a specific FXIa inhibitor. Although multiple anti-FXI and anti-FXIa agents of either a small molecule or macromolecular nature are in pre-clinical or clinical development, none has as yet reached licensure (21), and a serpin-based FXIa inhibitor could have advantages with respect to low toxicity and relatively rapid off-set of action.

Mechanistically, serpins present their RCL residues to attacking proteases, forming an encounter complex that proceeds through a tetrahedral intermediate stage to cleavage of the reactive center peptide bond (2, 22, 23). Bond scission releases free energy and powers a large conformational change in which the protease, still connected to the serpin RCL via an acyl ester bond between the RCL and its active site, is translocated to the opposite pole of the serpin, as the cleaved RCL inserts into the central β-sheet of the serpin as a new strand (2, 22, 23). The trapped protease is distorted in its active site and its ability to complete catalysis is severely impaired. Structural biological support for this model has been provided by crystal structures of multiple serpins, including the non-covalent complex of AAT M358R and active site-mutated S195A trypsin (24) and the covalent complexes of AAT with trypsin (25) or elastase (26). In terms of reaction outcomes, serpins exhibit a branched reaction pathway leading either to covalent complex formation or to the release of active protease and inactive serpin cleaved within its RCL (27, 28). While antithrombin is the principal serpin regulating coagulation, other serpins such as C1 inhibitor, protein C inhibitor, and protease nexin 1 also play some roles in this pathway (29).

While many previous investigations [reviewed in Scott and Sheffield (30)] have attempted to change the specificity of AAT to react with different target proteases, via directed mutagenesis or loop exchanges, few have addressed re-orienting AAT to inhibit FXIa specifically. Previously, we divided the AAT RCL into three sectors, screening hypervariable phage display or bacterial lysate libraries for motifs that provided more specific inhibition of FXIa than other proteases (31). We found that the combination of the second and third motifs, in AAT variant AAT-RC, containing five substitutions additional to M358R in its RCL (F352C/A355V/I356E/I360T/P361E), increased FXIa selectivity over thrombin, FXIIa, FXa, activated protein C, and kallikrein by 279-, 143-, 63-, 58-, and 36-fold, respectively, vs. AAT M358R. AAT-RC inhibited FXIa 2.3-fold less rapidly than AAT M358R and exhibited a 3.3-fold increase in reaction stoichiometry (31). In the current study, we back-mutated AAT-RC, one residue at a time, toward AAT M358R, to ascertain if less mutated variants retained or exceeded AAT-RC's activity against and selectivity for FXIa. We report that a variant with three substitutions additional to M358R, AAT-RC-2 (I356E/M358R/I360T/P361E), retained not only the full activity and reaction stoichiometry of AAT M358R but also the FXIa selectivity of AAT-RC.

## METHODS

### Reagents

The following coagulation and coagulation-related proteases were purchased from Enzyme Research Labs (USA): kallikrein and thrombin [also called factor (F) IIa, FXa, FXIa, and FXIIa]. The following chromogenic substrates were purchased from Diapharma (USA): for thrombin, S-2238; for FXa, S-2765; for FXIa and APC, S-2366; and for kallikrein and FXIIa, S-2302. Custom synthetic double-stranded DNA fragments (gBlocks) were bought from Integrated DNA Technologies (Canada). Restriction endonucleases and glutathione agarose were purchased from Thermo Fisher Scientific (Canada). Nickel chelate affinity resin Ni-NTA agarose was bought from Qiagen (Canada). PreScission Protease [a glutathione sulfotransferase (GST)–human rhinovirus (HRV) 3C protease fusion protein] was purchased from GE Healthcare (Canada). Normal human pooled plasma (NHPP) was produced in-house. FXI-deficient plasma was purchased from Affinity Biologicals (Canada). STA PTTA reagent, STA Neoplastine CI Plus reagent, and Owren-Koller buffer were bought from Diagnostica Stago (Canada).

### DNA Manipulations

Synthetic DNA fragments of 941 bp in length were designed to encode each of the AAT variants listed in **Table 1**: AAT-RC-1, AAT-RC-2, and AAT-RC-3. Following restriction with *Kpn2I* and *EcoRI*, the resulting 925-bp fragments were each separately ligated to the 5256-bp *Kpn2I*/*EcoRI* double digestion product of pGEX-AAT M358R (31) to create pGEX-AAT-RC-1, −2, or −3, respectively. Ligation reactions were used to transform *E. coli*

**TABLE 1 |** RCL sequences of AAT M358R and variants examined or considered in this study.

| Protein name | RCL sequence (P13-P3') |
|---|---|
| AAT-RC[a] | EAAGAM**C**LE**VE**PRS**TE** |
| AAT-RC-1 | EAAGAMFLE**VE**PRS**TE** |
| AAT-RC-2 | EAAGAMFLEA**E**PRS**TE** |
| AAT-RD[a] | EAAGAMFLEAIPRS**TE** |
| AAT-RC-3 | EAAGAMFLEAIPRSI**E** |
| AAT M358R | EAAGAMFLEAIPRSIP |

*Primary amino acid sequences of various AAT M358R derivatives between P7 (F352) and P3' (P361) are shown. Regular font indicates AAT M358R sequence; bolded and underlined font indicates mutated residues.*
[a]*Previously reported (31).*

DH5α to ampicillin resistance, and subclones were screened by standard methods as described (32, 33). Each plasmid construct was validated by DNA sequencing using the Sanger method by Mobix Lab, a central facility of the Faculty of Health Sciences, McMaster University. These plasmids encoded a 630-amino acid glutathione sulfotransferase (GST)–AAT fusion protein linking GST C-terminal K218 to Glu1 of AAT via peptide SDLEVLFQ-GPMGH$_6$, which specified a PreScission Protease recognition site and a hexahistidine tag. Validated plasmids were transferred to *E. coli* BL21 for protein expression as previously described (31).

## Protein Expression and Purification

*E. coli* BL21 transformed with pGEX-AAT M358R, -RC, -RC-1, −2, or −3 was grown at 37°C to mid-log phase while shaking at 225 rpm, and GST–AAT fusion protein expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). AAT proteins were purified from lysates liberated from cell pellets by sonication and purified via glutathione agarose chromatography with proteolytic elution of AAT variants with PreScission Protease and polishing via nickel chelate affinity chromatography as described (31).

## Kinetic Analysis

Pseudo-first-order conditions, comprising at least a 10:1 molar ratio of AAT variant to protease, were employed to determine the second-order rate constant of inhibition ($k_2$) of different proteases (5–75 nM) by AAT variants at 37°C, employing a previously described discontinuous method (31–33). Briefly, this approach involved dividing the observed rate constant $k_{obs}$, which was the slope of the plot of the natural logarithm of the ratio of initial protease activity to residual protease activity vs. time, by the initial AAT variant molar concentration. Similarly, the stoichiometry of inhibition (SI) for FXIa was determined as previously described (31–33). Briefly, this approach involved incubating varying ratios of AAT variants to FXIa at ambient temperature for 2 h, determining the residual FXIa activity at 37°C, plotting it vs. the ratio of AAT/FXIa, and regressing the resulting line of best fit to zero residual FXIa activity to yield the SI.

## Gel-Based Analysis

The electrophoretic profile of the reaction of AAT variants with thrombin or FXIa was visualized on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, except where indicated, under reducing conditions. In reactions with thrombin or FXIa with a total volume of 15 μl, AAT variants (1.0 μM final concentration) were reacted with 100 nM protease in 20 mM sodium phosphate, pH 7.4, 0.1% polyethylene glycol 8000, 100 mM NaCl, and 0.1 mM EDTA (PPNE buffer) for 0 (no protease control), 1 or 5 m at 37°C. Reactions were stopped by the addition of 1/3 reaction volume of concentrated SDS sample buffer as described (32), and samples containing the entire reaction volume were electrophoresed. In some reactions with FXIa, the reaction time was fixed at 5 min and molar ratios of AAT/FXIa were varied (10:1, 2:1, 1:1, and 1:2) while keeping the AAT variant concentration fixed at 1.0 μM. Gels were stained with Coomassie Brilliant Blue and destained as described (31). Gels were scanned using a model XR Gel Doc system manufactured by Bio-Rad Laboratories (Canada). Captured images were labeled and saved in a Tagged Image File (TIF) format using PhotoShop CS6 version 13 software from Adobe Systems Incorporated (USA). Image brightness was not manipulated unless so stated in the figure legend.

## Diluted Prothrombin Time Assay

The prothrombin time (PT) assay was modified by dilution of test reagents essentially as done by de Maat and co-workers (34). PT reagent STA Neoplastine CI Plus (Diagnostica Stago) was diluted 1:500 with 16.6 mM CaCl2, and 0.1 ml of the diluted reagent was combined with 0.05 ml of normal human pooled plasma (NHPP) supplemented with purified AAT variants. Both test solutions were pre-warmed to 37°C prior to combination in a STA-IV clotting analyzer (Diagnostica Stago) and determination of clotting time.

## Diluted APTT Assays

Standard APTT assays were modified in by dilution of the APTT reagent. The APTT reagent (STA PTTA) was diluted 1:15 with Owren-Koller buffer. NHPP supplemented with AAT variant protein (0.05 ml) was then combined with 0.05 ml of diluted reagent and pre-warmed to 37°C. Clotting was then initiated by the addition of 0.05 ml of 25 mM $CaCl_2$, and clotting time was determined on the analyzer specified above (31).

## Protein Modeling

The encounter or Michaelis complexes between AAT M358R, AAT-RC, and AAT-RC-2 were separately modeled using PyMOL Molecular Graphics System 2.3.4 (https://pymol.org) and ClusPro 2.0 (https://cluspro.bu.edu) (35–37). Protein Data Bank (PDB) file 1OPH of the non-covalent complex between AAT M358R and active site-mutated S195A trypsin (24) was first manipulated to select chain A (AAT M358R) and then to introduce the five mutations of AAT-RC or the three mutations of AAT-RC-2, using the backbone-dependent rotamer feature in PyMOL to minimize steric clashes. In ClusPro 2.0, AAT M358R from 1OPH or mutated PDB files based on 1OPH were designated the receptor, and the catalytic domain of FXIa (FXIac)

**TABLE 2 |** Kinetic parameter AAT variants compared to AAT M358R.

| Protein | $k_2$ for FXIa ($\times 10^5$ $M^{-1}s^{-1}$) | $k_2$ for kallikrein ($\times 10^5$ $M^{-1}s^{-1}$) | Selectivity ($k_2$ for FXIa/$k_2$ for kallikrein) | Fold increase in kallikrein selectivity vs. AAT M358R | SI vs. FXIa | SI vs. kallikrein |
|---|---|---|---|---|---|---|
| AAT M358R | $1.4 \pm 0.2^b$ | $0.21 \pm 0.06^a$ | 6.67 | – | $2.7 \pm 0.4^a$ | $3.4 \pm 0.1^b$ |
| AAT-RC | $0.54 \pm 0.09^b$ | $0.0050 \pm 0.0002^a$ | 108 | 16.2 | $7 \pm 1^d$ | ND |
| AAT-RC-1 | $1.7 \pm 0.2^b$ | $0.018 \pm 0.002^b$ | 94 | 14.1 | ND | ND |
| AAT-RC-2 | $1.85 \pm 0.05^b$ | $0.013 \pm 0.002^b$ | 142 | 21.3 | $2.7 \pm 0.8^c$ | $64 \pm 1^b$ |
| AAT-RC-3 | $1.0 \pm 0.1^b$ | $0.066 \pm 0.006^b$ | 15.2 | 2.3 | ND | ND |

*The mean of 4[a], 5[b], 6[c], or 8[d] determinations ± one standard deviation is shown, for kinetic parameters for AAT variants, compared to AAT M358R.*

from chain A of PDB 1ZJD was designated the ligand (38). Residues between AAT E346 and P361 or mutated equivalents and FXIac residues 57, 98, 102, 143, 151, 189, 192, 193, and 195 were selected for attraction based on crystal structural (38) or mutational information (39) identifying them as forming ionic or hydrogen bonds with the co-crystalized Kunitz protease inhibitor domain of protease nexin 2 (KPI-PN2) in PDB 1ZJD; H57, D102, and S195 form the catalytic triad of the FXIac active site. The structures were then docked using ClusPro 2.0. Resulting models of the best conformational fit were chosen based on the balanced scoring scheme, using the central model from the largest sized cluster. Hydrogen bond lengths between P3 and P3' residues on AAT and K192 on FXIac (or distances if hydrogen bonding was not possible) were determined using PyMOL. Rendering of figures was performed using PyMOL.

## Statistical Analysis

Statistical analysis was facilitated using InStat version 3.06 (GraphPad Software, San Diego CA). Prism 4.03 (GraphPad Software) was employed to generate graphs. Multiple comparisons were performed using ANOVA with Tukey post-tests for data sets passing tests of normality and similarity of standard deviations and with non-parametric ANOVA with Dunn's post tests for those failing one or more tests. Comparisons returning a $p < 0.05$ were considered statistically significant.

# RESULTS

## General Approach

**Table 1** shows the amino acid sequence of the RCL of AAT variants analyzed in this study. The general mutational approach was to start with AAT-RC, containing five amino acid substitutions compared to AAT M358R between P7 (F352) and P3' (P361), and reverse the mutations, one at a time, back toward the AAT M358R sequence. In so doing, we generated novel proteins AAT-RC-1, −2, and −3 and compared them to previously described proteins AAT-RC and AAT-RD. The latter were selected from phage display or bacterial expression libraries via biopanning or lysate screening (31). All variants were expressed in *E. coli*, purified to homogeneity, and reacted with different proteases to assess inhibition.

It should be noted that we did not characterize each variant exhaustively. Rather, we employed a lean design in which we first compared the rates of FXIa inhibition of AAT-RC-1, −2, and −3 to AAT M358R and AAT-RC (31) and the selectivity for FXIa over kallikrein, as a selected representative of other proteases inhibited less rapidly by AAT-RC than AAT M358R (31). We then chose the most selective of these three variants for more detailed characterization.

## AAT RC-2 Selectively Inhibits FXIa Over Kallikrein More Effectively Than Other Variants

We first investigated the rate of inhibition of FXIa of the variants. As shown in **Table 2**, while AAT-RC-1, −2, and −3 each exhibited a greater mean $k_2$ of FXIa inhibition than AAT-RC, the most rapid inhibitor of FXIa was AAT-RC-2. Reverting the F352C mutation to the native phenylalanine at P7 restored the decrease in the rate of FXIa inhibition observed between AAT M358R and AAT-RC; indeed, the mean $k_2$ of AAT-RC-2 for FXIa was 32% greater than that of AAT M358R. With respect to inhibition of another serine protease inhibited by AAT M358R, kallikrein, the AAT-RC-1, −2, and −3 variants were 3–42-fold slower inhibitors of kallikrein than AAT M358R, but all inhibited kallikrein more rapidly than AAT-RC. The selectivity index, or the ratio of the rate constants for FXIa inhibition over those for kallikrein inhibition, was greatest for AAT-RC-2, at 142, and this variant was therefore selected for further study.

With respect to reaction stoichiometry, as shown in **Table 2**, bacterially expressed AAT M358R exhibited a mean SI of 2.7, indicating that 2.7 molecules of AAT M358R were required, on average, to inhibit a molecule of FXIa, under steady-state conditions. This value rose to 7 for AAT-RC or -RD, indicating a decreased efficiency of inhibition. In contrast, reducing the number of mutations to four in AAT-RC-2 restored the SI to the same mean value observed for AAT M358R, 2.7. Having demonstrated that AAT-RC-2 and AAT M358R had identical SI values, we asked if this was also true for kallikrein inhibition. In contrast to the results with FXIa, we found substantial elevation of SI for the reaction of kallikrein with AAT-RC-2 compared to FXIa (mean values of 64 vs. 3.3, **Table 2**).

**Figure 1** shows plots used to determine $k_2$ and SI values for the reaction of AAT M358R, AAT-RC, and AAT-RC-2 with FXIa, which are representative of all such kinetic determinations in this report and which correspond to the data shown in **Tables 2**, **3**.

## AAT-RC-2 Selects FXIa Over Five Other Serine Proteases as Inhibitory Targets

The rates of inhibition of six serine proteases were next assessed for AAT M58R and AAT-RC-2. As shown in **Table 3**, mean $k_2$ values for inhibition of kallikrein, thrombin, APC, FXa, and FXIIa were all substantially reduced for AAT-RC-2 compared to AAT M358R. Combined with the slight increase in the $k_2$ of AAT-RC-2 for FXIa inhibition, these rate constants yielded selectivity factors of between 142 (kallikrein) and 3,700 (for FXIIa) for AAT-RC-2, which corresponded to increases in selectivity for the different proteases of AAT-RC-2 compared to AAT M358R ranging from 25-fold for kallikrein to 470-fold for thrombin.

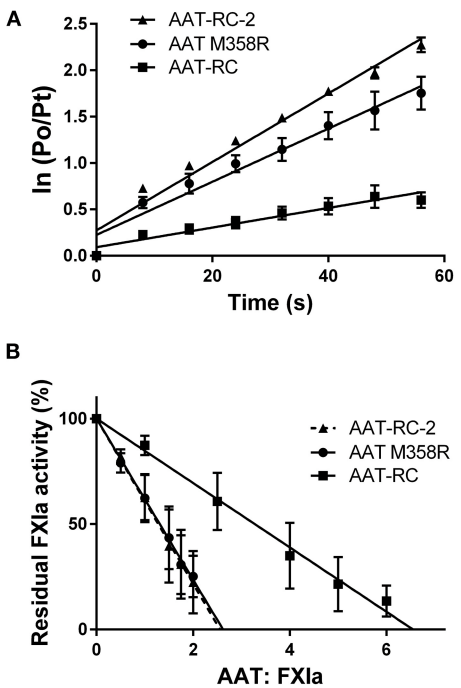


**FIGURE 1 |** Kinetic parameters of AAT variants. **(A)** The natural logarithm of the ratio of the initial protease (FXIa) activity ($P_0$) to the residual activity at time $t$ ($P_t$) vs. the time in seconds [time (s)]. Points are the mean ± SD of five determinations; lines are lines of best fit determined by linear regression. **(B)** The residual FXIa activity as a percentage of initial activity after incubation of AAT variants with differing molar ratios of FXIa for 2 h. Points are the mean of four (AAT M358R), six (AAT-RC-2), or eight (AAT-RC) separate experiments; lines are lines of best fit determined by linear regression. Note that the regression line for AAT-RC-2 is dashed to distinguish it from its superimposed neighbor, the line for AAT M358R.

## AAT-RC-2 Forms Denaturation-Resistant Inhibitory Complexes With FXIa but Not Thrombin

We next used gel-based assays to gain additional insights into the reaction of AAT-RC-2 with FXIa. We first examined the reaction products under pseudo-first-order conditions of 10-fold molar excess serpin over protease. As shown in **Figure 2**, and as anticipated based on the $k_2$ observations in **Tables 2**, **3**, AAT RC-2 and AAT M358R formed an SDS-stable complex with the light chain of FXIa, as shown by the appearance of an ∼78-kDa protein band following incubation of excess AAT under conditions for 1 min [**Figure 2**, compare no addition ("NA") lanes to "+FXIa" and "FXIa only" lanes]. Similarly, an SDS-stable complex was formed on incubation of AAT M358R with thrombin; no such complex formation was observed when AAT-RC-2 was incubated with thrombin, despite the lengthening of the reaction time to 5 min.

While the reactions shown in **Figure 2** provided information about initial reaction products, they were uninformative concerning whether the different AAT variants were fully reactive with FXIa because of consumption of all the FXIa in the reaction. We therefore examined the reaction products at a range of FXIa concentrations, including molar excesses. As shown in **Figure 3**, decreasing the ratio of AAT/FXIa from 10:1 (same conditions as **Figure 2**) to 5:1 to 1:1 resulted in complete reaction of AAT M358R (**Figure 3A**) and AAT-RC-2 (**Figure 3B**); in the case of AAT-RC, the unreacted protein and the heavy chain of FXIa co-migrated, preventing visualization (**Figure 3C**). For all three AAT variants, formation of SDS-stable 78-kDa complexes was readily visualized, as was AAT cleavage to a protein species of ∼45 kDa. Complex formation increased up to 1:1 ratios of AAT M358R or AAT-RC-2, although complex bands were less intense for AAT-RC. Conversely, cleaved AAT species were detectable at lower levels of FXIa for AAT-RC than the other two proteins, and the amount of cleaved product was noticeably greater at all ratios. Although the conditions used in the kinetic determination of SI

**TABLE 3 |** Kinetic parameters for AAT-RC-2 and AAT M358R with various proteases.

| Protease | $k_2$ AAT M358R (× $10^5$ $M^{-1}s^{-1}$) | $k_2$ AAT-RC-2 (× $10^5$ $M^{-1}s^{-1}$) | Selectivity of AAT M358R ($k_2$ for FXIa/$k_2$ for protease) | Selectivity of AAT-RC-2 ($k_2$ for FXIa/$k_2$ for protease) | Fold difference in selectivity (AAT-RC-2/AAT M358R) |
|---|---|---|---|---|---|
| FXIa | 1.4 ± 0.2[c] | 1.85 ± 0.05[e] | – | – | – |
| Kallikrein | 0.24 ± 0.02[b] | 0.013 ± 0.02[c] | 5.8 | 142 | 25 |
| Thrombin | 1.8 ± 0.2[b] | 0.005 ± 0.001[d] | 0.78 | 370 | 470 |
| APC | 0.25 ± 0.03[a] | 0.0007 ± 0.0003[b] | 5.6 | 2,440 | 440 |
| FXa | 0.29 ± 0.03[d] | 0.003 ± 0.001[d] | 4.8 | 620 | 130 |
| FXIIa | 0.23 ± 0.04[d] | 0.0005 ± 0.0002[d] | 8.8 | 3,700 | 420 |

*The mean of 3[a], 4[b], 5[c], 7[d], or 11[e] determinations ± one standard deviation is shown, for kinetic parameters for AAT-RC-2 and AAT M358R with various proteases.*

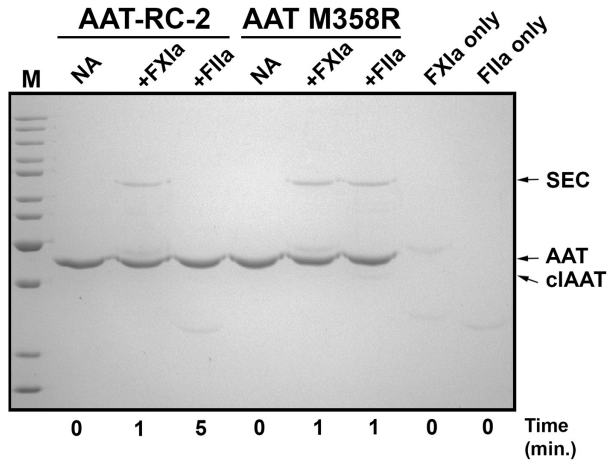


**FIGURE 2 |** AAT-RC-2 forms sodium dodecyl sulfate (SDS)-stable complexes with FXIa but not with thrombin. A 10% SDS-polyacrylamide gel electrophoresed under denaturing and reducing conditions and stained with Coomassie Blue is shown. Reactions contained purified AAT-RC-2 or AAT M358R (identified above horizontal bars indicating the relevant lanes) in the absence (no addition, NA) or presence of FXIa (+FXIa) or thrombin (+FIIa). AAT variants (1.0 μM) were reacted with protease (0.1 μM) for the time, in minutes, indicated below the lanes, at 37°C. Arrows, at the right, highlight the position of intact AAT (AAT), cleaved AAT (cIAAT), and serpin–enzyme complexes (SEC) of AAT-FXIa or AAT-thrombin. M denotes molecular weight markers, in kDa: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, and 25. The scanned image of the gel was uniformly darkened in Adobe Photoshop by setting the brightness to −50 (full scale 0 to −150) to maximize band visibility.

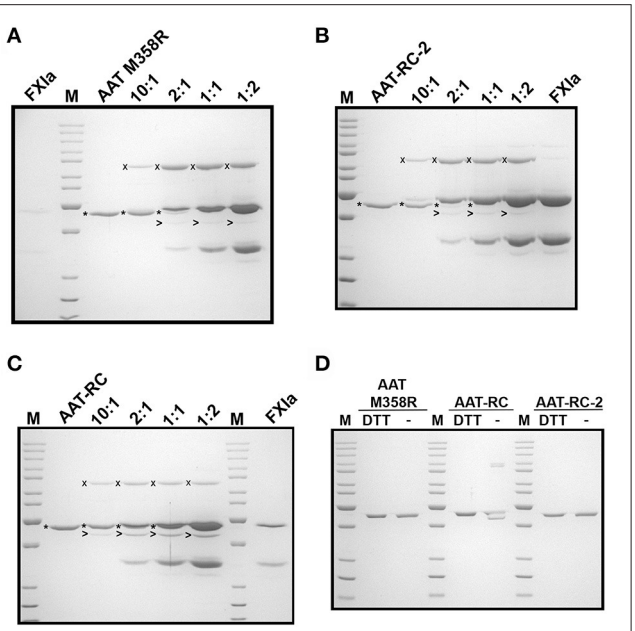


**FIGURE 3 |** Effects of increasing amounts of FXIa on reaction outcomes with AAT variants and electrophoretic profiles. A 10% SDS-polyacrylamide gel electrophoresed under denaturing and reducing **(A–C)** or reducing and non-reducing conditions **(D)** and stained with Coomassie Blue is shown. AAT variants (1.0 μM) were incubated with or without 0.1–2.0 μM FXIa, yielding molar reaction ratios of AAT/FXIa shown above the lanes. The position of AAT-FXIa serpin–enzyme complexes (x), unreacted AAT (*), and cleaved AAT (>) is indicated to the left of the respective bands, where reactions with AAT M358R, AAT-RC-2, and AAT-RC are shown in **(A–C)**, respectively. FXIa-only lanes in **(A–C)** correspond to 0.1, 2.0, and 0.5 μM concentrations, respectively. **(D)** AAT variants identified above the lanes (0.7 μg per lane) were electrophoresed under reducing conditions (with dithiothreitol, DTT) or non-reducing conditions (–). M, molecular weight markers, are the same as those in **Figure 1**.

and in these gel-based experiments were not identical, the results were qualitatively consistent in that the anti-FXI reactivity of AAT M358R and AAT-RC-2 was greater than that of AAT-RC, and the substrate behavior of AAT-RC (leading to cleaved AAT) exceeded that of these other two AAT variants.

AAT-RC exhibited a lower $k_2$ value than AAT M358R or AAT-RC-2 and formed less complex under conditions in which more FXIa was added. As shown in **Figure 3D**, when AAT-RC was electrophoresed under non-reducing conditions, only a portion of the preparation co-migrated with its reduced counterpart. Other fractions of the preparation exhibited retarded migration (i.e., doublet band migrating between 85- and 100-kDa markers) consistent with intermolecular disulfide bond formation and accelerated migration consistent with intramolecular disulfide bond formation (i.e., band migrating more rapidly than that co-migrating under reduced and non-reduced conditions). These properties of AAT-RC were dependent on the presence of the F352C mutation, since both AAT M358R and AAT-RC-2 preparations were comprised of single bands that co-migrated irrespective of whether reducing agents were added (**Figure 3D**). Linkage of two RCLs by a disulfide bond would be expected to prevent inhibition of target proteases.

## AAT-RC-2 Prolongs Modified APTT but Not PT Clotting Times in Human Plasma

Having demonstrated using kinetic and gel-based assays that AAT-RC-2 maintains or exceeds the rapid rate of FXIa inhibition of AAT M358R, with the same reaction stoichiometry but greatly enhanced selectivity, we addressed the effects of AAT-RC-2 on the clotting of human plasma *in vitro*. We employed modified forms of the hemostasis screening assays (PT and APTT). **Figure 4A** shows that supplementing pooled human plasma with buffer or 0.5 μM AAT-RC or AAT-RC-2 failed to increase the clotting time provoked by recalcification and provision of diluted tissue factor-containing PT reagent. In contrast, supplementation with 0.5 μM AAT M358R significantly prolonged this clotting time from a baseline of 65 ± 2 s to 73 ± 3 s (mean ± SD, $p < 0.001$).

In an APTT assay modified by dilution of the silica activator reagent, supplementation with 0.5 μM AAT M358R, AAT-RC-2, or AAT-RC significantly prolonged the clotting time compared to buffer alone (**Figure 4B**). The prolongation elicited by AAT-RC-2 or AAT-RC did not differ but was less than that elicited by AAT M358R. While the experiments shown in **Figures 4A,B** were performed at a single 0.5-μM AAT dose, in **Figure 4C**, we demonstrated a dose response for both AAT M358R and AAT-RC-2 in the diluted APTT assay. The dose response of AAT-RC-2 was shallower than that of AAT M358R, as expected based on the greater effect of AAT M358R than AAT-RC-2 in the assays shown in **Figure 2B**.

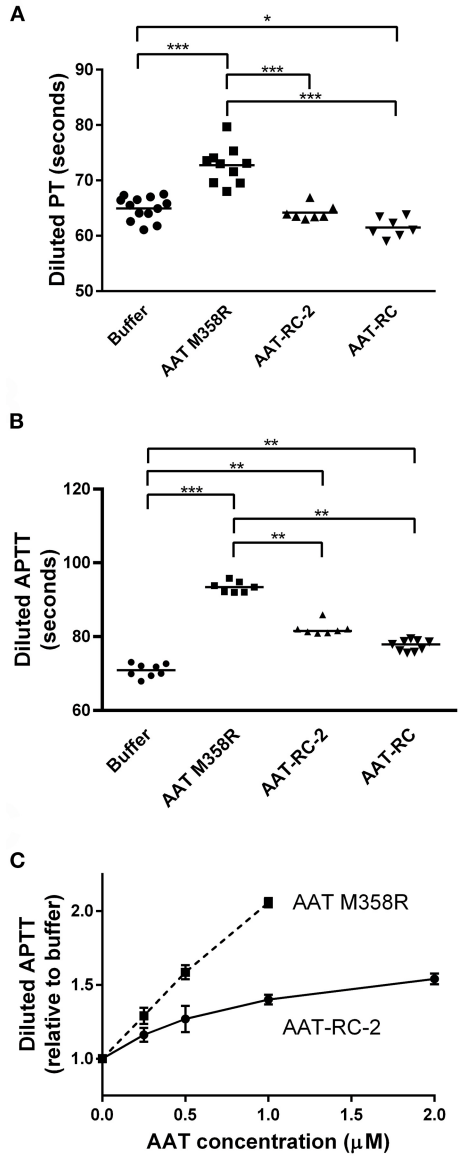


**FIGURE 4 |** Effects of AAT variants on modified hemostasis screening tests. Buffer or purified AAT M358R was introduced at 0.5 μM final concentrations into diluted PT **(A)** or diluted APTT **(B)** assays. Note that the y-axis does not commence at 0 s on any graph. Each point represents a single determination. Horizontal capped lines that link different groups indicate statistical differences by ANOVA with post-tests: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$. **(C)** A dose-response curve that shows diluted APTT values (normalized to buffer-only controls) as a function of increasing variant AAT concentration (μM). The mean ± SD of three determinations for each point is shown: squares, AAT M358R; circles, AAT-RC-2.

## Molecular Modeling Suggests Hydrogen Bonding Between P3 and P3' Glutamates and K192 in FXIac for AAT-RC-2 and AAT-RC but Not AAT M358R

We sought molecular explanations for the selectivity of AAT-RC-2 and AAT-RC as FXIa inhibitors. We used the ClusPro 2.0 web server, which carries out iterative rigid body docking simulations coupled to energy minimizations to construct a model of such a complex (35, 36). Essentially, the atomic coordinates of FXIac and AAT M358R were extracted from their crystal structures in complex with the KPI-PN2 domain (PDB 1ZJD) (38) or trypsin S195A (PDB 1OPH) (26), respectively, and input as receptor and ligand for *in silico* docking. **Figure 5A** shows a model of the initial, non-covalent encounter (also called Michaelis) complex of the light chain catalytic domain of FXIa (FXIac, cyan) docked to AAT M358R (RCL, yellow, and rest of the serpin, gray). Inspection of this structural model revealed close contacts between the active site of FXIac and portions of the AAT M358R RCL, as expected. Following *in silico* mutation of the RCL to create models of AAT-RC and AAT-RC-2 and docking to FXIac, these areas (see dashed box, **Figure 5A**) were examined in greater detail. As shown in close-up **Figure 5B**, the side chains of I356 (P3) and P361 (P3') in AAT M358R and K192 of FXIac possess no significant electrostatic or hydrophobic interactions that would contribute to binding the RCL in the FXIa active site. In contrast, in AAT-RC (**Figure 5C**) or AAT-RC-2 (**Figure 5D**), mutation of I356 (P3) and P351 (P3') to glutamate in both AAT-RC and AAT-RC-2 predicts similarly docked models. Both models suggest the I356E and P361E mutations create a hydrogen bonding network that would stabilize the interaction between the FXIa active site and AAT-RC or AAT-RC-2.

We followed an analogous approach to that described above to focus on AAT residues 354–358 in AAT M358R, AAT-RC, and AAT-RC-2, using the covalent complex crystal structure of AAT and trypsin (PDB 1EXZ) and focusing on the AAT moiety alone (25). However, in contrast to the results in the encounter complex with K192, no differences between these structural models were found (data not shown).

## DISCUSSION

The starting point for this investigation was our discovery of AAT-RC, a variant of AAT M358R containing five additional substitutions (F352C/A355V/I356E/I360T/P361E) at the P7, P4, P3, P2', and P3' positions of the serpin RCL (31). The variant residues between P7 and P3 inclusive were selected by biopanning an AAT M358R phage display library hypervariable at that portion of the RCL, while the rest of the AAT-RC additional substitutions were selected by lysate screening of a bacterial expression library of AAT M358R hypervariable at P2' and P3'. The two motifs were then combined in AAT-RC. In this investigation, we reversed the additional changes, one at a time, back toward the "parental" AAT M358R molecule. Our objective was to determine if AAT-RC was optimally selective for FXIa, and if one cost of its increased specificity, a 2.3-fold decrease in the rate of FXIa inhibition vs. AAT M358R, was unavoidable. If so, then each stepped reversal of mutation might have been expected to decrease FXIa selectivity and increase FXIa activity. Our experimental results, however, did not follow this linear path.

Reverting the P7 mutation F352C to the wild-type Phe residue led to a 13% decrease in selectivity for FXIa over kallikrein

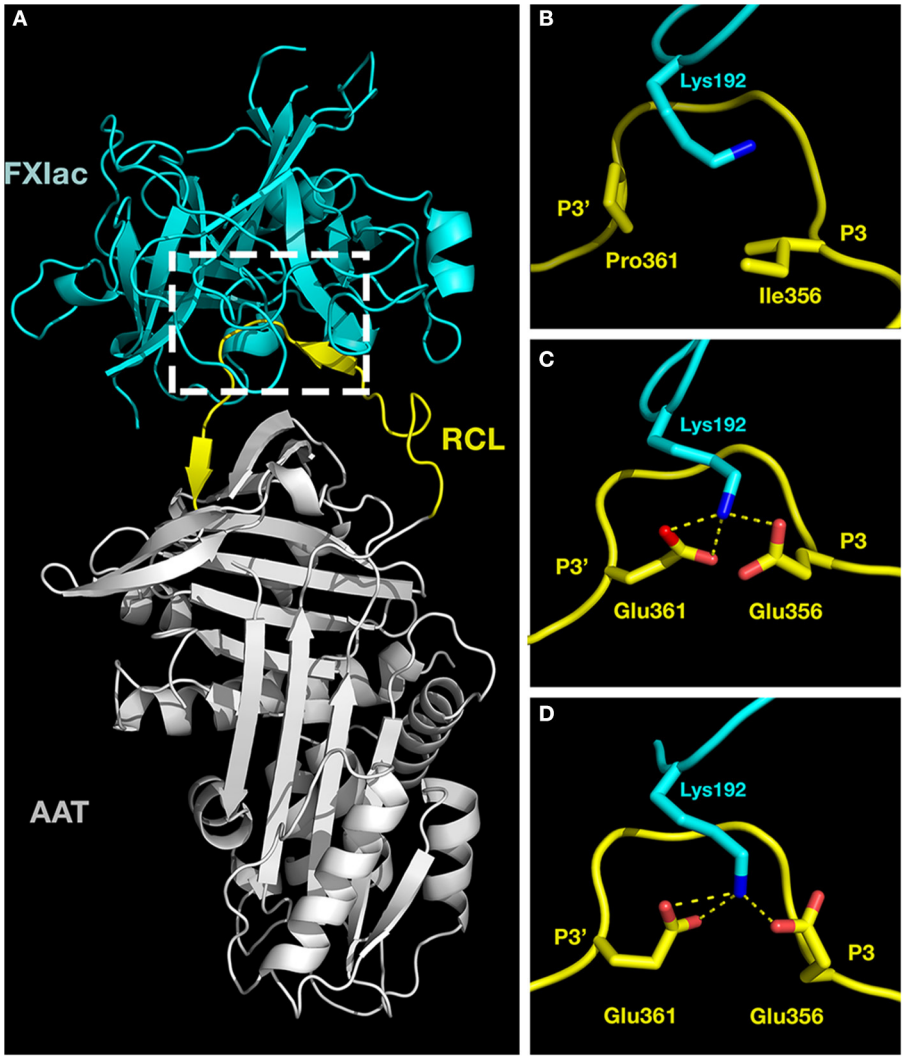


**FIGURE 5 |** Molecular models of activated factor XI docked to AAT variants. Crystal structures of the catalytic domain of FXIa (FXIac, cyan, PDB 1ZNT) and AAT M358R (PDB 1OPH, RCL yellow, rest of AAT gray) were docked using ClusPro 2.0 as described in the *METHODS* section. **(A)** The full structure of the docked proteins. **(B–D)** Exploded close-ups of the AAT RCL and the active site of FXIac [corresponding to the white dashed box in **(A)**] for AAT M358R **(B)**, AAT-RC **(C)**, and AAT-RC-2 **(D)**. Yellow dashed lines indicate hydrogen bonds predicted to form between FXIac K192 nitrogen atoms (blue) and Glu 356 and Glu 361 oxygen atoms (red) in AAT-RC and AAT-RC-2 **(B,C)** but not in AAT M358R **(A)**. More remote portions of the FXIac chain were deliberately reduced in visibility using PyMOL in order to maximize the visibility of K192 of FXIac and the AAT RCL between P3 and P3' in **(B–D)**.

in AAT-RC-1, followed by a 31% increase in selectivity when the P4 mutation A355V was also reverted in AAT-RC-2 and a substantial 711% drop in selectivity when I356E and I360T were reversed in AAT-RC-3. Although in this study we did not examine AAT-RD, intermediate in mutation reversal between AAT-RC-2 and AAT-RC-3, its 7-fold decrease in the rate of FXIa inhibition (31), to the lowest $k_2$ value of any of the variants in the mutational series depicted in **Table 1**, reinforces the non-additive nature of RCL mutations in AAT M358R. Such cooperativity was previously noted by Hopkins et al. who substituted the RCL of antithrombin for that of AAT M358R in AAT and then back-mutated this variant to generate an array of less modified AAT constructs (40). These investigators noted that changes in different parts of the RCL had greater effects when combined than when separate, with respect to APC inhibition, just as we observed with FXIa inhibition.

Our approach in this study and its predecessor (31) relied heavily on the determination of $k_2$ values and the reporting of selectivity as the ratio of the rate constants for the inhibition of two different proteases by a particular AAT variant. It should be noted that $k_2$ is an aggregate term reflecting the association rate constant diminished proportionately by non-productive turnover (e.g., substrate behavior leading to AAT cleavage and protease escape). We assessed non-productive turnover by measuring the SI. SI elevations could have resulted from a subset of inhibitor molecules being unable to react with the inhibitor,

impairments in the inhibitory mechanism leading to serpin cleavage, or instability of the serpin–enzyme complex. Gel-based analyses using a range of AAT/FXIa ratios showed that both AAT M358R and AAT-RC-2 reacted fully with FXIa, proceeding down the branched pathway to either complexed or cleaved serpin outcomes. In contrast, AAT-RC did not appear to react fully and was non-productively cleaved to a greater extent than the other two AAT variants. These relative deficiencies of AAT-RC likely arose from the formation of intermolecular disulfide bonds linking two AAT-RC molecules via the F352C cysteine residue in their RCLs. Such RCL-linked dimers, whose formation we demonstrated via non-reducing electrophoresis, would be expected to be unable to form serpin–enzyme complexes. Two dimeric forms of AAT-RC were detected via non-reducing electrophoresis; these likely represented intermolecular disulfide bonding between C352 and C352 and between C232 and C352, since wild-type AAT contains a single Cys residue at position C232 (41). Intramolecular disulfide bond formation was also suggested by the presence of a more rapidly migrating AAT-RC species not observed under reducing conditions, likely intramolecular C232–C352. Such a misfolded species would also be highly unlikely to form productive complexes with proteases; a precedent exists in antitrypsin I (R39C) (42). Taken together, these intermolecular and intramolecular oxidized forms of AAT-RC likely account for most of the difference in SI between AAT-RC and AAT-RC-2 or AAT M358R. Those AAT-RC molecules not diverted into non-productive dimers might also been converted into cleaved form to a greater extent than AAT M358R or AAT-RC-2 because F352C would disrupt an interface formed by F352 and a patch of hydrophobic residues underlying helix F observed in crystal structures of RCL-inserted AAT, either free or complexed to proteases (25, 26, 43).

AAT-RC-2 differs from AAT M358R at three positions: I356E (P3), I360T (P2'), and P361E (P3'). While inspection of the aligned RCL sequences of 30 human serpins shows Thr at P2' in two serpins (SERPIN A12, vaspin, and SERPIN D1, heparin cofactor II), Glu is not found at P3 or P3' in any of these serpins (44). Given the more conservative nature of the I360T substitution, our previous finding that an I360T/P361Q substitution enhanced thrombin reactivity of AAT M358R (45), and the diametrically opposed decreases in thrombin reactivity of AAT-RC and AAT-RC-2 and increases in FXIa reactivity of these proteins, it is likely that I356E and P361E (P3') are primarily responsible for the desirable properties of AAT-RC-2 as a selective FXIa inhibitor. While there is some precedent for FXIa favoring Glu at P3, in that FXIa cleaves the bond following EPR in an amyloid beta protein precursor (46), in engineered activatable hirudin-based thrombin inhibitors (47, 48), and in a chloromethyl ketone chromogenic substrate with specificity for FXIa (49), no precedent exists for a favorable interaction with Glu at P3'.

To gain greater understanding into the interactions of AAT-RC-2 with FXIa, we turned to *in silico* protein structural modeling. No crystal structure of any form of AAT in complex with FXIa can be found in the Protein Data Bank. However, AAT has been crystallized in two complexed forms: in M358R form, as an encounter complex with active site-mutated S195A trypsin (24), and in wild-type form, as a cleaved, covalently bonded serpin-enzyme complex with trypsin (25) or porcine pancreatic elastase (26). We chose to model an initial encounter complex between inhibitor and protease to understand how the changes we had engineered improved AAT M358R reactivity with and specificity for FXIa. The catalytic domain of FXIa has been crystallized with a number of inhibitors; we chose the complex of FXIac with the small protein Kunitz protease inhibitor domain of protease nexin 2 (38). We extracted the AAT M358R structure (24) and the FXIac structure (38) from these complexes and docked them using ClusPro 2.0 (35–37). We also introduced the mutations from AAT-RC and AAT-RC-2 *in silico*, minimizing steric clashes using PyMOL, docked them, and compared the three modeled complexes. The main difference that we noted was with respect to interaction of the variant AAT protein RCLs with K192, a residue N-terminally adjacent to the conserved GD*S*GGP motif surrounding the active site serine (S195 in chymotrypsin convention numbering, underlined) in serine proteases (50). We noted the stabilization of the interaction between E356 and E361 in AAT-RC and AAT-RC-2 by three hydrogen bonds not capable of forming in AAT M358R between K192 and the native P3 I356 and P361 residues. Notwithstanding slight differences in predicted hydrogen bond lengths and angles between FXIa K192 and AAT E356 and E361 in AAT-RC and AAT-RC-2, these models suggest that the encounter complex between AAT-RC and FXIa would have been favored to a similar extent as that between AAT-RC-2 and FXIa, had the former been able to form without interference from disulfide bonded inter-RCL dimerization.

It should be noted that ClusPro is limited in its predictive ability compared to molecular dynamics with free energy perturbation calculations. While molecular dynamics would provide a higher quality model, such approaches are beyond the scope of this study. We improved the docking model outcome from ClusPro by partially defining the interacting interface (AAT RCL and residues of FXIac) based on experimental evidence of the FXIac interacting interface from the co-crystal structure of FXIac with KPI-PN2 and mutagenesis studies (38, 39). Regardless, the proposed interactions of AATA-RC-2 E356 and E361 with FXIa K192 should be regarded as a hypothesis suggested by one modeling approach, one that could be tested in future via either molecular dynamics or experiments with recombinant FXI molecules altered at K192. Inspection of 79 proteins representing the S1A trypsin subfamily of human serine proteases revealed that Lys residues were uncommon at position 192 in coagulation-related proteases (51). Q192 is found in FXIIa and FXa and E192 in APC and thrombin. The latter residues might be expected to repel E356 and E361 in AAT-RC and AAT-RC-2. Of the proteases tested in this study, only kallikrein contains a Lys residue at position 192. However, we observed that AAT-RC-2 had a greatly elevated SI for kallikrein compared to that of AAT M358R. Thus, had the encounter complex formed efficiently between AAT-RC-2 and kallikrein, it is highly unlikely that it would have proceeded to form a stable covalent complex. Other spatial conflicts must have prevented the rapid insertion of the AAT-RC-2 RCL, but not the AAT M358R RCL into underlying β-sheet A, since failure to retain captured protease in

a stable complex correlates with a slowed speed of cleaved RCL (N-terminal to P1) insertion (30).

AAT-RC-2 is unusual among recently engineered AAT M358R variants in that it appears to exhibit enhanced selectivity for its target protease, without the cost of decreased activity. Polderdijk et al. reported a 7-fold decrease in the rate of APC inhibition for KRK AAT, an AAT M358R variant engineered to inhibit APC selectively, which was unreactive with thrombin and which inhibited FXIa and FXa with rate constants reduced by factors of 850 and 360, respectively (52). De Maat et al. exchanged tripeptide SMT for AIP in the P4-P2 positions of AAT M358R as well as S359V in AAT SMTRV to generate a variant AAT engineered to inhibit contact pathway proteases. While this variant inhibited kallikrein 2.5-fold more rapidly than AAT M358R, and was 20-fold less effective at inhibiting plasmin, these enhancements came at the cost of a 1.8-fold reduction in its rates of FXIa inhibition (34).

The effects of AAT-RC-2 in human plasma correlated with its retained AAT M358R-like rate of FXIa inhibition and its loss of AAT M358R's ability to inhibit other coagulation proteases. Neither AAT-RC nor AAT-RC-2 prolonged the diluted PT; the prolongation by AAT M358R likely arose due to its anti-FXa and anti-thrombin capacities, since the PT is affected by the extrinsic and common pathways of coagulation (53). Some prolongation of the diluted APTT by AAT-RC-2 and AAT-RC was observed, consistent with their anti-FXIa capacities, but it again did not surpass that of AAT M358R because of that inhibitor's effects on both contact factor and common pathways (53). The greater anticoagulant activity of AAT-RC-2 than AAT-RC in the FXI-dependent APTT likely reflected the more rapid inhibition of FXIa by AAT-RC-2 than AAT-RC in this more sensitive assay, as well as the diminished reactivity of AAT-RC arising from intermolecular or intramolecular disulfide bond formation. de Maat et al. reported a similar intermediate effect of AAT variant SMTRV on the diluted APTT, greater than buffer and lesser than AAT M358R, consistent with its anti-FXIa and anti-FXIIa activities (34).

AAT-RC-2 marks the culmination of our mutagenic campaign to engineer AAT M358R into a specific FXIa inhibitor. This goal was achieved with the alteration of only three additional residues. We employed phage display and bacterial lysate screening to probe three sectors of the RCL and then combinatorial mutagenesis to arrive at AAT-RC (31) and back-mutation to define the minimum mutations necessary to maintain FXIa specificity. While complex, this strategy permitted the engineering of the inhibitor without *a priori* assumptions and was vindicated by the cooperativity observed between changes in different residues in the AAT M358R RCL. AAT-RC-2 should function as a specific FXIa inhibitor *in vivo* when it is tested in animal models. The effectiveness of KRK AAT and SMTRV AAT *in vivo*, specifically in countering the hemorrhagic tendency of FIX knockout mice for KRK AAT (52) and in reducing the thrombotic and inflammatory responses of normal mice treated with SMTRV AAT (34), may bode well for future *in vivo* experimentation with AAT-RC-2.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

WS conceived and designed the study, secured competitive funding to support it, and wrote the manuscript. MH contributed to writing the manuscript. MH and VB performed the experiments. MH and WS created the figures. MH conducted all protein modeling studies under the direction of SA. All authors contributed to the data analysis, involved in the editing and revision of the manuscript, and approved its final version.

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REVIEW
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# Fibrinolytic Serine Proteases, Therapeutic Serpins and Inflammation: Fire Dancers and Firestorms

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The making and breaking of clots orchestrated by the thrombotic and thrombolytic serine protease cascades are critical determinants of morbidity and mortality during infection and with vascular or tissue injury. Both the clot forming (thrombotic) and the clot dissolving (thrombolytic or fibrinolytic) cascades are composed of a highly sensitive and complex relationship of sequentially activated serine proteases and their regulatory inhibitors in the circulating blood. The proteases and inhibitors interact continuously throughout all branches of the cardiovascular system in the human body, representing one of the most abundant groups of proteins in the blood. There is an intricate interaction of the coagulation cascades with endothelial cell surface receptors lining the vascular tree, circulating immune cells, platelets and connective tissue encasing the arterial layers. Beyond their role in control of bleeding and clotting, the thrombotic and thrombolytic cascades initiate immune cell responses, representing a front line, "off-the-shelf" system for inducing inflammatory responses. These hemostatic pathways are one of the first response systems after injury with the fibrinolytic cascade being one of the earliest to evolve in primordial immune responses. An equally important contributor and parallel ancient component of these thrombotic and thrombolytic serine protease cascades are the *ser*ine *p*rotease *in*hibitors, termed *serpins*. Serpins are metastable suicide inhibitors with ubiquitous roles in coagulation and fibrinolysis as well as multiple central regulatory pathways throughout the body. Serpins are now known to also modulate the immune response, either via control of thrombotic and thrombolytic cascades or via direct effects on cellular phenotypes, among many other functions. Here we review the co-evolution of the thrombolytic cascade and the immune response in disease and in treatment. We will focus on the relevance of these recent advances in the context of the ongoing COVID-19 pandemic. SARS-CoV-2 is a "respiratory" coronavirus that causes extensive cardiovascular pathogenesis, with microthrombi throughout the vascular tree, resulting in severe and potentially fatal coagulopathies.

**Keywords: serpin, thrombolysis, fibrinolysis, coagulation, inflammation, serine protease, infection, virus**

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## INTRODUCTION

Hemostatic control of bleeding by clot formation (thrombosis) and the subsequent dissolution through fibrinolysis (also termed thrombolysis) are essential components in the front line response to trauma (1). In the past decade, intensive research has revealed that thrombosis and fibrinolysis are extensively involved in immune pathologies not directly linked to clotting or hemorrhage, including disorders related to sterile inflammatory diseases and microbial and viral infections (2). Interactions between coagulation pathways and the inflammatory immune response are now known to be essential to maintaining health and limiting disease.

The interaction between coagulation and inflammation is bidirectional, a "two-way street," and one begets the other (3). Coagulation is used here to refer to thrombosis (clot formation) and thrombolysis (clot breakdown). It is well-understood that unregulated clotting or bleeding can have severe adverse consequences. Too much clotting causes occlusion of circulating blood (e.g., blocking the circulation), while too much fibrinolysis leads to hemorrhage and blood loss, and an excess of either can be fatal. To further complicate this interaction, in some cases severe vascular damage or infection causes excess thrombosis, consumption of clotting factors and eventual deficit in the homeostatic balance and excess fibrinolysis. This consumptive coagulopathy (CC) or disseminated intravascular coagulation (DIC) markedly increases mortality in viral and bacterial sepsis. Unregulated inflammation also causes severe tissue disruption, endothelial damage, microthrombotic occlusions, vascular leakage, hemorrhage, and shock with death. Elucidation of the cross-talk of these pathways (termed "thromboinflammation") makes eminently clear that inflammation can cause clotting, and clotting can cause inflammation, thus the regulation of one pathway affects the other (4).

Serine proteases are highly active and one of the most prevalent protease classes, driving the thrombotic and thrombolytic cascades. Dysregulation of these coagulation pathway proteases leads to onset and/or exacerbation of numerous diseases, including rare bleeding disorders, chronic lung disease, septic shock (whether viral, bacterial, or fungal in origin), DIC (or CC), and neurodegeneration. The thrombotic and thrombolytic cascades are intrinsically regulated by the *ser*ine *p*rotease *in*hibitor (*serpin*) superfamily (5). Recent investigations have identified numerous immune modulating functions for serpins, clearly demonstrating that these complex inhibitors directly interact with and influence immune cell responses and regulate inflammation beyond direct effects on the thrombotic and thrombolytic pathways (6). Thus, a complete understanding of the role of the thrombotic and thrombolytic proteases, and the serpins that regulate their function in the circulating blood, may lead to novel therapeutic avenues for treating a diverse array of immunopathologies.

The role of the thrombotic pathway in inflammation has been extensively highlighted in numerous reviews (4, 7–10). In this review, we will discuss the interaction of the fibrinolytic pathway with inflammatory responses and the bidirectional regulation of these responses, both fibrinolytic and inflammatory, by serpins. We begin with an exploration of the evolutionary roots of the coagulation-associated pathways, both thrombotic and thrombolytic, and the serpins that regulate these pathways, as evidence for their origins in primordial immune responses. We then focus specifically on the fibrinolytic/thrombolytic pathway, the interaction of serine protease activity in fibrinolysis and inflammation, and their contributing roles in disease. Next, we discuss the evidence for utilizing serpins as therapeutics designed to modulate the fibrinolytic response in disease. We will conclude with a brief discussion of the fibrinolytic pathway and serpins in the pathogenesis (and potentially treatment) of the ongoing COVID-19 pandemic caused by SARS-CoV-2.

## IMMUNE ORIGINS AT THE ANCIENT ROOTS OF COAGULATION PROTEASES AND SERPINS

### Thrombosis and Thrombolysis–Evolutionary Roots

Serine proteases involved in coagulation are functionally conserved across the Kingdom *Animalia* and represent an ancient class of proteins. Emerging evidence suggests that independent evolution has occurred for at least two separate functions for these pathways: (i) control of thrombotic/thrombolytic responses and (ii) regulation of the immune response. While some pro-thrombotic (clotting) enzymes appear to have emerged as early as 700 million years ago, the genes and proteins required for the conversion of fibrinogen to fibrin did not appear until 500–600 million years ago (11). This timeline appears to have coincided with the emergence of fibrinolysis 570 million years ago (in the Precambrian period) (12). While much has been discovered in the developmental biology-based study of clotting across *Animalia*, the clotting "toolkit" has been found to differ greatly amongst animals. Exploration of the ancient roots of clotting now reveals that the coagulation pathways may originally have had central roles in innate immune responses, or inflammation (**Figure 1**).

Mammals (Class *Mammalia*) harbor the most complex coagulation system, with the classically defined contact activation or "intrinsic" pathway and the tissue factor/factor VII system or the "extrinsic" pathway (13, 14). The pathways converge, resulting in a complex downstream cascade of protease activation events leading to the activation of factor X and thrombin, conversion of fibrinogen to fibrin, and ultimate generation of a stable fibrin clot (**Figure 1**). In the vascular system, thrombi form on the surface of activated platelets, damaged endothelium in the lining of the arterial wall, and activated macrophage cells adherent to damaged endothelium. This entire interactive complex both activates and is activated by the kallikrein-kinin system. While apparently important to the activation of thrombosis, deficiency of this pathway affects thrombosis and modifies immune responses.

A careful examination of other animals reveals a distinct role for coagulation in the immune response. For example, despite the presence of components of the kallikrein-kinin system

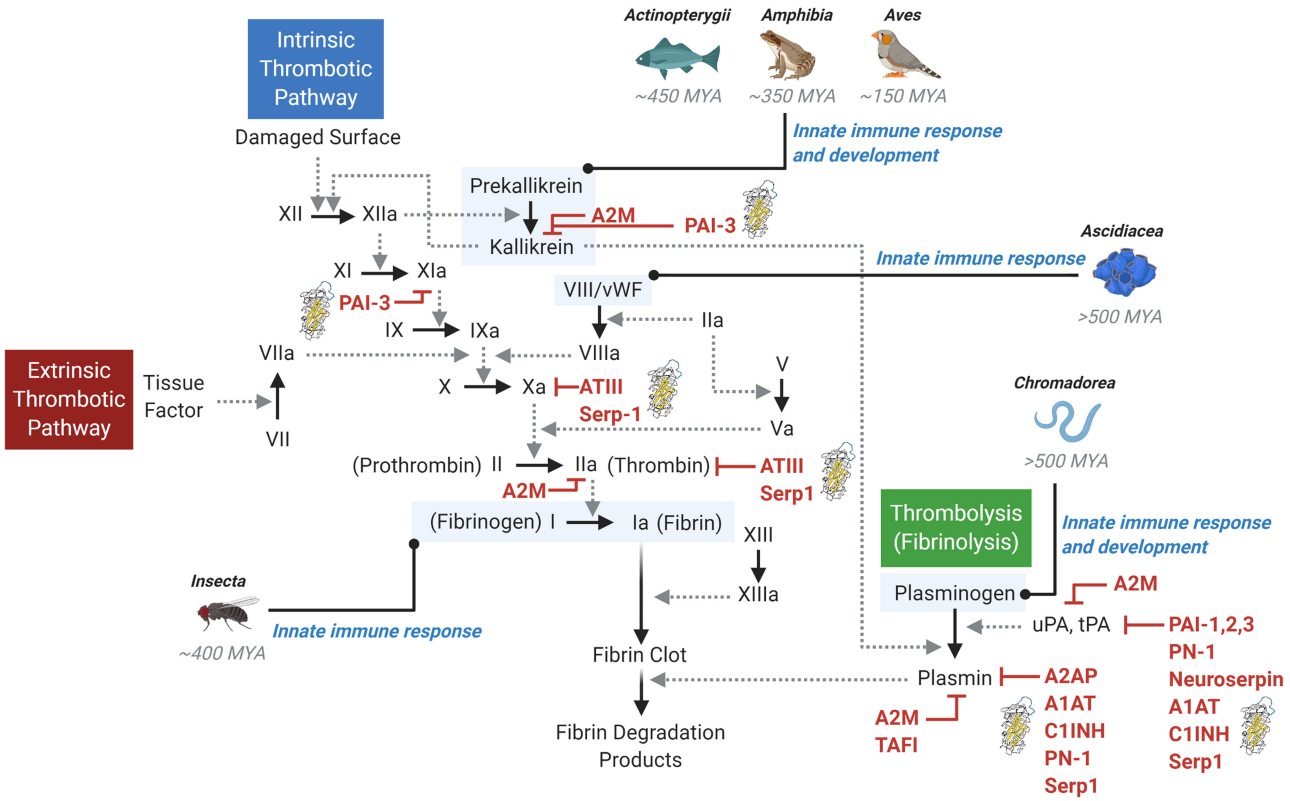


**FIGURE 1 |** The thrombotic and thrombolytic cascades and primordial immune response. The thrombotic pathways (intrinsic and extrinsic) and the thrombolytic (fibrinolysis) pathway involve a complex cascade of protease activation. Solid arrows indicate the conversion to an active protease, while dotted line arrows indicate the activity of the activating upstream protease. A variety of inhibitors are shown, with serpin inhibitors denoted by a serpin protein structural image. Examples of early primordial immune response origins are noted in context of the pathways. MYA, million years ago.

in birds (Class *Aves*), amphibians (Class *Amphibia*) and fish (Class *Actinopterygii*), evidence suggests these components do not drive clot formation, but rather, regulate angiogenesis and the immune response system (**Figure 1**). The process of clotting in these Classes is regulated by the extrinsic tissue factor-directed pathway (15). Looking further, lower-level animals (e.g., invertebrates) also contain mechanisms for regulating clotting. Due to their open circulatory system and propensity for massive loss of hemolymph (the equivalent of combined blood and lymphatic fluid), clotting evolved very efficiently in insects (Class *Insecta*) and is central to innate immunity in *Drosophila* (16). Class *Ascidiacea*, which include sac-like marine invertebrate filter feeders, is among the most ancient coagulation systems investigated. While their plasma contains some blood clotting components (such as von Willebrand factor, vWF), blood in *Ascidacea* animals does not clot, and these components are predominantly used to regulate innate immune responses (**Figure 1**) (17, 18).

A similar primordial role in innate immunity can be found for fibrinolysis, a cascade that balances and is complementary to the coagulation cascade which is responsible for dissolution of a fibrin clot. In *Mammalia*, fibrinolysis is initiated by the conversion of inactive, circulating plasminogen into active plasmin by serine proteases referred to as the plasminogen activators, tissue- and urokinase-type plasminogen activators (tPA and uPA, respectively) (**Figure 1**). Activated plasmin then breaks down cross-linked fibrin, resulting in dissolution of the thrombus. Investigations into lower-level organisms distinctly reveal the immune response origins of the thrombolysis pathway. For example, *Caenorhabditis elegans* (Class *Chromadorea*), despite its lack of vasculature, blood, or hemolymph, expresses a functional plasminogen-like protease required for organ development and innate immunity (19, 20). Thus, fibrinolysis represents another component of the primordial innate immune response that has been preserved for millions of years.

## Serpins–Evolutionary Roots

As counterparts to the proteases that mediate fibrinolysis, serpins are the equally-ancient, intrinsic protease inhibitors that arose in *Animalia* some 650–700 million years ago. Serpins are highly metastable proteins characterized by two key structural components: a reactive center loop (RCL) and a 4-stranded, core beta-sheet (termed the "A" beta-sheet). The reactive center loop contains a protease recognition sequence which acts as a bait for activated serine proteases (**Figure 2**). Cleavage of the protease recognition sequence by the appropriate protease creates a

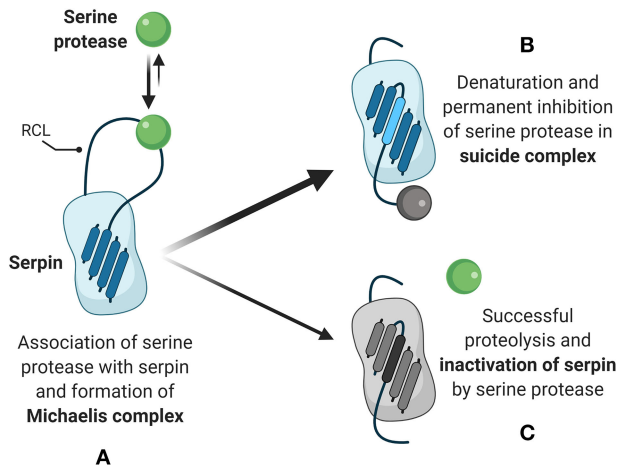


**FIGURE 2 |** Serpin-dependent mechanism of protease inhibition. **(A)** Natively folded serpins present a reactive center loop (RCL) which acts as a bait for interaction with active serine proteases. Upon association, initiation of proteolytic activity by the protease triggers the formation of a Michaelis complex and covalent bonding of the serpin with the protease. **(B)** The primary outcome of Michaelis complex formation is reconfiguration of the serpin, with the RCL inserting as the third of five strands in the core beta sheet and permanent denaturation of the target serine protease. **(C)** The secondary and less frequent outcome is completion of proteolytic activity and dissociation of the active serine protease, while the RCL continues to insert as the third of five strands in the serpin core beta sheet.

transient Michaelis complex, as occurs in any generalized enzyme-substrate interaction, where the protease and the serpin are covalently bonded at the active site of the protease (**Figure 2**). Upon cleavage of the recognition site, the metastability inherent in the serpin is released and the serpin-protease pair initiates a dramatic (on a molecular scale) conformational change (**Figure 2**). The reactive center loop "swings" 70 angstroms across the protein and inserts itself as the third strand in a now five-stranded beta sheet. The conformational change of the serpin induces a deformation of the active site in the protease, disallows completion of the protease-substrate interaction, and permanently denatures both the serpin and protease in what is referred to as a "suicide complex" (**Figure 2**). Because serpins are tuned to their inhibitor by changes in the protease recognition sequence in their RCL, multiple gene duplications have resulted in the ability for serpins to become tailored to a wide variety of proteases. Interestingly, in some cases individual serpins have lost their canonical protease inhibitory function, as in the case of Maspin, which does not form suicide complexes upon protease digestion with any tested protease and which is associated with tumor suppression by a mechanism that is still poorly defined (21). Accordingly, the genomes of most members of *Animalia* contain a multitude of serpins with human and mouse genomes encoding 37 and 60 serpins, respectively (22, 23). At the other end of the evolutionary spectrum, the genome of *Caenorhabditis elegans* encodes nine serpins (24).

The evolutionary origins of immune regulation by serpins are exemplified by *Drosophila*. Persephone is a circulating serine protease in *Drosophila* upstream of the fly Toll pattern recognition receptor pathway and activates the protease Spätzle (25). Deficiency in the Spn43Ac serpin, which regulates Persephone, leads to constitutive activation of Spätzle in the *Drosophila* innate immune response and results in developmental lethality (the *nec* phenotype) (25). Thus, both serine proteases and their serpins have evolutionary roots in regulating inflammation and the innate immune response.

## OVERVIEW OF THE FIBRINOLYTIC MACHINERY

### Serine Proteases of the Fibrinolysis System

Fibrinolysis is ultimately mediated by the activity of the serine protease plasmin (**Figure 3**). Initiation of fibrinolysis requires the conversion of the inactive zymogen plasminogen to active plasmin, which then subsequently breaks down cross-linked clots (**Figure 3**). Plasminogen is predominantly synthesized in the liver and secreted at a plasma concentration of ∼150 μg/mL (26). Plasminogen is converted by plasmin primarily by the activity of two plasminogen activators, tPA and uPA, as discussed in the next paragraph. Forward feedback of plasmin activity on its own activators results in increased processing of plasminogen to accelerate the generation of additional active plasmin (27). Plasmin activation occurs *in situ* when plasminogen co-localizes with its activators in a "ternary complex" at C-terminal lysine residue binding sites on fibrin (28). Active plasmin then directly cleaves the cross-linked fibrin to dissociate the clot. Plasminogen coordination at the cell membrane, and therefore localized generation of plasmin at cell surfaces, is mediated by a number of receptors, with the predominant plasminogen receptor being Plg-$R_{KT}$ (29, 30). Plg-$R_{KT}$ is expressed by a wide variety of cell types in all tissues, including migrating immune cells, and co-localizes with the receptor for uPA (discussed below) (29). The expression of Plg-$R_{KT}$ enhanced plasminogen activation by more than 12-fold, in part by coordinating its localization with uPA and tPA at the cell surface (29).

The serine proteases tissue- and urokinase-type plasminogen activators, tPA and uPA, respectively, are the key components of the plasminogen activation system. While tPA and uPA share only about 40% amino acid similarity, their basic structure is highly similar (31). The basal circulating levels of tPA and uPA are low compared to other circulating proteins, with tPA reported in the range of ∼1–10 ng/mL and uPA at 2–10x lower levels (∼0.1–0.3 ng/mL) (32–36). Synthesis of tPA occurs in abundance in both the vasculature and the central nervous system. In the nervous system, tPA is synthesized and released by neurons and glial cells and is constitutively active in a number of regions of the brain where its activity has been associated with neural plasticity (37). tPA has been identified in secretary vesicles after membrane depolarization and is rapidly localized to neuronal synapses (38). Further studies have identified that the activity of tPA is essential for the late phase of long-term potentiation and is a driver of synaptic growth (39). In the vasculature, synthesis of tPA occurs predominantly in endothelial cells and is stored in granules called regulated secretory organelles (RSOs). *In vitro* and *in vivo* experiments indicate that RSOs are

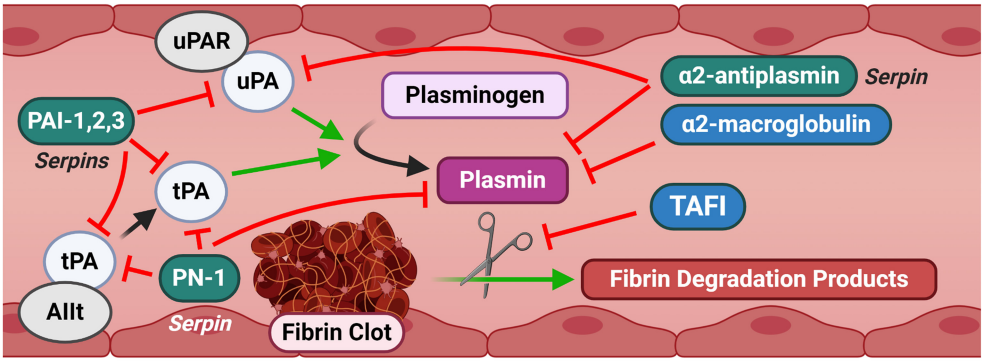


**FIGURE 3 |** Canonical signaling of the fibrinolysis pathway. Fibrinolysis is characterized by the degradation of a fibrin clot into degradation products by plasmin. Plasmin is generated from plasminogen by uPA and tPA. Several serpins and other inhibitors provide a tight regulation of this cascade. Substantial promiscuity exists across multiple elements of the pathway, providing redundant controls against inappropriate activation. AIIt and uPAR are shown as representative canonical fibrinolytic receptors for brevity. AIIt, Annexin II tetramer; PAI-1,2,3, Plasminogen Activator Inhibitor-1, 2, 3; PN-1, Protease Nexin-1; TAFI, Thrombin activatable fibrinolysis inhibitor; tPA, Tissue-type plasminogen activator; uPA, Urokinase-type plasminogen activator; uPAR, Urokinase-type plasminogen activator receptor.

trafficked and secreted rapidly in response to diverse physiologic stimuli, such as histamine (40), activated thrombin (41) and bradykinin, a metabolic product of the kinin-kallikrein system (42). While secreted tPA is active in the open circulation, its plasmin generation activity is enhanced by interaction with its receptor, the annexin A2 heterotetramer (AIIt), composed of two units of annexin A2 bound by a dimer of S100A10 (43). Binding of tPA to AIIt enhanced plasmin generation by 77-fold and mice deficient in S100A10, and therefore deficient in functional AIIt receptors, have 40% reductions in plasmin generation and reduced clearance of batroxobin-induced vascular thrombi (43, 44).

In contrast to the activity of tPA, uPA functions predominantly in innate, inflammatory immune responses and the tissue responses to injury, rather than the coagulation response (45). uPA is synthesized in a wide variety of tissues and cell types including vascular endothelial cells (46), hepatocytes (47), keratinocytes (48), renal tubular epithelial cells (49), neurons (50), and immune cells of both monocytic (51) and lymphocytic (52) lineages. Basal release of uPA is in the form of a single-chain zymogen with little or no proteolytic activity. uPA is further processed to a double-chain active enzyme that has a several hundred-fold higher activity by plasmin (53), kallikrein (54), activated factor XII (54), or trypsin-like proteases, for example, as released from tumors (55). While uPA can act in the open circulation, its enzymatic efficiency substantially increases by interaction with the urokinase-type plasminogen activator receptor (uPAR, also called CD87) (56). Interestingly, the activity of uPA itself is partially lowered by interaction with uPAR, but complex local coordination of uPA and its substrates (e.g., plasminogen) results in a net total increase in processivity by means of concentration and spacial protein-protein coordination effects (57). Interestingly, uPAR interacts promiscuously with components of the kallikrein pathway, FXII and AIIt, suggesting a broader, yet unexplored, role in regulating fibrinolytic and immune responses (43, 58, 59).

In addition to their function in fibrinolysis, plasmin, tPA, uPA and their receptors—Plg-Rs, the LDL receptor-related protein-1 (LRP1), and uPA receptor (uPAR), respectively,—have important functions in tissue remodeling and cell invasion. These thrombolytic proteases alter the extracellular matrix and modify cellular phenotype conversion via induction of intracellular signaling cascades. Thus far there have been 12 Plg-Rs identified, some of which are expressed on the cell surface and others are localized intracellularly (60). The dominant Plg-R expressed by macrophages is the plasminogen receptor Plg-$R_{KT}$, which is a surface expressed receptor where plasminogen binds and is activated (61). The activation of plasminogen when bound to the macrophage-expressed receptor is required for efficient invasion and clearance of dead cells (62, 63). uPAR is moderately expressed in most tissues in a healthy organism, including the lungs, kidneys, spleen, blood vessels, uterus, bladder, thymus, heart, liver, and testes (64). However, uPAR expression is strongest in tissue actively undergoing extensive remodeling (65). For example, keratinocytes at the migrating edge of cutaneous wounds exhibit potent upregulation of uPAR and wounds heal poorly in uPAR-deficient mice (66, 67). Upon immunological activation, neutrophils, monocytes and T cells markedly upregulate uPAR expression (68–70). Exposure of uPAR to uPA enhances the differentiation of monocytes to macrophages (71). Expression of uPAR also dictates the interaction of macrophages and neutrophils in efferocytosis (the clearance of apoptotic cells), as well as phagocytosis of viable cells by macrophages. Deficiency of uPAR in either macrophages or viable neutrophils enhances phagocytosis, but deficiency of uPAR in both cell types blocks phagocytosis (72). Independent of its fibrinolytic activity, recombinant uPA elicits an anti-apoptotic response in cultured endothelial cells by specific induction of the X-linked inhibitor of apoptosis (XIAP) protein (73). Similarly, uPA attenuates macrophage apoptosis induced by Ox-LDL and ER stress by activation of ERK1/2 and downregulation of Bim (71). Likewise, recombinant tPA dose-dependently rescued

cultured neurons from serum deprivation-induced apoptosis via a mechanism involving the PI-3 kinase pathway (74). Thus, serine proteases of the fibrinolytic cascade have essential fibrinolysis-independent functions in tissues and the immune system.

## Regulators of Fibrinolysis in Mammals

Serpins have evolved into a large class of regulatory proteins with extensive functions throughout circulating hematological and immune pathways in a wide range of organ systems from the cardiovascular tree to endocrine and neurological organs (75). As our knowledge of serpin sequences and structures have progressed, it is evident that widespread exchanges and combinations enabled serpins to target more than one pathway. As serpin functionality has evolved, some serpins retained immune regulating functions while concurrently expanding to target the thrombolytic cascades. In this section, we will discuss the known regulators of the thrombolytic/fibrinolytic cascades and in subsequent sections discuss their additional capacity to cross interaction and regulate immune and inflammatory responses with potential for providing new therapeutic reagents.

Serpin-dependent regulation of the fibrinolytic serine proteases in mammals is mediated by the plasminogen activator inhibitor (PAI)-1 (SERPINE1), PAI-2 (SERPINB2), and PAI-3 (SERPINA5; also called Protein C inhibitor, PCI) and protease nexin-1 (PN-1 or SERPINE2) against uPA and tPA, and by alpha-2-antiplasmin (SERPINF2) against activated plasmin. tPA is also regulated by a central nervous system-specific serpin, neuroserpin (SERPINI1). Two other serpins, alpha-1-antitrypsin (AAT or A1AT, SERPINA1) and Complement C1 inhibitor (C1INH, SERPING1), target fibrinolytic proteases but are better known for inhibition of other protease and inflammatory systems outside of the coagulation pathways will be briefly discussed.

Classical serpin-mediated inhibition of fibrinolytic serine proteases leads to permanent inactivation of both the serpin and protease via formation of a classic suicide complex (described above). Thus, tight control of fibrinolysis requires that circulating serpins are present in molar excess, or pre-synthesized and rapidly released from stores, without the need for transcription and translation. Accordingly, serpins account for up to 10% of circulating proteins in the circulation (76).

PAI-1, the principal member of the PAI protein family, is primarily produced by hepatocytes and secreted into the circulation by the liver. To a lesser extent, PAI-1 is synthesized and secreted by the kidney, spleen, heart, lung and adipose tissues (77). Additionally, circulating platelets continuously synthesize PAI-1, which is actively and rapidly released upon platelet activation and contributes to the stability of clots by limiting fibrinolysis (78). Circulating PAI-1 is usually present in a concentration range of 20–30 ng/mL, which is in three-fold excess of basal circulating tPA and up to 300-fold excess of basal circulating uPA (79). PAI-2 expression is restricted to keratinocytes, macrophages, activated monocytes, the placenta, and adipocytes (80). Circulating PAI-2 in healthy individuals is essentially undetectable, but drastically increases in pregnancy to over 250 ng/mL and rapidly declines postpartum (81). The skin is a major site for PAI-2 expression, where PAI-2 cross-links to the cornified cell membrane via transglutaminase during the terminal differentiation of keratinocytes to inhibit over-proliferation (82, 83). PAI-2 has been called the "undecided serpin" because its specific endogenous biological role has remained elusive, despite associations with regulating fibrinolysis and inflammation (84–86). PAI-1 is an efficient inhibitor of both uPA and tPA ($2 \times 10^7$ $M^{-1}$ $s^{-1}$ each) while PAI-2 effectively inhibits uPA ($2 \times 10^6$ $M^{-1}$ $s^{-1}$) and is a poor inhibitor of tPA ($2 \times 10^5$ $M^{-1}$ $s^{-1}$) (87, 88). PCI is predominantly an inhibitor of proteases in the thrombotic pathway, but detection of kidney-derived PCI complexed with uPA in the urine resulted in its identification as the third member of the PAI family as PAI-3 (89). Further justification for designating PCI as PAI-3 is the ability to inhibit plasma kallikrein and activated Factor XI (90), which are alternative activators of plasminogen (91, 92). Subsequent discussion of the roles of PAI serpins in disease will focus on PAI-1, as it is the principal serpin inhibitor of the fibrinolytic cascade.

Protease nexin-1 (PN-1 or SERPINE2) is expressed in diverse tissues during development, including cartilage, lung, skin, the urogenital tract and the nervous system, where it was originally identified as Glia-derived nexin (93). PN-1 is nearly undetectable in circulating plasma, amounting to ∼1 ng/mL or 20 picomolar amounts (94). In contrast to the low levels in circulation, PN-1 is endogenously synthesized and stored in abundance in the alpha-secretory granules of platelets, from which it is rapidly released upon platelet activation (95). As the second-order rate constant of PN-1-tPA interaction is three orders of magnitude lower than PAI-1-tPA, it was expected that PN-1 is not the primary inhibitor of fibrinolysis (96). However, systematic *in vivo* studies by Boulaftali et al. demonstrated that PN-1 inhibits both fibrin-bound tPA and auto-activation of plasminogen by fibrin-bound plasmin and is an important regulator of fibrinolysis (97).

Alpha-2-antiplasmin (SERPINF2) is the major serpin inhibitor of activated plasmin. Alpha-2-antiplasmin is synthesized in the liver and kidney at nearly equivalent levels (98). Alpha-2-antiplasmin is present at significantly higher concentrations than the PAI serpins, with circulating levels at 70 μg/mL (99, 100). Thus, alpha-2-antiplasmin, in coordination with the PAI family and PN-1, mediate the multi-stage, tightly controlled serpin-dependent regulation of the fibrinolytic proteases.

Neuroserpin (SERPINI1) is expressed predominantly in the central nervous system and was originally isolated from chicken ventral spinal cord neurons (101). Neuroserpin, a highly specific inhibitor of tPA, is expressed from the growth cone of neurons and is a poor inhibitor of uPA and plasmin (102). Neuroserpin is found at a concentration of ∼7.4 μg/L in the cerebrospinal fluid (CSF) and is significantly elevated in the CSF of patients with Alzheimer's Disease (103). Indeed, numerous neuropathologies are associated with dysregulation of neuroserpin. For example, familial encephalopathies with neuroserpin inclusion bodies are associated with mutations such as S49P-Syracuse (late onset encephalopathy) and S52R-Portland

(early onset encephalopathy) (104). Polymers of misfolded neuroserpin stimulate inflammation via NF-kappaB and oxidative stress signaling in an unfolded protein response-independent manner and may contribute to neurodegeneration (105, 106). The spatiotemporal patterning of neuroserpin suggests a role in neuronal development and synaptogenesis via homeostatic maintenance of tissue by limiting excess tPA activity which can lead to cerebral ischemia and epilepsy (104). The critical and sensitive role of neuroserpin in regulating tPA activity outside of the circulating blood highlights the importance of effective control of these serine proteases in diverse tissues.

C1INH and A1AT act primarily as regulators of complement and neutrophil elastase, and mutations in both of these serpins cause severe genetic disorders (107, 108). C1INH deficiency causes angiogenic edema which can be life threatening and A1AT deficiency resulting from aggregating mutations which deplete circulating levels of A1AT causes severe lung damage and emphysema. Both serpins are believed to primarily regulate serine proteases outside of the coagulation cascades and have established inhibitory functions for uPA and plasmin (109, 110).

In addition to serpin-dependent regulation of fibrinolysis, there are other non-serpin regulators/inhibitors of fibrinolysis. Thrombin-activatable fibrinolysis inhibitor (TAFI; also called carboxypeptidase B2, CPB2) is a non-serpin negative inhibitor of plasmin activity and acts as the terminal enzyme of the thrombotic cascade. TAFI is synthesized by the liver and megakaryocytes as an inactive zymogen (111, 112) and is processed to the functional form by thrombin, the thrombin-thrombomodulin complex, or plasmin (113–115). TAFI is secreted at circulating concentrations of 4–15 μg/mL (116). Processed TAFI exerts its anti-fibrinolytic effect by cleaving the C-terminal lysine residues which act as the plasminogen-binding site mediating plasminogen-to-plasmin conversion (117). Thus, active TAFI reduces fibrinolysis by suppressing the *in situ* activation of plasmin.

Alpha-2-macroglobulin (A2M) is a large (720 kDa) broad-spectrum inhibitor of an expansive array of proteases across all catalytic classes, including trypsin, chymotrypsin, thrombin, plasmin, kallikrein, uPA, cathepsins, papain, and matrix metalloproteinases among others (118). Accordingly, A2M can act as an inhibitor of both thrombosis and fibrinolysis. A2M is primarily synthesized in the liver, but *in vitro* experiments indicate that cultured cells from the lung as well as macrophages and microglia can also synthesize and secrete A2M (119–121). In the fibrinolytic cascade, A2M inhibits the activity of plasmin and its upstream activator uPA (122).

## FIBRINOLYSIS PATHWAY-ASSOCIATED SERPINS AS THERAPEUTICS IN INFLAMMATORY DISEASE

The dysregulation of fibrinolytic signaling is now identified as an important component of numerous pathologies. Genetic deficiencies in fibrinolytic regulation lead to bleeding disorders, organ dysfunction, and damage, and acquired disorders cause tissue fibrosis, dysregulated bleeding, cirrhosis, amyloidosis, and certain cancers (123). Several recent reviews discuss the role of the fibrinolytic system in inflammation and the immune response (124, 125). Similarly, small molecules inhibitors of fibrinolytic serine proteases such as tranexamic acid are under investigation for therapeutic modulation of the fibrinolytic processes that are associated with effects on the immune response (126). However, the highly evolved and potent inhibitory mechanisms of serpins have led to a growing interest in using serpins themselves as therapeutics. In the following section, we will discuss examples of the therapeutic use of fibrinolysis pathway-associated serpins, limiting our overview to examples which have been tested in preclinical models.

### PAI-1: Therapeutic Applications

Considering the delicate balance of the canonical targets of PAI-1—tPA and uPA—and the detrimental consequences of their dysregulation after injury, PAI-1 is a natural choice for therapeutic modulation of fibrinolysis dysregulation and has been demonstrated as such in numerous studies. PAI-1 is frequently seen as a mediator of injury and has been experimentally targeted to limit disease, especially cancer (127). The use of PAI-1 as a therapeutic at first may be unexpected. A large portion of studies investigating the delivery of PAI-1 for therapeutic purposes center on the cardiovascular system where thrombotic occlusion of infarction is treated with tPA, a target for PAI-1. However, PAI-1 has been extensively studied in numerous preclinical non-thrombotic animal disease models with demonstrated benefit. Unexpectedly, Carmeliet et al. demonstrated that adenovirus-mediated gene transfer of human PAI-1 in mice prior to induction of electric or mechanical vascular injury of the femoral or carotid arteries, respectively, reduced arterial neointima formation, a precursor to occlusive arterial plaque (128). This finding was supported by work from Schäfer et al. showing that bone marrow-derived PAI-1 reduces neointimal formation and luminal stenosis in bone marrow transplantation after carotid injury with ferric chloride (129). In subsequent work, Wu et al. demonstrated that recombinant PAI-1 prevented intimal hyperplasia in a model of carotid artery injury in rats, thus further supporting a potential therapeutic role for PAI-1 to prevent vascular restenosis (130). Interestingly, their systematic investigation involving constitutively active, inhibition-defective, and vitronectin binding-deficient forms of PAI-1 demonstrated that the ability to therapeutically limit intimal hyperplasia was mediated by either the ability for the serpin to inhibit proteases, or to bind to vitronectin, but did not require both. Zhong et al. similarly demonstrated that recombinant PAI-1, differentially through binding to vitronectin or protease inhibitory activity, mediates a therapeutic reduction of cardiac fibrosis in a model of cardiac fibrosis in uni-nephrectomized mice fed a high salt diet and angiotensin II (131). Using an adenovirus-5 (Ad5) with a CMV promoter, Qian et al. found that overexpression of human PAI-1 protected ApoE-deficient mice from abdominal aortic aneurysm induced by angiotensin II when delivered directly into the perivascular tissue of the aorta, but not when delivered systemically by tail-vein injection (132). Ad5-mediated gene transfer of human PAI-1 was also shown by Heymans et al. to preserve pump function in mice

after acute pressure overload by attenuation of left ventricular remodeling (129). In a highly rational translation of its natural function, Jankun et al. reported that a modified version of PAI-1 with a half-life increased from 2 h to more than 700 h (very long half-life, termed VLHL PAI-1) was therapeutically effective at promoting hemostasis in reducing total blood loss induced by tail clipping when given by systemic or topical administration in mice (133).

PAI-1 has also demonstrated therapeutic efficacy in models outside of the conventional cardiovascular system. Yang et al. identified a role for tPA in the breakdown of the blood-brain barrier during neonatal cerebral hypoxia-ischemia in rats (134). Administration of recombinant PAI-1 dose-dependently preserved brain tissue and reduced edema, axonal degeneration and cortical cell death. The same group further adapted the treatment to an intranasal delivery format, which reached the cerebral cortex and reduced ∼75% of brain atrophy in hypoxic-ischemic brain injury of newborns and in lipopolysaccharide-sensitized hypoxic-ischemic brain injury (135). Swiercz et al. demonstrated that exogenous delivery of the 14-1b active mutant of PAI-1 with an extended half-life limited angiogenesis and LNCaP prostate cancer tumor growth in SCID mouse xenografts when delivered by continuous infusion with subcutaneously implanted osmotic pumps (136). Praus et al. reported that liver adenoviral delivery of PAI-2, but not PAI-1, reduced the incidence of lung and brain liver tumor metastases but did not increase mouse survival (137).

PAI-1 therapy may also have a role in treating non-sterile conditions, as the pro-inflammatory functions of PAI-1 are found to be critical in numerous animal models of difficult-to-treat infections (138–140). Renckens et al. identified an essential role for PAI-1 in the host response to the respiratory pathogen *Klebsiella pneumoniae*, a Gram-negative bacteria, demonstrating an enhanced immune response, reduced lethality and prevention of sepsis and distal organ injury in mice transgenically overexpressing human PAI-1 delivered by Ad5 vector (141). Interestingly, the authors found that intranasal Ad5 delivery of PAI-1, but not Ad5 delivery alone in healthy mice also induced pulmonary inflammation and suggested that increased PAI-1 levels in the lungs may prime a protective inflammatory response in the context of infection.

## PN-1: Therapeutic Applications

PN-1 is expressed by many tissues in the body and thus exhibits broad potential regulatory functions outside of a role in modulating fibrinolysis in the circulation. Several features of PN-1 function have been translated to a potential for therapeutic development. Activation of fibrinolytic machinery, such as uPA and tPA, in tissues activates downstream matrix metalloproteinases (MMPs), leading to degradation of collagen and elastin in the extracellular matrix architecture (142). Stevens et al. demonstrated that intraarticular administration of recombinant PN-1 prevents articular cartilage degradation in rabbits subjected to interleukin-1 beta/basic fibroblast growth factor insult (143). Curiously, McKee et al. discovered that the PN-1 can engage the canonical serpin-enzyme complex receptor, lipoprotein receptor-related protein-1 (LRP-1), in the form of a PN-1:uPA complex to downregulate the activity of the sonic hedgehog (SHH) pathway which is involved in the malignant transformation of numerous tissues (144). Treatment of PC3 prostate cancer cells with recombinant PN-1 or combinatorial treatment with SHH pathway inhibitors significantly reduced xenograft tumor growth in SCID mice and was associated with alterations in angiogenesis (144). In a subsequent study, the same group identified that PN-1 may act via inhibition of X-chromsome-linked inhibitor of apoptosis (XIAP) and reported that therapeutic treatment of xenograft tumor growth in SCID mice was also synergistic with XIAP inhibitors (145). A potential limitation of both of these studies, however, is the use of a pre-treatment regimen for investigation. It will be valuable to know whether PN-1 has anti-tumor activity after tumor engraftment and growth. The utility of PN-1-mediated targeting of the SHH pathway was recently demonstrated by Li and Wang et al. using a model of Alzheimer's disease in APP/PS1 transgenic mice (146). Hippocampal delivery of lentivirus particles to overexpress PN-1 resulted in improved cognitive function, reduced amyloid deposition and preserved neuronal cell viability.

## Alpha-2-Antiplasmin (A2AP): Therapeutic Applications

Owing to the fact that alpha-2-antiplasmin (A2AP) is the primary inhibitor of plasmin, the majority of research on A2AP in the therapeutic context has focused on diminishing the inhibitor activity of A2AP, thereby enhancing fibrinolysis (147). In contrast, less focus has been spent on investigating A2AP to limit bleeding. In early work, Weitz et al. found that supplementation with A2AP inhibited tPA-induced fibrinogenolysis and bleeding, but did not affect thrombolysis in a model of jugular vein thrombosis in rabbits (148). Nieuwenhuizen et al. investigated the therapeutic administration of A2AP in a model of joint bleeding-induced arthropathy, which can persist even after the administration of clotting factor (149). Using a model of needle-induced arthropathy in Factor VIII-deficient mice, the authors found a reduction in both synovitis and cartilage damage over a period of 5 weeks when antiplasmin was given by direct intraarticular administration, whereas the uPA inhibitor amiloride was ineffective (149, 150). A2AP has also been found to have efficacy in limiting cancer burden. Hayashido et al. reported a drastic reduction of SCCKN squamous cell carcinoma tumor growth in SCID mice when the cells overexpressed A2AP vs. a mock-expressing control. A2AP-mediated reductions in tumor growth occurred by limiting E-cadherin processing by the fibrinolysis pathway (151). Similarly, Paquet-Fifield et al. reported restricted lymphatic remodeling and reduced metastases in SCID mice harboring 293-EBNA cells overexpressing A2AP and VEGF-D vs. LacZ (152). A limitation in these studies investigating the role of A2AP on tumorigenesis is again the prior delivery of A2AP to cells before implantation. Investigations determining if post-implantation

delivery of the A2AP gene sequence or recombinant protein will have a similar effects could advance A2AP as a therapeutic.

## Neuroserpin: Therapeutic Applications

The therapeutic use of neuroserpin has primarily been investigated in neuropathologies. In early studies Yepes et al. found that intracerebral administration of neuroserpin was protective after in a model of middle cerebral artery occlusion (MCAO) stroke and prevented basement membrane proteolysis and cellular apoptosis in the ischemic penumbra of rats by more than 50% (153). In subsequent work, Zhang et al. used neuroserpin as an adjuvant treatment and found that intracisternally-injected neuroserpin increased the therapeutic window for therapeutic tPA administration after MCAO in rats by as much as 4 h with reduced brain edema and ischemic lesion volume (154). This work was recently confirmed in a similar study by Cai et al. (155). Interestingly, Wu et al. showed that the neuroprotective effect of neuroserpin in experimental MCAO was independent of its ability to inhibit tPA because protection was observed even in tPA-deficient mice, suggesting broader protective mechanisms in cerebral ischemia potentially involving less efficiently-inhibited serine proteases such as plasmin (156). Yepes et al. further reported that administration of neuroserpin into the ipsilateral hippocampus enhances neuronal survival and delays the progression of seizure activity in rats and mice subjected to kainic acid-induced seizures (157). Labeurrier et al. also showed neuroprotection against NMDA-induced excitotoxicity when neuroserpin was co-injected with NMDA into the left striatum or left cortex of mice (158).

Leveraging the neuroserpin therapeutic benefits on brain-associated pathologies, several studies have expanded investigations into the broader therapeutic effects of neuroserpin. In a rat model of spinal cord injury induced by clip compression, neuroserpin immediately injected intrathecally increased numbers of anterior horn motor neurons associated with restoration of autophagy and improved functional recovery as determined by the Basso Beattie Bresnahan scoring system (159). Upon intravitreal administration, neuroserpin protected against retinal ischemia-reperfusion injury induced by elevated intraocular pressure associated with attenuation of apoptosis (160). In other studies examining neuroserpin as an immune modulating therapeutic, Munuswamy-Ramanujam et al. reported that intravenous administration of recombinant neuroserpin prevented vasculopathy in a mouse aortic allograft transplant model. In this model, neuroserpin reduced plaque growth and T-cell invasion and T helper cell responses (161). In contrast, neuroserpin was ineffective when evaluated as a treatment for severe gammaherpesviral (MHV68) infection and associated vasculitis in interferon gamma receptor-deficient ($IFN\gamma R^{-/-}$) mice (162). The highly specific endogenous sequestration of neuroserpin to the central nervous system may provide certain advantages to its therapeutic administration as it may not be subjected to the same degree of negative regulation in extra-neural tissues. While currently under-explored, studies investigating neuroserpin efficacy in other serpin-sensitive therapeutic scenarios are warranted.

# ALPHA-1-ANTITRYPSIN: A PROMISCUOUS SERPIN WITH POTENT THERAPEUTIC PROPERTIES

Alpha-1-antitrypsin (A1AT or AAT, SERPINA1) is the prototypical and best studied member of the serpin superfamily (163). While neutrophil elastase is the prominent and most characterized target of A1AT, leading to a reduction in neutrophilic inflammation (164), early investigations of A1AT activity identified a broad serine protease reactivity with cathepsins, caspases, metalloproteases, and coagulation cascade-associated serine proteases thrombin and plasmin (165). Interestingly, Talens et al. reported that A1AT is the most abundant non-covalently bound protein in fibrin clots and remains functionally active as a serpin *in situ* (166). The exclusion of A1AT from classical descriptions of fibrinolytic regulation may thus be an oversight, due to a focus on current understanding of therapeutic benefit in lung disease and underrepresenting the local control of plasmin activity by A1AT directly in the fibrin clot.

A1AT deficiency is a potentially severe, chronic condition characterized by unregulated inflammation primarily in the lungs, leading to COPD and emphysema, and in the liver leading to cirrhosis. Given A1AT potency as an inhibitor of serine proteinases, A1AT deficiencies may also be associated with other under recognized complications, such as during post-surgical healing (167, 168). A1AT recombinant protein therapy (augmentation therapy) is clinically eficacious, thereby prompting A1AT gene therapy, systemically administered via viral vectors, to advance into clinical trials and yield promising results (169).

Beyond treatment of serpin genetic deficiencies, A1AT is a broad and potent immune modulator. In early work, Libert et al. demonstrated a protective role for A1AT, which they first identified as an acute phase reactant, in lethal TNF insult in mice by a mechanism dependent on reducing platelet-activating factor and associated with reversals of body temperature drop, liver injury and increased clotting time when given recombinantly by intraperitoneal or intravenous administration (170). Later, the therapeutic effect of A1AT was demonstrated by Churg et al. in a model of cigarette smoke-induced emphysema in mice, which the authors suggested may be related to inhibition of both matrix metalloproteinases as well as TNF signaling (171). The protective effect of A1AT in the lungs may underscore the distinct physiological role observed with genetic deficiency. Similarly, Wang et al. found that A1AT treatment limited pulmonary apoptosis and necrosis in a rat model of ventilator-associated acute respiratory distress syndrome (ARDS) (172). Akbar et al. found that gene therapy with A1AT delivered by adeno-associated virus-8 (rAAV8) ameliorated bone loss in an ovariectomy-induced osteoporosis mouse model of post-menopause osteoporosis which was associated with inhibition of IL-6 and RANK levels (173).

A1AT has demonstrated repeated therapeutic efficacy in various models of cellular transplantation. Lewis et al. reported pancreatic islet transplantation survival was extended by

treatment with recombinant clinical-grade human A1AT, associated with a reduction of inflammatory cell infiltration and abrogation of TNF signaling (174). This effect was extended to preservation of islet cell viability in streptozotocin-treated mice. In a related study, Zhang et al. described that A1AT-dependent protection of islet viability after cytokine- and streptozotocin-induced diabetes in mice was in part due to dramatic reduction of beta cell apoptosis (175). Recently, the protective effect of A1AT in islet cell transplantation was demonstrated by Gou et al. in an intrahepatic transplant model in NOD-SCID mice by suppressing macrophage activation with reduced TNF, iNOS, IL-6, and CD11c signals (176). A1AT has been effective in preventing graft rejection in other cellular transplant models, as well. Marcondes et al. showed that A1AT prevented graft-vs.-host disease (GVHD) in an allogeneic murine transplantation model in both a preconditioning and post-conditioning treatment regimen (177). In a similar follow-up study, Tawara et al. confirmed the protective effect of A1AT in bone marrow transplant GVHD and described an associated reduction in TNF, IL1b, IL-6, and NF-kappaB signaling (178). Lee et al. showed that intravenous administration of A1AT reduced short-term engraftment of hepatocytes in rats which remained significant at 48 h (179). While significance was lost at longer timepoints, there was evidence of viable engrafted hepatocytes up to 1 month after transplant, which was not apparent in control mice. Recent work by Emtiazjoo et al. showed a remarkable reduction of acute lung allograft injury in an orthotopic single left lung transplantation model from Lewis to Sprague-Dawley rats at 8 days post-transplantation (180). Of crucial importance, protection was achieved in the absence of any systemic immunosuppression.

Addressing another complication of diabetes, Ortiz et al. reported that A1AT treatment alleviated the progression of diabetic retinopathy in mice by suppressing TNF signaling in both the serum and retina and promoting an M2-polarized macrophage population, which ultimately delayed ganglion cell loss and retinal thinning (181). In another study examining A1AT efficacy for ophthalmological disorders, Yang et al. demonstrated protection of iPSC grafts after subretinal transplantation into the eye of mice with preexisting ocular hypertension by inhibition of microglial activation (182). Interestingly, Zhou et al. reported that suppression of microglial inflammation and neurodegeneration in the eye was achieved by intraperitoneal injection of A1AT in a Rd1(FVB/N) mouse model of retinal degeneration (183).

Ischemia-reperfusion injury is characterized by a transient loss of blood and oxygen to a tissue, followed by a period of reoxygenation which paradoxically accelerates damage caused during the hypoxic period (184). Ischemia-reperfusion injury can occur in any tissue, whether by pathogenic etiology or by complications of surgical procedure, and there is an unmet need for novel therapeutics to address the condition (185–188). Moldthan et al. first demonstrated the therapeutic efficacy of A1AT therapy in a rat model of ischemic stroke which resulted in a drastic reduction of infarct volume and preservation of sensory motor system function (189). Toldo et al. generated a recombinant A1AT-Fc fusion protein and demonstrated efficacy in reducing inflammation following myocardial ischemia-reperfusion in mice which they found was independent of the capacity to inhibit elastase (190). In translation of this work, the VCU-a1RT clinical trial (NCT01936896) was undertaken to investigate the potential protective effect of A1AT therapy (Prolastin®) in patients with ST-segment elevation myocardial infarction (STEMI) (191). Abbate et al. reported that the VCU-a1RT trial found no in-hospital adverse effects of A1AT therapy and that a blunted initial inflammatory response resulted in significantly reduced CRP levels 14 days after admission. In further analysis of the trial, Abouzaki et al. also described a shorter time-to-peak in CK-MB levels indicating an inhibition of the onset of inflammatory injury (192). In investigations of other tissues, Maicas et al. found a limited therapeutic efficacy for clinical grade human A1AT (Prolastin®) in a mouse model of renal ischemia-reperfusion injury where they reported a significant decrease in kidney injury molecule-1 levels in urine but no effect on renal fibrosis (193). This finding contrasts later work by Jeong et al. who reported a significant protection against renal ischemia-reperfusion injury upon treatment with A1AT, including attenuated tubular injury and fibrosis (194). The design of these two contrasting studies are similar in the use of FDA-approved clinical grade A1AT at a dose of 80 mg/kg/day, however Jeong et al. administrated A1AT for 3 days prior to surgery and only followed up at 24 h post-procedure, whereas Maicas et al. first administrated A1AT at 24 h pre-procedure and followed up at 8 and 15 days post-procedure. Thus, the fibrotic phenotype likely developed over a longer time course than observed by Jeong et al.

Lupus is an autoimmune condition characterized by dysregulated adaptive and innate immune responses in tissues and the vasculature which can have damaging and potentially lethal effects on end organs such as the kidneys and lungs (195). Elshikha et al. investigated the protective effects of A1AT therapy in a series of preclinical studies. In the first of the series, they showed that A1AT inhibits plasmacytoid dendritic cell activation and protects against nephritis in the MRL/lpr spontaneous lupus model (196). They went on to describe that gene therapy with A1AT delivered by rAAV8 prolongs lifespan in NZM2410 mice which develop spontaneous lupus with early-onset glomerulonephritis and that the protection was associated with reduced autoantibody levels (197). Recently, Elshikha et al. described that treatment with recombinant A1AT limited disease progression and suppressed TNF signaling in a pristane-induced model of acute lupus diffuse alveolar lung hemorrhage (DAH) (198). Thus, the broad immune modulating effects of A1AT highlight the significant therapeutic potential of this serpin with broad activity against a wide range of serine proteases in diverse diseases driven by dysregulated inflammatory processes.

## SERP-1: A VIRUS-DERIVED COAGULATION REGULATOR AND THERAPEUTIC IMMUNE MODULATOR

Viruses, especially DNA viruses with large genomes such as poxviruses and herpesviruses, have expertly evolved highly effective and potent immune modulating protein machinery that

evade host immune defenses (199). These proteins have become the focus of a growing field of research around the development of virus-derived therapeutics (200), some of which have led to increased interest in mammalian serpin therapeutics. The most thoroughly investigated virus-derived therapeutic protein is Serp-1, a serpin from Myxoma virus which targets the fibrinolytic serine proteases uPA, tPA, and plasmin as well as the thrombotic proteases FXa and thrombin (in the presence of heparin) (201, 202). The first demonstration of Serp-1 therapeutic efficacy was in a rabbit model of aortic balloon angioplasty injury, where protection was characterized by significantly reduced inflammation and plaque growth (203). The doses used in that original study were single intravenous injections in the *picogram* range given immediately after angioplasty injury. This therapeutic effect against inflammation and vasculopathy with plaque growth was further demonstrated in models of aortic, renal and heterotopic heart transplants in mice and rats and in a mouse carotid compression model (204–207). Serp-1 was also demonstrated to be protective in a collagen-induced arthritis model in rats, with generalized immune modulation outside of transplant and pro-atherogenic disease states (208). In more recent work, Serp-1 was found to be an effective therapeutic against severe vasculitis in both human temporal artery biopsy transplants from patients suspected to have Giant cell arteritis into SCID mice and in the lethal MHV68 gammaherpesvirus-induced vasculitis in interferon gamma receptor-deficient mice (162, 209). Interestingly, peptides derived from Serp-1 are also therapeutically effective in the gammaherpesvirus-induced vasculitis model and protection imparted by both the full protein and the peptide derivatives are dependent on composition of the gut microbiome (202, 210, 211). *In vitro* studies demonstrated that, these reactive center loop (RCL) peptides bound and inhibited mammalian serpins (202). While these studies all used an intraperitoneal or intravenous delivery of naked recombinant protein, Serp-1 is also amenable to drug delivery vehicles and is currently the only serpin demonstrated for delivery by such approaches. Serp-1 is capable of sustained delivery in a chitosan-collagen biocompatible hydrogel and has demonstrated therapeutic efficacy in models of full-thickness cutaneous wound healing in mice and in spinal cord injury in rats, with efficacy dependent on engagement of uPAR in the fibrinolytic signaling pathway (212–214).

Serp-1 is a *First-in-Class* therapeutic and the first virus-derived protein given to humans in an FDA-overseen Phase IIa clinical trial (NCT00243308) for patients with unstable coronary syndromes, unstable angina and small heart attacks (215). Serp-1 was safe and well-tolerated with a major adverse cardiac event score (MACE) of zero and with a dose-dependent reduction in heart damage markers Troponin and CK-MB. Importantly, there were no detectable neutralizing antibodies against Serp-1. Thus, Serp-1 represents a cross-pathway serpin targeting thrombolytic and thrombotic cascades which regulates inflammatory responses in part by engaging signaling in the fibrinolytic pathway with potent therapeutic efficacy in a wide variety of disease states.

## FIBRINOLYSIS AND SERPINS IN SARS-COV-2 INFECTION AND THE COVID-19 PANDEMIC

Understanding the bidirectional activation of the coagulation proteases and activation of immune responses indicates a clear target for serpin therapeutics in severe infections where both coagulopathy as well as excessive and damaging immune response cause increased damage and mortality. Serpins have been examined in preclinical models of severe viral infections with coagulopathy, one notable example being the use of Serp-1 treatment in the severe vasculitis/lung hemorrhage model in IFN$\gamma$R$^{-/-}$ mouse models. In these models, as noted, Serp-1 improves survival and reduces both lung consolidation as well as vascular inflammation. Similarly, PAI-1 has proven beneficial in models of severe Klebsiella pneumoniae. Antithrombin III (ATIII), a serpin inhibiting the clotting pathway and activated by heparin infusions, also has had variable benefit in clinical trials of bacterial sepsis in man.

In December 2019, a pneumonia of unknown origin was identified in Wuhan, the capital of Hubei province in China and identified as a Severe acute respiratory distress syndrome (SARS) coronavirus denoted as SARS-CoV-2 (216). The disease caused by SARS-CoV-2, is referred to as coronavirus disease 2019 (COVID-19) and is now a worldwide pandemic as declared by the World Health Organization (217). As of March 10th, 2021, >118 million cases of COVID-19 has been reported globally with a death toll >2.6 million worldwide (Worldmeters.info).

Symptomatically, COVID-19 commonly causes severe coughing and hemoptysis, shortness of breath and hypoxemia accompanied by widespread lung infiltrates, consolidation and in some cases hemorrhage, with fever, weakness and confusion. SARS-CoV-2 is therefore identified, along with SARS-CoV-1, as a severe acute respiratory disease (218). However, despite the commonality of ARDS in COVID-19 patients, mounting evidence suggests that infection with SARS-CoV-2 induces a hypercoagulable state (219). Spiezia et al. reported that severe hypercoagulability, but not a clearly defined consumptive coagulopathy (or disseminated intravascular coagulopathy, DIC), is present in COVID-19 patients with acute respiratory failure (220). Endothelial injury and dysfunctional coagulation, with associated resistance against fibrinolysis, may thus reclassify COVID-19 as a vascular disorder complicated by widespread microthrombotic occlusions rather than a respiratory disease (221). We direct readers to a thorough description of this hypothesis in a recent review by Siddiqi, Libby and Ridker (222).

The apparent dysfunction of fibrinolysis during COVID-19 infection would suggest that therapeutic administration of serpins of the fibrinolytic pathway against SARS-CoV-2 may not be beneficial. However, many factors are worth consideration in the use of serpins in severe viral septic states with imbalance in coagulation as well as excess aggressive immune responses in accompanying cytokine storm. First, on a molecular level, SARS-CoV-2, like other coronaviruses, requires proteolytic processing of its Spike (S) protein in order to appropriately dock with and enter host cells (223, 224). It is now known

that the SARS-CoV-2 S protein is processed sequentially by the subtilisin-like peptidase furin and the transmembrane serine protease TMPRSS2 (225). Indeed, mechanistic studies have shown that a clinically approved TMPRSS2 inhibitor, camostat mesylate, inhibited SARS-CoV-2 S-driven infection *in vitro* (226). A recent retrospective observational case series on a small cohort of ICU patients in Germany found reduced severity in patients treated with camostat mesylate vs. those who received hydroxychloroquine (227).

Several fibrinolysis pathway serpins may interfere with these mechanisms (**Figure 4**). A preprint by Azouz et al., first deposited in May 2020, demonstrated that A1AT inhibits TMPRSS2 in an HEK-293T overexpression system (228). In an important follow-up study deposited in July 2020, where Wettstein et al. produced SARS-CoV-2 S-protein pseudoparticles and performed *in vitro* infection of Caco2 cells in the presence of chromatographically fractioned bronchoalveolar lavage samples they found that the highest inhibition of infection occurred in the fraction containing A1AT (229). These findings agree with prior reports of the ability for A1AT to inhibit infectivity of TMPRSS2-dependent viruses. Beard et al. reported that A1AT inhibits *in vitro* and *in vivo* mouse infection by H1N1 Influenza, which requires hemagglutinin processing via TMPRSS2 (230). Similarly, Esumi et al. reported that hepatitis C virus infection proceeds by the activity of TMPRSS2, which was dose-dependently inhibited *in vitro* by A1AT (231).

Second, on a coagulation systemic level, serpins target areas with activated serine proteases. Thus serpins are predicted to target areas with active thrombosis and or thrombolysis. Dysregulated thrombosis and thrombolysis are now clearly an important component of COVID-19 disease (232). What is not clear is how or why these pathways become dysregulated, and some have proposed that increased amounts of active PAI-1 may induce a feed-forward loop of inflammatory events (233). This idea has led to the recent initiation of a clinical trial to test the PAI-1 inhibitor, TM5614, for treating high-risk patients hospitalized with severe COVID-19 (NCT04634799). Similarly, tranexamic acid, a uPA inhibitor, is under investigation to combat COVID-19 (NCT04338074, NCT04338126). In further support of this, several groups have suggested modulation of the fibrinolytic pathway by targeting plasmin/plasminogen based on imbalances in protease levels in COVID-19 patients (234, 235). However, sensitivity in the pathway imbalance urges caution in administration, and the dynamics of the disease may dictate appropriate timing for intervention (236, 237).

While it is firmly established that coagulopathy occurs in COVID-19 patients, there is a possibility that PAI-1 may in fact be a protective host factor against SARS-CoV-2. Dittmann et al. reported that PAI-1 can dose-dependently inhibit Influenza A infection by preventing hemagglutinin processing by TMPRSS2 (238). Thus, the ability for PAI-1 to inhibit TMPRSS2 suggests that the problem of dysregulation of fibrinolysis may be more complex than focusing on PAI-1 may solve. Colling and Kanthi propose that the ratios of active PAI-1 and tPA may be more indicative of the pathway activity in COVID-19 patients due to ongoing consumption and microvascular thromboses (239). In support of this hypothesis, a recent preprint by Zuo et al. on 118 hospitalized COVID-19 patients and 30 healthy controls found that patients who died had significantly higher levels of both PAI-1 and tPA (240). Importantly, the authors found that a higher ratio of tPA vs. PAI-1 was indicative of potential mortality, and was driven by an increase in tPA, not PAI-1.

Furin is a second protease involved in S-protein priming during SARS-CoV-2 infection, and also has a role in intracellular processing (241). Furin is present in both membrane-bound and secreted, soluble states with the latter usually associated with a variety of pathologies, such as diabetes or infection (242–244). Cheng et al. recently reported that small molecule inhibitors of furin prevent SARS-CoV-2 infection as well as intracellular processing *in vitro* (245). However, the significant role of furin in normal tissue development and homeostasis make it a difficult target for therapeutic modulation and there are no FDA-approved furin inhibitors for clinical use. On the other hand, there is precedent for experimentally targeting furin with serpins to limit viral infection. For example, Shapiro et al. reported A1AT-mediated inhibition of HIV infection *in vitro*, which is dependent on furin-mediated processing of the membrane protein gp160 (246, 247). Numerous groups have engineered A1AT to fine-tune its properties, such as the A1AT Portland variant with increased specific and activity against furin described by Jean et al. to have anti-pathogenic properties (248). Similarly, Anderson et al. reported another A1AT variant (α1-PDX) with 3,000-fold higher anti-furin activity which potently inhibited HIV gp160-dependent infection *in vitro* (249). Furin is also inhibited by PAI-1 (intracellular furin) and endothelial PN-1 (extracellular furin), but their ability to limit infection via furin inhibition-dependent mechanisms remains to be explored (250, 251).

Third, the now understood evolution of serine proteases and serpins as regulators of both thrombosis and thrombolysis, and also of inflammation, would suggest a potential for the use of serpins that target both coagulation as well as immune responses in severe viral infections. The immune system response to SARS-CoV-2 reveals a different perspective for COVID-19 and fibrinolysis. Numerous inflammatory pathologies are associated with increased circulating levels of soluble uPAR (suPAR), produced by the cleavage of the C-terminal glycosyl-phosphatidylinositol linker by phospholipases (252). Growing evidence has established suPAR as a useful diagnostic and prognostic indicator of severe, acute pathologies including sepsis (253). Based on similarities of COVID-19 complications with diseases associated with or exacerbated by elevated suPAR, D'Alonzo et al. proposed suPAR as a therapeutic target for treating SARS-CoV-2 infection (254). In support of this proposition, early in the COVID-19 pandemic Rovina et al. identified elevated suPAR in 57 Greek patients and 15 American patients as a highly significant early prognostic indicator of severe outcomes in SARS-CoV-2 infection (255). More recently, Azam et al. investigated the association of acute kidney injury (AKI) in COVID-19 patients with suPAR (256). AKI occurs in up to 50% of severe COVID-19 patients and significantly increases morbidity and mortality. Azam et al. found that the highest tertile of suPAR levels was associated with a more than nine-fold increase in AKI in COVID-19 patients and

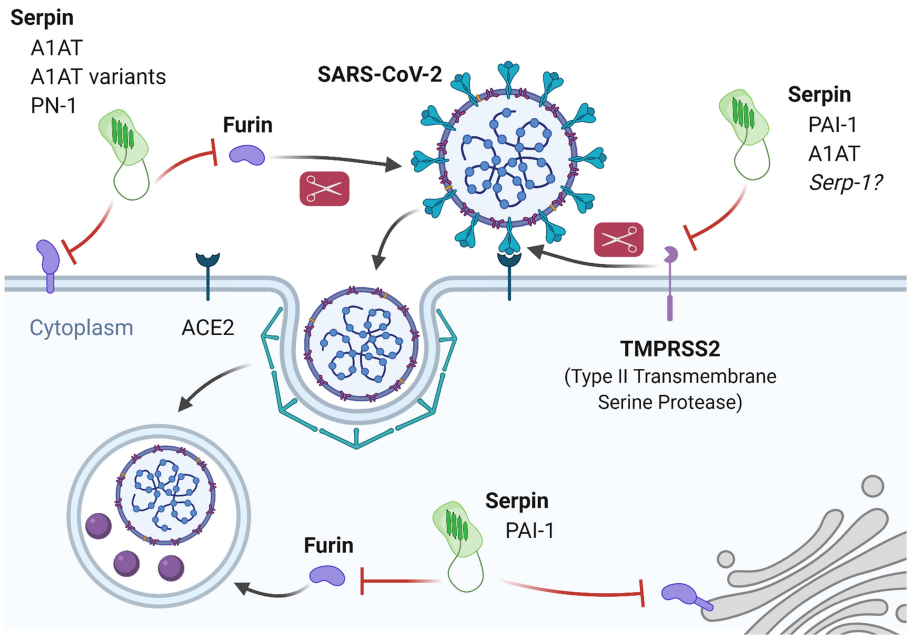


**FIGURE 4 |** Inhibition of SARS-CoV-2 infection and processing by fibrinolysis pathway-associated serpins. SARS-CoV-2 entry is dependent on proteolytic processing of the spike protein in order to engage the ACE2 receptor and internalize. Processing of the spike protein is performed primarily by TMPRSS2 and Furin. PAI-1 and A1AT are known inhibitors of TMPRSS2 and experimental evidence demonstrates that A1AT can inhibit SARS-CoV-2 infection *in vitro*. Based on similarities in inhibition properties, it may be predicted that Serp-1 will also inhibit TMPRSS2 with similar results. Furin has both an extracellular and intracellular role in the SARS-CoV-2 life cycle and there is evidence for extracellular furin inhibition by A1AT and A1AT variants (Portland and α-PDX) as well as endothelial PN-1, and for intracellular furin inhibition by PAI-1.

was independent of inflammatory markers or demographic subgroups. The uPAR system has other roles in SARS-CoV-2 infection in addition to suPAR elevation. Ly6E is a member of the uPAR family of proteins and a lymphocyte marker associated with immunological regulation and also recently associated with host responses to viral infection (257). Zhao et al. demonstrated that Ly6E restricts the entry of human coronaviruses in an ectopic expression model using both the common HCoV-O43 as well as SARS-CoV-2 (258). Importantly, Pfaender et al. performed crucial *in vivo* experiments in wildtype and Ly6E-deficient mice that revealed a critical Ly6E-dependent host defense against coronaviruses, including MERS-CoV, SARS-CoV, and SARS-CoV-2 (259). Mechanistic studies performed by the authors demonstrate that Ly6E prevents coronavirus entry into host cells by preventing S-protein-mediated membrane fusion. Of interest, the virus derived serpin that we have studied extensively, Serp-1, and also PAI-1 bind and block the uPA/uPAR complex (212, 213). With Serp-1 this leads to marked anti-inflammatory function. Thus, the uPA/uPAR/suPAR system represents an attractive therapeutic target for modulation to treat or limit the severity of immune disorders as well as potentially specific treatment for COVID-19 disease progression.

The role of serpins and serine proteases of the fibrinolytic system in COVID-19 is complex and investigations on the potential therapeutic modulation of these processes with natural, virus-derived or engineered serpins, expanding the consideration of these proteins beyond only regulation of the fibrinolytic system, may be a valuable pursuit as many of these modulators are already found to be safe and effective, and in some cases FDA-approved.

## DISCUSSION

The ancient roots of clot formation and clot dissolution speaks to the necessary role these pathways play in protection of the host from excess thrombosis and thrombolysis to immune-based disorders. In simplest terms, protection against loss of blood (or hemolymph in the case of lower organisms) after traumatic injury is an essential component of survival. However, the greater understanding of the role of these pathways in host responses to infection point to a more complex role for the coagulation and fibrinolysis cascades.

Focusing on the fibrinolytic cascade, we have reviewed the diversity of serine protease and serpin control of this essential pathway. The complexity of the pathway also underscores how critical this pathway is to maintain a normal homeostatic balance: dysregulation may easily lead to disease and therefore redundant control has evolved to maintain homeostasis and preserve host viability. A striking consequence of the evolution of serpin regulators of fibrinolysis is their potency and general safety. As we have discussed, these factors have led to the investigation of fibrinolysis-associated serpins as therapeutics. However, despite the dense and growing body of work justifying the application of serpins as therapeutics there are few, if any, indications outside of augmentation therapy for A1AT deficiency and related lung

and liver diseases which have received FDA approval. There is a growing need for novel therapeutics for inflammation-related disorders and naturally-evolved or improved, engineered serpins may provide higher potency and lower off-target selectivity than small molecule inhibitors. We would suggest that more attention should be given to the investigation of serpins as new potential therapeutics. This is particularly urgent in the context of severe cases of ARDS and microthrombotic vascular complications in the COVID-19 Pandemic (and potentially other viral pandemics), where serpins may provide a uniquely multifunctional role in modulating the host immune response as well as the virus life cycle toward improved outcomes.

## AUTHOR CONTRIBUTIONS

JRY wrote the first draft of the manuscript and produced all figures. LZ, QG, SEH, and ARL revised the manuscript. All authors approved the final version of the manuscript.

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**Conflict of Interest:** JRY, LZ, and ARL are inventors on several patents and patent applications relating to the use of Myxoma virus Serp-1 as a therapeutic. ARL is a co-founder of a small spin-out biotechnology company, Serpass Biologics Inc. which is developing Serp-1 as a therapeutic.

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# Therapeutic SERPINs: Improving on Nature

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Serine proteases drive important physiological processes such as coagulation, fibrinolysis, inflammation and angiogenesis. These proteases are controlled by serine protease inhibitors (SERPINs) that neutralize their activity. Currently, over 1,500 SERPINs are known in nature, but only 37 SERPINs are found in humans. Thirty of these are functional protease inhibitors. The inhibitory potential of SERPINs is in perfect balance with the proteolytic activities of its targets to enable physiological protease activity. Hence, SERPIN deficiency (either qualitative or quantitative) can lead to disease. Several SERPIN resupplementation strategies have been developed to treat SERPIN deficiencies, including concentrates derived from plasma and recombinant SERPINs. SERPINs usually inhibit multiple proteases, but only in their active state. Over the past decades, considerable insights have been acquired in the identification of SERPIN biological functions, their inhibitory mechanisms and specificity determinants. This paves the way for the development of therapeutic SERPINs. Through rational design, the inhibitory properties (selectivity and inhibitory potential) of SERPINs can be reformed and optimized. This review explores the current state of SERPIN engineering with a focus on reactive center loop modifications and backbone stabilization. We will discuss the lessons learned from these recombinant SERPINs and explore novel techniques and strategies that will be essential for the creation and application of the future generation of therapeutic SERPINs.

**Keywords: SERPIN, α1-antitrypsin, C1 esterase inhibitor, reactive center loop, therapy**

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## INTRODUCTION

Approximately one third of all proteases belong to the superfamily of serine proteases, which can be found throughout all kingdoms of life. In humans, ∼180 serine proteases govern essential physiological processes such as vascular hemostasis (1), inflammation (2), tissue remodeling (3) or angiogenesis (4). Many of these processes are regulated by chymotrypsin-like serine proteases, which are the most abundant class of serine proteases. These have a highly conserved proteolytic mechanism [reviewed in ((5))] and operate in "sequential activation" cascade mechanisms (e.g., coagulation or complement). The activity of serine proteases needs to be controlled, as excessive activity causes disease. This is where serine protease inhibitors (SERPINs) are of high importance.

### SERPINs in Human Physiology

The superfamily of SERPINs consists of ∼1,500 identified members (6). There is evidence for the existence of 37 human SERPINs at protein level. Thirty of these have proven inhibitory function, where they act as suicide substrate inhibitors. Loss of SERPIN function can have severe

pathological consequences. For example, patients with α1-antitrypsin (α1AT) deficiency develop pulmonary emphysema due to uncontrolled activity of neutrophil elastase (7). C1-esterase inhibitor (C1INH) deficiency leads to attacks of angioedema, due to excessive bradykinin formation by the plasma contact system (8), whereas patients with low levels of antithrombin (ATIII) have an increased risk of ischemic stroke, deep vein thrombosis or pulmonary embolism due to increased activity of the coagulation system (9). Currently, the majority of SERPIN therapeutics are meant as supplementation therapy to overcome these defects.

## SERPINs Mode of Action

SERPINS have a generally well-conserved secondary structure consisting of three β-sheets (A, B and C; highlighted in green in **Figure 1**) and nine α-helices (6, 10). Additionally, SERPINs contain an exposed reactive center loop (RCL; highlighted in red in **Figure 1**), which is a flexible loop structure on top of the SERPIN backbone. The RCL serves as a bait sequence for target proteases. The tertiary structure of native SERPINs is metastable, which can shift into a hyperstable conformation. This process is critical for the SERPIN function. While this shift can occur spontaneously, it becomes actively triggered when the RCL is cleaved by a protease (11). When the protease cleaves the RCL at the P1-P1'scissile bond, the serine (or a cysteine in case of cysteine proteases) of the catalytic triad of the protease attacks the carbonyl of the RCL, forming a tetrahedral intermediate (12). Hereafter, two situations may occur: (I) The SERPIN assumes its hyperstable state, pulling the protease to the opposite side of the SERPIN (13). Meanwhile, the N-terminal remainder of the RCL becomes inserted next to the five strands of β-sheet A, effectively making it the sixth strand (11, 14). During this process, the protease active site becomes distorted, and it can no longer hydrolyze the tetrahedral intermediate (15). When this occurs in the extracellular space, the SERPIN-protease complex will be cleared *via* scavenger receptors. II) The protease hydrolyzes the tetrahedral intermediate and releases it before active site disruption. The speed by which the C-terminal loop of the RCL is inserted into β-sheet A is critical and determines whether the SERPIN becomes a substrate or an inhibitor. Nonetheless, the SERPIN still folds into its hyperstable state (because its RCL has been successfully cleaved, which is irreversible) and will be rapidly cleared, while the regenerated protease remains active.

The ability of SERPINs to interact with multiple targets offers a unique opportunity for the therapeutic management of pathological enzyme systems. This review will explore the current state of SERPIN engineering, with a special focus on stabilizing SERPIN function and altering SERPIN specificity.

# SUPERCHARGING SERPINS

To supercharge SERPINs, co-factors can be administered to patients. Glycosaminoglycans can modulate the activity of several SERPINs by enhancing SERPIN functionality and therefore complex formation (16–21). For example, heparin (amongst others) enhances the efficiency of thrombin inhibition by endogenous antithrombin (from $7.2*10^3$ to $1.3*10^7$ $M^{-1} \cdot s^{-1}$ respectively) (22). More recently, polyphosphate was identified as a novel cofactor in the regulation of the complement system by enhancing the interaction between C1s and C1INH to an extent, similar to that of heparin (23). However, with the rise of recombinant protein technology, the doorway has been opened to change SERPINS for the treatment of disease states beyond SERPIN deficiency and can be used to control new therapeutic target proteases.

## Stability

Ideally, therapeutics are stable and in the case of life-long diseases should have a long half-life time in the human circulation. SERPINs are dependent upon their ability to shift from a metastable to a hyperstable conformation for function. This shift becomes problematic if it occurs spontaneously (i.e., without cleavage of the RCL by a target protease). This results in an inert SERPIN with the propensity to polymerize into pathogenic Z and S variants. Such behavior results in intracellular SERPIN accumulation and leads to tissue damage in the form of liver cirrhosis in the case of α1AT deficiency (24). Also for C1INH, intracellular accumulation has been reported for some mutations (25). However, there is little evidence to suggest that this is accompanied by liver cirrhosis. The differences in expression levels between both SERPINs are a logical explanation for this unwishful clinical phenotype. In order to avoid SERPIN polymerization during drug development, efforts are ongoing to achieve stabilization of the SERPIN backbone without it losing its inhibitory potential.

### Lessons From Antitrypsin

Kwon et al. increased the thermostability of α1AT 13-fold by using a single mutation (F51C) without harming its inhibitory activity (26). Similarly, mutation F51L increased thermostability, but reduced the misfolding and polymerization of the pathogenic α1AT Z variant (27). Interestingly, the naturally-occurring (non-pathogenic) mutation F51S leads to α1AT retention in CHO-cells and reduces its stability (28). All the above mutations are at the same position: residue 51, demonstrating that subtle differences have a large impact on backbone stabilization (28). Further mutagenesis of the α1AT-F51L backbone identified six additional mutations (T59A, T68A. A70G, M374I, S381A) that improve α1AT stability without influencing inhibitory activity (29, 30). Other stabilizing mutations influenced the inhibitory capacity and are therefore less interesting for the development of therapeutic SERPINs (31).

### Lessons From PAI-1

Not all SERPINs have a similar half-life and stability. Compared to other SERPINs, plasminogen activator I (PAI-1) has a short stability half-life of 1–2 h at 37°C and is considered relatively unstable (32). The binding of vitronectin to PAI-1 increases its stability 2–3 fold, but this complex would be considered unfavorable for therapeutic purposes. A single mutation (I91L) increases PAI-1 stability by nine-fold (32), whereas the combination of four mutations (N150H, K154T, Q319L and M354I) was able to increases the half-life 72-fold to 145 h (33). A set of 10 mutations (T50A, Q56R, A61V, G70D,

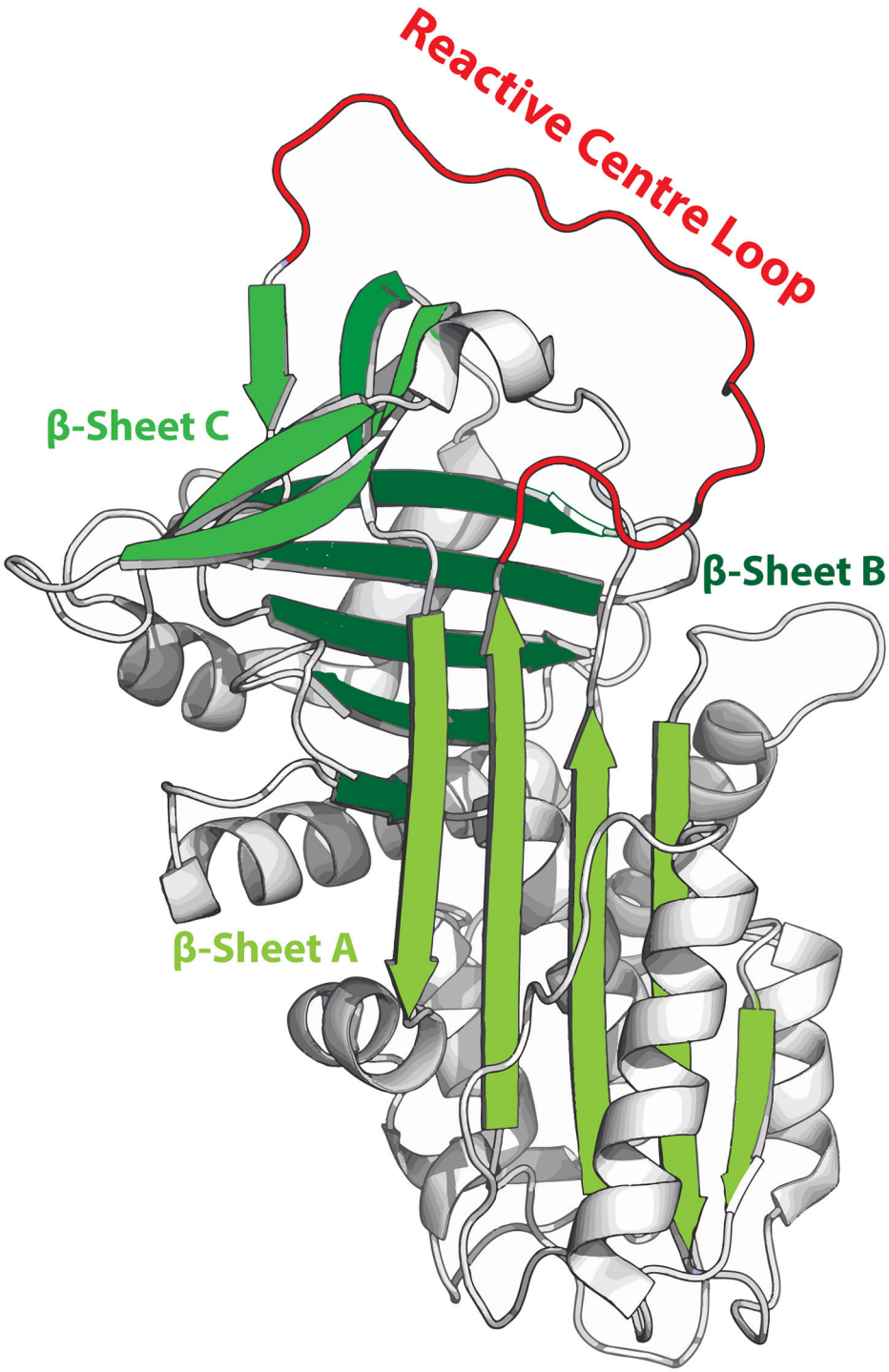


**FIGURE 1 |** Metastable structure of α1-antitrypsin. β-sheets are highlighted in green and the reactive center loop (RCL) is highlighted in red. When the RCL becomes cleaved it integrates into β-sheet A, effectively becoming the 6th strand of this sheet.

T94A, N150D, D222G, I223V, G264D, and S331G) increases the stability even further to 540 h (34). Remarkably, the introduction of additional disulfide bridge (Cys 197–Cys 355) in the original PAI-1 backbone increases the stability to 700 h (35).

## A Uniform Serpin Backbone

These results from work on α1AT and PAI-1 show the potential of engineering SERPIN stability, however these results cannot be directly extrapolated to other SERPIN molecules. To expedite

the development of therapeutic SERPINs, attempts have been undertaken to create uniform SERPIN backbones that have been optimized for stability. Hereto, the group of Porebski et al. aligned the sequence of various SERPINs to identify their consensus sequence (36). The resulting molecule "Conserpin," is stable up to temperatures of 110°C and shows resistance against polymerization. Furthermore, Conserpin is able to reversibly fold in response to chemical denaturation. Unfortunately, the inhibitory activity of Conserpin was found to be poor as it is unable to form stable covalent serpin-protease complexes (36, 37). While this behavior is improved by replacing nine amino acids of the Conserpin RCL (P7-P2') by that of α1AT, it still underperforms in comparison to wild type α1AT. As such, further insight in to SERPIN-protease interaction is required to allow therapeutic SERPINs to be optimized for stability without it affecting their efficacy. Although the RCL is very important, there are other motifs present in SERPINs that are important for target engagement such as exosites.

## Circulatory Half-Life

In humans, the circulatory half-life time between extracellular SERPINs differs quite significantly. For example, plasma-derived α1AT has a circulatory half-life of 4.5–8.7 days (38, 39). By comparison, its recombinant counterpart has a six-fold decrease in circulatory half-life (39, 40), which is thought to be the result of lacking, wrong or incomplete glycosylation. Although α1AT has been expressed in almost every host, the lack of proper glycosylation and circulatory half-life has been a major hurdle for any recombinant form of α1AT from reaching the market. Similar to α1AT, plasma-derived C1INH has a circulatory half-life of 22–56 h, but its recombinant variant (isolated from rabbit milk) only has a half-life of 2.4–3 h (41, 42). It is remarkable that prophylactic treatment with this molecule has therapeutic value (43), suggesting that it has biological properties unlike its natural counterpart which may facilitate alternative bio-distribution or cellular uptake which are beneficial to its therapeutic properties.

### Enhanced Glycosylation

These obstacles have motivated efforts to optimize recombinant SERPIN glycosylation. Introduction of an additional N-glycosylation site (introduced *via* the Q9N mutation) was able to further increase the circulatory half-life of α1AT in rats (44). Recently, a modified CHO cell line was presented that delivered full humanized N-glycosylation profiles for both α1AT and the C1INH (45). Here, ten genes were knocked out to prevent glycosylation errors by the CHO cell line. Furthermore, the α-2,6-sialyltransferase enzyme (ST6GAL1) was overexpressed to improve capping of the N-glycans with alpha-2,6-linked sialic acid. While these recombinant variants of α1AT and C1INH exactly match their plasma derived counterparts when it comes to N-glycosylation profiles, their circulatory half-life times remain to be investigated.

### Pegylation

To overcome the short circulation half-life of recombinant SERPINs, strategies have focused shielding the SERPIN *via* PEGylation (46–48). PEGylation of therapeutic proteins generally increases their biological stability and decreases their immunogenicity. Furthermore, PEGylation *via* a cysteine residue with an exposed thiol group (naturally present on certain SERPINs, including α1AT) is relatively straightforward and inexpensive. For recombinant α1AT, PEGylation did not influence its inhibitory potential *in vitro*, while pegylated α1AT variants (with 20 or 40 kDa PEG chains) showed increased circulation half-life, matching plasma-derived α1AT (48). Finally, in an *in vivo* elastase-mediated lung damage model, the PEGylated recombinant α1AT variants even outperformed its plasma derived counterpart.

### Fusion Proteins

As an alternative approach to increase the circulation half-life, SERPINs have been fused to the Fc domains from IgG. This results in a homo-dimeric protein that should increase both efficacy and extend circulatory half-life (49). Currently, a phase I trial with α1AT-FC fusion protein (INBRX-101) is ongoing (https://clinicaltrials.gov/ct2/show/NCT03815396).

### *In vivo* Expression

Trials with recombinant SERPINS are proven to be successful, patients would require weekly life-long therapy with injectables. As an attractive alternative, *in vivo* expression of SERPINs *via* gene therapy has been considered. For α1AT, both a phase I and II trial have been undertaken using a recombinant adeno-associated virus (AAV) vector which was administered to α1AT-deficient patients *via* intramuscular injection (50, 51). Patients tolerated the treatment and showed long term expression of α1AT. All subjects developed anti-AAV antibodies, but none developed antibodies against α1AT. While these studies confirmed the feasibility, patients only produced 20 μg/ml (0.38 μM) of α1AT in plasma serum, where therapeutic levels have to be at least 600 μg/ml (11.54 μM), where as normal levels are ∼1.5 mg/ml (28.85 μM). While improved delivery of the gene therapy and improved SERPIN expression might help to overcome this problem, increasing the inhibitory activity of α1AT through mutagenesis might help to lower the levels that are required for therapy.

# TAILORING SERPIN EFFICACY AND SPECIFICITY

The RCL together with exosites are the major regulators of SERPIN activity. Exosites can directly improve the SERPIN-protease interaction, whereas the RCL sequence determines the SERPIN specificity by controlling which proteases active sites can cleave it. Even a single amino acid mutation in the RCL can have functional consequences. For example, wild-type α1AT is a potent inhibitor of neutrophil elastase, trypsin, chymotrypsin, tissue kallikrein 7 & 14, cathepsin G, neutrophil proteinase 3 and pancreatic elastase (**Table 1**), but not of coagulation proteases. Contrastingly, the RCL P1 mutation M358R (α1AT-Pittsburgh) converts it into a potent inhibitor of thrombin, activated protein C (APC), plasmin, factor XIa, factor Xa, plasma kallikrein and factor XIIa (**Table 1**). As a net result, patients with α1AT-Pittsburgh suffer from a life-long bleeding disorder

**TABLE 1 |** The Pittsburgh (M358R) mutation dramatically alters α1-antitrypsin specificity.

| | α1-Antitrypsin inhibition kinetics ($k_2$: $M^{-1} \cdot s^{-1}$) | | | |
|---|---|---|---|---|
| | Wild Type | | Pittsburgh | |
| Neutrophil Elastase | $1.2\text{-}7*10^7$ | (52, 53) | | |
| Trypsin | $2.8*10^5$ | (53) | | |
| Chymotrypsin | $5.9*10^6$ | (54) | | |
| Tissue kallikrein 7 | $3.9*10^6$ | (55) | | |
| Tissue Kallikrein 14 | $2.6*10^5$ | (56) | | |
| Cathepsin G | $4.1*10^5$ | (57) | | |
| Neutrophil proteinase 3 | $9.24*10^5$ | (58) | | |
| Pancreatic elastase | $1.0*10^5$ | (57) | | |
| Thrombin | $4.8*10^1$ | (57) | $2.9\text{-}3.6*10^5$ | (59, 60) |
| Activated protein C | $1.1*10^1$ | (61) | $0.49\text{-}1.1*10^5$ | (59, 61) |
| Factor Xa | $2.26*10^2$ | (62) | $4.13*10^4$ | (59) |
| Factor XIa | $6.6*10^1$ | (63) | $4\text{-}5.1*10^5$ | (59, 63) |
| Plasmin | $1.9*10^2$ | (57) | $2.5*10^6$ | (64) |
| Plasma kallikrein | 4.2 | (63) | $6.9\text{-}8.9*10^4$ | (63, 65) |
| Factor XIIa | Not Detected | (63) | $2.5\text{-}3.5*10^4$ | (63, 65) |

*K2: second-order rate inhibition constant.*

(66, 67). This experiment of nature shows the impact of small RCL modifications.

Despite its pathological nature, α1AT-Pittsburgh has been investigated as treatment for coagulopathy and mortality in sepsis. While α1AT-Pittsburgh treatment decreased mortality and coagulopathy was reported in a piglet sepsis model (68), a baboon model was unable to confirm these results and even showed signs of increased coagulopathy (69). The overall consensus was that the inhibition of APC and plasmin in this setting were unfavorable.

## Redesigning RCL Specificity

Various groups have attempted to refine the specificity of α1AT-Pittsburgh. Initial redesign of SERPIN specificity started as an "exchange program" by grafting RCL sequences onto different SERPINs backbones. This led to some success (70, 71); but was limited by the inhibitory behavior of the initial donor sequences. Although APC inhibition is considered unfavorable in the treatment of sepsis-related coagulopathy, Polderdijk et al. recently demonstrated that a refined α1AT variant ($^{357}$KRK$^{359}$), which selectively inhibits APC, has therapeutic value for the treatment of hemophilia A- and B (59). This molecule is currently in clinical development (https://www.clinicaltrials.gov/ct2/show/NCT04073498).

To unlock the true potential of SERPIN engineering for diverse diseases, further mutagenesis of the RCL is warranted. Yet, with each position that is mutagenized, the amount of total possibilities rises exponentially. Indeed, to fully mutagenize a sequence of eight amino acids (stretching the P4-P4' region) and use all 20 naturally occurring amino acids for, leads to a total of $2.56*10^{10}$ RCL sequences variants. Specific RCL positions have been thoroughly researched, which provides valuable information. For example, Schapira et al. showed that a single mutation helps to refine the inhibitory potential of α1AT-Pittsburgh (60). By altering the P2 position from a proline to an alanine (P357A) in α1AT-Pittsburgh, the inhibition of thrombin was diminished to the extent that it had no effects on the *ex vivo* thrombin time in plasma of Wistar rats. Interestingly, this $^{357}$AR$^{358}$ mutation left the inhibition of FXIIa and PKa intact, protecting the rats in a model of bradykinin induced hypotensia. In 2002, Sulikowski et al. changed the RCL of α1AT-Pittsburgh into $^{356}$LGR$^{358}$ or $^{356}$PFR$^{358}$ to create a SERPIN to inhibit FXIIa, PKa and C1s (72). Where the $^{356}$LGR$^{358}$mutant inhibited its designated targets, it also potently inhibited APC. By comparison, $^{356}$PFR$^{358}$ showed an increase in specificity toward PKa. More recently, our group attempted to further improve the inhibition of the bradykinin producing proteases FXIIa and PKa. Based upon naturally occurring sequences and data from substrate peptide libraries, we created 18 α1AT variants. We found that only two new variants SMRT/V and SLLR/V (/ indicates RCL cleavage site) with a potent ability to inhibit FXIIa, PKa and FXIa, while showing negligible inhibition of thrombin, FXa and APC. These variants were effective in inflammatory models of carrageenan induced-paw swelling (driven by bradykinin) and dextran sulfate sodium-induced colitis as well as an injury-driven model of arterial thrombosis (73).

### Peptide Libraries

While data from synthetic substrate peptide libraries (74) can be used to guide selection of lead RCL sequences, we experienced that data from these libraries unfortunately poorly translates into the wanted inhibitory behavior of full-length SERPINS (73). This probably relates to the non-linear structure of the RCL loop. To overcome this obstacle, others have performed high throughput SERPIN screening studies with the T7 phage display system (75, 76). While this method allowed to find thrombin inhibitors that are twice more potent that a1AT-Pittsburgh, their specificity toward other proteases remains to be investigated.

## Viral SERPINs

Like humans, viruses also express SERPINs to inhibit targets in their respective hosts (77). Examples of viruses that express SERPINs are the Orthopoxviruses, Myxoma viruses, Cowpox virus, Baculovirus and the Swinepox virus. These "cross-class" SERPINs enhance infection and suppress host inflammatory responses. Deletion of these SERPINs dramatically reduces the lethality rates, showing that these SERPINs act as virulence factors (78, 79). The Myxovirus expresses the SERPIN Serp-1, which inhibits urokinase plasminogen activator (uPA), tissue plasminogen activator, factor Xa, plasmin, and thrombin (in the presence of heparin). Serp-1 requires the uPA receptor to function *in vivo* (80–82). Interestingly, Serp-1 effectively suppresses arterial inflammation and plaque growth (83–85). In addition, a peptide mimicking the Serp-1 RCL showed therapeutic benefits in a MHV68 virus-induced vasculitis mouse model (86). However, the activity and stability of this peptide was different from full-length Serp-1. Protein modeling studies were performed to improve the inhibitory (and antiplaque) activity of Serp-1-based peptides. The resulting peptides indeed displayed increased inhibitory activity and were able to increase the survival rate of the mice in a

MHV68 infection model of IFNγR KO mice (87). This work demonstrates the power of protein-modeling and shows its value for SERPIN design.

## DISCUSSION

Over the past years, new molecular insights have been rapidly acquired that help recombinant SERPINs to fulfill their therapeutic promise. While the first recombinant SERPIN variants (the α1AT-FC fusion protein and the APC inhibiting α1AT variant) are moving into clinical development, the design of new SERPIN variants is still a very specialized and labor-intensive exercise. Improvements in molecular cloning strategies combined with protein modeling approaches will be of great importance to efficiently unlock the potential of SERPINs as therapeutic agents.

## AUTHOR CONTRIBUTIONS

CM and SM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Protease Nexin-1 in the Cardiovascular System: Wherefore Art Thou?

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The balance between proteases and protease inhibitors plays a critical role in tissue remodeling during cardiovascular diseases. Different serine protease inhibitors termed serpins, which are expressed in the cardiovascular system, can exert a fine-tuned regulation of protease activities. Among them, protease nexin-1 (PN-1, encoded by *SERPINE2*) is a very powerful thrombin inhibitor and can also inactivate plasminogen activators and plasmin. Studies have shown that this serpin is expressed by all cell subpopulations in the vascular wall and by circulating cells but is barely detectable in plasma in the free form. PN-1 present in platelet granules and released upon activation has been shown to present strong antithrombotic and antifibrinolytic properties. PN-1 has a broad spectrum of action related to both hemostatic and blood vessel wall protease activities. Different studies showed that PN-1 is not only an important protector of vascular cells against protease activities but also a significant actor in the clearance of the complexes it forms with its targets. In this context, PN-1 overexpression has been observed in the pathophysiology of thoracic aortic aneurysms (TAA) and during the development of atherosclerosis in humans. Similarly, in the heart, PN-1 has been shown to be overexpressed in a mouse model of heart failure and to be involved in cardiac fibrosis. Overall, PN-1 appears to serve as a "hand brake" for protease activities during cardiovascular remodeling. This review will thus highlight the role of PN-1 in the cardiovascular system and deliver a comprehensive assessment of its position among serpins.

**Keywords: PN-1, atherosclerosis, aneurysm, fibrosis, serpinE2, heart failure, smooth muscle cell**

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## INTRODUCTION

Protease Nexin-1 (PN-1) is a 50-kDa glycoprotein encoded by the *SERPINE2* gene on human chromosome 2 (1). Phylogenetically, it is the closest relative to plasminogen activator inhibitor type-1 (PAI-1 or serpinE1) (2). The serpins comprise a superfamily of proteins that share a conserved tertiary structure. Serpins include inhibitors of serine and papain-like cysteine proteases and non-inhibitory members with other biological functions. PN-1 is a cellular serpin found within diverse organs, such as brain, male and female reproductive systems, kidneys and lungs. PN-1 is also largely expressed in the vessels and the heart (3). This review thus aims to focus on its role in the pathophysiological responses of the cardiovascular system.

## BIOCHEMICAL PROPERTIES OF PN-1

PN-1 inhibits a broad range of serine proteases explaining its physiological role in various processes ranging from coagulation and fibrinolysis to tissue remodeling and inflammation. *In vitro* kinetic assays showed that PN-1 reacts rapidly with trypsin and thrombin, with an association rate constant (Ka) of $\sim 2.10^6$ $M^{-1}.s^{-1}$ (4). The Ka values for the other target proteases including urokinase plasminogen activator (uPA) (4, 5), plasmin and factor XIa (6) are at least 10-fold lower, and 400-fold lower for tissue plasminogen activator (tPA) (4, 5) and activated protein C (7). PN-1 has also been shown to inhibit Factor VII-activating protease (FSAP) (8). As for many other serpins, PN-1 has a high affinity for heparin or heparan sulfate proteoglycans, which targets it to the pericellular space and strongly increases its ability to inhibit thrombin (9–11), thereby making this latter its preferred target. Indeed, unfractionated heparin is responsible for up to a 1,000-fold increase of the Ka value for thrombin, but only a ∼10-fold increase for most other proteases and has no impact on the Ka value for plasmin. The crystal structure of the complex between thrombin, PN-1 and heparin demonstrated that heparin acts as a bridge between the serpin and the protease, leading to a ternary complex and enhancing the rate of complex formation (12). The protease-binding site (named the reactive center loop) of PN-1 is situated at the carboxy-terminal end of protein. The reactive site (P1–P′1) represented by the Arg346–Ser347 bond is cleaved by the target serine protease, which results in the formation of a covalent SDS- and heat-stable enzyme–PN-1 complex where both the protease and PN-1 become inactivated (10).

## PN-1 IN THE VASCULAR SYSTEM

PN-1 does not circulate in plasma, but is present in blood cells, including platelets (13, 14) and monocytes (14). Active PN-1 is released from platelet α-granules during their activation. Platelet PN-1 displays anti-thrombotic properties *via* its ability to block thrombin generation and activity (15). This was illustrated by *in vivo* studies showing an important acceleration of the induction of thrombus formation after vascular injury in PN-1-deficient mice compared to wild-type mice (15). Platelet PN-1 also displays anti-fibrinolytic properties thanks to its ability to block plasmin generation and activity (16), as illustrated *in vivo* with PN-1-deficient mice that display accelerated and enhanced thrombolysis following treatment with tPA (16). Thus, both PAI-1 and PN-1 may play complementary roles in maintaining the fibrin clot, and therefore largely participate in the resistance of platelet-rich clots to thrombolysis.

The first report of the presence of PN-1 in the vasculature consisted of immunohistochemical studies demonstrating an abundance of PN-1 around cerebral blood vessels (17). Later, PN-1 expression was evidenced in the vascular wall where it is expressed by endothelial cells (18, 19), vascular smooth muscle cells (vSMCs) (20) and fibroblasts (21, 22). Importantly, it is retained at the cell surface of vascular cells and within the extracellular matrix (ECM) of the vessel wall due to its high affinity for heparin sulfate proteoglycans (22) and its ability to bind to the low-density lipoprotein receptor-related protein 1 (LRP1) of the scavenger receptor family (23, 24). PN-1 is expressed by endothelial cells and interacts with thrombomodulin, a high affinity thrombin ligand expressed on the endothelial cell membrane that plays an important role in the regulation of coagulation *via* the activation of the natural anticoagulant protein C. PN-1-thrombomodulin interaction favors the inhibition of fibrin formation and limits the generation of activated protein C and thrombin activatable fibrinolysis inhibitor (18). Endothelial PN-1 was also shown to protect the endothelial protein C receptor from endogenous shedding, thereby favoring the cytoprotective effects of activated protein C (25). Deficiency of PN-1 in mice does not generate a spontaneous vascular phenotype compromising their survival. However, endothelial PN-1 plays a role in physiological angiogenesis. Indeed, the retina from PN-1-deficient mice displayed increased vascularization with elevated capillary thickness and density, as well as an increased number of veins and arteries, compared to their wild-type littermates (26). Moreover, neovessel formation in Matrigel plug assays in PN-1-deficient mice, as well as the microvascular network sprouting from PN-1-deficient aortic rings, were both largely enhanced compared with their respective controls (27). These data clearly illustrate the important anti-angiogenic potential of vascular PN-1.

## PN-1 IN VASCULAR DISEASES

### PN-1 and Atherosclerosis

Atherosclerosis is a disease characterized by the thickening of the blood vessel wall due to the formation of plaques in the subendothelial intimal space. It involves endothelial cell dysfunction resulting in an alteration of endothelial permeability, allowing the penetration and accumulation of low-density lipoprotein (LDL) particles in the vessel wall where they are susceptible to oxidation. Monocytes are also implicated and transmigrate into the intima where they differentiate into macrophages, becoming foam cells after ingestion of oxidized LDL. VSMC proliferation and migration from the media to the intimal layer, as well as their phenotypic shift into foam cells, are also important features of atherosclerosis development. vSMCs present in the intimal layer form a fibrous cap that contains the plaque. The rupture of the fibrous cap leads to thrombus formation causing blockage of the blood flow (28).

An unbalanced ratio between proteases and their inhibitors is involved throughout the pathophysiology of atherosclerosis. Excessive thrombin, uPA/tPA or plasmin activities are indeed involved in the chronic evolution of the plaque. An important question thus concerns the regulation of these proteases in the vessel wall. In this context, serpins increasingly appear to be critical in regulating protease activity in arterial lesions. Among them, PN-1 has emerged as a key regulator in vascular biology even though its precise mechanism of action remains to be deciphered.

Immunohistochemical studies demonstrated the presence of PN-1 in the healthy vascular wall and particularly in vSMCs (20). PN-1 has also been shown to be associated with vSMCs in advanced carotid atherosclerotic lesions, but also

with macrophages and platelets (14, 29). Accumulation of PN-1 was detectable in very early lesions and was increased in complicated plaques: globally, PN-1 was present in the cap, in the necrotic core and in the mural thrombus (14, 30). In fact, the biological activity of PN-1 appears to be involved in the different stages of atherosclerotic plaque progression. During the early stage, PN-1 may be involved in endothelial dysfunction. Indeed, at the endothelial level, PN-1 has been shown to interact with thrombomodulin, a glycoprotein that transforms thrombin from a pro- to an anticoagulant protein (18). Thrombomodulin interaction with PN-1 accentuates the ability of the latter to inhibit thrombin. In advanced atherosclerotic plaques, PN-1 is largely expressed by platelets and inflammatory cells including monocytes/macrophages. In agreement with this observation, PN-1 has been shown to be up-regulated in lipopolysaccharide-activated monocytes and degraded in macrophages (14). Because monocytes/macrophages are exposed to an inflammatory environment in atherothrombotic lesions, PN-1 overexpression may represent a cell defense reaction against proteases present in the atherosclerotic plaque. Indeed, vSMCs synthesize and secrete tPA that is able to drive the conversion of plasminogen into plasmin at the cell surface, leading to matrix degradation, cell detachment, and death (31). However, PN-1 is also overexpressed by vSMCs in the advanced plaque where it is able to form covalent complexes with plasmin (30). Both endocytic LRP-1 and PN-1 are highly expressed in human atheroma, making PN-1 a crucial actor in plasmin internalization by vSMCs, *via* LRP-1 (30). PN-1 has also been shown to form covalent complexes with FSAP (8), a circulating protease found in human atherosclerotic plaques and supposed to play a regulatory role in their progression and vulnerability (32). The fibrous cap plays a crucial role in the development of atherosclerosis because its thickness is tightly related to the vulnerability of atherosclerotic plaques. PN-1 may also influence the thickness of the fibrous cap, by acting on the migration of vSMCs. Indeed, overexpression of PN-1 by vSMCs has been shown to significantly reduce their adhesion, spreading and migration on vitronectin, an adhesive protein found in atherosclerotic plaques (33). This effect is related to the high affinity of PN-1 to vitronectin, shown by direct-binding *in vitro* assays (11). Moreover, PN-1 can limit thrombin-induced vSMC proliferation (20) and (i) prevents the pro-apoptotic effect of high thrombin concentrations (34), (ii) inhibits plasminogen activation in the peri-cellular environment, and (iii) prevents plasmin-induced cell detachment (34). Taken together, these data raise the possibility that PN-1 overexpression during atherosclerosis could significantly influence the stability of the plaque. At the most complicated stage of atherosclerosis, rupture of the plaque can trigger localized, often occlusive, thrombus formation. PN-1 can thus also accumulate within thrombi generated during atherothrombosis since platelets are a reservoir of this serpin. Via its ability to inhibit plasmin generation and activity within the thrombus, platelet PN-1 is assumed to contribute to thrombus stabilization and is therefore also a non-negligible contributor to thrombus resistance to lysis (16).

Given its ubiquitous expression in the atheromatous lesions and its inhibitory activity against numerous deleterious proteases present in the atheroma, PN-1 can undoubtedly regulate the characteristics of the atherosclerotic plaque at different stages of development.

## PN-1 and Aneurysms

Aortic aneurysms are also diseases characterized by intense remodeling due to an imbalance in favor of proteolytic degradation of the vascular wall ECM, leading to progressive dilation and eventually to rupture. Despite various possible etiologies, all thoracic aneurysms of the ascending aorta (TAA) share common pathophysiological features leading to structural deterioration of the aortic wall. VSMCs apoptosis and the degradation of collagen and elastic fibers are the two principal modifications occurring within the medial layer characterizing TAA. The relevance of the antiprotease activity of PN-1 expressed by vSMCs has been emphasized by its ability to regulate *in vitro* pericellular plasminogen activation (35) and therefore cell resistance to proteolytic aggression, as observed during atherosclerosis. In human biopsies, PN-1 expression was found to be increased in the medial layer of TAA compared with the aortic medial layer from healthy donors and the protein colocalized with vSMCs. Interestingly, cultured vSMCs from TAA continued to display an increased level of PN-1 mRNA expression compared with control vSMCs (36). This was found to be due to the permanent epigenetic activation of the smad2 pathway *in vivo* in the arterial wall of TAA, an activation which persisted in cultures of vSMCs of TAA origin. Hence, human cultured vSMCs from TAA had a limited capacity to convert plasminogen into plasmin, and were therefore protected against apoptosis-induced detachment after plasminogen or plasmin treatment (36). Indeed, PN-1 overexpression was shown to be associated with aneurysmal dilatation, whereas the absence of PN-1 overexpression was associated with aortic dissections (36). Together, these data show that overproduction of PN-1 by vSMCs *in vivo* during TAA development may participate in the increased ability of the cells to resist the proteolytic environment.

The clearance of PN-1/plasmin complexes has also been addressed specifically in the TAA context. PN-1, LRP-1 and plasmin were shown to colocalize in the media of human TAA where PN-1 amounts correlated with plasmin activity (37). The uptake of PN-1/plasmin complexes was shown to be partly mediated by LRP-1 in vSMCs. These results strongly suggest that PN-1 might play a protective role *in vivo* during TAA development, as discussed for atherosclerosis, but further experimental animal models are required to fully understand its impact on TAA pathophysiology.

In contrast to TAA, the role of PN-1 in abdominal aortic aneurysms (AAA) has not yet been addressed. Previous reports have shown that the enzymes of the fibrinolytic system are also involved in AAA progression (38, 39) and local overexpression of PAI-1 in the mouse was accordingly reported to prevent the development of the disease (40). The role of the plasminergic system remains nevertheless incompletely understood (41) and the study of PN-1 in this context could provide new insights into

the understanding of how proteases and their counter-regulators participate in the evolution of AAA.

## PN-1 IN CARDIAC FIBROSIS

Myocardial fibrosis is an important pathophysiological process defined as an excessive accumulation of matrix proteins and is a well-established morbi-mortality marker. It increases myocardial stiffness, alters systolic function and contributes to malignant arrhythmias (42).

PN-1 in the heart has received less attention although it has been reported to be present in mouse heart (43). Moreover, in rats a high overexpression of PN-1 was described in *in vivo* heart failure models (44). Li et al. were the first to assess the role of PN-1 in cardiac fibrosis (45). They showed that both cardiomyocytes and myocardial fibroblasts express PN-1, even though the level of PN-1 expression in the former was only half that in the latter. They also found, in an *in vivo* mouse model of cardiac fibrosis induced by transverse aortic constriction (TAC), that collagen deposition was increased after 4 weeks, associated with a slight increase in PN-1 expression in the heart (45). Moreover, they showed that pro-fibrotic mediators like angiotensin II and transforming growth factor-β (TGF-β) could induce, in myocardial fibroblasts, an increased expression

**TABLE 1 |** Expected effect of PN-1 in cardiovascular diseases depending on its targets or partners.

| Pathology | PN-1 targets or partners | Expected effect | References |
|---|---|---|---|
| Thoracic and abdominal aortic aneurysms | Plasmin and LRP-1 | Protective | (36, 37) |
| Atherosclerosis | Plasmin and LRP-1 | Protective | (30) |
| Cardiac fibrosis | Thrombin | Protective | |
| | uPA, MMP, plasmin | Deleterious | (45) |

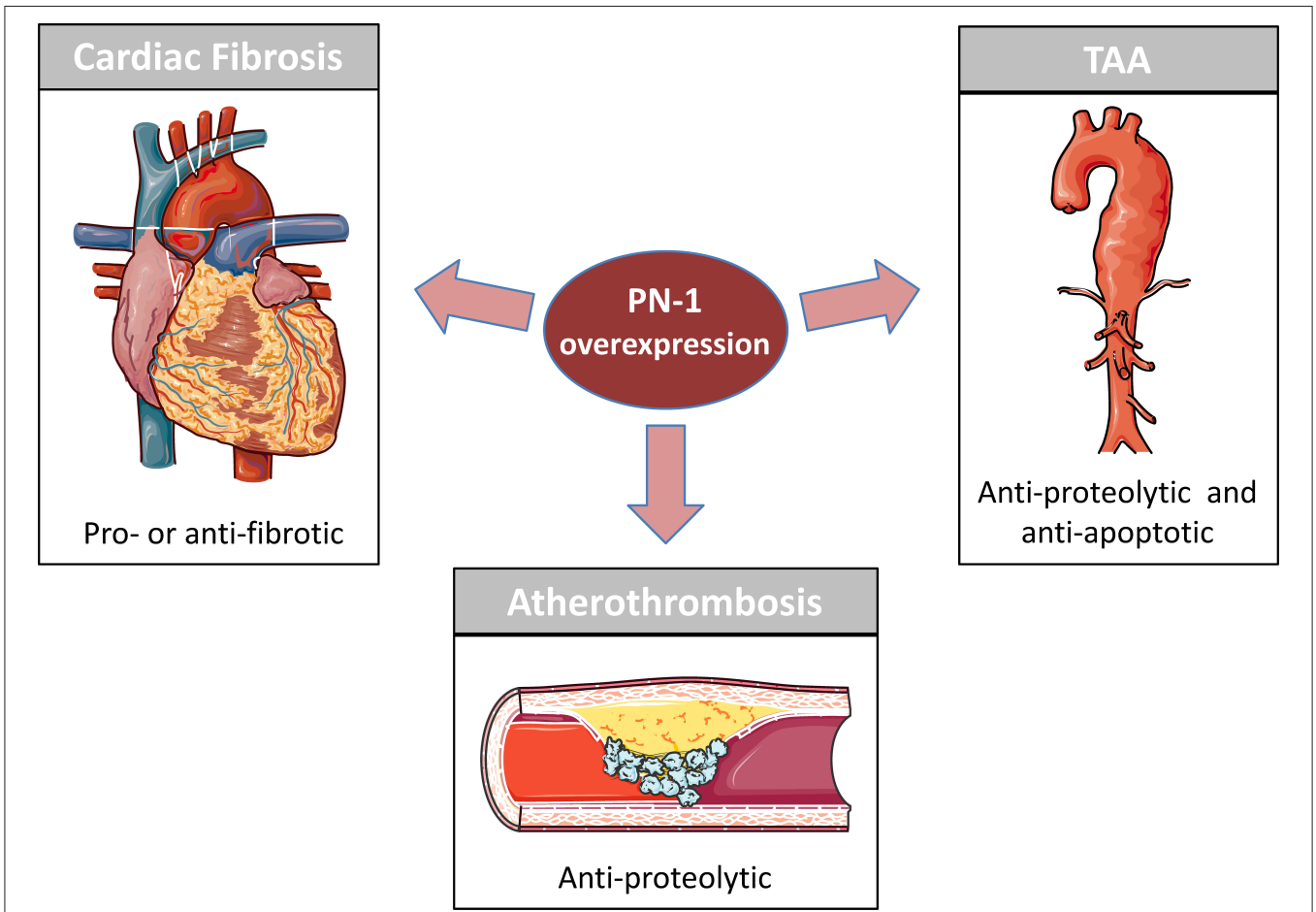


**FIGURE 1 |** PN-1 in cardiovascular disease. PN-1 is overexpressed in Atherosclerosis, Cardiac Fibrosis and Thoracic Aortic Aneurysm (TAA). Previous studies have shown PN-1 to be an important protective actor in atherosclerosis and TAA by reducing the impact of the proteolytic environment on the vascular cells. PN-1 is also involved in cardiac fibrosis but can be either anti-fibrotic and protective or pro-fibrotic and deleterious depending on its targets (see **Table 1**).

of collagen associated with PN-1 overexpression, at both the messenger and protein levels. Such an up-regulation of PN-1 induced by TGF-β has also been observed *in vitro* in human pulmonary fibroblasts (46). Reciprocally, the knockdown of PN-1 appears to partially attenuate cardiac fibrosis (45). However, cardiac expression of PN-1 is only partially impaired and these data do not allow us to draw clear conclusions as to the role of PN-1 in cardiac injury.

PN-1 appears to be importantly involved in fibrotic processes. Interestingly, depending on the affected tissue, PN-1 displays either anti-fibrotic properties as described in pulmonary fibrosis (47) or in contrast, pro-fibrotic properties as described here in cardiac fibrosis or as reported in scleroderma, a disease also characterized by ECM accumulation in skin and visceral tissue (48). The link between PN-1 and cardiac fibrosis can also be mediated, at least in part, by its antiprotease inhibitor activity, in particular by its ability to inhibit thrombin and uPA. Indeed, the direct inhibition of thrombin with dabigatran was shown to attenuate cardiac fibrosis and improve global cardiac function in a TAC murine model (49). The importance of the uPA/plasmin/matrix-metalloproteinase (MMP) system in collagen degradation has been well-characterized (50). PAI-1, a serpin close to PN-1, has also been shown to exert pro-or anti-fibrotic effects in different organs. The inhibition by PAI-1 of uPA- and tPA-mediated conversion of plasminogen to plasmin was shown to decrease plasmin-mediated MMP activation, and consequently to increase matrix accumulation and fibrosis in different tissues including lung, liver and kidney (51). In contrast, in the heart, PAI-1 protects mice from hypertension-induced cardiac fibrosis (52). Indeed, although PAI-1 is upregulated by TGF-β in numerous cell types (53), in the myocardium, PAI-1 was shown to inhibit TGF-β production specifically in cardiomyocytes (51).

More detailed studies are required to decipher the role of PN-1 in cardiac fibrosis. Indeed, in pathological conditions, such as pressure overload models or myocardial infarction, inflammation plays an important role in adaptative and inadaptive responses, where monocytes and macrophages are key components of the inflammatory pathophysiology (54). Because PN-1 is expressed by inflammatory cells and has been shown to be closely related to the inflammatory reaction in lung fibrosis, we can hypothesize that PN-1 can also participate in cardiac inflammation and consequently, in cardiac fibrosis.

## CONCLUSIONS

The close relationships between PN-1 and proteases of the coagulation and fibrinolytic systems, as well as between PN-1 and the endocytic receptor LRP1, explain the impact of this serpin in the cardiovascular system (**Table 1**). Essentially, PN-1 participates in maintaining the homeostatic function of the arterial wall and the cardiac tissue, as illustrated by its overexpression in the different cardiovascular pathologies mentioned in this review (**Figure 1**).

## AUTHOR CONTRIBUTIONS

CM and BR generated the figure and the table. VA and M-CB provided critical feedback and edited the review. All authors contributed to the review.

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# A Serpin With a Finger in Many PAIs: PAI-1's Central Function in Thromboinflammation and Cardiovascular Disease

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Plasminogen activator inhibitor 1 (PAI-1) is a member of the serine protease inhibitor (serpin) superfamily. PAI-1 is the principal inhibitor of the plasminogen activators, tissue plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA). Turbulence in the levels of PAI-1 tilts the balance of the hemostatic system resulting in bleeding or thrombotic complications. Not surprisingly, there is strong evidence that documents the role of PAI-1 in cardiovascular disease. The more recent uncovering of the coalition between the hemostatic and inflammatory pathways has exposed a distinct role for PAI-1. The storm of proinflammatory cytokines liberated during inflammation, including IL-6 and TNF-α, directly influence PAI-1 synthesis and increase circulating levels of this serpin. Consequently, elevated levels of PAI-1 are commonplace during infection and are frequently associated with a hypofibrinolytic state and thrombotic complications. Elevated PAI-1 levels are also a feature of metabolic syndrome, which is defined by a cluster of abnormalities including obesity, type 2 diabetes, hypertension, and elevated triglyceride. Metabolic syndrome is in itself defined as a proinflammatory state associated with elevated levels of cytokines. In addition, insulin has a direct impact on PAI-1 synthesis bridging these pathways. This review describes the key physiological functions of PAI-1 and how these become perturbed during disease processes. We focus on the direct relationship between PAI-1 and inflammation and the repercussion in terms of an ensuing hypofibrinolytic state and thromboembolic complications. Collectively, these observations strengthen the utility of PAI-1 as a viable drug target for the treatment of various diseases.

**Keywords: PAI-1, fibrinolysis, metabolic syndrome, obese, diabetes, thrombosis, inflammation**

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## INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is a fast-acting serpin that regulates the fibrinolytic system through inhibition of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). PAI-1 quenches the enzymatic activity of these proteases to constrain fibrin degradation and stabilize the hemostatic plug. Like other serpins, PAI-1 forms a 1:1 enzyme-inhibitor complex with its target proteases, rendering them enzymatically inactive and

resulting in rapid clearance from the circulation *via* the hepatic system. However, PAI-1 is an unusual serpin in that it can lose activity by spontaneous insertion of the reactive center loop into the body of the molecule, forming "latent" PAI-1 (1). The active form of PAI-1 is very unstable and has a short half-life of 1 h (2), whereas conversion to its thermodynamically stable latent form allows a prolonged half-life of 2–4 h (3). In healthy individuals' plasma, PAI-1 circulates in excess over its target protease tPA but at relatively low concentrations compared with other serpins, and is highly variable in normal individuals (1–40 ng/ml). PAI-1 is relatively unstable with a half-life of around 1–2 h in the circulation (4), however, binding to the extracellular matrix protein, vitronectin, stabilizes the active form of PAI-1 (5), and augments its half-life (6).

Circulating PAI-1 levels are under genetic control that is directly related to an insertion/deletion (5G/4G) polymorphism at position −675 of the promoter (7). The 4G allele gives rise to elevated plasma PAI-1 levels (8–11). PAI-1 displays circadian rhythm with a peak in early morning that coincides with the time of onset of myocardial infarction (MI) (12). The 4G/5G polymorphism differs according to ethnic group which has a direct impact on PAI-1 circulating levels (13). Interestingly, the levels of PAI-1 vary according to gender and show a positive correlation with increasing age (14).

Platelets contain the major pool of circulating PAI-1, which when activated following vessel injury, release this cargo thereby protecting the developing thrombus from premature fibrinolysis. Not surprisingly, the platelet precursor cell, megakaryocytes, are a major site of PAI-1 synthesis (15) and platelets themselves are now known to retain some PAI-1 mRNA which can produce functional protein (16). However, PAI-1 is also synthesized by other cells including endothelial (17), adipocytes (18–20), hepatocytes (21), and cardiomyocytes (22) (**Figure 1A**). Given the crucial role of PAI-1 in hemostasis, a deficiency in this serpin gives rise to a moderate bleeding diathesis (23). Conversely, increased levels of PAI-1 are associated with thrombotic complications. In addition to its hemostatic role, PAI-1 functions in several physiological processes such as inflammation, wound healing, and tumor progression. A strong relationship between PAI-1 and obesity, diabetes, and metabolic syndrome (MetS) was recognized many years ago with this serpin now being considered central to these pathophysiological processes (24). This review will focus on the impact and relationship of this unusual serpin in dictating and orchestrating the development of thromboinflammation and cardiovascular complications as a result of its participation in the pathogenesis of associated diseases.

## PAI-1 AND THROMBOSIS

The fate of a forming thrombus is determined by platelet deposition and the balance of coagulation and fibrinolytic factors. An increase in circulating levels of PAI-1 or augmented local release of this inhibitor due to platelet activation shifts the balance to a hypofibrinolytic state. PAI-1 has been recognized as a pivotal protein in the progression of vascular events and is linked to MI (25–27), stroke (28), deep vein thrombosis (DVT) (29), and microvascular thrombosis (30). Elevated levels of plasma PAI-1 precede the occurrence of MI (26), and survivors exhibit consistently high levels (25). Acute increases in plasma PAI-1 levels within 24 h in patients with acute ST-elevated myocardial infarction are associated with heart failure and death and are a strong independent predictor of mortality at 30 days (31). The renin-angiotensin II system (RAS) is strongly activated following acute MI, and angiotensin II triggers PAI-1 synthesis (32) (**Figure 1B**). The RAS has been linked to the circadian variation in PAI-1, suggesting that the use of angiotensin-converting enzyme (ACE) inhibitors to inhibit the RAS system may blunt PAI-1 levels thereby reducing the risk of "early morning" MI (33).

Elevated levels of PAI-1 have been detected in atherosclerotic plaques in humans (34–36), which are significantly inflated in type 2 diabetes mellitus (T2DM) subjects (37). Dysregulation of the fibrinolytic system appears to play a significant role in atherosclerotic plaque development by perturbing the wound-healing response and neointimal formation (38). This can in part be attributed to reduced vascular smooth muscle cell migration *via* inhibition of binding of vitronectin to integrin $\alpha_v\beta_3$ (39). Moreover, PAI-1 stabilizes the fibrin matrix within the developing plaque by attenuating plasmin formation. Increased serum levels of PAI-1 have been noted in patients with atherosclerotic disease, including coronary artery disease (40) and stroke (41). A recent large meta-analysis has indicated that PAI-1 is implicated in the pathogenesis of atherosclerotic disease (42). The two-pronged approach of PAI-1 inducing a hypofibrinolytic state in atherosclerotic diseases and impacting on lesion formation and progression indicates the perilous complications of this serpin in the pathogenesis of these diseases. Animal models of ablation (43, 44) of the PAI-1 gene and pharmacological inhibition of PAI-1 (45) in murine models have proved insightful in our understanding of these processes. Nevertheless, it is challenging to extrapolate all of these findings to humans, which combined with the lack of a licensed inhibitor, leaves a gap in our knowledge and understanding of this serpin in the intricate mechanisms of this complex disease.

Perioperative DVT has been linked to elevated levels of circulating PAI-1 (29). A higher incidence of DVT (46) and venous thrombosis (47) has been noted in Asian Indian patients harboring the 4G polymorphism, leading to a suggestion that it be included in all laboratory testing panels for thrombophilia. Similar studies in white Caucasian populations have described association of the 4G polymorphism with idiopathic DVT and inherited thrombophilia (48). More recently, preoperative plasma PAI-1 has been revealed as an independent risk factor for the onset of DVT in patients undergoing total hip arthroplasty (49). Elevated levels of plasma PAI-1 largely account for delayed clot lysis times in healthy individuals and are associated with first incidence of venous thrombosis (50). These lines of evidence highlight the importance of this serpin in predisposing individuals to a hypofibrinolytic state, which is directly linked to an increased frequency of venous thrombosis. Yet, the exact mechanisms underpinning this pathophysiological process and cellular source of PAI-1 remain to be elucidated.

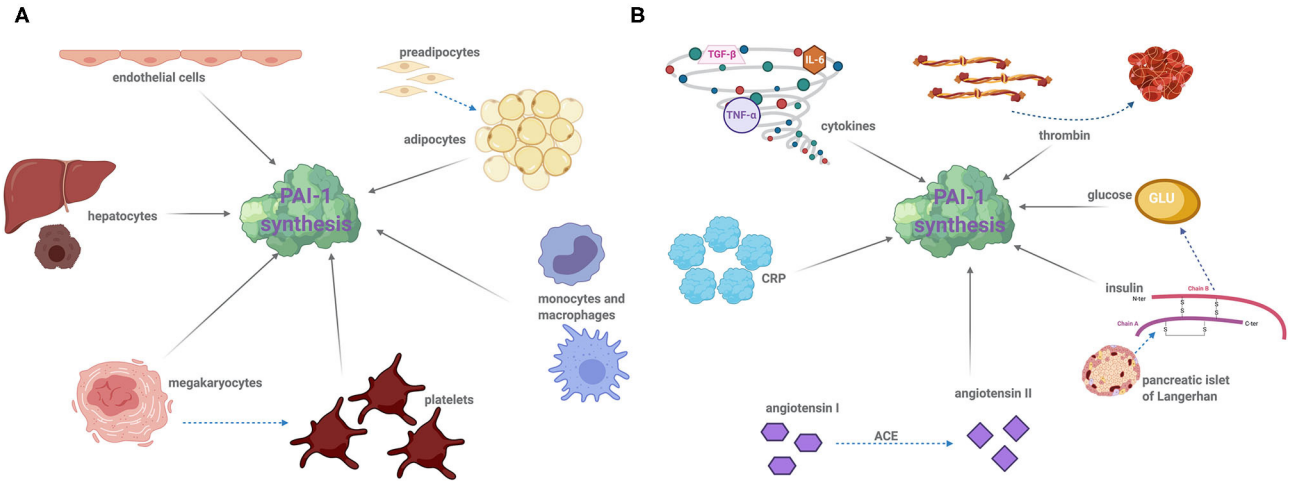


**FIGURE 1 |** Cellular sources of PAI-1 of modulators of synthesis: **(A)** PAI-1 is produced by a variety of cell types including the following: endothelial cells, adipocytes, hepatocytes, leukocytes (monocytes and macrophages), megakaryocytes, and platelets. Dotted arrows demonstrated cell differentiation and solid arrows indicate PAI-1 synthesis. **(B)** A number of factors induce PAI-1 synthesis and secretion. Proinflammatory cytokines, namely interleukin-6 (IL-6), tissue necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) significantly augment PAI-1 synthesis. Thrombin, insulin, glucose, angiotensin II, and C-reactive protein (CRP) can also stimulate PAI-1 expression. Drugs that target insulin and the angiotensin converting enzyme (ACE), such as metformin and captopril, are known to decrease plasma PAI-1 concentration. Dotted arrows represent the reaction of an enzyme or protein synthesis, and solid arrows indicate PAI-1 synthesis.

## INFECTION AND INFLAMMATION

PAI-1 is a positive acute phase protein that is dramatically elevated in the proinflammatory state, such as acute tissue injury, sepsis, and inflammation. The role of PAI-1 in this context is primarily considered a protective mechanism to limit dissemination of pathogens and promote tissue repair. Augmented levels of PAI-1 in non-typeable *Haemophilus influenzae* infection are associated with bacterial clearance and shortening of the disease duration (51). Pharmacological inhibition of PAI-1 in a *Pseudomonas aeruginosa* pneumonia mouse model attenuates neutrophil migration, thereby dampening the innate immune response (52). PAI-1 modulation of neutrophil migration has also been demonstrated in *Escherichia coli* infection (53). However, aberrant activation of this defense mechanism produces a hypofibrinolytic state which promotes thrombotic complications.

Sepsis occurs due to overreaction of the host defense mechanism, most commonly in response to bacterial infection, but can also be caused by viral and fungal pathogens. Sepsis leads to enhanced exposure of the coagulation protein, tissue factor, inciting fibrin deposition and microthrombi throughout the vasculature (54). Multiorgan failure is a frequent complication in sepsis patients, and the development of disseminated intravascular coagulation (DIC) is a major contributor (54) and is associated with aberrant thrombin generation. Endothelial dysfunction induces release of proinflammatory cytokines (55, 56) which combined with augmented levels of thrombin provoke PAI-1 synthesis (**Figure 1B**). Endothelial cells produce enhanced levels of PAI-1 in response to C-reactive protein (CRP) (57, 58) (**Figure 1B**), which is a proinflammatory marker in critically ill patients such as sepsis patients (59–61). A recent meta-analysis has reported PAI-1 as a predictor of disease severity in sepsis and overall mortality (62), but the prognostic value of this biomarker in disease progression requires further attention.

A hypofibrinolytic state has been observed in multiple viral infections and is associated with elevated PAI-1 levels (63–67). Most recently, elevated PAI-1 and tPA antigen levels have been described in patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) which causes coronavirus-19 disease (COVID-19) (64, 68). However, the net effect of the increased PAI-1 and tPA levels may differ between patients, with variations in this axis being attributed to both a hypo- (64) and hyperfibrinolytic (68) phenotype. In the severe acute respiratory syndrome coronavirus (SARS-CoV) epidemic in 2002 and 2003, the hypofibrinolytic state was attributed to overexpression of PAI-1 which inhibited plasminogen activator activity causing persistence of fibrin deposition (63, 69).

Several proinflammatory cytokines significantly augment PAI-1 synthesis (70–72). Interleukin-6 (IL-6) is an acute-phase inflammatory protein that has been reported to significantly increase PAI-1 and tPA antigen (73). Cytokine release syndrome (CRS) is an acute systemic inflammatory response that can be triggered by various infections and can be observed in sepsis and acute respiratory distress syndrome (ARDS). Endothelial IL-6 trans-signaling promotes IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) and PAI-1 synthesis (55). Inhibition of this trans-signaling circuit by the IL-6R antagonist, tocilizumab, has recently been shown to reduce PAI-1 expression in a small study of COVID-19 patients (55), and is now a recommended treatment for ICU patients after improved outcomes in patients on the Remap-Cap trial (74).

Tumor necrosis factor-alpha (TNF-α), acting *via* NFκB, is a strong stimulator of PAI-1 expression (56) (**Figure 1B**).

TNF-α is an important regulator of PAI-1 expression in adipose tissue, and neutralizing TNF-α significantly reduces both plasma and adipose tissue levels (75, 76). Several studies support the hypothesis that TNF-α may be responsible for expression of TGF-β (75, 77), another major stimulant of PAI-1 biosynthesis (77–80). Elevated levels of PAI-1 in turn block conversion of latent transforming growth factor beta (TGF-β) contributing to a self-regulation mechanism (80). The affiliation between proinflammatory cytokines and heightened PAI-1 synthesis provides a definitive link between this serpin and the inflammatory response.

## METABOLIC SYNDROME

Metabolic syndrome (MetS) is a multifaceted disorder that encompasses several conditions that considerably elevate the risk of CVD and T2DM. Definitions vary, but in general, diagnosis of MetS requires individuals to meet at least three of the following criteria (81, 82);

- Abdominal obesity
- Dyslipidemia—elevated triglycerides and apolipoprotein B and low levels of high-density lipoprotein (HDL)
- Hypertension
- Hyperglycemia
- Insulin resistance

Whether the clustering of these conditions elevates an individual's risk over that of a single disorder is a matter of ongoing debate (83, 84). Nonetheless, given the prevalence of MetS worldwide and the fact that this cluster of risk factors predicts CVD in multiple settings, it is clear that we require a stronger understanding of the pathophysiology to develop predictive tools and improve therapeutic options.

Abdominal obesity is an essential criterion in the development of MetS (85, 86). Adipose tissue is an endocrine organ composed of multiple cell types, that secrete adipokines of diverse biological function, such as adiponectin (87–90), leptin (91), and various cytokines, including IL-6 and TNF-α. PAI-1 is a known adipokine (8, 92–94). Adipokine secretion is dependent on the location of the fat store in the body and the composition of cells comprised within the adipose tissue. A correlation between MetS and PAI-1 levels was established in the late 1980s (95). Elevated levels of PAI-1 in individuals with MetS has been demonstrated using criteria defined by both the World Health Organization (WHO) (96) and the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (NCEP-ATPIII) (97). Elevated levels of PAI-1 in humans predict incidence of MetS in two prospective studies (98, 99). It is well-established that PAI-1 can predict the risk of future CVD (100) and onset of T2DM (101). Together, these data have led to the interpretation that PAI-1 is a true component of MetS (102) and could be an important clinical criterion for development of future CVD (103).

### Obesity

Obesity is a global epidemic (104) that is inextricably linked with increased risk of CVD, including arterial, venous, and microvascular thrombosis (105, 106); DVT (107, 108), coronary thrombosis (105, 106), pulmonary embolism (PE) (108, 109) and stroke (110). Obesity-related thrombosis is linked to decreased fibrinolytic activity (111–119) which can be largely attributed to escalating levels of plasma PAI-1 antigen and activity (112, 120). Increased PAI-1 synthesis by adipocytes in response to protractedly elevated levels of TNF-α, insulin, and TGF-β is primarily responsible (112, 114, 120–122) (**Figure 1B**). Intriguingly, elevated tPA levels have been reported in studies on MetS (96, 97) and obesity (114, 123), which could be the result of impairment of the endothelium; however, the dominant phenotype is hypofibrinolysis.

Plasma PAI-1 significantly correlates with a variety of adiposity measures, including body mass index (BMI), waist-to-hip ratio, total fat mass, and visceral and subcutaneous adipose tissue (124–126). The insulin resistance atherosclerosis study (IRAS) was the first to report that PAI-1 antigen and activity positively correlate with BMI ($r = 0.314/0.425$, respectively) (127). Adipocytes from obese humans harbor twice as much PAI-1 mRNA resulting in a ≥six-fold increase in secretion of PAI-1 and plasma PAI-1 activity compared with lean individuals (8). Weight loss in obese subjects reduces plasma PAI-1 (122, 125, 128), indicative that circulating levels are directly related to the degree of adipose tissue. In line with this, pharmacological inhibition of plasma PAI-1 in animal models results in weight loss, as well as a reduction in adipose tissue and adipocyte volume (19, 129–131). The number and size of lipid-containing vesicles in adipocytes are also decreased, as well as plasma glucose and triglyceride levels and insulin resistance (129, 132, 133). These data indicate that adipocyte-derived PAI-1 functions in an autocrine role, with one study indicating that PAI-1 inhibition limits differentiation of preadipocytes into mature adipocytes (129, 134).

Interestingly, PAI-1 synthesis is not uniform, with adipocytes from visceral fat depots harboring significantly more PAI-1 mRNA than subcutaneous or femoral fat depots (92, 135). Indeed, visceral fat has been suggested as a determinant of PAI-1 activity in overweight and obese women (136). To fully exploit PAI-1 as a biomarker of MetS, and future CVD, it may be necessary to correlate this serpin with additional factors such as visceral fat levels, rather than more general measurements of BMI.

### Type 2 Diabetes

T2DM is intrinsically linked to obesity (92, 137–139), and elevated levels of PAI-1 are strongly correlated with insulin resistance (137, 140), impaired glucose tolerance (137, 140), and T2DM (141, 142). Many studies have reported strong associations between PAI-1 and development of T2DM (143–150). Furthermore, lifestyle and pharmacological interventions to manage diabetes have been shown to decrease circulating plasma PAI-1 levels (151–153). Indeed, PAI-1 activity is significantly reduced upon treatment with the antidiabetic drug, metformin, with a corresponding improvement in glycemic control and reduction in insulin resistance (152).

The IRAS study revealed that PAI-1 was a reliable predictor for developing T2DM, despite adjustments for adiposity, body

fat distribution, and insulin sensitivity in patients ($p = 0.002$) (101). Interestingly, this study also found that PAI-1 activity was increased, but not correlated with insulin concentration, in plasma from non-obese children with T2DM parents (101). Various murine models have determined that PAI-1 plays a pivotal role in development of insulin resistance (132, 133). A mouse model of diet-induced obesity was used to study the relationship between PAI-1, obesity, and insulin in PAI-1 deficient PAI-$1^{-/-}$ and wild type (WT) mice (132). Obesity and insulin resistance that developed in WT mice in response to a high-fat diet was prevented in PAI-$1^{-/-}$ mice (132). Furthermore, PAI-$1^{-/-}$ mice showed increased resting metabolic rates and total energy expenditure, compared with WT. Treatment of WT mice with an angiotensin type I receptor antagonist reduced PAI-1 levels, attenuated diet-induced obesity, hyperglycemia, and hyperinsulinemia (132). Genetically obese (ob/ob) mice deficient in PAI-1 weigh significantly less than those with normal PAI-1 levels; as a result, these mice demonstrate a significant improvement in hyperglycemia and hyperinsulinemia (133). Intraperitoneal glucose administration markedly augments serum insulin levels in WT ob/ob mice; however, the increase in PAI-$1^{-/-}$ mice was dramatically reduced (133). *In situ* hybridization studies revealed that TNF-α expression was significantly reduced in PAI-$1^{-/-}$ ob/ob mice compared with WT ob/ob mice (133). Together with the well-documented role of TNF-α in stimulating PAI-1 expression (75), this study exposes a complex reciprocal relationship between PAI-1 and TNF-α which merits further study.

Insulin directly stimulates PAI-1 synthesis and secretion from adipocytes (154) (**Figure 1**), a process which is upregulated in hyperinsulinemia and hyperglycemia (154, 155). Glucose also upregulates PAI-1 expression in vascular smooth muscle cells, endothelial cells, and adipose tissue (156–159). Clinical studies consistently demonstrate a strong correlation between plasma PAI-1 and insulin resistance (154, 160); however, cause or consequence is less clear, that is whether elevated PAI-1 is a result of insulin resistance or if it occurs independently. The role of TNF-α and TGF-β in stimulating PAI-1 expression in adipose tissue (76, 154) suggests that the increase in plasma PAI-1 and insulin resistance may be bi-directional.

There is evidence to support that chronic inflammation and insulin resistance are linked (101, 143). It is hypothesized that this may be due to increased expression of proinflammatory cytokines, namely IL-6 and TNF-α, from adipose tissue (161, 162) which in turn can stimulate acute phase proteins, including PAI-1 (13, 163). Furthermore, several studies (143, 164, 165) have reported that tPA antigen and activity are associated with developing T2DM, and tPA and PAI-1 antigens are strongly correlated in plasma (166). Despite the concordant increase in tPA and PAI-1, a hypofibrinolytic state prevails in T2DM individuals.

### Hypertension

There is accumulating evidence implicating PAI-1 in the development of hypertension (167), and plasma PAI-1 is associated with several risk factors for hypertension, including obesity (168, 169), insulin resistance (140, 169), and inflammation (70) as discussed above. Genetic ablation of PAI-1 protects against hypertension and perivascular fibrosis induced by nitric oxide synthase (NOS) inhibition (170, 171). NOS plays a key role in regulating vascular tone and remodeling of the vessel wall (172–174), and inhibition of NOS induces progressive hypertension and vascular fibrosis (175–177). Furthermore, inhibition of PAI-1 with a novel small molecule inhibitor (PAI-039) protects a mouse model against angiotensin II-induced aortic remodeling and cardiac fibrosis (178).

Human studies indicate a direct correlation between plasma PAI-1 and hypertension and its associated conditions (179–185), such as arterial stiffness (186) and atherosclerosis (187). Interestingly, the 4G allele for PAI-1 is associated with increased systolic, diastolic, and mean arterial blood pressure (188), indicative of a direct link between plasma PAI-1 and blood pressure. A study examining two longitudinal cohorts of American Indians revealed that baseline PAI-1 is predictive of hypertension independent of other variables (189). Participants with the highest concentration of PAI-1 (>58 ng/ml) had a 63% increased risk of hypertension compared with those in the lowest group (<33 ng/ml) (189). A similar prospective study, the Framingham Offspring Study, confirmed that a higher concentration of plasma PAI-1 was associated with an increased risk of hypertension (odds ratio = 1.28) (190). Despite plasma PAI-1 correctly predicting the risk of hypertension in human studies, it did not provide a significant advantage over conventional risk factors, such as fasting glucose, alcohol consumption, BMI, cigarette smoking, or C-reactive protein (189). Given the close relationship of PAI-1 with the RAS system and the documented increase in the levels of PAI-1 in hypertension, the mechanisms underpinning this relationship warrant further investigation.

## POTENTIAL THERAPEUTIC OPTIONS AND DISCUSSION

The driving force of PAI-1 in thrombosis, inflammation, and metabolic syndrome is evident (**Figure 2**). In addition, this serpin functions in a variety of pathophysiological processes, outwith the subject matter of this review, including wound healing (191), cardiac fibrosis (192), cancer (193), and senescence (194). These numerous roles underscore the potential of PAI-1 as an attractive therapeutic target; nevertheless, to date, no PAI-1 inhibitors have been approved for clinical use.

Small molecules, peptides, monoclonal antibodies, and antibody fragments have all been used to modulate PAI-1 activity by interfering at different stages of the PAI-1/plasminogen activator interaction [(195–199), reviewed in detail by (200)]. A number of clinically approved drugs indirectly reduce plasma PAI-1; these include insulin sensitizing agents for management of T2DM, such as metformin, and ACE inhibitors (used to treat hypertension) (201). However, these drugs have been studied in experimental models (202–205), and as yet, there is limited information available from human studies.

Drugs targeting PAI-1 in the experimental phase have produced promising results (206–208). A potent neutralizing

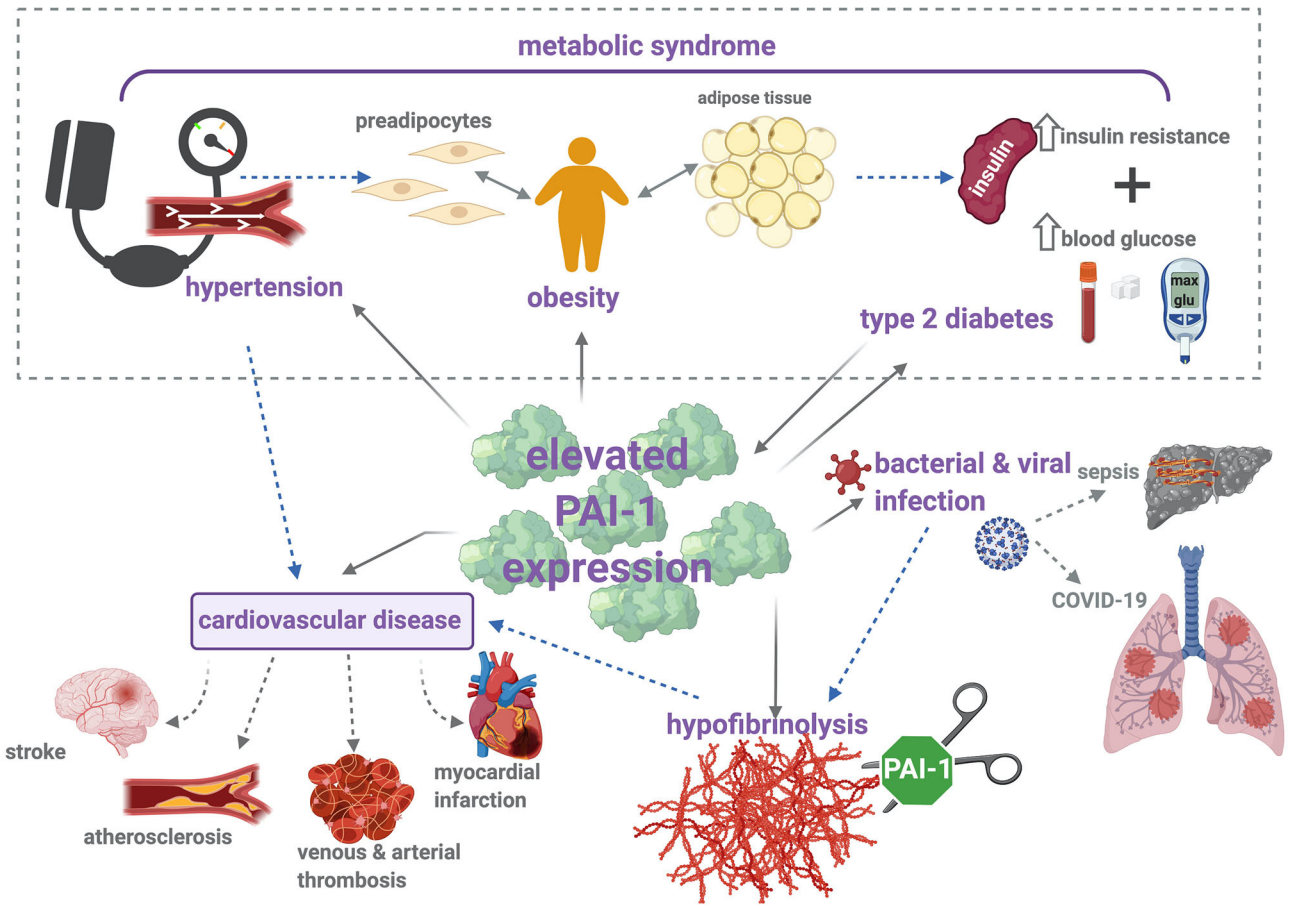


**FIGURE 2 |** PAI-1 modulates thrombosis and inflammation *via* multiple pathophysiological mechanisms. Metabolic syndrome is characterized by increased insulin resistance, obesity, and hypertension, which contribute to elevated risk of cardiovascular disease (CVD). Increased levels of PAI-1 antigen and activity are positively associated with hypertension, obesity, type 2 diabetes (T2DM), and CVD. Briefly, elevated levels of PAI-1 antigen and activity occur in obesity, which is a known risk factor for CVD and T2DM. Strong correlations between increased PAI-1 and development of T2DM have been identified, including increased insulin resistance and impaired glucose tolerance. Conversely, insulin and glucose can stimulate PAI-1 secretion from adipose tissue. Elevated levels of PAI-1 attenuate plasmin formation and downregulate fibrin degradation. This hypofibrinolytic state provokes thromboembolic complications, including stroke, atherosclerosis, myocardial infarction, and venous and arterial thrombosis. Hypofibrinolysis and elevated PAI-1 levels have been associated with bacterial and viral infections, including sepsis and COVID-19. Sepsis is characterized by the development of disseminated intravascular coagulation (DIC), a major contributor to resulting organ failure. In COVID-19 patients, disruption between coagulation and fibrinolysis leads to fibrin deposits in the lung parenchyma and thrombosis. Solid arrows represent (patho)physiological processes that arise as a result of increased PAI-1 levels, and dotted arrows illustrate the links between each of these individual pathologies.

diabody to PAI-1 and activated thrombin activatable fibrinolysis inhibitor (TAFIa) rapidly enhances clot breakdown (207). Simultaneous inhibition of PAI-1 and TAFIa may improve current thrombolytic therapy; e.g., co-administration with tPA thereby permitting a lower dose and thus enhancing its safety profile (207, 209). Tiplaxtinin, a PAI-1 antagonist, prevents venous thrombosis, angiotensin II-induced atherosclerosis, and obesity in a ferric chloride-induced vascular injury model in rats (206). More recently, a nanobody to PAI-1 has been developed that selectively stabilizes the active form of PAI-1, which may be used as a diagnostic or analytical tool (195, 208).

Other drugs that elicit pharmacological inhibition of PAI-1 have reached phases 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 (210). 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 (211, 212).

This review predominantly focuses on the role of PAI-1 in the thrombosis-inflammation axes and associated diseases (**Figure 2**). It is evident that a proinflammatory state, whether it arises from infection, vascular thrombotic complications, a condition such as MetS, or its associated cluster of diseases spark a dramatic elevation in plasma PAI-1. The underlying motive for this can be attributed to the many cytokines and proteins that can elicit PAI-1 synthesis, thereby inextricably bridging these conditions with this complex serpin. The cellular

source of PAI-1 may vary according to the disease processes, e.g., of platelet or endothelial origin during infection or venous thrombosis but of adipose tissue origin in obesity. Many of the conditions described herein predominantly affect the aging population, and it is noted that there is also a clear link between circulating PAI-1 levels and age (213). These observations further confound the relationship between elevated PAI-1 and thromboinflammation, leading to cardiovascular complications. This review serves as an aide-mémoire on the consequences of PAI-1 elevation and highlights the utility of this serpin as a potential therapeutic target in the treatment of various pathological conditions which are associated with a hypofibrinolytic state and development of thromboembolic diseases.

## AUTHOR CONTRIBUTIONS

GM and CW researched the data, wrote, and revised the manuscript. NM conceived the article, wrote the article, and edited the article. All authors contributed to the article and approved the submitted version.

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# Serp-1 Promotes Corneal Wound Healing by Facilitating Re-epithelialization and Inhibiting Fibrosis and Angiogenesis

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**Purpose:** Chemical corneal injuries carry a high morbidity and commonly lead to visual impairment. Here, we investigate the role of Serp-1, a serine protease inhibitor, in corneal wound healing.

**Methods:** An alkaline-induced corneal injury was induced in 14 mice. Following injury, five mice received daily topical saline application while nine mice received Serp-1 100 μL topically combined with a daily subcutaneous injection of 100 ng/gram body weight of Serp-1. Corneal damage was monitored daily through fluorescein staining and imaging. Cross sectional corneal H&E staining were obtained. CD31 was used as marker for neovascularization.

**Results:** Serp-1 facilitates corneal wound healing by reducing fibrosis and neovascularization while mitigating inflammatory cell infiltration with no noticeable harm related to its application.

**Conclusions:** Serp-1 effectively mitigates inflammation, decreases fibrosis, and reduce neovascularization in a murine model of corneal injury without affecting other organs.

**Translational Relavence:** Our study provides preclinical data for topical application of Serp-1 to treat corneal wounds.

**Keywords: corneal injury, neovascularization, Serp-1, PAI-1, corneal wound repair**

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## INTRODUCTION

Corneal injuries are a commonly cited reason for emergency department visits in the United States (1, 2). Although they are typically non-fatal, they can result in significant morbidity and change in quality of life. Ocular chemical burn in particular, represent an ophthalmic emergency and constitute about 11.5–22.1% of eye injuries (3). The most common causes of ocular chemical burns are household cleaners and building products such as ammonia, sodium hydroxide, and plaster (2). Accidents involving these chemicals require prompt diagnosis and frequently result in hospitalization for treatment (1, 2).

Under normal circumstances, the corneal epithelium acts as a protective barrier to the ocular bulb (2). Intercellular attachments and attachments of epithelial cells to the extracellular matrix through both junctional and non-junctional adhesions, maintain this barrier (4). With damage, epithelial cells undergo apoptosis and are shed in tear films (2). Intercellular junctions are disrupted, and cell-substrate junctions are temporarily replaced with weaker attachments and a provisional extracellular matrix is laid down in preparation for repair (5). Chemical ocular injuries are particularly more deleterious due to the additional oxidative stress they impose on the cornea which not only damages the corneal cells but will also trigger an immune response characterized by inflammation (2, 6). This damage to the cornea causes a keratocyte induced fibrosis which hardens and opacifies the cornea and results in varying degrees of blindness (7). Additionally, while acute inflammation is initially beneficial to the eye, long-term sequalae of inflammation in the cornea include neovascularization which can threaten vision (8).

Serp-1 is a myxoma virus derived SERine Protease INhibitor (SERPIN) with previously demonstrated roles in inflammation, cell migration, wound closure, tissue remodeling and fibrosis (9–12). It is a single-chain glycosylated protein composed of three β-sheets and nine α-helical domains with a strained reactive center loop (RCL) positioned in the carboxy terminus (13). SERPINs are important injury response factors that participate in all stages of injury repair [summarized in our recent review article (14)]. We have previously showed that recombinant Serp-1 protein could be a potential therapeutic agent to reduce aortic balloon angioplasty injury (15), suppress atherosclerotic plaque growth (16), prevent chronic renal (17), and cardiac (18) allograft rejection, promote spinal cord injury repair (19) and full thickness dermal wound healing (10) in rodent models. Here, we investigate the role of Serp-1 in corneal wound healing through immune system modulation.

# METHODS

## Animal Corneal Wound Healing Model

Animal husbandry and experimentations were conducted with approval from the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. All injuries were induced under anesthesia (Henry Schein Isothesia, Isofluorane). Four milliliters of Ibuprofen (Perrigo Basic Care, 100 mg per 5 mL Oral Suspension) was mixed into 400 mL $ddH_2O$ (Millipore Sigma Milli-Q IQ system) and made accessible to the mice 24 h before the injury and throughout the duration of the entire experiment. A solution of buprenorphine was made by triturating buprenorphine and saline solution (Baxter, 0.9% Sodium Chloride) in a 3:50 ratio by volume. Each mouse received a 100 μL subcutaneous injection of the buprenorphine solution every 12 h following the injury for 72 h.

Fourteen mice (C57BL/6J) were selected for this experiment. A piece of filter paper, 2-mm in diameter, was soaked in 1M NaOH solution and placed on the cornea of the mouse's right eye for 30 s and then rinsed off with 15 mL of saline solution (Baxter, 0.9% Sodium Chloride). The clinical opacity and neovascularization scores were determined using Modified Hackett-McDonald scoring methods for 10 days after the injury (20). After the analysis was complete, the mice were sacrificed, and the eyes were collected for flat-mount and paraffin-embedded staining. The size and depth of the corneal damage was monitored and recorded daily through fluorescein staining and imaging. Ten microliters of fluorescein was placed on the right eye of the mouse and the excess fluorescein was rinsed off with saline. The Kowa SL-17 slit lamp was set to the cobalt blue filter and used to illuminate the eye and excite the fluorescein particles so that they could be captured by a point and shoot camera (Samsung ST150F). Images were obtained once per day.

For treatment of the corneal wounds, the mice were divided into two experimental treatment groups. One group received a control treatment of 10 μL of phosphate-buffered saline (PBS) applied topically for 10 min. The second group not only received a 10 μL (0.1 μg/μl) topical treatment of recombinant Serp-1 protein but also a subcutaneous injection of recombinant Serp-1 protein (0.1 μg/μl) in a dosage equivalent to 100 ng per gram of body weight (recorded every morning) based on our previous publication (10). Treatments were applied twice daily for a total of 10 days following the injury. Mice body weights and eating habits were monitored daily and used as indicators of health status. Heart, lung, kidney, liver, splenic tissues were collected at the completion of the experiment and evaluated for pathology.

## Histopathology and Immunohistochemistry

The mice were sacrificed following 10 days of observations. The eyes were removed, set in paraformaldehyde (BioWorld, 4% in PBS, pH 7.4) overnight, and then transferred into 70% ethanol. The corneas were dissected from the ocular bulb and placed into a 96-well plate with 100 μL PBS (Thermo Fisher Scientific). Corneas were washed 3 times with 300 μL PBS for 10 min per wash and placed in blocking buffer solution for 2 h at room temperature. Anti-CD31 antibodies (1:100, BD Biosciences Pharmingen) was applied, followed by overnight incubation at 4°C. All corneas were washed 6 times with 1x washing buffer for 1 h at a time at room temperature. Secondary antibodies α-rat 488 (1:500, 1:500, Thermo Fisher Scientific) was applied for CD31(angiogenesis/neovascularization marker. The corneas were then washed 3 times with PBS for 1 h each. Four cuts were made on the cornea to "butterfly" the sample so that the sample could be laid down in a flat manner. Mounting was performed in mounting buffer containing 4′,6′-diamidino-2-phenylindole (DAPI). Additionally, full corneal cross-sections were obtained, embedded in paraffin, and stained using Hematoxylin and Eosin (H&E). Immunofluorescent staining of CD11b [anti-CD11b antibody (Invitrogen, 14-0112-82, 1:200)] was performed as follows: slides were deparaffinized and rehydrated by incubating sequentially in xylene, 100% ethanol, 95, 75, 50% ethanol and PBS. A pressure cooker was used for antigen retrieval. Slides were merged in Tris-EDTA buffer and cooked for 13 min. Primary antibody were applied and incubated at 4°C overnight. Goat anti-rat secondary antibody Alexa-488 (Invitrogen, A11006) were applied and incubated at room temperature for 1 h. All images were captured by a Zeiss LSM 780 confocal microscope and analyzed by ImageJ as described in our previous publication (21).

## Opacity and Neovascularization Scores

A modified Hackett-McDonald scoring was used (20). Image J was used for immunofluorescence image analysis. Vessel density was calculated by tracing the fluorescent, CD31 positive areas in ImageJ then dividing that area by the total cornea area to achieve a percentage for vessel coverage.

## Production of Recombinant Serp-1 Protein

Recombinant Serp-1 protein was produced by Chinese hamster ovary (CHO) cell line protein expression system (Viron Therapeutics Inc., London, ON, CA), as described by our previous publication (10). Sequential column chromatographic separation was employed to purify GMP-compliant recombinant Serp-1 protein. Purity of Serp-1 protein (>95%) was determined by Coomassie-stained SDS-PAGE and reverse-phase HPLC. Endotoxin was confirmed absent from purified Serp-1 by LAL assay (10).

## Statistical Analysis

The data are represented as mean ± standard deviation. Comparisons were made by Student's *t*-test when comparing two experimental groups. The standard deviation of the mean is indicated by error bars for each group of data. A value of $p < 0.05$ was considered significant. All of these data were analyzed using Prism 8 software.

# RESULTS

## Serp-1 Facilitates Corneal Wound Healing by Reducing Fibrosis and Neovascularization

To test the effectiveness of Serp-1 as a treatment for corneal injuries, an alkaline-induced corneal injury was induced in 14 mice. Following injury, a control group of five mice received daily topical saline application while nine mice received Serp-1 (100 ng/ μL concentration, 100 μL topically plus 100 ng/gram body weight subcutaneously) each day. Fluorescein dye and quantitative opacity and neovascularization scores were used to determine the level of fibrosis, neovascularization, and re-epithelialization in the corneas.

Fluorescein positive-areas and fluorescence intensity were used as indicators for corneal damage and re-epithelialization. Mice who received Serp-1 treatment exhibited smaller fluorescein-positive areas and lower intensity fluorescence when compared to the mice who received only saline (**Figure 1A**). A significant difference between the damaged areas was noted by Day 5 following injury ($p < 0.05$) (**Figure 1B**). Serp-1 treated mice corneas consistently showed reduced opacity and vascularization, with a significant difference in both opacity and neovascularization starting from Day 6 post-injury (**Figures 1C,D**, *: $P < 0.05$; **: $P < 0.01$). CD31 expression was used as an indicator of the presence of superficial and deep neovascularization in corneal sections. Evaluation of the CD31 staining revealed a reduction in neovascularization in the Serp-1 treated group (**Figure 2**, *: $P < 0.05$).

## Serp-1 Reduces Rates of Inflammatory Cell Infiltration Into the Cornea

Histochemical analysis of the enucleated globes of both Serp-1 and control-treated mice was performed following 10 days of observations. Cross-sectional image analysis following H&E staining revealed an obvious reduction of corneal swelling and decreased inflammatory cells infiltration in Serp-1 treated animals (White arrows in **Figure 3A**). Similarly, CD11b positive immune cells were stained. We found that Serp-1 treatment significantly reduced immune cell infiltration following alkali burn injury (**Figures 3B,C**, *: $P < 0.05$).

## Repetitive Application of Serp-1 Produces No Obviously Toxic Effects

Since Serp-1 protein is a virus derived protein, we also checked whether repetitive administration of Serp-1 could induce any adverse effects to the mice. We first checked mouse body weight change after Serp-1 injection. While the mice receiving saline gained body weight, we did observe Serp-1 treatment group had slight reduction of body weight (**Figure 4A**). However, when we checked major organs of the mice treated with Serp-1, we didn't observe pathologic inflammatory changes as result of the use of Serp-1 (**Figure 4B**). Thus, further studies will be required to identify the cause of slight body weight loss after repetitive administration of Serp-1 protein in mice.

# DISCUSSIONS

The application of Serp-1 following corneal alkali induced injury leads to reduced fibrosis and neovascularization, and a decrease in inflammatory cell infiltration. In order to maintain transparency, the cornea must remain avascular and unscarred (22). Since chemically induced corneal injuries rapidly progress to visual impairment, attention has recently turned toward developing treatments for rapid re-epithelialization of the cornea with minimal fibrosis and neovascularization. The novelty of this study is evident in the fact that it demonstrates the therapeutic efficacy of Serp-1 in promoting the natural healing process of the cornea following injury in a mouse model of corneal injury.

We hypothesized that Serp-1 facilitates corneal wound healing by reducing inflammation. An important facet of corneal wound healing is the rate at which the cornea re-epithelialize (22). To monitor re-epithelialization in the mice, fluorescein dye was used daily to stain areas of the mouse cornea where the tight cell-to-cell junctions of the epithelium were compromised. Analysis during a period of 10 days following injury revealed quick healing in mice treated with Serp-1. These results are consistent with the findings from previous work done in evaluating the role of Serp-1 in dermal wound healing (10). The reduced rates of opacity and neovascularization in the Serp-1 treatment group demonstrate a reduced inflammatory response. Additionally, observations from our paraffin-embedded cross sections revealed reduced corneal swelling and improved morphology with Serp-1 treatment following injury which points to an improved rate of corneal epithelialization.

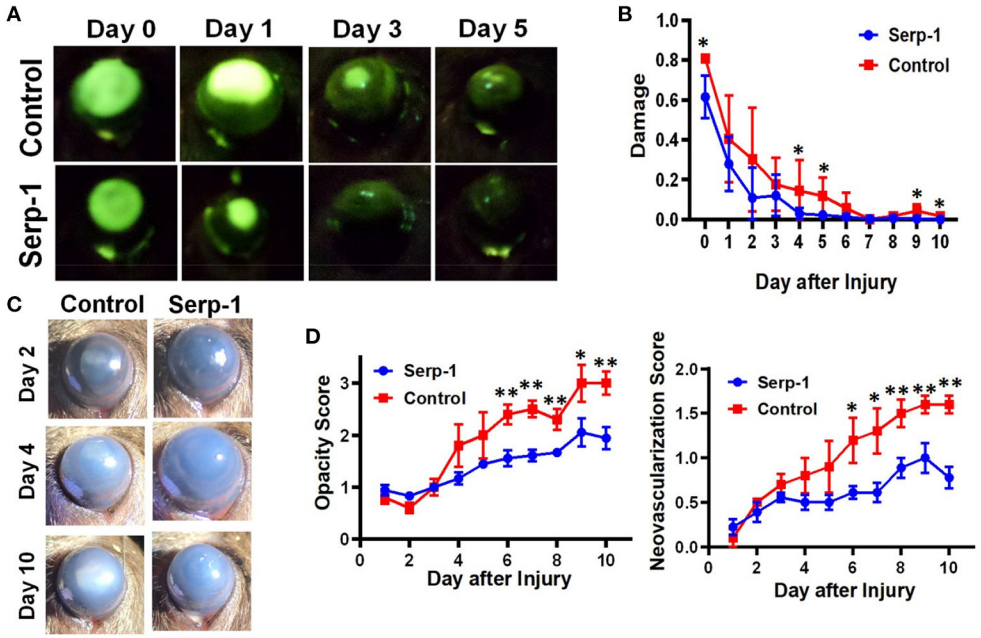


**FIGURE 1 |** Serp-1 contributes to the corneal wound healing process by limiting the inflammatory response. **(A)** Treatment with Serp-1 shows improved re-epithelialization in mouse corneas with an alkaline induced injury ($n = 5$ for control group and $n = 9$ for Serp-1 group). **(B)** Quantification of fluorescent signal in **(A)** by dividing the fluorescein positive area by total corneal area (data were presented as mean ± S.D. $^{*}P < 0.05$). **(C)** Bright-field imaging shows reduced fibrosis and encroachment of the cornea by neovascularization in mice treated with Serp-1. **(D)** Quantification of corneal fibrosis and neovascularization using a modified Hackett-McDonald scoring system (data were presented as mean ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$).

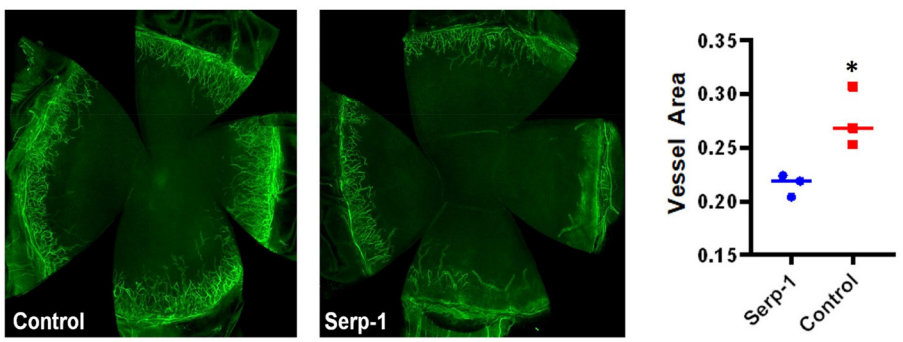


**FIGURE 2 |** Immunostaining with flat-mounted corneas shows reduced inflammation in Serp-1 treated mice. Immunostaining with flat-mounted corneas shows reduced inflammation in Serp-1 treated mice. Anti-CD31 antibody was applied and samples were mounted in buffer containing 4′,6′-diamidino-2-phenylindole (DAPI). Total vessel area was quantified by dividing the CD31 positive areas by the total cornea area using FijiWin's ImageJ software, with a significant difference reported between the two treatment groups ($n = 3$/group; $^{*}p < 0.05$).

In addition to a fast recovery time, it is essential for the cornea to heal without excessive fibrosis or neovascularization which impede light transmission. Due to its anti-inflammatory properties, it was hypothesized that injured corneas treated with Serp-1 would have reduced amounts of fibrosis and neovascularization. Our data points toward a significant reduction in opacity and neovascularization in animals treated with Serp-1. This was further confirmed through quantitative analysis of the total area of neovascularization in flat-mount staining. Together, these data support the hypothesis that Serp-1 inhibit the inflammatory response in a positive manner that limits fibrosis and scarring that impairs vision.

The immunogenicity of Serp-1 is a legitimate concern due to the fact that it is virally derived. Daily monitoring of mice's body weights and staining of other major organ tissue in these experiments revealed no cytotoxic impact of the repeated application and injection of Serp-1. This finding is entirely consistent with prior research both in pre-clinical or clinical studies wherein Serp-1 demonstrated no significant toxicity and no neutralizing antibodies were detected in the Phase

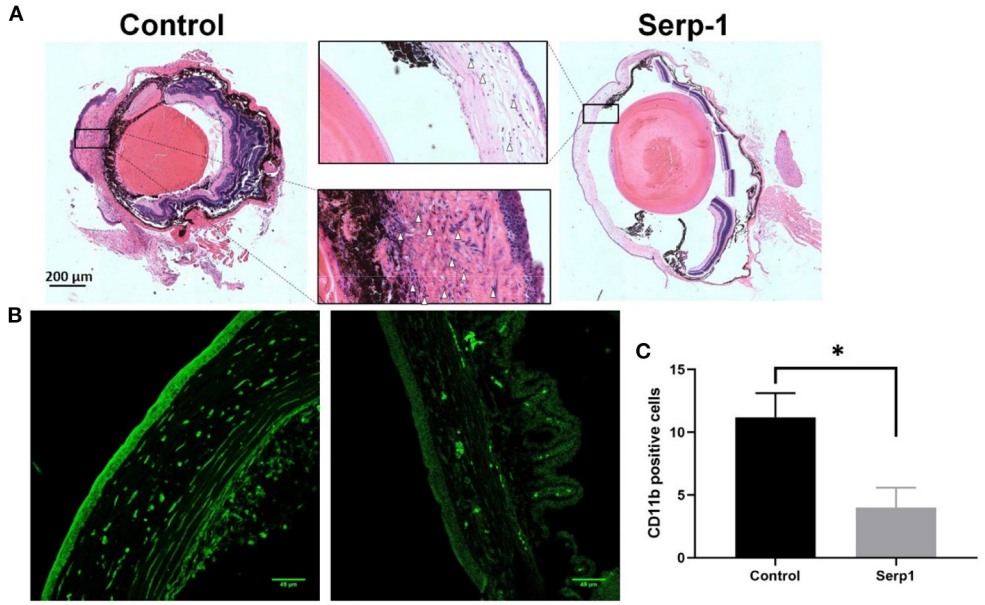


**FIGURE 3 |** Histochemical analysis of eye cross-sections show reduced rates of inflammatory cell infiltration and swelling of the cornea in Serp-1 treated mice. **(A)** Histochemical analysis of eye cross-sections shows reduced rates of inflammatory cell infiltration and swelling of the cornea in Serp-1 treated mice. Enlarged images of the corneal region of two mice visibly show more swelling and a higher presence of inflammatory cells in the control mice. Inflammatory cell nuclei are stained by the deep-purple spots marked with white triangles in the light pink stromal layer of the cornea. **(B)** To further identify infiltrating immune cells, the slides were stained with CD11b (an immune cell marker, green). **(C)** CD11b positive cells (cells/0.1 $mm^2$) were quantified ($n = 3$/group; $^*p < 0.05$).

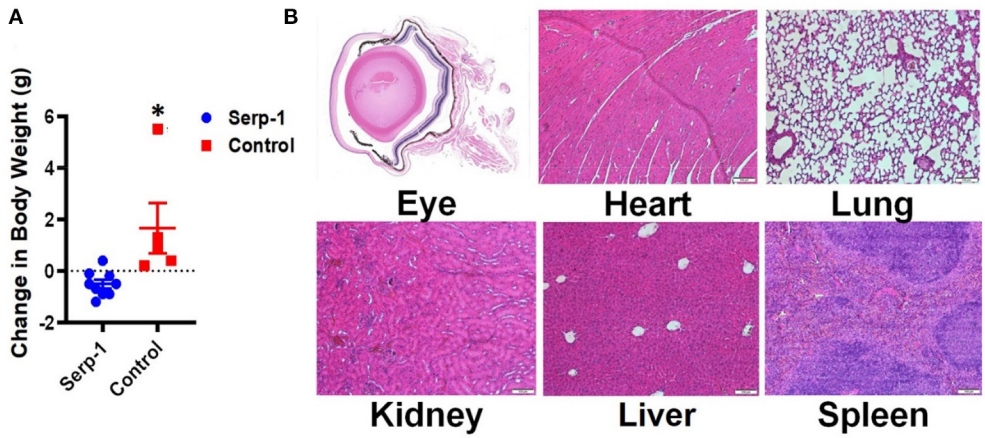


**FIGURE 4 |** Repetitive application of Serp-1 produces no obviously toxic effects. **(A)** Changes of body weight before and after experiments were measured and calculated ($n = 5$ for control group, $n = 9$ for Serp-1 group. $^*P < 0.05$). **(B)** Histochemical analysis of eye cross-sections shows reduced rates of inflammatory cell infiltration and swelling of the cornea in Serp-1 treated mice. Enlarged images of the corneal region of two mice visibly show more swelling and a higher presence of inflammatory cells in the control mice. Inflammatory cell nuclei are stained by the deep-purple spots marked with white triangles in the light pink stromal layer of the cornea.

2 clinical trial in patients with coronary stent implant (23). Future directions as a result of this analysis should include evaluation in larger samples, evaluation of topical applications alone, analysis of interactions with the uPA, MMP proteases after corneal injury and serp-1 treatment, evaluation in other primates, and consideration for clinical trials in humans for an improved understanding of the safety and efficacy of the protein. Further studies should also aim to investigate the molecular mechanism of action of Serp-1 in its anti-inflammatory role. Finally, a recent publication from our group demonstrated that administration of recombinant M-T7 protein (another Myxoma virus protein) could also accelerate dermal wound healing (24). This provides an additional example of a Myxoma virus derived protein in facilitating tissue repair. Thus, future investigations are needed to explore potential Myxoma virus proteins with tissue repair properties.

There is a similar study by Liu et al. elegantly showed topical application of serpinA3K could promote alkali induced corneal wound healing via inhibiting neovascularization and inflammation (25). Thus, it would be interesting to compare the beneficial effects between mammalian serpin and viral serpin in corneal wound healing.

While our study showed beneficial effects of administration of Serp-1 to treat corneal wound in mice, there are some limitations that we will address in our future studies. First, our control treatment group did not use an inactive mutant protein, thus, the beneficial effect we observed in Serp-1 group might be simply due to general protein effect. Our previous publication identified an inactive Serp-1 mutant (26), which would be a preferred negative control for our future study. We are currently working on development of purification protocol for the GMP level mutant Serp-1 for our *in vivo* animal studies in the future. Second, one of direction of our future studies will focus on dissecting the weight loss observe in Serp-1 treatment group. For example, different doses of Serp-1 should be tested and different administration methods should be compared (topical application, intravenous injection, intraperitoneal injection and subcutaneous injection, etc.). Finally, comprehensive toxicology analysis should be included, such as inflammatory cytokine analysis, liver function analysis, potential cardiovascular function analysis and systemic/metabolic disruption of adipose tissue functions or circulating lipids level should be measured.

## CONCLUSIONS

Serp-1 can modulates and enhance the corneal wound healing response. The protein can effectively mitigate inflammation, decrease fibrosis, and reduce neovascularization in the cornea following alkaline-induced injuries in a murine model. Further studies to investigate the biologic mechanism of action of Serp-1 are needed to further outline its role, its safety profile, and define its potential as an alternative to standard of care in the treatment of corneal injuries.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by IACUC of the Ohio State University.

## AUTHOR CONTRIBUTIONS

BJ, OG, MS, BG, SW, and HZ performed the experiments. ARL and HZ designed the studies. BJ, OG, and HZ analyzed the data. BJ, OG, DB-M, JRY, ARL, and HZ wrote the paper. All authors contributed to the article and approved the submitted version.

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# The Higher Prevalence of Venous Thromboembolism in the Hungarian Roma Population Could Be Due to Elevated Genetic Risk and Stronger Gene-Environmental Interactions

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**Background:** Interactions between genetic and environmental risk factors (GxE) contribute to an increased risk of venous thromboembolism (VTE). Understanding how these factors interact provides insight for the early identification of at-risk groups within a population and creates an opportunity to apply appropriate preventive and curative measures.

**Objective:** To estimate and compare GxE for VTE risk in the general Hungarian and Roma populations.

**Methods:** The study was based on data extracted from a database consisting of results previously obtained from a complex health survey with three pillars (questionnaire-based, physical, and laboratory examinations) involving 406 general Hungarian and 395 Roma subjects. DNA was genotyped for rs121909567 (SERPINC1), rs1799963 (F2), rs2036914 (F11), rs2066865 (FGG), rs6025 (F5), and rs8176719 (ABO) polymorphisms. After allele frequency comparisons, the odds ratio (OR) was calculated for individual SNPs. Furthermore, genetic risk scores (weighted GRS, unweighted GRS) were computed to estimate the joint effect of the genetic factors. Multivariable linear regression analysis was applied to test the impact of GxE on VTE risk after interaction terms were created between genetic and VTE risk factors [diabetes mellitus (DM), cancer, chronic kidney diseases (CKD), coronary artery diseases (CAD), migraine, depression, obesity, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein (HDL-C), triglyceride (TG), and smoking].

**Results:** Interestingly, the rs121909567 (SERPINC1, ATBp3 mutation) SNP was not present in the general population at all. However, the risk allele frequency was 1% among the Roma population, which might suggest a founder effect in this minority. This polymorphism multiplicatively interacted with CAD, CKD, cancer, DM, depression,

migraine, and obesity. Even though interactions were not statistically significant, the trend of interaction showed the probability of an incremental VTE risk among the Roma population. The risk of VTE was 4.7 times higher ($p > 0.05$) for Roma subjects who had ≥3 wGRS (median value) compared with individuals having lower wGRS values but lower for the general subjects (OR = $3.1 \times 10^{-8}$). Additionally, the risk of VTE was 6.6 times higher in the Roma population that had ≥3 risk alleles (median value) than in individuals with the 0–1 risk allele, and the overall risk was much higher for the Roma population (OR = 6.6; $p > 0.05$) than for the general Hungarian population (OR = 1.5; $p > 0.05$). Five positive and significant GxE interactions were identified in the Roma population. The risk of VTE was higher among depressive Roma subjects who carried the risk variant rs2036914 ($\beta = 0.819$, $p = 0.02$); however, this interaction was not significant for the general subjects. The joint presence of high levels of LDL-C and rs2066865 (FGG) increased the VTE risk only among Roma individuals ($\beta = 0.389$, $p = 0.002$). The possibility of VTE risk increment, as a result of a multiplicative interaction between rs8176719 (ABO) and cancer, was identified, which was higher for the Roma population ($\beta = 0.370$, $p < 0.001$) than for the general population ($\beta = -0.042$, $p = 0.6$). The VTE risk increased in the Roma population ($\beta = 0.280$, $p = 0.001$), but was higher in the general population ($\beta = 0.423$, $p = 0.001$) as a result of the multiplicative interaction between CAD and rs2036914 (F11). The presence of a multiplicative interaction between rs2066865 (FGG) and CAD increased the VTE risk for the Roma population ($\beta = 0.143$, $p = 0.046$) but not for the general population ($\beta = -0.329$, $p < 0.001$).

**Conclusions:** rs121909567 (SERPINC1, ATBp3) was confirmed as a founder mutation in the Roma population. Our study revealed some evidence on the burden of the joint presence of genetic and environmental risk factors on VTE, although the finding is highly subjected to the selection and observational biases due to the very small number of VTE cases and the observational nature of the study design, respectively. As a result of higher genetic load and GxE interactions, this minority Roma population is at higher risk of VTE than the general Hungarian population. Thus, our results suggest the need for an intensive search for the rs121909567 (SERPINC1; ATBp3) founder mutation, which might be an important factor for the assessment of thrombotic disease susceptibility among the Roma population. In addition, we strongly recommend further studies among a large number of VTE cases to explore the more precise impact of genetic and environmental risk factors on VTE in the study populations.

**Keywords: VTE, GxE interactions, ATBp3 mutation, SERPINC1, Roma population, general Hungarian**

## INTRODUCTION

Venous thromboembolism (VTE) is a multifactorial disease that occurs due to a combination of environmental, behavioral, and genetic risk factors. It contributes to relatively high morbidity and mortality within a short period after its occurrence (1, 2). The genetic basis of VTE is robust, and 50–60% of VTE is attributed to genetics. Family and twin studies confirmed the contribution of inheritable factors to VTE risk (3–5). The risk of recurrent VTE hospitalization among individuals with affected families was 1.92-fold that of the general population (6). An estimated incidence rate of VTE in subjects of European ancestry was 1–2 per 1,000 person/year, of which ∼60% of the cases presented with deep venous thrombosis (DVT), whereas the remaining 40% presented with pulmonary embolism (PE) with or without DVT (7).

The presence of chronic diseases such as cancer, diabetes mellitus (DM), chronic kidney disease (CKD), and coronary artery disease (CAD) increases the likelihood of VTE among individuals (8–12). Furthermore, other personal and environmental risk factors, such as migraine (13–15), obesity, cigarette smoking, depression, high levels of lipoprotein, and antithrombin deficiency, also increase VTE risk. Previously conducted studies indicated that the risk of VTE was two times higher for obese individuals than for normal weight individuals (BMI 20–24.9 kg/m$^2$) (16–18). Additionally, the risk of PE

increases 6-fold among obese individuals compared with normal weight individuals (19). VTE risk is 1.3–1.7 times higher among current cigarette smokers than among non-smokers (20–22). Similarly, cigarette smoking was related to an absolute risk increase of 24.3 VTE cases per 100,000 person/year (22).

Studies have indicated that depression is a plausible risk factor for VTE (23–25); in a cohort study, Lee et al. revealed that the risk of VTE was 1.38 times higher among depressive than non-depressive individuals (23). Another systematic review and meta-analysis study showed that the risk of VTE was 1.3 times higher among depressed subjects than among non-depressed subjects (24).

In addition, a high level of low-density lipoprotein cholesterol (LDL-C) contributes to the occurrence of VTE. González-Ordóñez et al. reported that the risk of VTE was 2-fold higher among individuals with a high level of LDL-C than among individuals with a normal level of LDL-C (26). Another meta-analysis study of randomized control trials (RCTs) indicated that the risk of VTE was reduced among patients who received statin treatment for a high level of LDL-C (27). However, a cohort study reported no statistically significant association between lipoproteins [triglyceride (TG) and LDL-C] and VTE risk (28).

Furthermore, studies have found that antithrombin deficiency plays an important role in the pathogenesis of VTE. Antithrombin is an important inhibitor of blood coagulation proteases; individuals with hereditary AT deficiency have elevated thrombotic risk (29–31). Studies have revealed that the mutation profile of the AT gene (SERPINC1) is heterogeneous (32–35). Formerly, it was found that the prevalence of ATBp3 mutation was relatively high in the Roma population, but not in the general Hungarian population (36).

Likewise, there are populations that are susceptible to cardiovascular diseases due to the coexistence of genetic and environmental risk factors. The Roma are the most marginalized ethnic group in Central–Eastern European countries, with an estimated population of 8–12 million. The Roma experience social exclusion, which intensely affects their health outcomes (37). A higher burden of disease, low life expectancy, low socioeconomic status, low education, and harmful behavior are common among Roma minorities (38–42). As a result, cardiovascular risk factors are prevalent in the Roma population (43, 44). On the other hand, due to the restriction of health-related data collection by ethnicity in the Hungarian Roma population (45), to date, there is no incidence or prevalence data of VTE for this population. However, recent studies indicate that the Roma population is at higher risk of VTE due to an elevated prevalence of metabolic syndrome (46) and several inheritable risk factors (47).

Our previous study concluded that the Roma population seems to have increased genetic susceptibility to VTE. Further investigation has also suggested the necessity of comparing the gene–environmental interaction (GxE) for VTE risk in the general Hungarian and Roma populations (47). Understanding how genetic and environmental risk factors interact provides insight for the early identification of risk groups within populations, allowing appropriate preventive and therapeutic measures to be taken (48, 49). To date, no GxE comparison study has been conducted in the general Hungarian and Roma populations. Thus, the main aim of our current study was to explore the interaction of environmental risk factors with six prothrombotic SNPs [(rs121909567 (SERPINC1), rs1799963 (F2), rs2036914 (F11), rs2066865 (FGG), rs6025 (F5), and rs8176719 (ABO)] (36, 47, 50). In addition, we aimed to investigate the distribution of the rs121909567 (ATBp3) mutation in the SERPINC1 gene in the Hungarian population, which accounts for the vast majority of AT deficiencies in the Hungarian population due to its founder effect.

# METHODS AND MATERIALS

## Study Design

A total of 832 (415 Roma and 417 Hungarian generals) subjects were culled from a comprehensive database created from the data obtained from a complex health survey for comparative and association studies (51). A total of 801 (395 Roma and 406 general Hungarian) subjects who had complete genotype and phenotype data were selected from the current study to assess and compare GxE and VTE risk.

## Study Populations and Data Used

Recently, a complex, three-pillar (questionnaire-based, physical examination, laboratory investigations) health survey was carried out to develop a database for the comparative and association studies to explore the underlying reasons for the very unfavorable health of Roma individuals when compared with the general population, especially as regards their high burden of cardiometabolic diseases. Details of the survey and the database created were published recently (51). Briefly, Roma subjects were recruited randomly from two counties (Szabolcs- Szatmár-Bereg and Hajdú-Bihar) in northeastern Hungary, the place where the Hungarian Roma are predominantly found and where the majority of the segregated Roma colonies are located. The reference group (representing the general population) included randomly selected individuals who lived in private households in the same counties of northeastern Hungary. Individuals aged 20–64 years were included in both groups. Demographic and anthropometric characteristics of the study populations, as well as laboratory data, were published previously (51).

Considering previously published reports on environmental and personal lifestyle risk factors found to affect VTE risk (8, 9, 12, 24, 52–54), the following data were extracted from the database:

(i) For chronic non-communicable diseases (cancer, DM, CAD, and CKD) proven to be risk factors for VTE (8, 12, 55), data were collected by a self-report questionnaire that assessed the history of experiencing those chronic diseases in the last 12 months before the survey. Respondents who replied "Yes" to the question regarding those chronic diseases were considered diseased, and otherwise not. Those respondents who answered "Yes" for the current smoking status were considered smokers. Similarly, the survey questionnaires assessed VTE through three questions: (1) Did you have thrombosis in the last 12 months? (2) Have you been

diagnosed with thrombosis? (3) Have you received hospital treatment for thrombosis? Consequently, if the respondents replied "Yes" to either of those questions, we considered them "VTE cases," whereas those who replied "No" were considered "non-cases."

(ii) Lipid (total cholesterol, LDL-C, high density lipoprotein, and TG) levels were measured.
(iii) Other behavioral (smoking status) and psychosocial status data (depression and migraine histories), and
(iv) Anthropometric measures (BMI and WC) were obtained.

## DNA Isolation

A MagNA Pure LC system (Roche Diagnostics, Basel, Switzerland) with a MagNA Pure LC DNA Isolation Kit-Large Volume was used to isolate DNA from the blood sample according to the instructions of the manufacturer. Extracted DNA was eluted in a 200 μl MagNA Pure LC DNA isolation kit-large volume elution buffer.

## SNPs Selection and Genotyping

Six SNPs, five prothrombotic SNPs [rs1799963 (F2), rs6025 (F5, Leiden), rs2066865 (FGG), rs2036914 (F11), and rs8176719 (ABO)] from our previous study (47), due to their confirmed and large effect size, and the rs121909567 SNP in the SERPINC1 gene, from the so-called antithrombin Budapest3 (ATBp3 mutation) study (36), were included in the present study. ATBp3 is the common cause of antithrombin (AT) deficiency in Hungary, and the founder effect of this mutation was previously considered in the Roma population (36, 56).

The assay design and genotyping were performed by Karolinska University Hospital, Stockholm, Sweden Mutation Analysis Core Facility (MAF). A MassARRAY platform (Sequenom, CA, USA) with iPLEX Gold chemistry was used for genotyping. Quality control, validation, and concordance analysis were conducted by MAF.

## Genetic Risk Score Computations

The weighted and the unweighted genetic risk scores (wGRS, uGRS) were computed to identify the combined effect of the included SNPs on VTE risk. In the GRS, the individuals were assigned a score based on the number of risk alleles they carried. Consequently, "0," "1," and "2" codes were given for the absence of risk alleles and heterozygosity and homozygosity for risk alleles, respectively. When the risk allele was found to be protective, the coding for the homozygous risk allele became "0," whereas it became "2" for the other homozygous allele (47). Accordingly, uGRS was simply computed by adding all risk alleles in the loci assuming that all alleles had the same effect. However, wGRS was computed under the assumption that SNPs with larger effects contributed more to the GRS. Weights were derived from the risk coefficient because each allele depended on the odds ratio (OR) reported in the former genetic association study (50). For this study, only five SNPs that had an effect size in the previously conducted study were included for computing wGRS. Median values of wGRS and uGRS were used to compare the association between genetic risk score and VTE risk factors in the study populations.

## Statistical Analysis

Statistical tests were computed using IBM SPSS Version 25 statistical software. The Shapiro–Wilk normality test was used to test the distribution of quantitative variables. Non-normally distributed variables were transformed using a two-step Templeton's transforming approach (57). The presence of Hardy–Weinberg equilibrium (HWE) and allele frequency differences of all included SNPs in the two study populations were evaluated by using PLINK statistical software Version 1.9. A Bonferroni multiple testing was employed to prevent the problem associated with multiple comparisons. In our study, the level of significance for allele frequencies and individual SNPs comparison in the study populations was declared at an α level of 0.0083 ($n = 6$, $\alpha = 0.05$; $0.05/6 = 0.0083$).

Logistic regression analysis was used to determine the associations between individual SNPs, environmental risk factors, and VTE. A multivariate linear regression analysis with 95% CI was used to test the impact of GxE on VTE risk. An interaction term was created between each SNP and environmental risk factors to assess their combined effect on VTE risk.

Three categories of ORs were defined based on the GxE assumption models: subjects who were unexposed to environmental risk factors and free from risk variants (also known as wild type) (E=G=0), were used as reference groups ($OR_{00}$); $OR_{11}$ represented subjects with both genetic and environmental risk factor exposure (E=G=1); $OR_{10}$ = subjects exposed to environmental risk factors but not with genetic risk (E=1, G=0); and $OR_{01}$ = subjects with only genetic exposure but not environmental risk exposure (E=0, G=1). Thus, under the multiplicative interaction model, if $OR_{11} = OR_{01} \times OR_{10}$, there was no interaction between genetic and environmental risk factors; however, if $OR_{11} \neq OR_{01} \times OR_{10}$, there was a multiplicative interaction between the given environmental risk factors and genetic risk factors, which was either a synergistic interaction ($OR_{11} > OR_{01} \times OR_{10}$) or an antagonistic interaction if $OR_{11} < OR_{01} \times OR_{10}$ (58, 59). For the additive model, no interaction was concluded based on the null hypothesis ($H_0$) of $OR_{11} = OR_{01} + OR_{10} - 1$; however, the interaction would be considered synergistic when $OR_{11} > OR_{01} + OR_{10} - 1$ or antagonistic if $OR_{11} < OR_{01} + OR_{10} - 1$ (58, 59).

During the analysis, the general Hungarian and Roma population samples were combined, and ethnicity was included as a variable in the model to differentiate its effect on VTE risk. For this study, the general Hungarian population was used as a reference population to compare the GxE interaction and VTE risk in the two populations. All analyses were adjusted for age, and the reported $p$ values were two-sided. An α level of 0.05 was used to define the statistical significance.

## Ethical Approval

The Committee of the Hungarian Scientific Council on Health Research approved the protocol (61327-2017/EKU). Written informed consent was obtained from all study subjects.

# RESULTS

## Characteristics of the Study Participants

The 801 study subjects (395 Roma population and 406 Hungarian generals) who had complete genotype and phenotype data were involved in this study. The female proportion in the Roma sample was higher than that in the Hungarian general sample (55.4 vs. 73.9%; $p < 0.001$). Further details on the study population characteristics are presented in **Table 1**.

## VTE Morbidity in the Study Populations

In the current study, only 6 (1.5%) and 12 (3.0%) of the Hungarian general and Roma populations, respectively, reported VTE during the survey. The proportion of VTE cases was higher among the Roma population; in particular, the VTE risk was higher among female subjects (**Table 2**).

## Allele Frequency Comparison of the Study Populations

All included SNPs were checked for HWE; no significant deviation from HWE was observed in the study populations. The allele frequencies of the five prothrombotic SNPs [(rs1799963 (F2), rs2036914 (F11), rs2066865 (FGG), rs6025 (F5), and rs8176719 (ABO)] of the current study (**Table 3**) were not significantly different from those of our previous study (47). Before multiple corrections testing, allele frequencies of rs121909567 (SERPINC1) and rs1799963 (F2) were significantly different among the two populations. However, after adjustment for multiple testing using Bonferroni correction rs121909567 (SERPINC1) allele frequency remains significant among the study populations. The genotype distribution was used to calculate the allele frequencies in the study populations.

## Comparison of GRS

In this study, the wGRS was computed using only five SNPs (**Supplementary Table 1**), which showed a strong association with VTE from previously conducted studies (47, 50). Due to the absence of a published external weight (60) for rs121909567 (ATBp3 mutation), the wGRS computation did not consider this particular SNP. The wGRS ranged from 0.0 to 4.7 and 0.0 to 4.6 for the general Hungarian and Roma populations, respectively. The mean wGRS was $1.8 \pm 0.8$ (95% CI [1.7, 1.9]) for the general Hungarian population and $1.9 \pm 0.76$ (95% CI [1.8, 1.9]) for the Roma population (**Figure 1**). The uGRS was calculated for six SNPs, and it ranged from 0.0 to 7.0 for both populations, with a mean of $2.7 \pm 1.2$; 95% CI [2.6, 2.8] for the general Hungarian population and $2.8 \pm 1.2$; 95% CI [2.7, 2.9] for the Roma population (**Figure 2**).

Although it was not statistically significant, the risk of VTE based on the joint effect of prothrombotic risk alleles was higher for Roma subjects who had ≥3 wGRS (OR = 4.74; 95% CI [0.45, 50.3]) than for individuals with 0–1 wGRS values, but not for the general Hungarian population ($OR = 3.1 \times 10^{-8}$ $p > 0.05$). Additionally, the risk of VTE was 6.6 times higher in the Roma population who had ≥3 risk alleles than in individuals with 0–1 risk alleles, and the risk was much higher for the Roma population (OR = 6.6; $p > 0.05$) than for the general Hungarian population (OR = 1.5; $p > 0.05$).

## Associations of Individual SNPs With VTE

As shown in **Table 4**, only the Leiden mutation (rs6025) and rs2066865 (FGG) were significantly associated with VTE risk and found to be nominally significant for the Roma population, but not for the general population. Study subjects who had homozygous risk alleles for the FGG gene were 5.9 times more at risk for VTE than subjects without a risk allele (OR = 5.9;

**TABLE 1 |** Characteristics of the study participants selected from the database of the complex survey.

| Characteristics | General Hungarian (*N* = 406) (Mean, 95% CI) | Roma (*N* = 395) (Mean, 95% CI) | *p* |
|---|---|---|---|
| Age (years) | 44.3 (43.1–45.5) | 43.5 (42.2–44.7) | 0.35 |
| **Female (%)** | **55.4** | **73.9** | **<0.001** |
| BMI (kg/m$^2$) | 27.2 (26.7–27.7) | 27.5 (26.8–28.2) | 0.48 |
| WC (cm) | 96.0 (94.5–97.5) | 95.0 (93.3–96.7) | 0.38 |
| Cholesterol (mmol/l) | 5.0 (4.9–5.1) | 4.9 (4.8–5.0) | 0.45 |
| LDL-C (mmol/l) | 3.1 (3.0–3.2) | 3.1 (3.0–3.2) | 0.31 |
| **HDL-C (mmol/l)** | **1.4 (1.3–1.4)** | **1.3 (1.2–1.3)** | **<0.001** |
| TG (mmol/l) | 1.6 (1.5–1.7) | 1.7 (1.6–1.9) | 0.29 |
| FBG (mmol/l) | 5.3 (5.1–5.5) | 5.2 (5.0–5.4) | 0.51 |

*BMI, Body mass index; WC, waist circumference; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High density lipoprotein cholesterol; TG, Triglycerides; FBS, Fasting blood glucose. Significance differences are highlighted in bold.*

**TABLE 2 |** Distribution of VTE cases by population and sex among the study subjects.

| Sex | General Hungarian (*N* = 406) | | Roma (*N* = 395) | |
|---|---|---|---|---|
| | Yes (%) | No (%) | Yes (%) | No (%) |
| Male | 4 (2.2) | 177 (97.8) | 4 (3.9) | 99 (96.1) |
| Female | 2 (0.9) | 223 (99.1) | 8 (2.7) | 284 (97.3) |
| Total | 6 (1.5) | 400 (98.5) | 12 (3.0) | 383 (97.0) |

**TABLE 3 |** Comparison of effect allele frequencies (%) in the general Hungarian and Roma populations.

| Gene | SNPs | Risk allele | General Hungarian (*N* = 406), frequency (%) | Roma (*N* = 395), frequency (%) | *p* |
|---|---|---|---|---|---|
| **SERPINC1** | **rs121909567** | **A** | **0 (0%)** | **0.01 (1%)** | **<0.001** |
| F2 | rs1799963 | A | 0.02 (2%) | 0.01 (1%) | 0.04 |
| F11 | rs2036914 | C | 0.54 (54%) | 0.51 (51%) | 0.2 |
| FGG | rs2066865 | A | 0.23 (23%) | 0.28 (28%) | 0.05 |
| F5 | rs6025 | T | 0.07 (7%) | 0.09 (9%) | 0.2 |
| ABO | rs8176719 | G | 0.47 (47%) | 0.48 (48%) | 0.6 |

*Significant differences are highlighted in bold.*

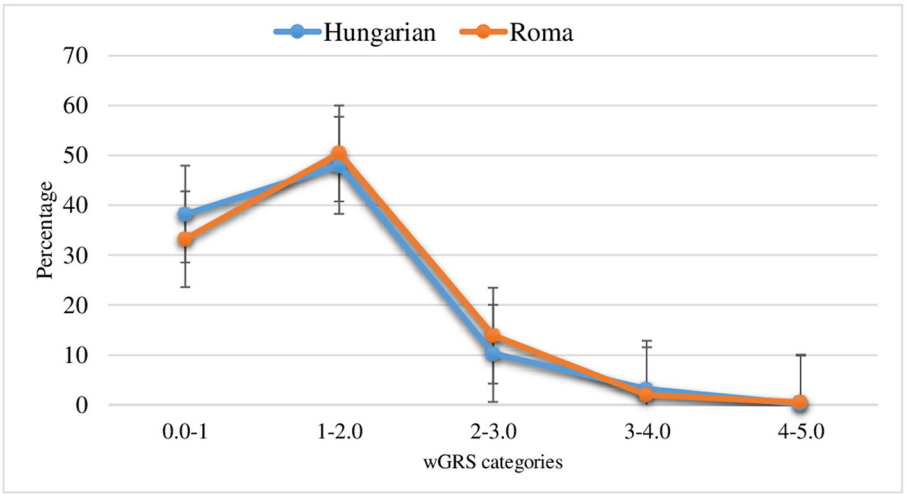


**FIGURE 1 |** Distribution of wGRS in the general Hungarian and Roma population (The error bars indicate the standard error of the mean).

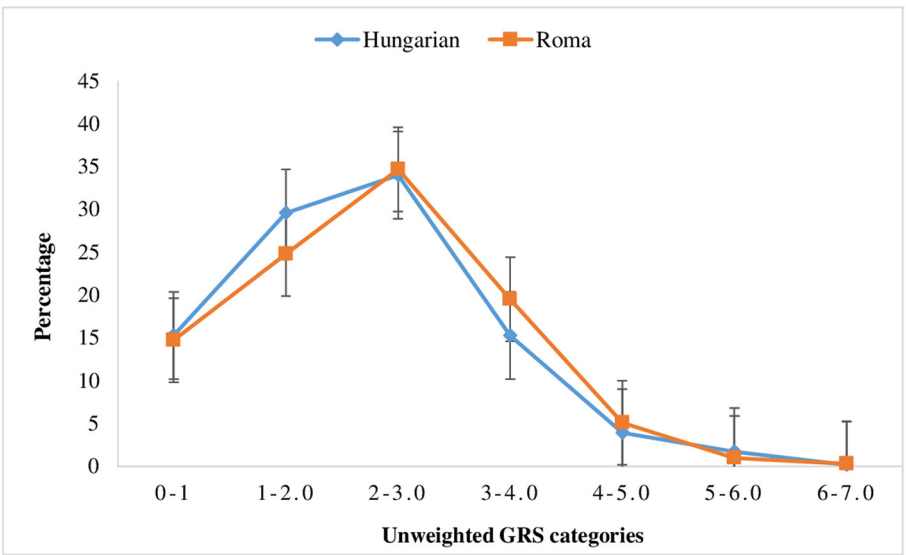


**FIGURE 2 |** Distribution of un-weighted genetics risk scores in the general Hungarian and Roma population (the error bars indicate the standard error of the mean).

95% CI: [1.23, 28.4]). Furthermore, people with the heterozygous risk allele of the Leiden mutation (rs6025) were 3.8 times more likely to develop VTE than individuals without the risk allele, and the risk was higher for the Roma population. However, this significance does not exist ($p > 0.0083$) after we adjust the test using Bonferroni multiple correction testing. Furthermore, our study reveals that some individuals who had VTE were carriers for homozygous risk variants of multiple SNPs. In our study, three Roma subjects who had VTE were carriers of the homozygous risk variant rs2036914 (F11), and another three individuals carried the homozygous variant of rs2066865 (FGG), as depicted in **Table 4**. However, two out of the six Roma subjects, who had VTE and were carriers for homozygous risk variants of rs2036914 (F11) and rs2066865 (FGG), also carried homozygous risk variants of both SNPs.

## GxE and VTE Risk

As indicated in **Supplementary Table 2**, multivariate linear regression analysis was performed to explore the impact of GxE on VTE risk among the study subjects. The finding of the GxE and VTE risk in our study was reported using the standardized beta value of the multivariate linear regression analysis.

We found several statistically significant multiplicative interactions on VTE risk for the majority of the included SNPs, such as rs2036914 (F11), rs2066865 (FGG), rs6025 (F5, Leiden), and rs8176719 (ABO). Since the ATBp3 mutation was not

**TABLE 4 |** Comparisons of associations between individuals SNPs and VTE in the general Hungarian and Roma populations.

| | | VTE cases | | | | | |
|---|---|---|---|---|---|---|---|
| | | General Hungarian | | | Roma | | |
| Gene (SNPs) | Genotype | Yes | No | OR (95% CI) | Yes | No | OR (95% CI) |
| SERPINC1 (rs121909567) | G G | 6 (1.5) | 400 (98.5) | NA‡ | 12 (3.1) | 372 (96.9) | 1.00 |
| | G A | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 11 (100) | 2.2E-8 |
| F2 (rs1799963) | G G | 6 (1.5) | 384 (98.5) | 1.00 | 12 (3.1) | 377 (96.9) | 1.00 |
| | G A | 0 (0.0) | 16 (100) | 4.4E-8 | 0 (0.0) | 6 (100.0) | 2.9E-8 |
| F11 (rs2036914) | T T | 1 (1.2) | 83 (98.8) | 1.00 | 3 (3.0) | 97 (97.0) | 1.00 |
| | C T | 4 (2.0) | 201 (98.0) | 1.4 (0.13, 13.9) | 6 (3.2) | 184 (96.8) | 0.96 (0.22, 4.2) |
| | C C | 1 (0.9) | 116 (99.1) | 0.49 (0.03, 9.5) | 3 (2.9) | 102 (97.1) | 1.15 (0.21, 6.4) |
| FGG (rs2066865) | G G | 2 (0.9) | 233 (99.1) | 1.00 | 5 (2.4) | 201 (97.6) | 1.00 |
| | G A | 3 (2.0) | 149 (98.0) | 2.5 (0.39, 16.2) | 4 (2.5) | 156 (97.5) | 0.95 (0.24, 3.8) |
| | A A | 1 (5.3) | 18 (94.7) | 5.6 (0.43, 73.9) | 3 (10.3) | 26 (89.7) | 5.9 (1.23, 28.4)* |
| F5 (rs6025) | C C | 4 (1.1) | 347 (98.9) | 1.00 | 8 (2.4) | 323 (97.6) | 1.00 |
| | C T | 2 (3.7) | 52 (96.3) | 3.5 (0.57, 21.3) | 4 (6.7) | 56 (93.3) | 3.8 (1.01, 14.2)* |
| | T T | 0 (0.0) | 1 (100) | 1.1E-7 | 0 (0.0) | 4 (100.0) | 1.1E-8 |
| ABO (rs8176719) | C C | 5 (4.2) | 114 (95.8) | 1.00 | 5 (4.7) | 101 (95.3) | 1.00 |
| | C DEL | 1 (0.5) | 193 (99.5) | 0.11 (0.01, 0.9) | 5 (2.5) | 192 (97.5) | 0.49 (0.13, 1.9) |
| | DEL DEL | 0 (0.0) | 93 (100.0) | 1.6E-8 | 2 (2.2) | 90 (97.8) | 0.43 (0.08, 2.5) |

‡*No risk allele found in the general Hungarian for rs121909567 SNP,* $*p < 0.05$.

present in the general population, regression analysis was only performed for the Roma population. Although the trend of their relationships indicated the possibility of higher VTE risk, the multiplicative interaction was not statistically significant for the SERPINC1 (rs121909567) and F2 (rs1799963) genes ($p$-value for interaction >0.05). According to the regression coefficient of the multivariate linear regression analysis, the likelihood of VTE was higher among the study subjects who had cancer, DM, CAD, and CKD, plus experienced migraine and depression in addition to their genetic susceptibility to VTE. Furthermore, cigarette smoking, a high level of LDL-C, and obesity increased the VTE risk for the study subjects.

**Figure 3** shows that the standardized beta ($\beta$) values of the GxE and VTE risk from the linear regression analyses were statistically significant in both the study populations. The observed multiplicative interaction and VTE risk were bidirectional: a positive beta ($\beta$) value indicated a VTE risk increment as a result of GxE (red color), whereas a negative beta ($\beta$) value indicated the reverse (green color).

The risk of VTE was higher ($\beta = 0.819$, $p = 0.02$) among depressive Roma individuals with the rs2036914 risk variant, and it was statistically significant, but not for the general Hungarian individuals ($\beta = 0.343$, $p = 0.33$). The presence of high levels of LDL-C and the rs2066865 (FGG) risk variant makes Roma subjects at higher risk of VTE ($\beta = 0.389$, $p = 0.002$); however, the joint presence of those risk factors did not increase the VTE risk in the general subjects ($\beta = 0.048$, $p = 0.70$). The existence of a multiplicative interaction between CAD and rs2036914 (F11) increases the VTE risk among both the populations: the Roma population ($\beta = 0.280$, $p = 0.001$) and the general Hungarian population ($\beta = 0.423$, $p = 0.001$).

As a result of the multiplicative interaction between rs2066865 (FGG) and CAD, VTE risk was higher for the Roma population ($\beta = 0.143$, $p = 0.046$), but not for the general Hungarian population ($\beta = -0.329$, $p < 0.001$). Nonetheless, the interaction between this particular SNP and depression was not positively related to VTE risk ($\beta = -0.160$, $p = 0.046$) for the Roma or general Hungarian population ($\beta = -0.119$, $p = 0.11$). The interaction between rs6025 (F5, Leiden) and smoking ($\beta = 0.172$, $p = 0.008$), and also Leiden and LDL-C ($\beta = 0.368$, $p = 0.001$) increased the risk of VTE for the general population only, but not for the Roma population ($\beta = -0.014$, $p = 0.86$ and $\beta = -0.150$, $p = 0.55$, respectively).

Our study also identifies the higher risk of VTE as a result of a multiplicative interaction between rs8176719 (ABO) and cancer, and the risk was higher for the Roma population ($\beta = 0.370$, $p < 0.001$) than for the general Hungarian population ($\beta = -0.042$, $p = 0.6$). Nevertheless, the interaction of rs8176719 (ABO) with CAD, ($\beta = 0.197$, $p = 0.009$), migraine ($\beta = 0.287$, $p = 0.001$), and depression ($\beta = 0.342$, $p < 0.001$) significantly increased VTE risk only for the general Hungarian population. The risk of VTE was higher for general Hungarian subjects ($\beta = 0.194$, $p < 0.01$) who had diabetes mellitus and non O blood type, but not for the Roma subjects ($\beta = -0.039$, $p = 0.63$) (**Figure 3**).

## Association Between GRS and VTE Risk Factors

Weighted GRS was computed for five SNPs that were strongly associated with VTE in former studies. Logistic regression analysis was performed to test the relationships between wGRS, uGRS, and VTE risk factors. The effects of the combined genetics

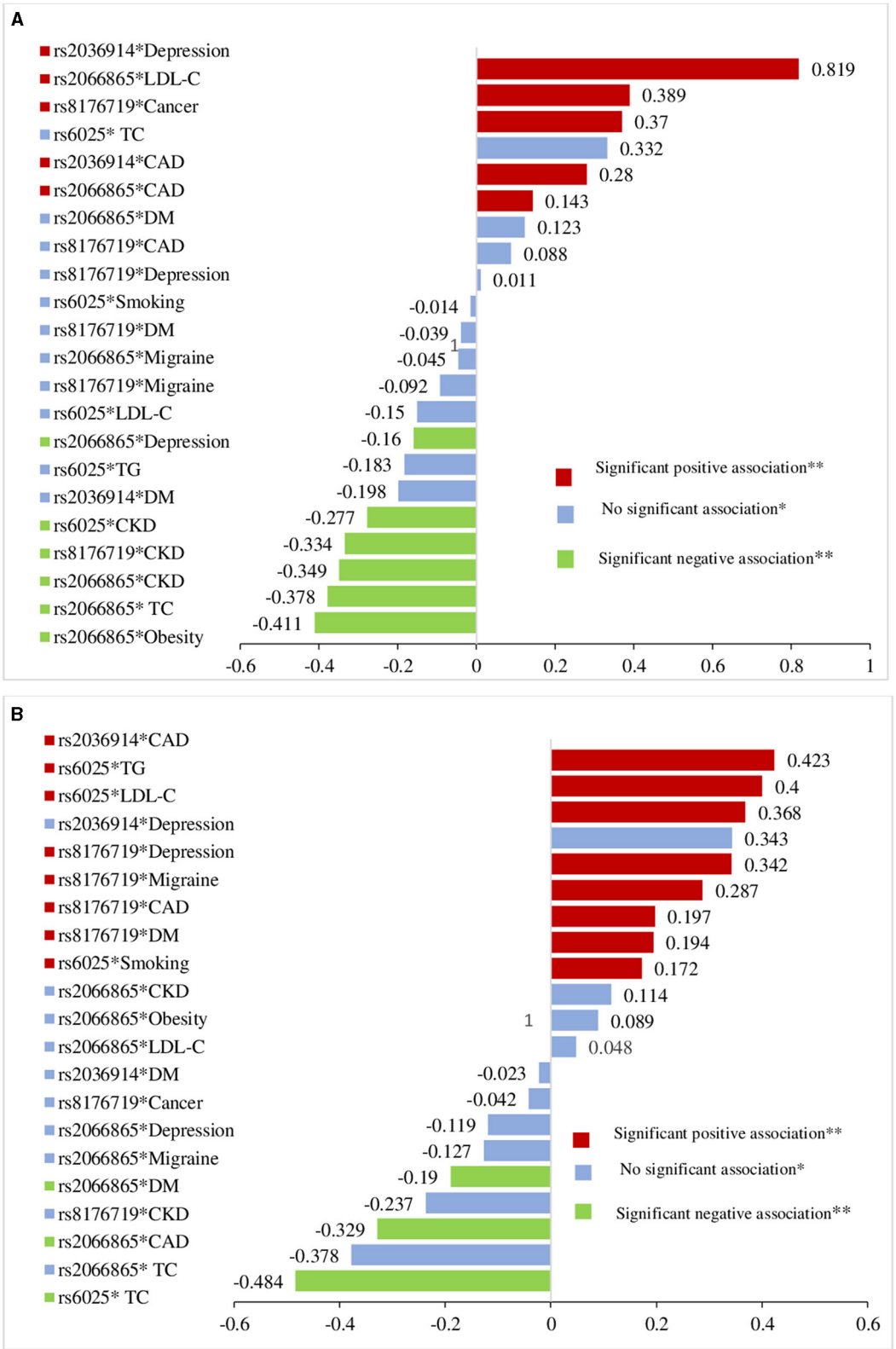


**FIGURE 3 |** Comparison of G × E on the VTE risk among Roma population **(A)** and general Hungarian **(B)** based on standardized linear regression coefficients from multivariate linear regression analysis after interaction terms included between genes and VTE risk factors.

risk factors and a high level of LDL-C, migraine, and current cigarette smoking were additive and statistically significant for the Roma population. As revealed in **Table 5** below, a high level of LDL-C (OR = 3.2; 95% CI [1.2, 8.8]) and migraine (OR = 3.9; 95% CI [1.1, 12.3]) increased the risk of VTE in the Roma population but not in the general population.

Furthermore, this study indicated that the risk of VTE was reduced among study participants who had ever smoked cigarettes but quit cigarette smoking <1 year (OR = 0.1; 95% CI [0.0, 0.9]) and more than 1 year (OR = 0.2; 95% CI [0.1, 0.6]). The risk of VTE among Roma individuals who had >3.0 wGRS, but not currently smoking was reduced by 10 and 20%, respectively, but not for the general Hungarian individuals. However, cigarette smoking did not have a statistically significant additive effect on the VTE risk (OR = 1.9; 95% CI [0.9, 3.8]).

For the unweighted GRS, only a high level of LDL-C (OR = 2.2, 95% CI [1.0, 4.7]) was associated with VTE risk in an additive model, and the risk was higher for the Roma population but not for the general Hungarian population (OR = 1.5; 95% CI [0.7, 3.2]) (**Table 6**).

## DISCUSSION

A total of 395 Roma and 406 general subjects with full clinical and genotype records were involved in the analyses. We assessed interactions between six prothrombotic SNPs [rs121909567 (SERPINC1), rs1799963 (F2, prothrombin G20210A), rs6025 (F5, Leiden), rs2066865 (FGG), rs2036914 (F11), and rs8176719 (ABO)] and environmental factors that were proven to be risk factors for VTE (8–10, 12, 16–24, 53, 61). Our study is the first to investigate and compare GxE in populations of Hungarians (Roma vs. general) and found evidence for higher GxE and VTE risk among Roma individuals.

The current study revealed that GxE and VTE risk was predominantly common among a group of populations with a specific SNP but not among others; a statistically significant multiplicative interaction was observed between the rs8176719 (ABO) gene and diabetes mellitus, migraine, depression, and CAD for the general Hungarian population, but only with cancer for the Roma population. The coefficient of multiplicative interactions of diabetes mellitus and rs8176719 (ABO) was positive and significant, indicating that non-O blood type general Hungarian individuals who had diabetes mellitus were more likely to have a higher VTE risk. This finding was supported by a systematic review and meta-analysis of cohort studies that indicated that diabetes mellitus increases the VTE risk 1.4 times compared with subjects without diabetes mellitus (62). Conversely, another systematic review and meta-analysis of case-control studies (63) reported no significant association. This discrepancy might be due to the study design and sample size they considered for analysis.

Our study demonstrated that subjects experiencing migraine and non-O blood types were at higher risk of developing VTE. This finding is in line with studies that indicated that the presence of migraine increased VTE risk by 1.3-(14), 1.5-(13, 15), and 2.5-(61) fold. The present study also suggests that VTE risks increased among depressive individuals with risk variants for rs2036914 (F11) among Roma subjects and rs8176719 (ABO)

**TABLE 5 |** Association between wGRS and VTE risk factors.

| | General Hungarian ($N = 406$) | | Roma ($N = 395$) | |
|---|---|---|---|---|
| **VT risk factors** | **OR (95% CI)** | ***p*** | **OR (95% CI)** | ***p*** |
| TC[a] | 1.6 (0.3–8.0) | 0.57 | 1.5 (0.5–4.8) | 0.47 |
| **LDL-C[b]** | 1.8 (0.4–9.1) | 0.47 | **3.2 (1.2–8.8)** | **0.02** |
| HDL-C[c] | 0.7 (0.1–3.8) | 0.69 | 1.2 (0.4–3.7) | 0.81 |
| TG[d] | 3.1 (0.5–18.9) | 0.22 | 2.3 (0.6–8.6) | 0.22 |
| **Migraine[e]** | 3.0 (0.7–13.8) | 0.15 | **3.9 (1.1–12.3)** | **0.03** |
| Currently smoking cigarettes[f] | 1.0 (0.5–2.0) | 0.91 | 1.9 (0.9–3.8) | 0.06 |
| **Cessation of smoking for <1 year[g]** | 1.1 (0.1–8.9) | 0.95 | **0.1 (0.0–0.9)** | **0.04** |
| **Cessation of smoking for >1 year[h]** | 1.9 (0.4–8.2) | 0.42 | **0.2 (0.1–0.6)** | **0.004** |
| Obesity (BMI > 30 kg/m$^2$) | 1.2 (0.5–3.0) | 0.64 | 1.7 (0.7–3.8) | 0.2 |

[a] *Total cholesterol ≥ 5.2 mmol/L.*
[b] *LDL-C level ≥ 3.4 mmol/L.*
[c] *HDL-C < 1.3 mmol/L.*
[d] *TG ≥1.7 mmol/L.*
[e] *Reported as they had migraine in the past 12 month.*
[f] *Currently smoking cigarette.*
[g] *Ever smoked cigarette but stopped smoking for less than a year.*
[h] *Ever smoked cigarette but stopped smoking for more than a year.*
*Significant differences are highlighted in bold.*

**TABLE 6 |** Association between uGRS and VTE risk factors.

| | General Hungarian ($N = 406$) | | Roma ($N = 395$) | |
|---|---|---|---|---|
| **VT risk factors** | **OR (95%CI)** | ***p*** | **OR (95% CI)** | ***p*** |
| TC[a] | 1.5 (0.3–7.7) | 0.60 | 1.5(0.5–4.8) | 0.48 |
| **LDL-C[b]** | 1.5 (0.7–3.2) | 0.36 | **2.2 (1.0–4.7)** | **0.04** |
| HDL-C[c] | 0.7 (0.1–3.6) | 0.62 | 1.2 (0.4–3.8) | 0.79 |
| TG[d] | 3.3 (0.5–20.0) | 0.19 | 2.3 (0.6–8.5) | 0.22 |
| Migraine[e] | 1.0 (0.5–2.3) | 0.96 | 1.4 (0.7–2.7) | 0.32 |
| Currently smoking cigarettes[f] | 1.1 (0.6–1.8) | 0.76 | 1.5 (0.9–2.4) | 0.09 |
| Cessation of smoking for <1 year[g] | 1.6 (0.3–8.9) | 0.6 | 0.2 (0.02–1.8) | 0.2 |
| Cessation of smoking for >1 year[h] | 1.2 (0.5–2.8) | 0.7 | 1.1 (0.4–3.1) | 0.9 |
| Obesity (BMI >30 kg/m$^2$) | 1.6 (0.9–3.1) | 0.1 | 1.1 (0.6–1.8) | 0.8 |

[a] *Total cholesterol ≥ 5.2 mmol/L.*
[b] *LDL-C level ≥ 3.4 mmol/L.*
[c] *HDL-C < 1.3 mmol/L.*
[f] *TG≥1.7 mmol/L.*
[e] *Reported as they had migraine in the past 12 month.*
[f] *Currently smoking cigarette.*
[g] *Ever smoked cigarette but stopped smoking for less than a year.*
[h] *Ever smoked cigarette but stopped smoking for more than a year.*
*Significant differences are highlighted in bold.*

among general Hungarian subjects. This result is in accordance with previous investigations indicating that depression increases VTE risk (23, 24). Moreover, the risk of developing VTE was six to seven times higher in cancer patients (7, 64, 65). This result was consistent with our study findings which reveal that the VTE risk is higher for the Roma subjects who had cancer.

The VTE risk was higher among non-O blood type Roma subjects who had cancer as well. Our study also confirmed other studies (10, 66) which showed that the presence of cancer increased the risk of VTE in addition to other VTE risk factors. Further studies on cancer and prothrombotic genotypes point out that VTE risk increased by 11–12-fold as a result of the simultaneous presence of cancer and rs8176719 (ABO) risk variant (67, 68). The authors also found that 39% (67) and 30% (68) of VTE risk was attributed to the joint presence of cancer and non-O-blood type.

An earlier study (64) found that the presence of cancer and the Leiden mutation (rs6025) variant increases the VTE risk by 2-fold; this concurred with our result, although the association was not statistically significant. The lack of significance might be due to the small proportion of individuals with cancer and VTE, the study design, and the relatively small sample size.

The multiplicative interaction coefficient of Leiden mutation (rs6025) and cigarette smoking was positive and statistically significant. The possibility of VTE risk was higher in the general Hungarian subjects who smoke cigarettes and carried the risk variant for the Leiden mutation (rs6025). Prior studies also revealed that the combined effect of rs6025 (F5) and smoking increased the risk of VTE (69, 70). A large population-based case-control study also supported our finding, where the joint effect of rs6025 (F5) and current smoking resulted in a 5-fold increased VTE risk (71). Another cohort study revealed that the simultaneous presence of smoking in addition to rs6025 (F5) increased the VTE risk by 51 and 10% at 10 years for homozygous and heterozygous risk variants, respectively (72). Crous-Bou et al. also found an additive interaction between prothrombotic SNPs and smoking that increased VTE risk (53).

Study subjects with coronary artery diseases and rs2036914 (F11) or rs8176719 (ABO) were at higher risk of VTE than subjects without CAD and those variants. The presence of an interaction between rs2036914 (F11) and CAD increased the VTE risk among both the study populations who carried those risk factors. Sejrup et al. found that myocardial infarction patients with $\geq$1 risk allele at rs2036914 (F11) had a 1.8-fold higher risk of PE (73). Furthermore, the risk of VTE was 1.5-fold higher among individuals with non-O blood type and myocardial infarction (73). Our finding was also consistent with this study.

Additionally, the current study also presented an additive interaction between a high level of LDL-C, migraine, current cigarette smoking, and $\geq$3 wGRS value. The risk of VTE increased by 3.2-fold for the Roma individuals with a high level of LDL-C and $\geq$3 risk alleles. This result was in line with a GWAS (74) which revealed that one standard deviation (SD) of elevated LDL-C was associated with an increased risk of VTE.

The finding of current cigarette smoking was in agreement with the study of Crous-Bouet al., in which the relationships between current smoking and VTE genetic factors were additive (53). In our study, individuals who had experience with migraine in addition to a wGRS value of $\geq$3 had 3.9 times higher risk for VTE than individuals with either a wGRS$\geq$3 or migraine. This finding was in agreement with a study by Peng et al., who revealed that migraine headaches increased the risk of VTE (61).

Although the multiplicative interaction between rs121909567 (SERPINC1), rs1799963 (F2), and VTE risk factors was not statistically significant, and their regression coefficient indicated the possibility of higher VTE risk among individuals who have dual exposures. Interestingly, rs121909567 (SERPINC1) (ATBp3 mutation) multiplicatively interacted with CAD, CKD, cancer, DM, depression, migraine, and obesity. Even though their relationships were not statistically significant, the trend of interaction showed the probability of VTE risk increment among the Roma population. The lack of statistical significance between GxE and VTE risk for rs121909567 (SERPINC1) and rs1799963 (F2) might be due to the very small number of VTE cases.

Similarly, studies have shown that the prevalence of cardiovascular risk factors was higher in the Roma population (43, 44), and we found that in addition to environmental factors, genetic susceptibility contributes to high cardiovascular mortality/morbidity (75). In a relatively isolated population such as the Roma, the consanguinity rate is high (76); consequently, it was assumed that a founder mutation had an impact on the development of thrombotic diseases (36). A recent study also identified a high prevalence (2.74%) of ATBp3 mutation in the Roma population; however, no ATBp3 mutation was found in the general Hungarian population (56). This finding was in line with our study results, which revealed that ATBp3 mutations were found only in the Roma population but not in the general Hungarian population.

Altogether, rs121909567 (SERPINC1, ATBp3) was confirmed as a founder mutation among the Roma population. The present study also reveals some clues about the burden of the joint presence of genetic and environmental risk factors on VTE risk, although the finding was highly subject to selection and observational biases due to the very small number of VTE cases and the observational nature of the study design, respectively. As a result of higher genetic load and GxE interactions, this minority Roma population is at higher risk of VTE than the general Hungarian population. Thus, our results suggest that an intensive search for the rs121909567 (SERPINC1; ATBp3) founder mutation might be an important factor for the assessment of thrombotic disease susceptibility among the Roma population. In addition, we strongly recommend further studies among a large number of VTE cases to explore the more precise impact of genetic and environmental risk factors on VTE in the study populations.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee of the Hungarian Scientific Council on Health Research (61327-2017/EKU). Written informed consent was obtained from all study subjects. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

RÁ: conceptualization. SN and SF: data handling, writing, and interpreting the results, and preparing the manuscript. SN and MM: statistical analysis. ZK, PP, and JS: data collection and management. SN, SF, ZB, and RÁ: review, editing, and finalizing the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.647416/full#supplementary-material

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