

# Recent Advances on the Molecular Mechanism and Clinical Trials of Venous Thromboembolism

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**Abstract:** Venous thromboembolism is a condition that includes deep vein thrombosis and pulmonary embolism. It is the third most common cardiovascular disease behind acute coronary heart disease and stroke. Over the past few years, growing research suggests that venous thrombosis is also related to the immune system and inflammatory factors have been confirmed to be involved in venous thrombosis. The role of inflammation and inflammation-related biomarkers in cerebrovascular thrombotic disease is the subject of ongoing debate. P-selectin leads to platelet-monocyte aggregation and stimulates vascular inflammation and thrombosis. The dysregulation of miRNAs has also been reported in venous thrombosis, suggesting the involvement of miRNAs in the progression of venous thrombosis. Plasminogen activator inhibitor-1 (PAI-1) is a crucial component of the plasminogen-plasmin system, and elevated levels of PAI-1 in conjunction with advanced age are significant risk factors for thrombosis. In addition, it has been showed that one of the ways that neutrophils promote venous thrombosis is the formation of neutrophil extracellular traps (NETs). In recent years, the role of extracellular vesicles (EVs) in the occurrence and development of VTE has been continuously revealed. With the advancement of research technology, the complex regulatory role of EVs on the coagulation process has been gradually discovered. However, our understanding of the causes and consequences of these changes in venous thrombosis is still limited. Therefore, we review our current understanding the molecular mechanisms of venous thrombosis and the related clinical trials, which is crucial for the future treatment of venous thrombosis.

**Keywords:** venous thromboembolism, miRNA, neutrophil extracellular traps, plasminogen activator inhibitor-1, EVs, inflammatory factor

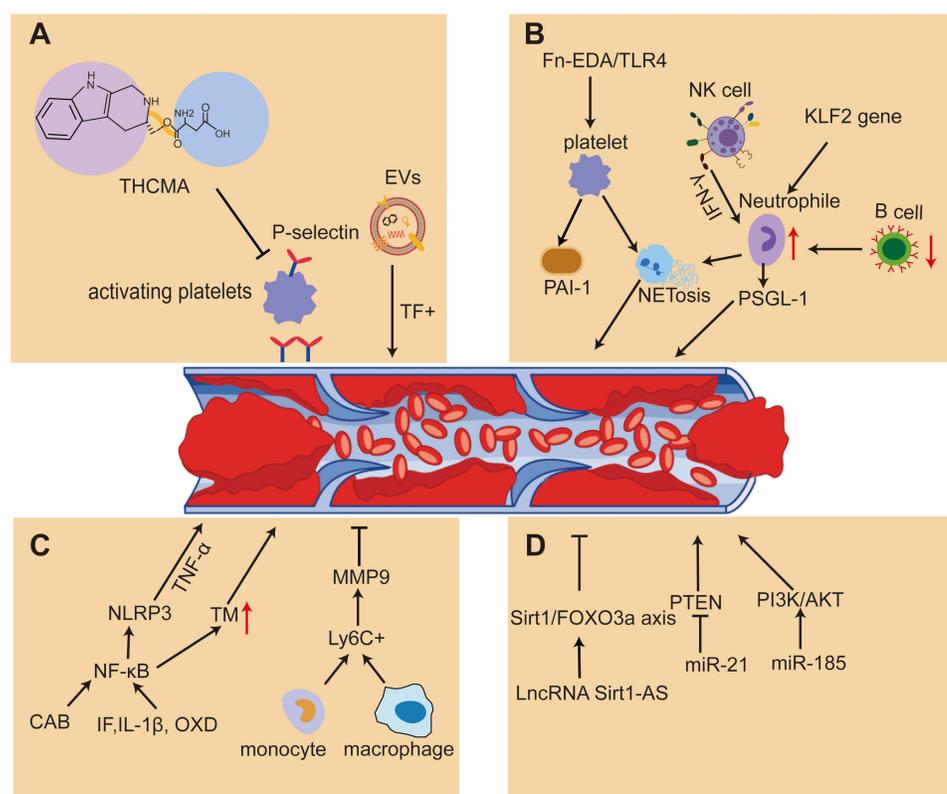
## Introduction

Venous thromboembolism (VTE) is a multifaceted, potentially fatal event that activates coagulation and fibrinolysis.<sup>1</sup> It is a condition that includes pulmonary embolism (PE) and deep vein thrombosis (DVT).<sup>2</sup> In addition, it is also the third most prevalent cardiovascular disease after stroke and coronary heart disease (CHD).<sup>3</sup> Venous thrombosis (VT) is a leading cause of mortality as well as morbidity worldwide, occurring in approximately one case in 1000 people per year in affluent nations.<sup>4</sup> DVT is the thrombosis that blocks the deep venous cavity, disrupting the venous reflux network and resulting in chronic deep venous dysfunction.<sup>5,6</sup> Furthermore, DVT is a consequence of a complicated interaction between enzymes and cellular processes, in which the endothelium, platelets, and leukocytes coordinate a pro-inflammatory state that ultimately leads to the clot formation<sup>7</sup> with higher mortality rate for acute DVT.<sup>8</sup> To date, vascular ultrasound and digital subtraction

angiography are the primary techniques for diagnosing DVT. The lower extremity deep venous thrombosis (LEDVT) is diagnosed clinically based on venography, the gold standard.<sup>9</sup> Even though both techniques have a high diagnostic value, the latter is quite intrusive and expensive, while the former lacks the ability to diagnose intraperitoneal venous embolism.<sup>10</sup> In addition, ultrasound is not a routine and is only performed before discharge for patients with symptoms following major orthopedic surgery.<sup>11</sup> The absence of specific clinical signs and non-specific symptoms associated with venous thrombosis may lead to a delayed or inaccurate diagnosis, ultimately resulting in poor patient prognosis.<sup>12,13</sup> Both genetic and acquired factors can lead to the formation of venous thrombosis, which is a complex process whose molecular mechanism remains poorly understood. Therefore, exploring the mechanism is essential for effective treatment of venous thrombosis. Recently, there has been a lot of discussion on the mechanisms of venous thrombosis, including inflammatory, immunological, and neutrophil extracellular trap formation aspects, other factors under investigation include age, p-selection, MicroRNA(miRNA), PAI-1-induced venous thrombosis and extracellular vesicles (EVs). We have outlined these relevant molecular mechanisms involved in VTE (Figure 1), which may be an important target for future therapeutic interventions.

## Plasminogen Activator Inhibitor-1

The main protein of plasminogen-plasminase system, plasminogen activator inhibitor-1 (PAI-1), is a significant inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (U-PA).<sup>14</sup> Inflammation stimulates



**Figure 1** Mechanisms of venous thrombosis through PAI-1, inflammatory factors, miRNA, age-related changes, NETs formation, P-selectin activation and immunological processes. **(A)** THCMA inhibits platelet activation and aggregation by P-selectin to reduce thrombosis and tissue factor-positive (TF<sup>+</sup>) EVs are associated with VTE **(B)** Exogenous cellular Fn-EDA stimulated NETosis in neutrophils via TLR4-stimulated thrombin-activated platelets and TLR4 contributes to Fn-EDA-mediated DVT. B-cell deficiency leads to an increase in circulating neutrophils and an increased abundance of NETs within the thrombus. NK cells promote venous thrombolysis by activating neutrophils and producing IFN- $\gamma$ -dependent NETs. **(C)** Ly6C<sup>+</sup> monocytes and macrophages release IL-6 that induce mononuclear cell sources of MMP 9, and MMP 9 participates in the process of thrombolysis. IL-1 $\beta$ , TF, XOD, GAB, and TNF- $\alpha$  regulate venous thrombolysis through the NLRP 3 / IL-1 / NF- $\kappa$ B signaling mechanism. **(D)** Mir-185 inhibits thrombolysis by regulating PI3K/Akt signaling pathway, which inhibits inflammation-induced tissue factor expression in deep vein endothelial cells. Mir-21 inhibits the expression of PTEN, increases the proliferation of endothelium and promotes the formation of new blood vessels and venous thrombolysis. LncRNA SIRT1-AS reduces the incidence of aging-associated DVT by Sirt1/FOXO3a axis.

**Abbreviations:** THCMA, 3S-1,2,3,4-tetrahydro- $\beta$ -carboline-3-methyl aspartyl ester; EVs, extracellular vesicles; Fn-EDA, fibronectin containing extra domain A; TLR4, toll-like receptor 4; DVT, deep vein thrombolysis; NETs, neutrophil extracellular traps; MMP 9, matrix metalloproteinase 9; TF, tissue factor; XOD, xanthine oxidase; GAB, Grb2-associated binding.

endothelial cells to release tPA and PAI-1 locally. Besides, since platelets are the major circulating pool of PAI-1, activated platelets release a great number of PAI-1, leading to locally high levels of PAI-1 at growing fibrin clot sites,<sup>15,16</sup> thus increased PAI-1 is a risk factor for thrombosis. In addition, the polymorphism of RS1799889 in the promoter region of the PAI-1 gene has been detected in patients with thrombosis.<sup>17</sup> However, there is no association between elevated levels of PAI-1 and the risk of venous thrombosis in recent study.<sup>18</sup> The 5G allele is associated with reduced PAI-1 transcript levels compared with 4G allele, so the presence of the 5G allele may lead to a reduced risk of thrombotic events. However, the risk of thrombosis for the 4G allele carrying the PAI-1 gene is controversial.<sup>19</sup> Another study showed that PAI-1-siRNA strengthens the cavity-forming capacity of endothelial progenitor cells (EPCs) and significantly accelerates EPCs homing. After PAI-1 gene silencing, PAI-1 mRNA and protein expression decreased, vascular endothelial growth factor (VEGF) expression increased, and light-like structure enhanced in inferior vena cava tissue. PAI-1 gene silencing could promote VT recanalization by enhancing the lumen-forming capacity of the rat EPCs.<sup>20</sup>

## Inflammatory Factor

There is growing evidence showed that inflammatory factors linked to venous thrombosis. The role of inflammation and inflammation-related biomarkers in cerebrovascular thrombotic disease is a subject of ongoing debate.<sup>21,22</sup> Vascular cell adhesion molecule 1 (VCAM-1) plays an important role in leukocyte adherence and migration among vascular endothelial cells and the level are elevated in endothelial cell inflammatory areas.<sup>23</sup> Research has revealed that (lymphocyte antigen 6 complex)+Ly6C<sup>+</sup> monocytes and macrophages are the primary myeloid cell source of interleukin-6 (IL-6) in lytic thrombus, and IL-6 was known to induce monocyte-derived matrix metalloproteinase 9 (MMP9) production and that MMP9 has a weakened engagement in the process of thrombus lysis. The monocyte-IL6-MMP9 axis reflects a prospective non-anticoagulant target that may promote thrombolysis in individuals with completely occlusive DVT because of its crucial role in the IL-6 signaling pathway, which is essential for venous thrombosis and is influenced by the amount or lack of blood flow around the thrombus.<sup>24</sup> Another research also revealed that inflammatory Ly6C<sup>hi</sup> monocytes regulate the development, proliferation, and lysis of thrombus, which could be treated with transcription factor Nur77 (NR4A1) agonists at any stage of the illness.<sup>25</sup> However, investigators were unable to establish any correlation between plasma tissue factor (TF), IL-6, VCAM-1 or D-dimer levels and the development of DVT, but it should be noted that blood samples were only taken at recruitment within three days of injury, which may be too early to detect a prethrombotic status.<sup>23</sup> Another study has demonstrated that elevated levels of inflammatory factors interleukin-1 $\beta$  (IL-1 $\beta$ ), tissue factor (TF), xanthine oxidase (XOD) and nuclear factor kappa B (NF- $\kappa$ B) may accelerate thrombosis.<sup>26</sup> The NLRP3/IL-1/NF- $\kappa$ B signaling mechanisms regulate IL-1 and tumor necrosis factor alpha (TNF- $\alpha$ ), which may be essential signs of the prethrombotic condition due to slowed blood flow, impaired vascular endothelium, and elevated tissue factor expression. Therefore, VTE is the result of the coagulation system's cascade reaction.<sup>27-29</sup> Coagulation factor XII (FXII), an essential coagulation factor, was found to be associated with thrombosis, the study revealed that the knockdown of FXII markedly raised superoxide dismutase (SOD) concentrations, reduced the thrombosis and apoptosis, and raised the malondialdehyde (MDA) concentrations in DVT mice. Moreover, TNF $\alpha$ , IL-6, interleukin-8 (IL-8), and phosphatidylinositol 3-kinases (PI3K)/protein kinase B (AKT) signaling activation were all markedly decreased by FXII knockdown. The stimulation of PI3K/AKT signaling by the FXII protein causes an inflammatory response, which in turn leading to DVT. Therefore, focusing on FXII protein may show promise as a DVT therapy strategy.<sup>30</sup> As we know, AKT2 is a subtype of AKT, in a mouse model of venous thrombosis, AKT2 could modulate endothelial cell-mediated blood coagulation homeostasis as well as facilitate endothrombotic recanalization and thrombus resolution. Besides, AKT2 could increase the expression of thrombomodulin (TM) and decrease the expression of TF in cultivated endothelial cells.<sup>31</sup> According to another research, TM is thought to be a valuable marker for assessing endothelial impairment and plays a crucial role in DVT. The activation of the NF- $\kappa$ B signaling pathway leads to an increase in plasma TM levels and thrombus size.<sup>32</sup> As for endothelial cells, study found that prothrombotic procoagulant phospholipids was found on the surfaces of activated endothelial cells.<sup>33</sup> The endothelium procoagulant action is supported by phospholipid-disrupting enzymes, TMEM16E and TMEM16F (Ca<sup>2+</sup>-activated phospholipid-disrupting enzyme), which externalize phosphatidylserine (PS), in the mice model of thrombosis caused by laser damage, PS externalization was inhibited and fibrin production in the vessel wall was decreased without affecting platelets when TMEM16E or

TMEM16F were deleted genetically or treated with TMEM16 inhibitors, the results demonstrate the involvement of endothelial TMEM16E in thrombosis and suggest TMEM16E as a possible target for therapeutic intervention to inhibit the development of thrombus.<sup>33,34</sup> Besides, another recent research has demonstrated the Grb2-associated binding 2 (GAB2), a signal adapter protein, plays a vital part in the dissemination of the inflammatory signals in endothelial cells induced by IL-1 $\beta$  and other cytokines of inflammation.<sup>35</sup> In endothelial cells, GAB2 contributes to the activation of NF- $\kappa$ B and Rho.<sup>36</sup> According to the study, greatly reducing IL-1-induced Rho-dependent exocytosis of Von Willebrand factor (VWF) and P-selectin then following adhesion of neutrophils to vascular cells was achieved by either gene silencing of GAB2 or mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), the effector signals molecule in the CBM (CARD recruited membrane-associated protein 3-B cell lymphoma 10 - MALT1) signalosome, or by pharmacologically inhibiting MALT1 with a particular inhibitor, mepazine.<sup>36</sup> Additionally, IL-1-induced NF- $\kappa$ B-dependent production of tissue-related factors and VCAM-1 was decreased by MALT1 suppression. Gab2 loss or pharmacological suppression of MALT1 decreased venous thrombosis brought on by inferior vena cava-ligation-induced stenosis or stasis in mice and reduced the concentration of monocytes and neutrophils at the wound area, which was in accordance with the *in vitro* data.<sup>36</sup> Furthermore, the findings of another investigation demonstrated that the neutrophil count, monocyte level, c-reactive protein (CRP) concentration, lymphocyte-to-monocyte ratio (LMR), and neutrophil-to-lymphocyte ratio (NLR) were significantly altered in accordance with the duration of cerebral venous thrombosis (CVT). Patients with CVT exhibit distinct inflammatory patterns throughout the course of their illness: higher levels of NLR and lower levels of LMR during the acute phase; higher levels of LMR and lower levels of CRP during the chronic phase.<sup>37</sup>

## Mechanism of miRNA Leading to Venous Thrombosis

MicroRNA is an endogenous, highly conserved 19–22 short nucleotide fragment of a non-coding RNA molecule.<sup>38</sup> As a protein that is directly affected by AKT, glycogen synthase kinase 3 (GSK3) is a crucial part of the PI3K/AKT signaling cascade and has the ability to influence cell survival, death, motility, and migration.<sup>39,40</sup> Study has demonstrated that miRNA-185 was relevant with the proliferation and apoptosis of vascular endothelial cells by controlling the late glycation end product receptor (RAGE). MiR-185 can inhibit the expression of tissue factor in rat. The rat deep vein endothelial cells were induced by lipoderma endothelium, which was regulated by PI3K/AKT signaling pathways. MiR-185 inhibits thrombosis by reducing inflammation-induced tissue factor expression.<sup>41</sup> In addition, another study found that miR-150 is an important microRNA that plays a key part in all kinds of cell functions. Moreover, miR-150 plays a crucial role in endothelial progenitor cells (EPCs), and its expression is downregulated in EPCs induced by DVT. The upregulation of miR-150 promotes angiogenesis and proliferation of EPCs through the targeting of SRC kinase signaling inhibitor 1 (SRCIN1) both *in vitro* and *in vivo* for thrombolysis.<sup>42</sup> The Fas ligand (FASLG) gene is a target of miR-21, knockdown of FASLG can impair EPCs function, while the expression of miR-21 may stimulate EPCs proliferation and angiogenesis. In addition, In the EPCs of the DVT model rat, miR-21 expression is diminished. By targeting FASLG, miR-21 may promote the proliferation of endothelial progenitor cells and the creation of new blood vessels, which imply that miR-21 could be a potential indicator of thrombosis.<sup>43</sup> In other side, it has been proved that miR-21 is closely related to PTEN (phosphatase and tensin gene), which inhibit cell proliferation and promote apoptosis under normal physiological conditions and therefore plays an important role in thrombosis.<sup>44</sup> MiR-21 can increase the proliferation of vascular endothelial cells and promote the formation of new blood vessels by inhibiting the expression of PTEN.<sup>45</sup> A new study has uncovered that the function of miR-21 is to increase the rate of angiogenesis and cardiomyocyte survival by target to PTEN in heart failure.<sup>46</sup> In summary, miR-21 plays a complex regulatory role in thrombosis, including inhibition of PTEN and FASLG, promotion of endothelial cell proliferation and neovascularization, and contribute to keratinocyte migration, angiogenesis and cardiomyocyte survival in heart failure. Further study should focus on the mechanism of miR-21 in thrombosis and provide a theoretical basis for the development of new treatments.

## Mechanisms of Venous Thrombosis Associated with Aging

The risk of venous thrombosis increases with age,<sup>47</sup> but the mechanisms underlying the increased risk of thrombosis with age are not well understood. Silent information regulator 1 (Sirt1) is associated with endothelial cell senescence, inflammation, oxidative stress and platelet adhesion. Sirt1antisense (Sirt1-AS) is an antisense long non-coding RNA

(lncRNA) of Sirt1, DVT development is related to endothelial cell senescence and low lncRNA expression of Sirt1-AS and Sirt1. Sirt1 delays senescence to reduce the incidence and production of age-related thrombosis, and the Sirt1 antisense lncRNA (lncRNA Sirt1-AS) mitigates DVT by modulating the Sirt1/FOXO3a axis. Specifically, it reduces the incidence of senescence-associated DVT by enhancing human vascular endothelial cell (HUVEC) viability and proliferation while decreasing HUVEC apoptosis.<sup>48</sup> In a cross-sectional research of mice and humans, researcher found that Deoxyribonuclease 1 (DNase 1) could inhibit age-induced increases in endogenous thrombin generation and venous thrombosis. The work revealed that circulating cell-free DNA increased with aging via NETosis-independent processes.<sup>49</sup> The NETosis, which involves the release of NETs following the activation of neutrophils *in vitro*, has been demonstrated to be mediated by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>).<sup>50</sup>

## The Mechanism of Neutrophil Traps Promoting Thrombosis

Vascular Willebrand factor or glycoprotein Iba-dependent platelet “Priming” triggers the activation of integrin  $\alpha_{IIb}\beta_3$ , which in turn regulates neutrophil and T cells binding. Neutrophil binding of platelet  $\alpha_{IIb}\beta_3$  to SLC44A2 results in the production of highly prothrombotic NETs.<sup>51</sup> Therefore, it is believed that one of ways in which neutrophils encourage venous thrombosis is by forming NETs.<sup>52</sup> Additionally, NETs utilize transforming growth factor- $\beta$  (TGF- $\beta$ ) in thrombi to up-regulate fibroblasts and facilitate fibrotic thrombus remodeling.<sup>53</sup> It has long been thought that arterial and venous thrombosis involve different mechanisms,<sup>54,55</sup> but recent studies have revealed that the neutrophil play a crucial role in both arterial and venous thrombosis. The transcription factor Kruppel-like Factor 2 (KLF2) is the key regulator of neutrophil activation, which can be triggered by anti-phospholipid antibodies or be lost through KLF2 gene expression. This leads to the aggregation of P-selectin glycoprotein ligand-1 (PSGL-1) via reconstitution of cortical actin, thereby increasing adhesion potential at thrombotic sites.<sup>56</sup> Another study also revealed that in the progression of heart failure and myocardial hypertrophy, KLF2 controls thrombosis and activation of neutrophil.<sup>57</sup> In addition, recent research demonstrated that under a live microscope, the neutrophil “plucked” an extension of megakaryocytes in blood vessels, known as pre-platelets, to regulate the platelet production. Along with cxcr4-cxcl12-dependent migration to the periatrial megakaryocytes, the neutrophil activate platelets via reactive oxygen species and trigger activation of myosin light chain and extracellular signal-regulated kinases. Through these mechanisms mentioned above, neutrophils accelerate platelet growth and promote stable platelet release. After myocardial infarction, neutrophils lead to excessive release of young reticular platelets and increase the risk of re-ischemia, and on the contrary, ablation neutrophil can normalize platelet and reduce the thrombus burden of recurrent and venous thrombosis after myocardial infarction.<sup>58</sup>

Cellular fibronectin containing extra domain A (Fn-EDA) is a toll-like receptor 4 (TLR4) endogenous ligand that promotes thrombotic inflammation.<sup>59–63</sup> Overweight and obesity are known to increase the chance of developing VTE.<sup>64,65</sup> In a diet-induced obesity mouse model, researchers found increased plasma levels of Fn-EDA in high-fat (HF) fed animals.<sup>59</sup> Additionally, individuals with VTE had high plasma levels of Fn-EDA and were linked to body mass index (BMI). Under co-morbid diet-induced obesity circumstances, genetic ablation of Fn-EDA decreased susceptibility to DVT. Besides, the research revealed that exogenous cellular Fn-EDA stimulated NETosis in neutrophils via TLR4-stimulated thrombin-activated platelets and that TLR4 contributes to Fn-EDA-mediated DVT. Therefore, Fn-EDA/TLR4 axis may be involved in NETosis and the development of DVT in mice. The elevated levels of Fn-EDA in plasma may be an important mechanism for promoting DVT in the context of diet-induced obesity.<sup>59</sup>

Defibrotide, a heterogeneous mixture of polyanionic oligonucleotides, has been currently approved for the treatment of transplant-related venous occlusive disease. Recently, researchers have conducted *in vitro* experiments and mouse models to determine the mechanism by which defibrotide inhibits NET formation and venous thrombosis in antiphospholipid syndrome (APS). This study revealed the role of NETs in the thrombotic complications of APS. At a dose of 1–10  $\mu\text{g mL}^{-1}$ , defibrin significantly inhibited NET formation in IgG-stimulated control neutrophils isolated from APS patients.<sup>66</sup> Defibrotide elevates intracellular cyclic AMP levels in neutrophils, thereby mitigating the inhibition of NET formation through blockade of adenosine A<sub>2A</sub> receptors or suppression of cyclic AMP-dependent kinases. In a model where antiphospholipid antibodies accelerated thrombosis, defibrin at doses ranging from 15–150 mg/kg/day inhibited both NET formation and venous thrombosis and the effect was diminished in adenosine A<sub>2A</sub> receptor knockout mice.

This study has demonstrated the mechanism by which defibrin can counteract thrombotic inflammation mediated by neutrophils in APS.<sup>66</sup>

## Selection

P-selectin is an adhesion molecule expressed on the surface of activated platelets and endothelial cells,<sup>67,68</sup> leading to platelet-monocyte aggregation and stimulating vascular inflammation and thrombosis.<sup>69</sup> Therefore, inhibiting the expression of p-selectin is a good option for reducing the thrombosis. 3S-1,2,3,4-tetrahydro- $\beta$ -carboline-3-methyl aspartyl ester (THCMA) is a new small molecule inhibitor of p-selectin, which can remarkably restrict platelet aggregation in vitro and down-regulate serum p-selectin and TNF $\alpha$  expression levels in vivo. THCMA has been successfully developed as a nanomedicine and is 100-fold more effective in inhibiting arterial and venous thrombosis and 10-fold more effective in suppressing inflammation than PSI-697, a drug in clinical trials.<sup>68,69</sup> The doses of THCMA that inhibit thrombosis do not produce clotting disorders and no risk of bleeding, the drug significantly improves oral efficacy, which could be used for oral therapy of arterial and venous thrombosis, cancer-related thrombosis and inflammation.<sup>68</sup>

## Immunological Mechanism of Venous Thrombosis

Over the past few years, growing research suggest that the production of venous thrombosis also involves the immune system. As thrombosis originates from severe hypoxia in deep venous environment, endothelial cells are subjected to oxidative stress. This encourages the binding of additional pattern recognition molecules or mannose-binding lectin (MBL) to the surface of endothelial cell via the lectin pathway, which in turn activates mannose-binding lectin-associated serine protease 2 (Masp-2), then Masp-2 can cleave prothrombin to thrombin and forms fibrin.<sup>70-74</sup> According to the study by Damoah et al, having elevated levels of the complement-activating enzyme Masp-2 raises the chance for developing venous thromboembolism in the future.<sup>75</sup> Furthermore, the absence of B cells in mice indirectly contributes to venous thrombosis by elevating neutrophil counts and increasing fibrinogen levels. The inferior vena cava (IVC) stenosis model demonstrated an augmented incidence of venous thrombosis due to B-cell deficiency, which was hypothesized by investigators to be caused by a rise in circulating neutrophils leading to an increased abundance of NETs within the thrombus and upregulation of fibrinogen production.<sup>76</sup> Through the production of Interferon- $\gamma$  (IFN- $\gamma$ )-dependent NETs, natural killer (NK) cells contribute to the development of venous thrombosis, which DVT decreases with NK cell depletion.<sup>77</sup> Moreover, the activation of innate effect-memory T cells plays a crucial role in regulating venous wall inflammation and thrombus lysis after thrombosis.<sup>78</sup> Besides, a study demonstrated that regulatory T cells (Treg) clustered in venous clots of blood, where they formed the stromal cell protein SPARC (secreted protein acidic and rich in cysteine) which promoted the MMP activity of monocytes. Treg thrombolysis is through the control of monocyte recruitment, differentiation, and regulation of the activity of MMP, which is possible to use clot Tregs therapeutically to speed up thrombus clearance.<sup>79</sup> Mast cell protease-4 (MMCP-4) is a chymase-type particle-localized protease that has been discovered to be crucial in the development of DVT, in the deep vein thrombosis-affected mice, chymase decreased the activity of plasmin within thrombus, the inhibition of chymase could eliminate and avoid deep vein thrombosis without lengthening the bleeding duration, which reduce chymase activity without disrupting the coagulation cascade, these findings offer a potential pharmaceutical approach to treat or prevent DVT.<sup>80</sup>

Immobilization is known to be an important risk factor for the VTE development, but a protracted state of inactivity of paralyzed spinal cord injury (SCI) patients and free-ranging hibernating brown bears is protected from VTE. Thienel et al has demonstrated that mass spectrometry-based proteomics can identify antithrombotic properties in the platelets of hibernating brown bears, with heat shock protein 47(HSP47) being most markedly diminished. The rationale behind this is that the downregulation or ablation of HSP47 leads to a decrease in immune cell activation and neutrophil trap formation, thereby promoting thromboprotection in patients with spinal cord injury and bears and mice. This cross-species conservation of platelet characteristics may provide antithrombotic therapy as well as prognostic indicators.<sup>81</sup>

Immune thrombosis refers to the innate immune response triggered by the formation of blood clots in blood vessels and serves as a framework for the interaction between the immune system (innate and adaptive) and endothelial dysregulation-mediated thrombotic response caused by inflammation. The immune cells support the related molecules and produce specific intravascular scaffolds that promote pathogen recognition, containment, and destruction. These

mechanisms preserve the integrity of the host without resulting in serious side effects.<sup>82,83</sup> The previous study showed IL-6, IL-8, and monocyte chemoattractant protein (MCP-1) were the independent predictors of accelerated VTE development and they concluded that systemic inflammation is a key driver of VTE risk after major trauma.<sup>84</sup> Future work will need to further determine the importance of immunothrombosis for host protection and characterize in more detail the host molecules involved in this process, without causing significant collateral damage to the host.

## Extracellular Vesicles (EVs) in the Coagulation Mechanism of VTE

Many clinical cases of VTE have unknown causes and lack typical clinical symptoms, so there is an urgent need to develop reliable biomarkers for assisting in the prediction and diagnosis of VTE. Extracellular vesicles (EVs) produced by activated, damaged, or apoptotic cells carry a variety of bioactive substances and play diverse physiological roles while participating in the pathological processes of numerous diseases. The procoagulant specificity of EVs is also associated with the expression of TF. The previous study showed tumor cells constitutively release EVs that may contribute to thrombosis in cancer patients. Clinical studies have found that levels of circulating tumor-derived, tissue factor-positive (TF<sup>+</sup>) EVs in pancreatic cancer patients are associated with VTE.<sup>85</sup> Besides, the activity of PS and TF expressed by EVs is much higher than that of the mother cell, which can significantly shorten mice's bleeding time and promote thrombosis formation.<sup>85</sup> The researchers discovered that inhibiting CD36 reduced the binding of endothelial, monocyte, and platelet EVs to resting platelets.<sup>86</sup> The binding of EVs to platelets was also reduced by annexin V or an antibody to PS, suggesting that EV PS plays a role in the binding process.<sup>86</sup> In addition, EV also carries P-selectin glycoprotein ligand-1 and glycoprotein GpIb, which respectively mediate EVs activation and platelet activation. Endothelial cells and vWF interact to rapidly deposit TF-associated EVs at the site of thrombus formation, thus more effectively promoting initiation and amplification of the coagulation cascade.<sup>87</sup>

In addition to directly promoting coagulation, EVs can also promote thrombosis through indirect mechanisms. For example, activated platelets can transfer "cargo" such as arachidonic acid via EVs, thereby inducing the activation of other platelets.<sup>88</sup> There is a close relationship between inflammation and thrombosis, and it has recently been discovered that EVs can indirectly promote coagulation by influencing the inflammatory system. High-level EVs can significantly up-regulate the expression of pro-inflammatory signaling molecules, activate systemic coagulation response and induce inflammation and apoptosis.<sup>89</sup>

## Summary and Future Perspectives

In this review, we provide a comprehensive overview of the mechanisms underlying PAI-1, inflammatory factors, miRNA, age-related changes, neutrophil extracellular traps formation, P-selectin activation, immunological processes and extracellular vesicles (EVs) in relation to venous thrombosis (Figure 1). We reviewed venous thrombosis biomarkers from ongoing clinical trials, as shown in Table 1, as well as published clinical trials related to venous thrombosis as shown in Table 2. With regard to venous thrombosis, we have outlined the important points as follows: firstly, increased PAI-1 is regarded as a thrombosis risk factor, although there is debatable evidence about the thrombosis risk association

**Table 1** Ongoing Clinical Trials Involving Biomarker in VTE

Biomarker	Trail ID	Study Population	Target	Status
Platelet	NCT05240508	Cancer patients are starting therapy	Platelet FcγR1a expression	Recruiting
Neutrophil extracellular traps (NETs)	NCT03781531	Consecutive patients age >18 years with objectively confirmed venous thromboembolism diagnosed < 2 days	DNA and histones	Recruiting
D-dimer combined with other thrombus molecular marker	NCT05515549	Surgical patients with high risk of venous thrombosis		Not yet recruiting
New Genetic Markers	NCT03977870	Patients with VTE	Whole Genome Analysis	Not yet recruiting

**Table 2** Published Literature Involving Venous Thrombosis

Disease	Model	Study Population	Mechanism	Ref
Venous thrombosis	Mice	JAK2-V617F-positive chronic myeloproliferative neoplasia patients	AK2-V617F activates $\beta$ 1/B2 integrin to accelerate the venous thrombosis.	[90]
Lower extremity deep venous thrombosis (LEDVT)	Mice	LEDVT patients	Mir-185 activates the PI3K/AKT signaling pathways to inhibit the expression of tissue factor and fibrin to reduce thrombosis.	[41]
Deep vein thrombosis (DVT)	Mice	DVT patients	LncRNA Sirt1-AS reduce DVT by modulating the Sirt1 / Foxo3a axis.	[48]
Venous thromboembolism (VTE)	Spinal cord injury patients, bears and mice	Spinal cord injury patients	Downregulation or ablation of HSP47 reduces the activation of immune cells and the formation of neutrophil traps, which is conducive to thromboprotection of spinal cord injury patients, bears and mice.	[81]
VTE	Mice	VTE patients	Through PI3K/AKT signaling, FXII controls the development of thrombosis	[30]

with the 4G allele in the PAI-1 gene.<sup>19</sup> Besides, vein thrombosis is influenced by inflammatory factors, such as IL6, IL-1, and TNF- $\alpha$ , as well as other factors mediated by various signaling pathways like VCAM-1, TF, TM, VWF, and P-selectin. Venous thrombosis is also connected to the immune system and miRNA. Importantly, although immobility is a known risk factor for venous thrombosis, the downregulation or ablation of HSP47 in patients with SCI and bears leads to a decrease in immune cell activation and neutrophil trap formation, thereby promoting thromboprotection in SCI patients and bears. This offers a crucial concept for the management of venous thrombosis. The connection between inflammation and the immune system in venous thrombosis warrants more research as well. Additionally, NETosis is also involved in the mechanism of venous thrombosis caused by aging, which may share similar linked mechanism. Notably, age-related and immunological venous thrombosis have been linked to neutrophil trap development; hence, one potential treatment for venous thrombosis might be to inhibit neutrophil trap formation. Besides, EVs involved in the pathological process of various clinical VTE-related diseases, and has potential applications in indicating the risk of VTE and aiding in the diagnosis and treatment of VTE.

However, venous thrombosis is a complex illness, there are still unknown mechanisms. Therefore, inactive venous thrombosis may not be predicted even after testing all available indicators. It is critical to continue to explore of the underlying mechanisms of venous thrombosis. The more clearly the mechanisms of venous thrombosis are understood, the greater therapeutic and diagnostic targets for venous thrombosis are identified.

## Data Sharing Statement

The authors declare that the submitted data is available. This paper does not contain any other individual or collective published or written works data except those specifically annotated and cited in the paper.

## Consent for Publication

We declare that all authors agreed to publish the manuscript at this journal based and followed publication ethics.

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## Disclosure

The authors declare that they have no known competing commercial interests or personal relationships that could have appeared to influence the work reported in this paper.

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