Review article

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ANTITHROMBOTIC MEDICATIONS AND THEIR IMPACT ON FIBRIN CLOT STRUCTURE AND FUNCTION

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Fibrin constitutes a major protein component of intravascular thrombi in all locations. Fibrin formation and its functions are essential for physiological hemostasis and the pathologic thrombosis. Formation of dense fibrin networks which are relatively resistant to lysis is observed in patients with venous or arterial thromboembolism, including myocardial infarction, ischemic stroke and venous thromboembolism. Measures of clot characteristics, in particular clot permeability and clot lysis time, may predict arterial and venous recurrent thromboembolic events. Medications, including vitamin K antagonists (VKA), direct oral anticoagulants (DOAC), and parenteral direct or indirect thrombin or activated factor X inhibitors increase clot permeability, reflecting fibrin network density, in association with enhanced efficiency of fibrinolysis. These effects are only in part related to decreased thrombin generation. There is evidence that aspirin can also favorably alter fibrin clot properties probably through acetylation of fibrinogen. No such effects were observed for P2Y12 inhibitors. Of note, plasma fibrin clot permeability has been shown to predict adverse clinical outcomes in patients receiving oral anticoagulants, which might have practical implications. The current review summarizes data on effects of antithrombotic agents on fibrin clot phenotype in cardiovascular disease.

Key words: direct oral anticoagulants, fibrin clot, antplatelet, anticoagulant, fibrinolysis, thromboembolism, aspirin, coagulation factors

INTRODUCTION

Fibrin formation is the final step of blood coagulation (1). Fibrinogen, the soluble fibrin precursor, is a multifunctional 340-kDa glycoprotein that consists of two sets of three polypeptides: \( \alpha \), \( \beta \), and \( \gamma \), linked in a dimeric structure by 29 disulfide bonds. The central E region contains the N termini of all 6 chains. The chains in the form of 2 coiled-coils composed of 3 chains represent two symmetrical globular D domains containing the \( \beta \)-nodules and \( \gamma \)-nodules formed by the carboxy termini of the \( \beta \) and \( \gamma \)-chains, respectively. The C-terminal segment of the \( \alpha \) chain goes through the D region and folds back to join the coiled-coil for several residues (\( \alpha \) residues 213 to 610). About 10% of fibrinogen molecules contain \( \gamma \)-chains, in which the 4 C-terminal residues are replaced with a 20-residue sequence with a large negative charge (1-3).

Fibrin formation initiates thrombin that splits off two pairs of fibrinopeptides A and B from the N termini of the \( \alpha \) and \( \beta \) chains, respectively, in the central nodule, leading to the exposure of binding sites ‘A’ and ‘B’. Polymerization of fibrin monomers involves formation of half-staggered and double-stranded protofibrils through binding of knobs ‘A’ and ‘B’ in the central nodule of fibrin monomer to complementary holes ‘a’ and ‘b’ in the \( \gamma \) and \( \beta \)-nodules, respectively, of another monomer, which is followed by the assembly of protofibrils into fibers through lateral aggregation promoted mainly by intermolecular \( \alpha \)C: \( \alpha \)C interactions between protofibrils and probably also by interactions between both \( \alpha \)- and \( \gamma \)-chains (3, 4). Fibrin fibers are on average composed of thousands of protofibrils arranged side-by-side (5). Fibrin fibers are known to display the largest extensibility among protein fibers because they can be strained 180% (2.8-fold extension) without sustaining permanent lengthening, and to 525% (average 330%) before rupturing (6).

When bound to fibrin, thrombin activates factor (F)XIII which enhances the formation of e-(\( \gamma \)-glutamyl)lysyl covalent bonds between \( \gamma \)-\( \gamma \), \( \gamma \)-\( \alpha \) and \( \alpha \)-\( \alpha \) chains of adjacent fibrin molecules, along with binding several proteins, including \( \alpha \)-plasmin inhibitor, into a fibrin clot (7). Fibrinogen binds a variety of proteins related to coagulation, inflammation, platelet activation and many others as shown in our recent report that provided evidence for the presence of almost 500 proteins within plasma clots in humans (8).

Fibrinolysis induced by plasmin that is responsible for thrombus removal and maintaining blood flow leads to digestion of fibrin at specific lysine residues. Fibrin enhances the activation of plasminogen by tissue plasminogen activator (tPA). Plasmin cleaves a number of Lys-X and Arg-X bonds in the fibrin molecule, thereby breaking down the structure of the fiber. Fibrin clot properties affect the rate of clot lysis in part due to their impact on the distribution of lytic enzymes, which results in faster lysis of less compact fibrin meshworks (9). Fiber thickness has a strong impact on the rate of fibrinolysis induced by tPA, with its increase in regions composed of thicker fibers (10).

Since in vivo fibrin structure and function can be modified by blood flow, cellular blood components, endothelial cells, circulating proteins and other molecules, as well as...
posttranslational modifications e.g. oxidation, or glycation (11), plasma fibrin clots and those made from purified fibrinogen may have different characteristics and extrapolation of in vitro findings could be challenging. To evaluate clot characteristics in vitro, the permeability assay measuring the volume of percolating buffer through a clot under different hydrostatic pressure is commonly used and attempts to standardize it have been made (12). Other measures of clot structure in plasma-based and purified fibrinogen-based systems involve the fiber diameter and the size of the pores in the fiber network on scanning electron microscopy (SEM) or confocal microscopic images. Efficiency of fibrinolysis is assessed using a variety of assays with various final concentrations of tPA added to plasma samples together with thrombin or tissue factor (TF) and phospholipids. Recently an assay that concomitantly measures fibrin clot formation and lysis has been introduced and supported by the International Society on Thrombosis and Haemostasis Scientific Standardization Subcommittee (13).

Growing evidence indicates that patients with a history of arterial and venous thrombotic events have altered fibrin clot phenotype, involving the formation of more compact fibrin meshworks displaying impaired permeability and lysability (14-16). Fibrin formation during blood coagulation is influenced by genetic and to a larger extent, environmental factors, which explain a substantial interindividual variability of clot properties, regardless of the methodology used. Pharmaceutical agents affecting blood coagulation have been postulated to act in part through modification of clot features.

This review is focused on the effects of antithrombotic agents used in cardiovascular patients on fibrin clot formation, structure and degradation. Potential clinical implications of modified fibrin properties will be highlighted.

**FIBRIN CLOTS AND THROMBOSIS AT VARIOUS LOCATIONS**

The pathogenesis of venous thromboembolism (VTE) encompassing deep-vein thrombosis (DVT) and pulmonary embolism (PE), that occurs in 1–2 per 1000 adult individuals in Europe, is complex and multifactorial (17). Analysis of pulmonary thromboemboli in acute PE shows the predominance of fibrin (up to 80%) over cellular components (18, 19). It has been reported that clots prepared in vitro after addition of 1 U/ml human thrombin to platelet-poor plasma (PPP) obtained from patients with a history of first-ever idiopathic DVT assessed at 4–20 months of anticoagulant therapy displayed 34% lower clot permeability, 40% longer lysis time and 8% lower rates of the D-dimer release from fibrin clots compared with controls (20).

Scanning electron microscopy of intracoronary thrombi from patients with ST-segment elevation myocardial infarction (STEMI) demonstrated that fibrin content within thrombi increases with time up to 66.9% at six hours since the chest pain onset (21). A 2-fold increase in fibrin content per each ischemic hour has been estimated in STEMI patients (21). Compared to stable coronary artery disease (CAD), in acute coronary events (ACS) the in vitro formation of less permeable and lysable PPP clots (1 U/ml human thrombin was used as a clotting activator) in association with oxidative stress and inflammation has been shown within the first 12 hours from the onset of chest pain (22).

The histological analysis of thromboemboli retrieved during thrombectomy from the middle cerebral artery and intracranial carotid artery of patients with acute ischemic stroke showed random fibrin fibers mixed with platelet deposits with a large heterogeneity (23). It is estimated that fibrin constitutes about 60% of the retrieved thrombi obtained from stroke patients and the most common type of the thrombi (44%) is fibrin-dominant (24). Acute ischemic stroke within the first 72 hours of symptom onset has been found to be associated with 30% lower in vitro PPP clot permeability and prolonged lysis time by 11%, coupled with faster fibrin formation by 8% (1 U/ml human thrombin was used as the clotting activator in all assays) compared with healthy controls (25). Fibrin clot compaction correlated with neurological deficit both on admission and at discharge of patients admitted for acute ischemic stroke (25). More compact plasma clots resistant to lysis were shown when clots were generated from plasma samples of patients with cryptogenic stroke at 3 to 19 months (26).

Atrial fibrillation (AF), the most common cardiac arrhythmia, causes about 15% of ischemic strokes with poor clinical outcomes, including 25% mortality within 30 days. It has been shown that patients with chronic AF and previous stroke have prolonged clot lysis time (CLT) assessed in PPP in vitro in the presence of 0.6 µM human TF, combined with higher thrombin-activatable fibrinolysis inhibitor (TAFI) antigen than those free of stroke (27). Of note, CLT, plasminogen activator inhibitor (PAI-1), TAFI activity and soluble thrombomodulin were positively correlated with CHA2DS2-VASe scores, reflecting stroke risk in AF (27). Experimental studies in a swine model demonstrated that cerebral arteries occluded by fibrin-rich clots have a lower recanalization rate and a longer mean recanalization time than did arteries occluded by erythrocyte-rich clots, suggesting that the histology of the occluding thromboembolus may affect the angiographic outcome of mechanical thrombectomy (28).

Antithrombotic drugs include antiplatelet agents and anticoagulants, both oral and parenteral.

**ASPIRIN**

The strongest evidence from several groups shows favorable alterations in clot structure following administration of aspirin. Aspirin acts by irreversible inhibition of platelet cyclooxygenase-1 (COX-1) due to acetylation of serine 529 residue, which inhibits the synthesis of the pro-aggregatory thromboxane A2 (29). In large primary prevention trials aspirin use was associated with reduced by 12% occurrence of serious vascular events, including 20% lower incidence of MI (30). Acetyl salicylic acid is the mainstay of the secondary prevention of cardiovascular disease (30, 31), however, non-adherence is still a major problem (32).

In ACS patients, low dose of aspirin (75–100 mg per day) has not been less effective than doses of 300–325 mg/day (33). Beyond inhibition of platelet activation by aspirin, which is a main mechanism related to reduced risk of arterial thrombotic events, aspirin may provide many additional antithrombotic effects, including reduced thrombin generation, attenuation of FXIII activation or acetylation of fibrinogen or antithrombin (34, 35). Administration of low-dose aspirin was shown to be associated with improved clot properties probably through fibrinogen acetylation (36, 37). Fibrinogen is acetylated at several lysine residues, which also are involved in the FXIII-mediated crosslinking of fibrin (37). Human fibrinogen treated in vitro with aspirin (0.8 mM for 24 hours) shows using mass spectrometry 12 acetylation sites (lysines) in all three polypeptide chains (35). In healthy men the intake of aspirin at a daily dose of 37.5, 320 and 640 mg increased permeability and density of clots prepared in vitro from PPP in the presence of 0.4 U/ml thrombin by 44%, 31%, and 21%, respectively, and treatment with low-dose aspirin led to formation of thicker fibrin fibers with larger pores (38). Using a cell model with transfection with fibrinogen and the analysis of clots made in vitro from purified fibrinogen after addition of 0.5 U/ml thrombin, aspirin has been demonstrated to render fibrin networks looser and their
fibers thicker, leading to lower clot rigidity by 30% and enhanced clot lysis (36). In healthy subjects treated with aspirin for 1 week, final turbidity of clots prepared in vitro from purified fibrinogen (clotting mixture as above) increased by 18% in response to aspirin 50 mg daily for 7 days, with two-fold increase in fiber thickness (36). Improved properties of plasma clots in response to low-dose aspirin have been observed in CAD patients as well as patients with VTE (39). Taken together, aspirin-induced alterations to clot characteristics might be additional antithrombotic action of this drug.

P2Y12 INHIBITORS

Inhibitors of platelet P2Y12 receptor (i.e. ticlopidine, clopidogrel, prasugrel or ticagrelor), with preference to the two latter agents are recommended in patients after coronary intervention or MI, usually in combination with aspirin (40). P2Y12 is a chemoreceptor for adenosine diphosphate (ADP) mainly found on platelets surface and involved in platelet aggregation. Binding of ADP to P2Y12 receptor activates the glycoprotein (GP)IIb/IIa receptor resulting in enhanced platelet degranulation, thromboxane production and aggregation. The thienopyridines (ticlopidine, clopidogrel and prasugrel) are indirect platelet inhibitors, which metabolites covalently and irreversibly bind to the P2Y12 receptor. Direct P2Y12 receptor inhibitors (ticagrelor and cangrelor) can change P2Y12 receptor conformation, resulting in its reversible inhibition (40). Available data show no effect of clopidogrel on plasma clot properties. In subanalysis of the PLATO trial comparing ticagrelor with standard therapy in acute MI, in vitro fibrin clot formation and lysis (PPP mixed with 0.03 U/ml thrombin) assessed few days since the symptom onset were unaffected by P2Y12 inhibitors, however clot lysis time predicted clinical outcomes (41). Precisely, after adjusting for cardiovascular risk factors, each 50% increase in lysis time was associated with cardiovascular death or spontaneous MI (hazard ratio [HR] 1.17, P < 0.01) and cardiovascular death alone (HR 1.36; P < 0.001). There was no effect of randomized antiplatelet treatment or bleeding events on clot characteristics (41).

GPIIb/IIIa INHIBITORS

The αIIBβ3 (GPIIb/IIIa) is an integrin family receptor on the platelet surface, which plays a critical role in thrombosis and hemostasis and interacts with many ligands, including fibrinogen. GPIIb/IIIa antagonists inhibit platelet aggregation independently of the type of platelet agonist. Currently, among GPIIb/IIIa antagonists abciximab, eptifibatide and tirofiban are available. In contrast to platelet studies, in the fibroblast model neither αβ3, αIIBβ3, nor other tripeptide Arg-Gly-Asp (RGD)-binding integrins caused the spatially-dependent morphology seen in clots formed during in situ thrombin generation (42). This suggests that a major mechanism causing the spatially heterogeneous fibrin clot morphology is likely attributable to differential rates of thrombin generation at and above the cell surface (42). Moreover, αIIBβ3 determines fibrin structure in platelet-rich clots, whereas integrin interactions play a minor to no role in clots formed by fibroblasts, smooth muscle cells and human umbilical vein endothelial cells (HUVECs) (42). In this model, extracellular cells and tumor necrosis factor (TNF)-α-stimulated HUVECs produce stable fibrin networks resistant to lysis (43). Analysis of fibrin clots formed at increasing red blood cell counts shows a decrease in fiber diameter, with its increase following inhibition of GPIIb/IIIa, while red blood cells impair clot lysis, with no effect of aggregation inhibition (44).

VITAMIN K ANTAGONISTS (VKA)

Vitamin K antagonists (VKA), including warfarin, acenocoumarol and phenprocoumon, have been the standard of care in anticoagulated patients regardless of indication, for many years. Until now this class of drugs is often used in patients with AF for stroke prevention (45, 46). VKAs still play the important role in acute and extended management of VTE. The molecular target of VKAs is vitamin K epoxide reductase complex subunit 1 (VKORC1) and their effects are governed by genetic and environmental factors strongly modifying anticoagulant actions. Mutations in the genes encoding VKORC1 and cytochrome P450 2C9 (CYP2C9) largely contribute to the interindividual variations in VKAs dose requirements. VKA-induced impairment of gamma-carboxylation of FII, FVII, FIX and FX results in reduced thrombin generation (47).

Warfarin at a concentration adjusted to obtain the therapeutic range (international normalized ratio 2 – 3) in a commercially available PPP resulted in higher in vitro clot permeability by about 35% (in a model with 5 PM recombinant human TF used as the clotting activator) (48). Warfarin and acenocoumarol have been shown to increase plasma clot porosity in patients with AF as early as after 3 days of treatment, reaching the plateau value after 7 days (Fig. 1 left panel, Fig. 2A) (49). Improvements in plasma clot properties have been found to be related to lower activities of vitamin K-dependent clotting factors and lower protein C activity (49). Importantly, activated coagulation factor concentrate can completely reverse the changes in fibrin clot properties following warfarin (48). Drabik et al. have reported that low permeability of a fibrin clot prepared in vitro after addition of 1 U/ml human thrombin to PPP, is an independent predictor of both thromboembolic events and major bleedings in AF patients anticoagulated with VKA for at least 3 months and observed for about 4 years (50). Patients with lower clot permeability (< 6.8 × 10^-9 cm^2) had increased risk of ischemic stroke or transient ischemic attack (HR, 6.55; 95% confidence interval [CI], 2.17-19.82) and major bleedings (HR, 10.65; 95% CI, 3.52 – 32.22), while those with elevated clot porosity (≥ 6.8 × 10^-6 cm^2) had an increased rate of minor bleeding compared with the remainder (11.63% versus 3.55% per year) (50). Increased clot permeability predisposed to minor bleedings in the same patients receiving VKA, which appears to agree with findings in several bleeding disorders because less compact clots are more prone to lysis and they disintegrate before mucosal or skin rupture is fully healed.

DIRECT ORAL ANTICOAGULANTS (DOACS)

Non-vitamin K antagonist oral anticoagulants (NOACs) or direct oral anticoagulants (DOACs), including dabigatran, rivaroxaban, apixaban or edoxaban, have demonstrated favorable efficacy and safety compared with VKAs. Large randomized controlled trials in VTE patients indicate that the NOACs were at least as effective as the conventional treatment in preventing recurrent VTE (51). Recurrent VTE occurred in a similar proportion of AF patients (2.0% on NOACs versus 2.2% on VKA) whereas use of NOACs is associated with a 40% reduction in the risk of major bleeding (51). Similar results have been reported for patients with nonvalvular AF. Compared with warfarin, there was a 19% reduction in the occurrence of stroke or systemic embolism, 10% reduction in all-cause mortality and 50% reduction in both hemorrhagic stroke and intracranial bleeding in NOAC users with AF (52).

Dabigatran, a direct thrombin inhibitor, has been shown to enhance clot lysisability and this effect was in part mediated by TAFI (53). Reduced thrombin formation and suppressed thrombin-mediated reactions during NOAC treatment appear to
account for the formation of less compact and more lysable fibrin clots.

The strongest evidence shows that rivaroxaban, a direct activated FX (FXa) inhibitor, favorably alters fibrin clot properties (Fig. 1 right panel, Fig. 2B) (54, 55). Formation of fibrin networks composed of thicker fibers forming larger pores associated with a 2-fold increased clot permeability and 3-fold faster lysis has been observed compared with the control clots generated in the absence of rivaroxaban (Fig. 3) (54). Similar improvement in the presence of rivaroxaban has been reported in whole blood clots (54). Plasma clots formed in the presence of rivaroxaban solution were made of thicker fibrin fibres, had larger pores and were more permeable (54). Increased clot permeability has been shown at therapeutic plasma concentrations of apixaban by 36 – 53% (Fig. 3) (48). Of note, activated coagulation factor concentrate can only partly reverse changes in clot properties induced by DOACs (48). We have shown recently that in patients with a history of VTE, rivaroxaban treatment increases clot permeability by 40% (55).

Rivaroxaban treatment improved fibrin clot properties also in carriers of the G20210A prothrombin mutation (3% of the European population) associated with 30% higher prothrombin levels, but anticoagulant therapy with rivaroxaban cannot lead to return normal fibrin clot phenotype (55).

Very recently, Carter et al. (56) have shown that rivaroxaban and apixaban as FXa-specific DOACs can enhance fibrinolysis in plasma by a modulation of fibrinolytic effects of FXa. Moreover, a direct correlation between accelerated fibrinolysis and FXa conversion from FXαα to FXαβ has been shown (56). Although DOACs, including dabigatran, in different models of *in vitro* clot formation have reduced thrombin generation and/or promoted fibrin degradation, the direct influence of these anticoagulants on fibrin structure using clotting activators containing thrombin or TF needs to be elucidated.

It is unclear whether drug-induced changes in fibrin clot properties may contribute to bleeding risk in anticoagulated patients. Our preliminary data from a cohort study performed in AF patients suggest that low clot permeability measured off

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*Fig. 1. Scanning electron microscopic (SEM) images showing representative plasma clots from patients with: atrial fibrillation before and during warfarin treatment (left) and venous thromboembolism before and after rivaroxaban intake (right). INR, International Normalized Ratio. Plasma fibrinogen concentrations about 2.5 g/l. Magnification × 5000.*
anticoagulation independently predicts an increased risk of both stroke and clinically relevant bleeding events in rivaroxaban treated patients who were followed for a median 40 months (A. Undas, unpublished data). It is unknown whether clot permeability has a comparable prognostic potential in patients on dabigatran and apixaban.

OTHER ANTICOAGULANTS

Unfractionated and low-molecular-weight heparins have been shown to improve clot properties as evidenced by analysis of nanostructure of fibrin clots in the presence of antithrombin (57). Parenteral direct thrombin inhibitors, argatroban, bivalirudin and danaparoid, have been shown to increase fibrin clot permeability at therapeutic concentrations in in vitro models to a similar extent (Fig. 3) (58). Increased clot permeability has been reported at therapeutic plasma concentrations of a parenteral indirect FXa inhibitor, fondaparinux by 58 – 76% (48).

OTHER MEDICATIONS OF POTENTIAL ANTITHROMBOTIC ACTIVITY

Statins, potent cholesterol-lowering drugs, are recommended in subjects at high-risk of CAD (59). Statins have been reported in various models to produce several antithrombotic effects, including down-regulation of TF expression, resulting in suppressed thrombin generation and thrombin-mediated reactions, such as fibrinogen cleavage, FV and FXIII activation and increase in thrombomodulin expression in endothelial cells, resulting in increased protein C activation (60). Additional antithrombotic effect of statin use represent favorable alterations in fibrin clot phenotype (60). A 3-month simvastatin treatment can result in increased clot permeability in patients free of cardiovascular events and low-density lipoprotein cholesterol < 3.4 mmol/l (61). Increased clot permeability (+16%) has been reported in patients receiving simvastatin with no association with low-density lipoprotein cholesterol reduction (62). It has been reported that a 3-day use of atorvastatin reduces plasma clot density by 23% (63).

Fig. 2. Changes in fibrin clot permeability ($K_\text{s}$) and clot lysis time (CLT) in patients with atrial fibrillation (panel A) and venous thromboembolism (panel B) during vitamin K antagonist and rivaroxaban treatment, respectively. Boxes represent mean ± standard deviation or median and interquartile range. OAT, oral anticoagulant therapy; INR, International Normalized Ratio. *P < 0.05.
Properties of fibrin clots prepared in vitro from PPP obtained from advanced CAD patients taking simvastatin or atorvastatin correlated with a post-treatment decrease in thrombin-antithrombin complex levels, most likely induced by down-regulation of TF expression (64). It might be speculated that changes in fibrin clot structure observed in patients treated with statins are involved in clinical benefits of this class of drugs, as evidenced by reduced risk of thrombosis in individuals taking statins in primary and secondary prevention (60).

Regarding anti-hypertensive agents, data on fibrin-modulating effects of angiotensin-converting enzyme inhibitors (ACEIs) are most consistent. As shown for quinapril, ACEI administered in patients with CAD can improve in vitro assessed plasma fibrin clot properties (64). Depressed thrombin formation observed after treatment with ACEIs in CAD patients has been shown to be associated with improved clot permeability (64). Newer data on various classes of drugs indicates that most antihypertensive drugs may slightly improve clot properties measured in plasma-based assays (65).

Treatment with insulin has been shown to make fibrin more permeable despite no improvement in the control of glycaemia (66). In vitro experiments showed that metformin, the most commonly used antidiabetic agent in prevention and therapy of type 2 diabetes (67), interferes with the polymerization process of fibrin monomers and reduces FXIII-mediated cross-linking of fibers that show increased lysability (68). Metformin can increase clot permeability also in obese, nondiabetic individuals (69). In diabetic patients better glycaemia control render fibrin clots more permeable and lysable and the key mechanism altered by these interventions is glycation of fibrinogen and other proteins like plasminogen (37, 70). Limited data suggest that administration of omega-3 polyunsaturated fatty acids (71) might also increase permeability and susceptibility to lysis of clots prepared in vitro using 1 U/ml human thrombin and PPP obtained from patients with stable cardiovascular disease.

Conclusions

Venous or arterial thromboembolic events are associated with the so-called prothrombotic fibrin clot phenotype, reflected by denser networks that are formed quickly and are relatively resistant to lysis. Clot properties in thrombotic disease can be modulated by some antithrombotic drugs, in particular anticoagulants and aspirin. Therapies targeting fibrin clot properties might be a new class of agents able to improve outcomes in patients with arterial and venous thromboembolism.

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