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INVITED REVIEW

Pleiotropic actions of factor Xa inhibition in cardiovascular prevention: mechanistic insights and implications for anti-thrombotic treatment

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Abstract

Atherosclerosis is a chronic inflammatory disease in which atherothrombotic complications lead to cardiovascular morbidity and mortality. At advanced stages, myocardial infarction, ischaemic stroke, and peripheral artery disease, including major adverse limb events, are caused either by acute occlusive atherothrombosis or by thromboembolism. Endothelial dysfunction, vascular smooth muscle cell activation, and vascular inflammation are essential in the development of acute cardiovascular events. Effects of the coagulation system on vascular biology extend beyond thrombosis. Under physiological conditions, coagulation proteases in blood are pivotal in maintaining haemostasis and vascular integrity. Under pathological conditions, including atherosclerosis, the same coagulation proteases (including factor Xa, factor VIIa, and thrombin) become drivers of atherothrombosis, working in concert with platelets and vessel wall components. While initially atherothrombosis was attributed primarily to platelets, recent advances indicate the critical role of fibrin clot and plasma coagulation factors. Mechanisms of atherothrombosis and hypercoagulability vary depending on plaque erosion or plaque rupture. In addition to contributing to thrombus formation, factor Xa and thrombin can affect endothelial dysfunction, oxidative stress, vascular smooth muscle cell function as well as immune cell activation and vascular inflammation. By these mechanisms, they promote atherosclerosis and contribute to plaque instability. In this review, we first discuss the postulated vasoprotective mechanisms of protease-activated receptor signalling induced by coagulation enzymes under physiological conditions. Next, we discuss preclinical studies linking coagulation with endothelial cell dysfunction, thromboinflammation, and atherogenesis. Understanding these mechanisms is pivotal for the introduction of novel strategies in cardiovascular prevention and therapy. We therefore translate these findings to clinical studies of direct oral anticoagulant drugs and discuss the potential relevance of dual pathway inhibition for atherothrombosis prevention and vascular protection.

Keywords Factor Xa • Thrombin • Atherosclerosis • Cardiovascular • Anticoagulant

1. Introduction

Cardiovascular diseases (CVD) remain the leading cause of worldwide mortality, surpassing other communicable and non-communicable causes of death in the long term.[1] While CVD is influenced by multiple risk factors and mechanisms, acute cardiovascular events are largely triggered by thrombosis. Myocardial infarction, ischaemic stroke, and complications occurring in patients with peripheral artery disease (PAD),

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including major adverse limb events, are caused either by acute occlusive atherothrombosis, or by thromboembolism.[2] Atherothrombosis is the consequence of atherosclerosis, a chronic inflammatory arterial disease, in which haemostatic mechanisms are triggered by rupture or erosion of plaques to form a clot.[3] For many years, the initiation of atherothrombosis has been thought to result from an interaction between platelets and intraplaque material; hence, antiplatelet therapy (APT) with acetylsalicylic acid (ASA), or clopidogrel, has been the mainstay of secondary prevention for decades. However, atherothrombosis is the result of a more complex process, which involves not only platelets but also coagulation proteins, other blood cells, as well as extracellular vesicles. The resulting thrombus consists of a platelet-fibrin-rich clot in close association with atherosclerotic plaque. Recognition that fibrin is an essential element in thrombus formation (both in venous and in arterial thrombosis), changed the way we think about antithrombotic therapies in CVD. It also triggered new interest in combining APT with anticoagulants, to reduce the risk of recurrent atherothrombosis.

While several years ago, we postulated that pleiotropic actions of anticoagulants would provide additional vasoprotection,[4] recent results of the Cardiovascular Outcomes for People Using Anticoagulation Strategies (COMPASS),[5] a study to Assess the Effectiveness and Safety of Rivaroxaban in Reducing the Risk of Death, Myocardial Infarction or Stroke in Participants With Heart Failure and Coronary Artery Disease Following an Episode of Decompensated Heart Failure (COMMANDER HF),[6] and Efficacy and Safety of Rivaroxaban in Reducing the Risk of Major Thrombotic Vascular Events in Subjects With Symptomatic Peripheral Artery Disease Undergoing Peripheral Revascularization Procedures of the Lower Extremities (VOYAGER PAD)[7] trials not only confirmed the clinical importance of this approach but raised a number of key questions related to the mechanisms of vasoprotection potentially associated with an antithrombotic regimen comprising agents with dual pathway inhibitory properties.

In this article, we discuss the impact of coagulation proteases, in particular factor (F) Xa and thrombin, on physiology and pathophysiology of the vascular endothelium. We discuss the role of these proteases in atherothrombosis, and the potential clinical consequences of dual pathway inhibition (DPI), combining platelet inhibition and anticoagulation, to achieve a stronger antithrombotic and possibly vascular-protective effect, beyond classical indications for anticoagulation.[8]

2. The role of coagulation proteases and protease-activated receptors in haemostasis and vascular protection

2.1 Coagulation proteins and the physiological activity of coagulation

The coagulation cascade comprises a series of linked proteins, most of which are produced by the liver: procoagulant factors (F) I–XII, and the anticoagulant proteins antithrombin, proteins C and S, and tissue factor pathway inhibitor (TFPI). Factors II (prothrombin), VII, IX, X, and proteins C and S are dependent on vitamin K for their function, the result of vitamin K oxidase reductase mediated carboxylation of these proteins.[9] Some proteins like FVIII, protein S, TFPI, and thrombomodulin are also synthesized by vascular endothelial cells (ECs) in or outside the liver. Many coagulation proteins can also be produced by other, extravascular, cells in different organs, including heart, brain, bone marrow, intestines, kidney, and placenta. In the extravascular compartments, these proteins may have multiple roles in complex regulatory pathways, not related to haemostasis. An example is the effect of the thrombin–thrombomodulin–protein C pathway in directing retention and release of cells from the bone marrow.[10,11] Another example is the critical functions that the 'coagulome' appears to fulfil in the central as well as peripheral nervous system.[12–14] Discussion of most of these extravascular processes is beyond the scope of this article, with the exception of the significance of coagulation proteins within the vessel wall and in the context of atherosclerosis.

Adequate *levels* of coagulation proteins are essential to provide haemostasis in case of occurrence of any vascular defect. Essentially, haemostasis is important for wound healing, a process in which the platelets attract inflammatory cells such as macrophages and the fibrin matrix supports the generation of new extracellular matrices by fibroblasts.

A major role of circulating coagulation proteins is to maintain an ambient level of coagulation *activity*, involving the generation of enzymes and the end product fibrin (*Figure 1A*). This process derives from controlled and limited proteolysis of coagulation proteins in a cascade model. The fact that fibrin degradation fragments including d-dimer are detected in any healthy subject, illustrates the presence of fibrin formation and cleavage under physiologic conditions, *in vivo*.[15–17] Primate studies showed that this basal level of coagulation activity is TF/FVIIa dependent, suggesting that indeed minute amounts of TF support this coagulation activity; excess coagulation is prevented by several natural inhibitory mechanisms.[18] One can imagine that for effective haemostasis a limited degree of coagulation is helpful, in order to allow a quick response to injury. Another reason may be that several coagulation proteases engage in cell signalling mechanisms, conferring protection of ECs, and their barrier function.

2.2 Coagulation proteins regulate haemostasis and vascular integrity

Specific coagulation proteins are essential for maintaining endothelial vascular integrity, as shown in knockout mice for prothrombin, TF, FVII, and protein C. Transgenic mice with *homozygous* deficiency in TF, FVII, prothrombin, or protein C, die *in utero*. For a substantial part, this is caused by severely disturbed haemostasis, resulting in overt bleeding (deficiency in TF, FVII, FX, FV, or prothrombin). In addition, in case of homozygous deficiency in TF, prothrombin, FV, or the cellular protease-activated receptor 1 (PAR1), several defects in vascular integrity, blood vessel development, and/or embryonic growth were observed (reviewed in Ref.19). Three groups independently reported that $TF^{-/-}$ embryos died between E8.5–10.5 due to inability to establish or maintain vascular integrity, showing defective yolk sac development.[20–22] The primary defect is characterized by severely impaired vascular smooth muscle cell (VSMC)/pericyte accumulation and differentiation around ECs, resulting in loss of endothelial barrier formation and vascular leakage.[20] Transgenic mice expressing a cytoplasmic tailless form of TF are rescued from lethality; FVII binding to the extracellular domain of TF, as well as catalytic function of the FVIIa/TF complex, are important for normal embryogenesis and vascular integrity.[23] Low levels (±1%) of a human TF minigene rescue mice from lethality resulting in an apparently normal phenotype without signs of bleeding or loss in vascular integrity.[24]

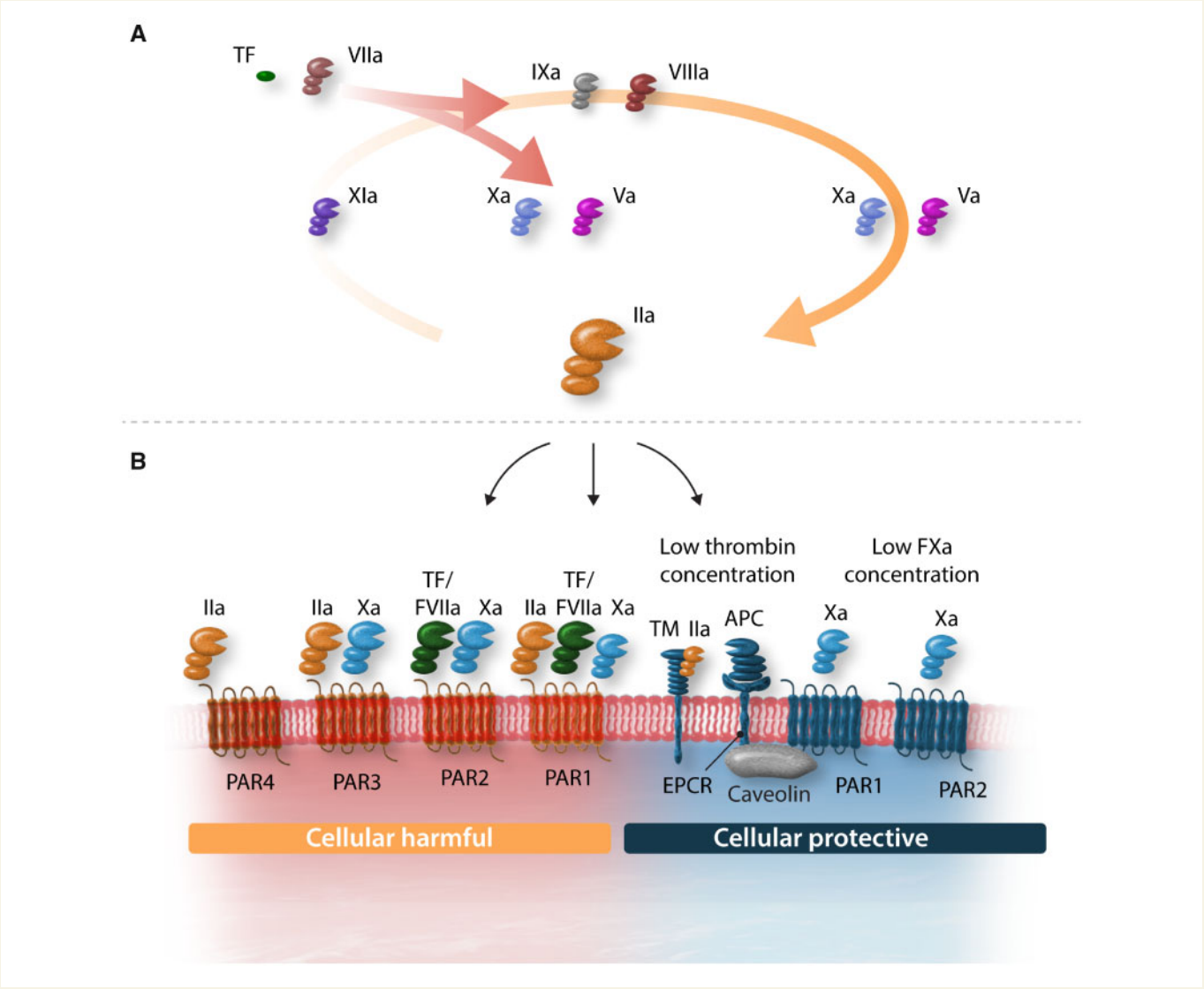

Figure 1 Tissue factor (TF) is the main physiological activator of the coagulation resulting in activated factors VIIa, Xa, and thrombin (IIa). Thrombin generation is further enhanced through the positive feedback loop via factors XIa, IXa, and subsequently Xa and thrombin. Through binding to thrombomodulin, thrombin activates endothelial protein C receptor (EPCR) bound protein C into activated protein C (APC). Besides the anticoagulant activity of APC on inhibition of the cofactors Va and VIII, APC has anti-inflammatory and antiapoptotic activities through activation of the protease-activated receptor 1 (PAR1). Thrombin can activate PAR1, PAR-3, and PAR-4 as well, with the notion that at low levels of thrombin generation effects are mainly cellular protective through either direct activation of PAR1 or via APC-PAR1 signalling, whereas at higher thrombin levels signalling through PAR1 is mainly cellular destructive. Factor Xa or the tissue factor: factor VIIa can activate PAR-2 and PAR-1 can be activated by the tissue factor: factor VIIa: factor Xa complex.

One half of $F7^{-/-}$ mice die in utero and the remainder within 24 h due to major bleeding; there are no indications for defects in vascular development.[25] Theoretically, however, survival of $F7^{-/-}$ mice may be the result of transfer of minute amounts of maternal FVII from the mother, as humans with FVII levels as low as 1% survive.[19] $F10^{-/-}$ mice die mostly in utero between E11.5 and 12.5 and as far as detectable, bleeding is the major cause of death, while there are no obvious signs of impaired vascular integrity. FX deficiency is not associated with disturbed yolk sac development, while this defect was reported in $F5^{-/-}$ and $F2^{-/-}$ mice.[26,27] The remarkable observation that fibrinogen$^{-/-}$ mice survive and develop normally[28] demonstrates that abnormal blood clotting is not sufficient to explain neither the above-mentioned severe bleeding nor impaired vascular development. This raises many questions regarding the role of coagulation proteases in other cellular functions, including endothelial barrier formation.

2.3 Coagulation proteases provide protective actions through activate PARs

2.3.1 PAR-mediated cell signalling

Protease-activated receptors belong to the G-protein-coupled receptors and are unique in their activation mechanism: a protease cleaves the extracellular N-terminal domain of the receptor, thereby generating a new N-terminus (the so-called tethered ligand) which folds back and

activates the receptor. Thus far, four subtypes are recognized in humans: PAR1 through 4 (*Figure 1B*). Early studies demonstrated that one half of PAR1 null mice ($F2r^{-/-}$) die between E9.5 and 10.5.[29,30] The observation that PAR1 is expressed on ECs and VSMCs, triggered further research into the link between coagulation proteases, PAR activation, and vascular properties. Many subsequent studies identified the cell signalling pathways triggered by PAR activation through thrombin, FXa, and FVIIa-TF.[31,32] In a previous paper in this journal, Borissoff and colleagues[33] summarized the known multiplicity in functions of thrombin, acting as an enzyme with vasoprotective properties (mediated through PAR1 activation) as well as anticoagulant functions (through activating protein C upon binding to the EC cofactor thrombomodulin). We will recapitulate some of thrombin's properties as they link to PAR signalling and vascular integrity/permeability.

2.3.2 Thrombin activates PAR1; its cellular effects depend on thrombin concentration and cellular cofactors

Thrombin is one of the mediators of endothelial-dependent changes in *tone* of underlying VSMC. This PAR1-mediated effect of thrombin comprises the release of endothelial-derived relaxing factors, including nitrous oxide (NO) and prostacyclin (various *in vitro* studies reviewed in Ref.34). Increased activity of endothelial nitric oxide synthase3 (eNOS) induced by local thrombin or platelet release products (serotonin, ADP) can be regarded as an endothelial defense mechanism. NO-induced VSMC relaxation improves local blood flow and counteracts the locally offensive effects of platelet released vasoconstrictive mediators like thromboxane A2. Impaired flow dilation can be regarded as an early sign of EC dysfunction (see further).[34]

Infusion of purified thrombin preparation in a dog hindlimb model showed dose-dependent *vasodilatation*, likely directly related to the effects of thrombin[35] producing a two-stage flow dilatational effect, which in part may be determined by platelet activation and release of mediators like ADP/ATP. Later studies in humans showed that PAR1 activation with the agonist peptide SFLLRN (tethered ligand sequence) has differential effects on venous and arterial vasculature, showing venous constriction and arterial dilation, associated with evidence of platelet activation and tissue-type plasminogen activator (tPA) release.[36] Previous studies in dogs with the same agonist peptide showed that in the coronary circulation SFLLRN causes dose-dependent transient coronary dilation, followed by more sustained vasoconstriction and signs of impaired myocardial perfusion.[37]

Thrombin and the PAR1 activating peptide have differential effects on calcium signalling in human brain microvascular cells,[38] effects that for thrombin are dependent on concentration, with a low dose (0.1 nM) preventing calcium rise, in contrast to a high dose (10 nM). The functional differences in G-protein signalling between agonist peptides and thrombin for PAR1 activation were further dissected by McLaughlin *et al.*[39] Bae and colleagues reported that thrombin displays endothelial-protective effects through PAR1, provided that EC protein C receptor (EPCR) is associated with caveolin-1 in lipid rafts and that its occupancy by protein C/activated protein C (APC) causes dissociation from caveolin-1 and recruitment of PAR1 to a protective pertussis-toxin sensitive G(i)-protein (*Figures 1B* and *2*). In this way, both thrombin and APC can induce protective effects via the same receptor, PAR1.[40] Whether this protective effect also requires transactivation of other PARs, for instance shown for PAR1-mediated protective effects in sepsis being dependent on PAR2 transactivation,[41] remains to be further established.

2.3.3 Thrombin-mediated protein C activation at the EC surface

Early studies showed that protective effects of low doses of thrombin infusion (1 unit/kg min) *in vivo* are associated with rapid protein C activation.[42] In addition, profibrinolytic effects were observed, caused by plasminogen activator release, but subsequent studies could not confirm a role for APC in fibrinolysis *in vivo*.[43] In a thrombosis model in primates, a 1 h infusion of low doses of thrombin (1–2 units/kg min) reduces both arterial graft platelet deposition and fibrin incorporation in a venous-type thrombus, an effect that could be abolished by infusion of a monoclonal antibody that prevented protein C activation.[44] APC formation *in vivo* depends on interaction between protein C and EPCR,[45] while the principal driver of protein C activation is the endothelial quaternary complex of protein C bound to EPCR and thrombin, captured by thrombomodulin at the EC surface.[46]

Both for thrombin and for APC, PAR1 is an important cellular signalling receptor. EC barrier function is maintained by APC in a PAR1-, as well as sphingosine 1-phosphate receptor-cross-activation, dependent manner.[47] Biased stimulation of PAR1 occurs through thrombin or APC, cleaving the extracellular domain at sites that are at five amino acids distance from each other (*Figure 2*). While thrombin cleaves at Arg41, inducing G-protein biased signalling via extracellular signal-regulated kinases (ERK1/2) and Ras homolog family member A (RhoaA) resulting in proinflammatory effects, APC cleaves at Arg46 in a beta-Arrestin-dependent manner thereby triggering Phosphoinositide 3-kinases (PI3Ks)/protein kinase B (PKB/Akt) and Ras-related C3 botulinum toxin substrate 1 (RAC1) mediated pathways providing cytoprotective effects.[48] Modifying factors include binding of thrombin to the hirudin-like sequence of PAR1, localization of PAR1 in caveolae, association with EPCR-bound protein C and dimerization with other PARs.

Administration of APC provides cytoprotective effects in a wide range of disease models including for sepsis and ischaemic stroke,[49] diabetic nephropathy,[50] myocardial ischaemia-reperfusion injury,[51,52] and atherosclerosis.[53] A recent clinical trial with a mutated form of APC (lacking anticoagulant activity but maintaining cytoprotective properties) suggests a protective effect on the brain following ischaemic stroke.[54]

2.3.4 Distinct roles for factor Xa

Factor X is another key coagulation protein as it links directly to thrombin generation but also because of its active form, FXa, has independent actions through PAR activation. Under physiologic conditions, FXa may, comparably to low concentrations of thrombin, provide endothelial-protective actions, either by PAR1- or by PAR2-mediated routes (*Figure 3*).[55] Low amounts of FXa (5 nM or higher) protect against high-dose thrombin-induced EC permeability. This effect was also seen with a PAR2 agonist peptide. Bae and colleagues[56] provided further insight in the protective effects of FXa, showing that pre-treatment of EC with catalytic inactive FX (FX-S195A) allows the dissociation of EPCR from caveolin-1 (similar to protein C) and recruitment of PAR1 towards a protective pathway. In addition, FVIIa/TF-activated FX also protects via a PAR2-activated mechanism, an effect that can be mimicked by both PAR1 and PAR2 agonist peptides. Rezaie and co-workers further showed that through activation of PAR2, FXa prevented thrombin-induced EC permeability.[57,58] Finally, Stavenuiter and Mosnier[59] determined that FXa, like APC, also activates PAR-3, resulting in prolonged Tie-2 activation and PAR3-dependent stabilization of EC tight junctions.

Summarizing, the ambient blood levels of coagulation proteins (zymogens) and their activated forms (proteases) serve at least two purposes:

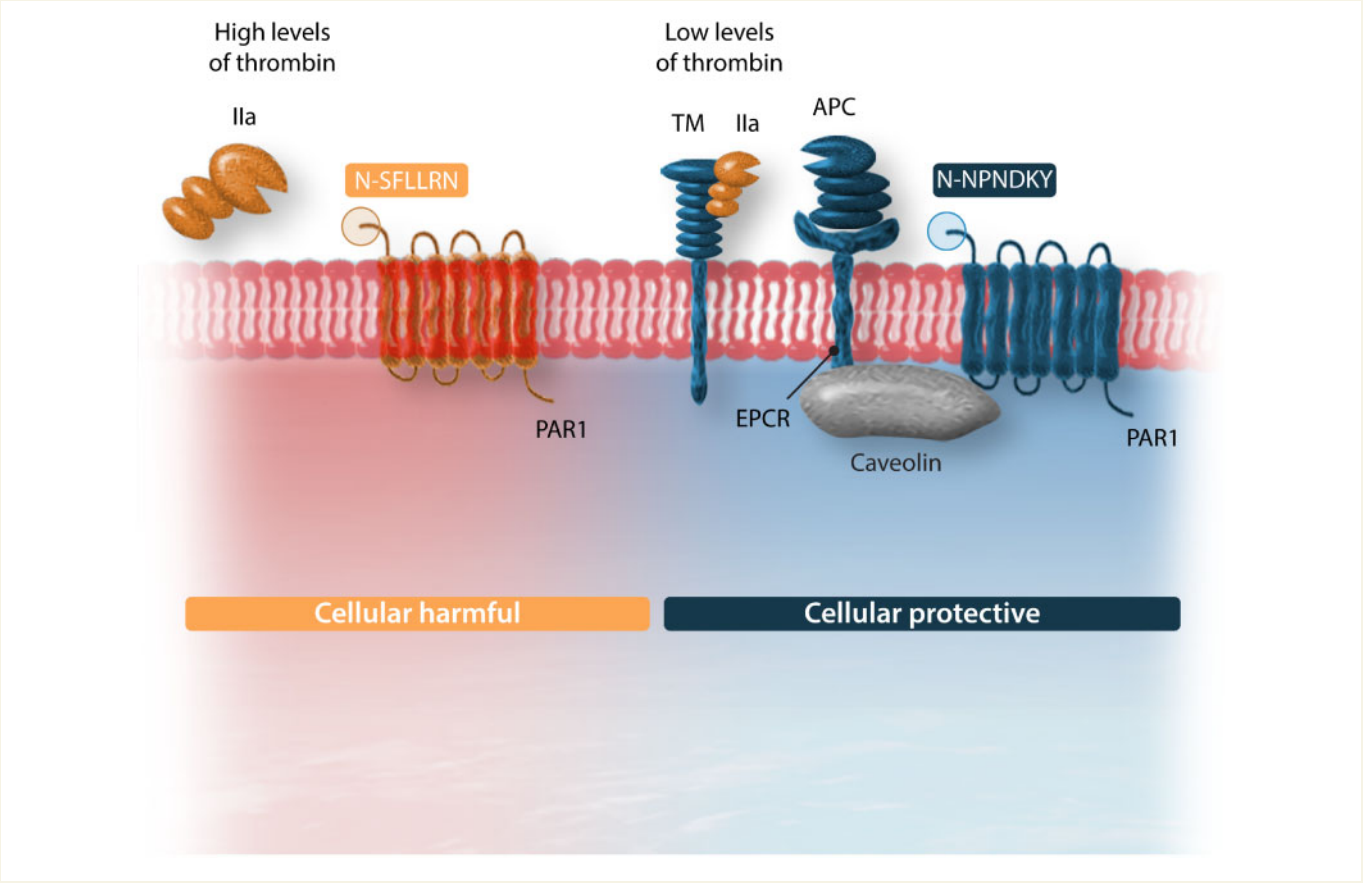

Figure 2 Summary of PAR-1 biased signalling. PAR-1 can be activated by thrombin (factor IIa) or by activated protein C (APC). Thrombin cleaves PAR-1 on position R41 generating the activation sequence SFLLRN, whereas APC cleaves at position R46 revealing the tethered ligand NPNDKY. Cytoprotective effects of APC through PAR1 are mediated through Caveolin-1 interaction with EPCR in lipid rafts. APC binding to EPCR causes dissociation of Caveolin-1 and recruitment of PAR1 to a protective pertussis-toxin sensitive G(i)-protein.

maintaining haemostasis and maintaining vascular patency, in particular endothelial barrier integrity.

Thrombin is a key enzyme in controlling haemostasis (platelet activation and regulating fibrin formation), while EC integrity is maintained in part by (low concentrations of) thrombin-activated PAR1-mediated vascular responses, including vasodilatation in a NO-dependent manner. More important, thrombin-mediated protein C activation yields APC-dependent cell-protective mechanisms via biased and EPCR-mediated PAR1 activation. FXa, similar to thrombin, provides cell-protective actions at relatively low concentrations in a PAR1- and PAR2-dependent manner. The presence of zymogens FX and protein C appears to enhance EC protection and the presence of EPCR provides another important protective cofactor.

3. Determinants of vascular protection: local coagulation protease concentrations and endothelial receptors EPCR and thrombomodulin

In the above vascular-protective scenarios, circulating coagulation proteins and specific cell receptors play major roles. Coagulation proteins will generally be present in sufficient amounts to serve all purposes (under *physiologic* conditions), although they may become rate limiting in severe deficiencies like haemophilia, where strongly reduced thrombin generation may conceptually contribute to loss in EC barrier integrity to provoke focal bleeding. However, based on the mouse studies discussed above, one may assume that such critical functions remain reasonably intact also at very low levels of clotting factors, compatible with a scenario in which low levels of FXa or thrombin activity at the EC suffice to mediate protective effects via PAR signalling, as discussed before.

When using systemic *anticoagulation*, levels of active proteases may be reduced (vitamin K antagonists), but remaining concentrations will probably still be sufficient to provide barrier protection, while even uncarboxylated proteins may retain some signalling properties.[4] Pathophysiological conditions like sepsis, may dramatically change the haemostatic balance and reduced synthesis, 'consumption' and cell receptor shedding may deplete the reservoir of cell-protective proteins; in the most dramatic phenotype, disseminated intravascular coagulation, this can result in a systemic bleeding diathesis and vascular permeation due to massive EC barrier loss, aggravated by thrombocytopenia and platelet dysfunction, as well as disturbed fibrinolysis.[60] Similarly, in anticoagulated patients the diminished coagulation protease 'reserve' may make the EC barrier function prone to perturbation in case of additional trauma. This may be an additional mechanism explaining the risk of major bleeding in anticoagulated patients.

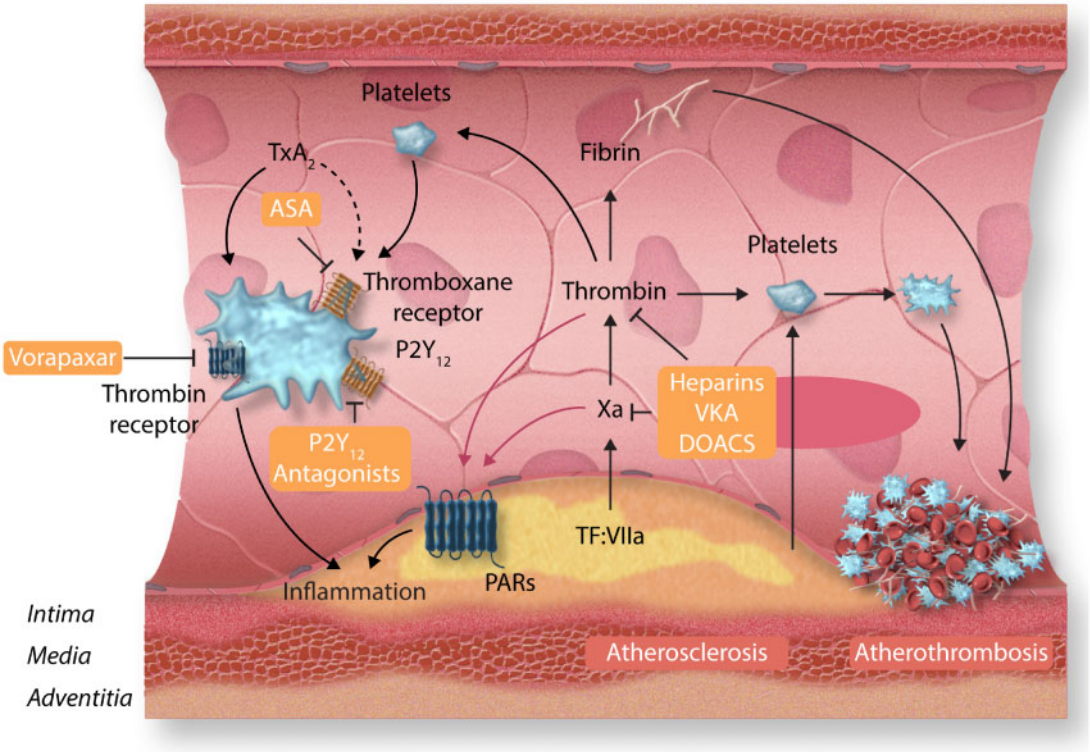

Figure 3 Antithrombotic and vascular-protective effects of anticoagulants and platelet inhibitors. Atherosclerosis-mediated vascular injury causes atherothrombosis through activation of coagulation and platelets. Fibrin formation can be diminished by anticoagulants including heparins, vitamin K antagonists, and direct oral anticoagulants such as the thrombin inhibitor dabigatran and the factor Xa inhibitors rivaroxaban, edoxaban, and apixaban. Platelets can be inhibited by aspirin (ASA, effecting the thromboxane A2 receptor TxA2), P2Y12 receptor antagonists clopidogrel, prasugrel, or ticagrelor, or via inhibition of the thrombin receptor PAR2 by Vorapaxar. Inhibition of thrombin, factor Xa, and platelets will diminish cellular effects through attenuated activation of the protease-activated receptors PAR1 and PAR2 on endothelial cells, vascular smooth muscle cells, and macrophages.

Physiologically, the expression of EC receptors like EPCR and thrombomodulin is heterogeneous throughout the vascular bed and highly regulated. EPCR is detected on ECs of arteries and veins, most arterioles, and some capillary venules, but is undetectable on capillary endothelium.[61] In contrast, thrombomodulin is expressed in the endothelium of large vessels as well as capillaries. Pro-inflammatory stimulation with endotoxin or thrombin induces mRNA for EPCR but also enhances receptor shedding in rodents.[62] Reduced expression of endothelial EPCR and thrombomodulin is reported in severe sepsis,[63] while EC are still morphologically intact. In contrast, overexpression of EPCR provides protection against endotoxemia.[64] FVIIa binding to EPCR enhances protection against endotoxin-induced vascular leakage.[65] Interestingly, recent studies suggest that FVIIa binding to EPCR, which also facilitates its uptake and transport to extravascular tissues where it remains catalytically active,[66] induces PAR1 and β-Arrestin-1-mediated anti-inflammatory signalling pathways.[67] In mice with low EPCR levels or wild-type mice treated with anti-EPCR antibodies, vascular permeability is markedly enhanced in particular in the brain, kidney, and lungs.[68] EPCR deficiency can be functionally restored (APC cofactor functions) by so-called EPCR painting with caveolae-targeted GPI-coupled EPCR.[69]

Thrombomodulin is a cellular receptor with a C-type lectin domain at its N-terminus, six copies of the epidermal growth factor-like motif and serine/threonine-rich domain carrying a glycosaminoglycan external to the membrane. Thrombomodulin binding to thrombin changes thrombin's actions towards anticoagulant and anti-inflammatory actions, through activation of protein C and thrombin-activatable fibrinolysis inhibitor. Thrombomodulin's lectin domain has independent anti-inflammatory activity through its interaction with HMGB1.[70,71] In addition to its cellular functions, recombinant thrombomodulin has anticoagulant and anti-inflammatory properties in pre-clinical models as well as clinical studies in sepsis.[72–74]

Shedding of (soluble) EPCR has been observed in patients with coronary heart disease,[75] renal injury in lupus.[76] In EC, EPCR shedding by cytokines is regulated by several MAP kinase signalling pathways.[77] Several compounds that may downregulate EPCR shedding have been reported including epi-sesamin,[78] piperlongumine,[79] rosmarinic acid,[80] and rutin.[81] Similarly, shedding of thrombomodulin from EC occurs at low levels in culture and is increased upon inflammatory or toxic challenges.[82,83] Many subsequent studies have documented increased levels of soluble thrombomodulin in various disease conditions.[84–86] Loss in endothelial EPCR and thrombomodulin may enhance the risk of atherosclerosis, by reducing local protection against inflammation. Whether in atherosclerotic vessels, local levels of these receptors remain reduced or functionally impaired cannot yet be established with certainty due to the scarce evidence[87,88] or vascular heterogeneity contributing to alterations in atherosclerotic lesion expressed thrombomodulin.[89]

Given their important roles in EC-regulated activation of protein C and in directing cell-protective effects of other coagulation proteases, it is likely that intact expression of EPCR and thrombomodulin is a very potent element of the endothelial barrier function. APC formation may be impaired in atherosclerosis, suggested by diminished anticoagulant response to thrombin infusion in primates,[90] probably related to reduced expression of cofactors EPCR and thrombomodulin, at least on large arteries.[46]

4. Coagulation and EC dysfunction: first step in atherogenesis

Endothelial dysfunction is a hallmark of early stages of vascular pathology.[91] It is characterized by loss of bioavailability of endothelium-derived protective factors, such as nitric oxide or prostacyclin.[92] This not only impairs vasodilatation and promotes vascular remodelling but also enhances platelet adhesion and aggregation.[93] In atherogenesis this primarily concerns the larger arteries that are susceptible to thromboinflammation, driven by flow conditions (velocity, shear stress) and anatomy, making arterial branching points the most vulnerable sites for atherosclerosis development.

In human vasculature, overproduction of superoxide anion by NADPH oxidases and eNOS, leading to rapid scavenging of NO, are primary mechanisms of endothelial dysfunction.[94] Importantly, thrombin has been identified as a main activator of endothelial and VSMC NADPH oxidases.[95] Thrombin has been shown to induce NADPH oxidases Nox2 and Nox5 in ECs and VSMCs and these oxidases are essential mediators of pathological responses of these cells to thrombin.[96] This is important, as Nox5 expression is particularly enhanced in unstable plaques.[97] These effects are heterogeneous but can be mediated by PAR1-dependent mechanisms.[98] Importantly, pro-remodelling effects of thrombin on VSMCs are mediated by NADPH oxidase (Nox) 2 and Nox5 generated reactive oxygen species (ROS). Nox5-dependent effects may be related to the fact that this oxidase is calcium dependent and thrombin, acting on PAR1, increases cytosolic Ca^{2+} concentration in ECs.[99]

Factor Xa can also stimulate endothelial as well as vascular fibroblast oxidative stress via PAR2 activation and these effects appear to be primarily mediated by Nox1 homologue of NADPH oxidase.[10,100] The role of PARs in mediating endothelial dysfunction has been widely discussed. In a number of conditions associated with endothelial dysfunction such as diabetes or atherosclerosis, increased expression of PAR1, PAR3, and PAR4 in the aorta has been identified.[102] Interestingly, in the same study authors reported that direct thrombin inhibition by dabigatran significantly attenuated endothelial dysfunction independently of blood glucose levels. Numerous features of endothelial activation linked to endothelial dysfunction were prevented including inflammatory activation (expression of MCP-1 and ICAM-1) through NFκB activation.[102] Thus, a proinflammatory coagulation-vascular circuit exists that is a major regulator of vascular tone, blood pressure, and endothelial function.[103] For example, in conditions associated with upregulation of TF along with the thrombin-dependent EC dysfunction, integrin αMβ2- and platelet-dependent leukocyte adhesion to endothelium is promoted. This leads to vascular inflammation that promotes dysfunction mediated by activation of thrombin-driven FXI (FXI) feedback, independent of FXII.[103]

These studies indicate that targeting coagulation may represent an important therapeutic avenue for preventing endothelial dysfunction so may actually be effective at very early stages of atherosclerosis and vascular disease.

5. Inflammation and hypercoagulability in atherosclerosis and atherothrombosis

5.1 Thrombo-inflammation and atherothrombosis

Both chronic and acute inflammatory challenges challenge the blood and vessel wall compartments.[104] This thrombo-inflammatory interaction contributes to endothelial dysfunction, loss in EC barrier function, while hypercoagulability due to concerted actions on inflammatory cells, platelets, extracellular vesicles (EVs), and coagulation proteases stimulate atherogenesis.[105,106] In blood, evidence of coagulation cascade hyperactivity is detectable in patients with atherosclerosis; increased markers like d-dimer, prothrombin fragment (F)1.2, or thrombin-antithrombin complexes are oftentimes detected in blood from patients at risk of (recurrent) atherothrombotic events.

TF-driven hypercoagulability is amplified through platelet-leukocyte and EVs mediated thrombin and fibrin formation and accelerated through the contact activation system in CVD.[107–109] Atherothrombosis is further determined by the potential of clots to stabilize and dissolve the net result of fibrin formation, polymerization, and fibrinolysis, as discussed elsewhere.[110–113]

5.2 Expression of proteins by vascular cells and the local 'coagulome'

The inflammation-driven interaction of platelets, leukocytes, EVs, and protease-PAR signalling induced EC barrier permeation, facilitates passage of cells, EVs, and proteins to the subendothelial space. Circulating cells including monocytes also express TF, FVII, and FX.[114–118] Other cells like neutrophils and anuclear platelets may either produce TF, or accumulate TF, by EVs transfer from other cells (discussed in Ref.119). In the intima, VSMCs that undergo the phenotypical switch from contractile to synthetic, also produce TF. Many coagulation proteins are expressed within the vessel wall; one of the first findings is the constitutive expression of TF by fibroblasts in the adventitia, previously referred to as a haemostatic envelope, protecting the larger arteries from bleeding upon trauma.[118,120] While this primary protective function may be relevant, also other cell signalling functions of TF, triggered by binding FVII(a), may be important. Despite the well-known function of tissue factor in activation of coagulation in arterial thrombosis, the role of tissue factor signalling in development and progression of atherosclerosis remains to be elucidated. Experimental atherosclerosis is not altered by low expression (1% human level on murine deficiency or 50% murine levels) of tissue factor, sufficient to maintain haemostasis. Overall, the contribution of tissue factor on development of atherosclerosis is mainly through the signalling pathway of tissue factor: factor VIIa via PAR-2, as reviewed by Grover and Mackman.[121]

TF and FVII are also locally expressed by VSMC and macrophages, transformed from monocytes during their migration into the vessel wall; administration of TFPI can inhibit monocyte chemoattraction.[122] This way many active coagulation components are either constitutively expressed during atherogenesis or may be induced through inflammatory stimuli, supporting local thrombin and fibrin formation, from fibrinogen transferred across the (permeable) EC barrier. Fibrin

formation and cleavage into fragments is a characteristic feature of atherosclerosis and probably modulates several inflammatory mechanisms.[123,124]

As discussed in previous work, various interactions may take place between proteases including FXa and thrombin with PARs expressed at all cells within the arterial vessel wall. Whereas relatively low concentrations of FXa and thrombin are cytoprotective, as discussed in previous sections, increased concentrations of the same proteases may become offensive if conditions allow. These conditions include a pro-inflammatory environment and loss or downregulation of protective receptors and anticoagulant molecules as discussed for the EPCR/TM/PC system.

PAR2 is one of the key receptors involved in atherogenesis, as PAR2 null mice are protected against atherosclerosis; also, those with only haematopoietic deficiency in PAR2 (macrophages) are similarly protected.[125] Activated FX or an agonist peptide based on a FXa amino acid sequence induces inflammatory molecules and stimulates lipid uptake by bone marrow-derived macrophages from wild-type mice, but not by PAR2-deficient macrophages. Genes induced by FXa-mediated degradation of the nuclear inhibitor IκBα in macrophages include an array of proinflammatory cytokines including Il-1β, IL-6, and TNFα.[125] Studies from the immunology field show that tumour-associated macrophages synthesize the TF ligands FVII and FX.[126,127] Recent studies show that FX produced by monocytes/macrophages activates PAR2 in a local tumour environment impeding anti-tumour activity; this reaction could be abolished by the direct FXa inhibitor rivaroxaban.[128] It should be noted that while coagulation proteases are important, other enzymes can be involved and several studies showed that trypsin,[129] cathepsin S,[130] and tryptase[131] also activate PAR2, probably in a tissue-dependent manner.

Extrapolating these data to atherosclerosis it seems likely that macrophage-derived FX also regulates proinflammatory mechanisms through PAR2 activation on these cells, as well as on VSMC. For instance, PAR2 deficiency on VSMC is associated with reduced Ccl2 and Cxcl1 mRNA expression and protein release, limiting migration of monocytes.[132] Inhibition of FXa, hence thrombin generation, may be an effective way of inhibiting a variety of thromboinflammatory reactions and associated disease, including atherosclerosis, fibrosis, heart, and kidney failure.

6. Atherothrombosis

6.1 Pleiotropic nature of atherothrombosis—from plaque rupture to plaque erosion

Increasing use of statins, reduced prevalence of smoking, advances in intravascular imaging, and a better understanding of the pleiotropic nature of the process of atherothrombosis led to reports of a shift in the natural history of thrombotic events developing as complications of atherosclerosis. While plaque instability and rupture have been the focus of our diagnostic and therapeutic attention for past decades, only recently, we realized the critical importance of plaque erosion in this process.[3,133–136] Erosion prone plaques are proteoglycan/glycosaminoglycan rich, have relatively small lipid cores but many smooth muscle cells with neutrophils and neutrophil extracellular traps (NETs).[137] Inflammation and metabolic changes in the EC leading to endothelial–mesenchymal transition[138,139] and detachment promoted by non-laminar flow in concert with basement membrane damage are the key factors contributing to plaque erosion. The rupture-prone plaque is characterized by a thin, collagen poor fibrous cap, large lipid core, macrophage-driven inflammation in plaque shoulder regions.[140] Importantly, while both plaque erosion and rupture are accompanied by intense coagulation activation, chronically eroded plaques are associated with platelet-rich white thrombus whereas ruptured plaques are associated with fibrin-rich red thrombus.[3]

The changing insight in atherosclerosis-induced thrombosis has profound clinical implications. In the past, the most common clinical presentation of acute coronary ischaemia was ST-elevation myocardial infarction (STEMI) but this is now less common than non-STEMI (NSTEMI).[3] Reflecting this shift, non-invasive therapies that target the pathology of plaque erosion pathology may become more important, including anti-inflammatory therapy with the use of canakinumab[141] or colchicine,[142] and DPI with the combination of APT and anticoagulant therapy.[5] This is because while the thrombus is platelet rich, plaque erosion is associated with local activation of coagulation. This includes not only substances released by platelets and damaged basal membranes but also generation of NETs, which can also acquire proteins from extra-neutrophilic sources, including tissue factor (TF) that through activation of FVII and formation of FXa lead to local thrombin generation (*Figure 1A*).[143–145]

6.2 Peripheral and coronary atherothrombosis—the same or different?

A related issue of importance is that while multi-level atherosclerosis is clinically considered a more advanced form of the same disease, an increasing body of evidence suggests that in different vascular beds mechanisms of atherothrombosis may be different. In a recent histopathological study of critical limb ischaemia, it has been shown that in PAD thrombotic luminal occlusion associated with insignificant atherosclerosis is much more commonly observed than in coronary arteries.[146] This links to earlier considerations of the role of plaque erosion as a primary mechanism in the peripheral circulation. Moreover, differences were observed within peripheral PAD itself, with a more prominent role of atherothrombosis in lower locations (infra-popliteal) of critical limb ischaemia. These clinical observations, while still poorly defined mechanistically, may support a greater preventive role of antithrombotic agents in PAD.[147]

7. Preclinical evidence for vascular protection

Many preclinical studies done in (transgenic) mice have elucidated the important role of blood coagulation proteins in atherogenesis. These studies, virtually all carried out in *apoE*$^{-/-}$ mice, showed a general pattern of increased atherosclerosis in *apoE*$^{-/-}$ mice backcrossed with animals expressing thrombophilic traits, while mice that expressed lower levels of procoagulant proteins were partially protected (discussed in Ref.148). While thrombin-triggered PAR activation may be an important mechanism, the impact of other ligands cannot be ruled out. First, as discussed, FVIIa, FXa, and thrombin each or in concert activate different PARs in a variety of cells; variations in the levels of these proteases may have considerable impact on degree and direction of PAR-mediated signalling. Second, crosstalk between the PARs and other cellular cofactors (e.g. see the important impact of thrombomodulin and EPCR expression at EC related to biased PAR signalling discussed before), is involved. Third, specific proteases may contribute unique procoagulant properties,

independent from thrombin; an example is FXIa that inactivates TFPI, enhanced by platelet-derived short-chain polyphosphates.[149,150] Another example is fibrinogen; its absence in null mice does not protect against atherosclerosis,[151] but it can affect the phenotype of lesions in susceptible mice, probably through loss of interactions with lipoproteins.[152]

Inhibiting FXa or thrombin with direct inhibitors consistently attenuates atherosclerosis in *apoE*$^{-/-}$ mice.[148] Studies with dabigatran revealed differences in plaque phenotypes linked to reduced or increased plaque stability,[53,153] probably related to age and timing of intervention. For FXa inhibition, data are consistent with reduced atherosclerosis and increased plaque stability, although the observed effects vary per study depending on the anticoagulant dose applied.[154–156] Inhibition of FXa in *apoE*$^{-/-}$ mice with existing atherosclerosis attenuates plaque volume and stabilizes plaque phenotype.[156] Collectively, the data point to a substantial influence of coagulation proteases on atherosclerotic plaque formation and plaque phenotype. Inhibiting FXa is one potent therapeutic avenue to attenuate and stabilize atherosclerotic lesions. Clinical studies also provide supporting evidence for these observations although much more and robust evidence needs to be obtained.[157] As recently reviewed, targeting both platelets and coagulation proteases is conceptually attractive. Platelets are key players in atherosclerosis and atherothrombosis, while the above preclinical studies indicate the importance of coagulation proteases.[158] The sum of antiplatelet and anticoagulant activities is likely to amplify the potency of antithrombotic therapy aimed at preventing atherothrombosis and thromboinflammation.

8. Clinical translation: trials of the combination of rivaroxaban and aspirin in chronic atherosclerosis

Several recent trials provide strong support for the results of experimental and animal studies that combining platelet inhibition with anticoagulation not only produces a more intense antithrombotic effect but also enhances vascular protection.[5–7] Previous studies evaluating the combination of long-term warfarin and aspirin suggested the potential of dual pathway therapy to reduce major adverse cardiovascular events in patients with a history of coronary artery disease.[159] However, the reduction in non-fatal cardiovascular events with combination of warfarin and aspirin compared with aspirin alone was accompanied by a large excess of serious bleeding and there was no overall mortality benefit. These results dampened enthusiasm for adopting a warfarin-based dual pathway regimen for long-term management of patients with atherosclerosis.

The advent of the direct acting oral anticoagulants (DOACs) provided for the first time the potential to selectively target individual coagulation proteases with agents that produced consistent and predictable response when given in fixed doses without routine coagulation monitoring.[160] Two FXa inhibitors, apixaban and rivaroxaban, have been tested on a background of APT in patients with a recent acute coronary syndrome but only rivaroxaban was shown to be effective. The APPRAISE trial tested apixaban using the same doses that were shown to be effective for stroke prevention in patients with atrial fibrillation (5 mg twice daily or 2.5 mg twice daily in those meeting prespecified criteria for dose reduction), but was stopped early because of excess bleeding.[161] The ATLAS ACS 2–TIMI 51 trial tested rivaroxaban at lower doses (2.5 or 5 mg twice daily) than those shown to be effective for stroke prevention in patients with atrial fibrillation, and demonstrated a reduction in both non-fatal cardiovascular events and mortality.[162] The magnitude of the benefit achieved using only a very low intensity of anticoagulation raised the possibility that the effects of rivaroxaban extended beyond effects on coagulation to provide vascular protection.

COMPASS tested rivaroxaban at the same low doses that were successfully evaluated in ATLAS, rivaroxaban 2.5 mg in combination with aspirin 100 mg once daily and rivaroxaban 5 mg twice daily alone, compared with aspirin 100 mg once daily, in patients with chronic coronary artery disease or PAD. Patients with a history of stroke ≥1 month ago could be included as long as they did not have a history of lacunar or haemorrhagic stroke.[163]

The trial randomized 27 395 patients from 602 centres in 33 countries, and the intent was to continue follow-up until 2200 patients had experienced a primary outcome, the composite of cardiovascular death, stroke, or myocardial infarction. However, after a mean of 23 months of follow-up, and when only just over 50% of primary outcome events had occurred, the Data Safety Monitoring Board recommended discontinuation of the antithrombotic arms of the trial because of clear evidence of benefit of the combination of rivaroxaban 2.5 mg twice daily and aspirin 100 mg once daily compared with aspirin alone.[164] Rivaroxaban alone given at a dose of 5 mg twice-daily was not superior to aspirin 100 mg once-daily.

The main results of the COMPASS trial[5] comparison between rivaroxaban 2.5 mg twice-daily and aspirin 100 mg once-daily are summarized in *Table 1*.

The combination of rivaroxaban and aspirin compared with aspirin reduced major adverse cardiovascular events by 24%, including a 22% reduction in cardiovascular death, 42% reduction in stroke, and a 14% reduction in myocardial infarction. Bleeding was increased by 70%, mainly from the gastrointestinal tract, and although there was a consistent pattern of increased fatal, critical organ and surgical site bleeding, these bleeds were infrequent and not significantly increased. Importantly, the combination produced a clear net clinical benefit, as evidenced by reductions in a composite outcome that included the primary efficacy outcome and serious bleeding, as well as reduction in all-cause mortality.[165] The greatest benefit of the combination was in patients at highest baseline risk, including those with polyvascular disease (i.e. both coronary artery disease and PAD), heart failure, diabetes, or chronic kidney disease.[166]

The COMMANDER HF trial compared rivaroxaban 2.5 mg twice daily with placebo in 5022 patients with recently decompensated chronic heart failure and underlying coronary artery disease.[6] The majority of patients was receiving APT (aspirin 93.1%, dual APT 34.8%). The trial involved 628 centres in 32 countries. The main results are summarized in *Table 2*. After a median follow-up of 21 months, rivaroxaban compared with placebo did not reduce the primary outcome, a composite of death, myocardial infarction, or stroke, and did not increase bleeding. There was a significant 34% reduction in stroke as well as a promising pattern of fewer myocardial infarctions, but in the primary composite outcome this signal was obscured by mortality which accounted for >80% of primary events and was not reduced by rivaroxaban. In a separate exploratory analysis, rivaroxaban compared with placebo significantly reduced the composite of arterial and venous events by 17%.

The VOYAGER PAD trial compared rivaroxaban 2.5 mg twice daily with placebo on background of standard APT (aspirin alone or dual APT) in 6564 patients with PAD who had recently undergone a lower limb revascularization procedure.[7] The trial was performed in 542 sites in 34 countries.

Table 1 COMPASS trial: outcomes comparing the combination of rivaroxaban 2.5 mg twice daily and aspirin 100 mg once-daily with aspirin 100 mg once-daily

Outcome	Rivaroxaban 2.5 mg twice-daily plus aspirin 100 mg once-daily vs. aspirin 100 mg once-daily *N* = 9152	Aspirin 100 mg once-daily *N* = 9126	HR (95% CI)
CV death, stroke, or myocardial infarction	379 (4.1%)	496 (5.4%)	0.76 (0.66–0.86)
CV death	160 (1.7)	203 (2.2%)	0.78 (0.64–0.96)
Stroke	83 (0.9%)	142 (1.6)	0.58 (0.44–0.76)
Myocardial infarction	178 (1.9%)	205 (2.2%)	0.86 (0.70–1.05)
Mortality	313 (3.4%)	378 (4.1%)	0.82 (0.71–0.96)
Venous thromboembolism	25 (0.3%)	41 (0.4%)	0.61 (0.37–1.00)
CV hospitalization	1303 (14.2%)	1394 (15.3%)	0.92 (0.86–1.00)

Table 2 COMMANDER HF trial: overall results comparing rivaroxaban 2.5 mg twice-daily and with placebo on a background of standard care

Outcome	Rivaroxaban 2.5 mg twice-daily *N* = 2507	Placebo twice-daily *N* = 2515	HR (95% CI)
Death, myocardial infarction, or stroke	626 (25%)	658 (26.2%)	0.94 (0.84–1.05)
Death	546 (21.8%)	556 (22.1%)	0.98 (0.87–1.10)
Myocardial infarction	98 (3.9%)	118 (4.7%)	0.83 (0.63–1.08)
Stroke	51 (2.0%)	76 (3.0%)	0.66 (0.47–0.95)
Composite of thromboembolic events: myocardial infarction, ischaemic stroke, sudden/unwitnessed deaths, symptomatic PE, symptomatic DVT	328 (13.1%)	390 (15.5%)	0.83 (0.72–0.96)

Table 3 VOYAGER PAD trial: overall results comparing rivaroxaban 2.5 mg twice-daily with placebo on a background of antiplatelet therapy

Outcome	Rivaroxaban 2.5 mg twice-daily *N* = 3286	Placebo twice-daily *N* = 3278	HR (95% CI)
Acute limb ischaemia, major amputation for vascular causes, myocardial infarction, ischaemic stroke, or CV death	508 (15.5%)	584 (17.8%)	0.85 (0.76–0.96)
Acute limb ischaemia	155 (4.7%)	227 (6.9%)	0.67 (0.55–0.82)
Major amputation for vascular causes	103 (3.1%)	115 (3.5%)	0.89 (0.68–1.16)
Myocardial infarction	131 (4.0%)	148 (4.5%)	0.88 (0.70–1.12)
Ischaemic stroke	71 (2.2%)	82 (2.5%)	0.87 (0.63–1.19)
CV death	199 (6.1%)	174 (5.3%)	1.14 (0.93–1.40)
Mortality	321 (9.8%)	297 (9.1%)	1.08 (0.92–1.27)
Venous thromboembolism	25 (0.8%)	41 (1.3%)	0.61 (0.37–1.00)

The main results are summarized in *Table 3*. After a median follow-up of 28 months, rivaroxaban compared with placebo reduced the primary outcome, a composite of acute limb ischaemia, major amputation for vascular causes, myocardial infarction, ischaemic stroke, or CV death, by 15%, including a 33% reduction in acute limb ischaemia. There was no reduction in mortality and no significant increase in TIMI major bleeding although ISTH major bleeding was increased by 42%. Results were consistent when separately examined in patients receiving single compared with dual APT.

It is not possible to establish with certainty from the results of these trials whether the benefits of combination therapy are explained solely by increased intensity of antithrombotic therapy or whether the combination also provides vascular protection through an effect on the vascular endothelium. The reduction in venous thromboembolism obtained by using rivaroxaban on top of APT in both the COMPASS and the VOYAGER trials is strongly suggesting of a direct antithrombotic effect as this result is unlikely to be explained by 'vascular protection'.

However, the context of previously summarized experimental and animal research, as well as the results of the ATLAS ACS 2–TIMI 51trial,[162] the unexpectedly large reduction in major adverse cardiovascular events of adding a low dose of rivaroxaban to aspirin in the COMPASS trial and the consistent benefits of low-dose rivaroxaban in other trials is consistent with the conclusion that rivaroxaban has effects beyond inhibition of coagulation.

9. Conclusions and future perspectives

Atherothrombotic complications of atherosclerotic vascular disease can at least be limited by a strategy of DPI, aimed at both platelets and coagulation proteases, in particular FXa (and consequent thrombin formation) (*Figure* 2). The fact that this strategy provides substantial clinical benefit may be due to a combination of factors: (i) patients with atherosclerosis have signs of active thrombo-inflammation, not only in blood but also in the arterial vessel wall compartment; (ii) pre-clinical data provide robust evidence for a causal association between hypercoagulability and atherosclerosis towards an unstable phenotype, mediated by PAR directed cell signalling; (iii) attenuating thrombo-inflammation likely dampens plaque instability and in doing so, may actually reverse the risk of atherothrombotic complications; and (iv) DOACs inhibit or even reverse severity of atherosclerosis in experimental mouse models through attenuating thrombo-inflammatory mechanisms.

It is an important question whether *higher doses* of DOACs such as used to prevent ischaemic stroke in AF also provide such atheroprotective effects? Although some studies suggest this may be the case, such extrapolation may not be obvious. Maybe low concentrations of anticoagulants are just sufficient to dampen thromboinflammation, while higher doses prevent thrombosis but may theoretically undermine some of the protein C activating mechanisms that are so critical in host defense. An additional advantage of DOACs over VKA may be the fact that while VKA also lowers functional activity of proteins C and S, DOACs do not directly affect these natural anticoagulants.

Preventing atherothrombosis through a potent anticoagulant action remains obviously a key property of any anticoagulant and irrespective of the differences, DOAC and VKA are equally potent in that regard. Potency however comes at a price of bleeding and while this may relate to haemostasis impairment, undermining the vascular-protective properties of coagulation and in particular the protein C system, may be an additional reason for the dose dependent increases in bleeding and the difference in organ specific bleeds, between DOACs and VKA.

Given the complexity of the coagulation-PAR-EC-vessel wall interactions, where even a limited number of proteases and cell receptors may have sheer infinite different signalling interactions, depending on systemic and local conditions, simple answers to some of the raised questions are impossible to give. Still, appreciating the complexity of coagulation proteases and their cellular effects provides a starting point for better targeted and tailored vascular-protective medication, ultimately removing bleeding side effects that remain critically linked to any type of antithrombotic strategy.

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