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New Insights into Modulation of Thrombin Formation

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Abstract Thrombin is a pleiotropic enzyme that regulates hemostasis and nonhemostatic functions, including an array of actions within and on the vasculature. Physiologically, thrombin generation serves mainly to protect against thrombosis, but also to maintain vascular endothelial integrity. This protective effect is mediated in part through generation of anticoagulant enzymes, including activated protein C, formed on the action of thrombin on the endothelial receptor thrombomodulin. Partly, thrombin's vascular effects are effectuated through interaction with protease-activated receptors on various cell types. Pathophysiologically, downregulation and shedding of anticoagulant-acting receptors such as thrombomodulin and endothelial protein C receptor may contribute to a shift in activities of thrombin towards thrombogenic and proinflammatory actions. This shift may typically occur in the process of atherosclerosis, leading to a proatherogenic direction of the effects of thrombin. Therapeutically, the long-term inhibition of thrombin may create new ways of reducing atherosclerosis burden, altering the plaque phenotype.

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Introduction: Thrombin and Hemostasis

The blood coagulation (reviewed in [1]) cascade primarily serves the role of protecting against bleeding on tissue trauma. This protective effect is crucial in the evolutionary conservation of all mammals, starting at limiting blood loss during delivery, which explains the conservation of this complex system in time. This function of the blood coagulation system is referred to as "hemostasis," and is based on effective arrest of bleeding by platelets interacting with the damaged vessel wall (reviewed in [2•, 3]). It also involves the generation of thrombin, facilitated by phospholipid surfaces provided by platelets and other cells, as well as microparticles released on cell activation [4]. Thrombin formation results from a rapid cascade of limited proteolytic reactions, started by the formation of a cell surface tissue factor (TF)–factor VIIa complex, which activates factor X, either directly (extrinsic route [5]) or indirectly (through the intrinsic route, via factor IX [6]). Thrombin is the principal enzyme that converts fibrinogen to fibrin, which, on polymerization, forms the clot that arrests bleeding [7]. In the blood coagulation cascade, thrombin formation is a key step. Thrombin not only controls fibrin formation, but also activates factor XIII to cross-link fibrin, and amplifies thrombin formation via the activation of the cofactors factor V and factor VIII [8]. In addition, thrombin is probably key to activation of factor XI, upstream in the intrinsic route [9]. Although the intrinsic route is formally started by activation of factor XII (contact system), the contribution of this pathway to hemostasis is a matter of debate, mostly because of the absence of a bleeding phenotype in patients lacking factor XII. Activation of factor XI by thrombin as part of a positive-feedback loop is considered to be of more importance for hemostasis [9], whereas activation of factor XII and subsequently activation of factor XI by factor

XIIa is involved in triggering and/or propagation of thrombotic coagulation [10]. Thrombin also activates platelets through the protease-activated receptor (PAR) 1 [11], resulting in activated platelets that also generate a phospholipid surface on which coagulation reactions are localized and amplified (in tenase and prothrombinase complexes).

Although all these thrombin-mediated reactions are primarily procoagulant in nature, i.e., contributing to the formation of a fibrin clot, thrombin can also dampen coagulation activity under physiological conditions, by binding the cell surface receptor thrombomodulin [12]. The thrombin–thrombomodulin complex facilitates activation of protein C to activated PC (APC), which, in conjunction with endothelial protein C receptor (EPCR), potently inhibits the procoagulant intrinsic formation of thrombin and fibrin [13]. Thus, thrombin has procoagulant and anticoagulant roles, depending on the required local condition [8]. Physiologically, an ambient level of thrombin is maintained in blood, which most likely acts in APC formation to slow down any unwanted fibrin formation in the intact vasculature. At the same time, this basal level of thrombin forms the engine that can quickly accelerate to produce fibrin in the case of hemostatic damage.

The rate of thrombin formation is different for each individual and is well controlled by several mechanisms, including, in addition to the protein C and protein S mechanism, the natural anticoagulants TF pathway inhibitor, which attenuates the TF-mediated coagulation route [14], and antithrombin, which directly inhibits serine proteases, including thrombin and factor Xa [15]. To limit excess fibrin formation and to take care of fibrin clearance following clot formation ("wound healing"), fibrinolytic proteins generate plasmin, which degrades cross-linked fibrin in a stepwise proteolytic reaction [16]. Thrombin is also involved in this cascade, by activating thrombin-activated fibrinolysis inhibitor [17]. Thus, thrombin is a pluripotent enzyme that controls several coagulation mechanisms under physiological conditions as well as during hemostatic emergencies resulting in bleeding.

Thrombin Formation in the Vasculature: Hemostasis and Thrombosis

Basal levels of thrombin are formed in each healthy person; the degree differs among subjects but remains quite constant in time. The origin of the traces of thrombin required to maintain hemostasis is essentially unknown. Physiologically, the vascular endothelium is postulated to act in an anticoagulant manner, generating APC and other substances, including prostacyclin and nitrous oxide, that prevent clotting [18]. Since the contact system including factor XII is not essential in hemostasis, it is likely that the extrinsic, or TF-dependent pathway generates ambient levels of thrombin. Although the presence of TF is not directly demonstrable under physiological conditions, we observed that coagulation activity induced by infusion of recombinant factor VIIa was blocked by an antibody against TF in healthy primates [19], suggesting the presence of so-called blood-borne TF in the circulation [20]. Possible sources of TF are vascular microtraumata exposing or generating minute amounts of TF, and circulating microparticles expressing TF [2]. Normal apoptotic processes, involved in the physiological turnover of blood cells, may produce the latter.

Hemostatic challenge by vessel wall damage (trauma) triggers the hemostatic defense to rapidly arrest bleeding. This controlled reaction is followed by similarly controlled clot removal by fibrinolysis and blood flow. In the case of congenital hemostatic defects, the clot formation, stabilization, or removal may be impaired and a bleeding tendency is the result (not discussed in this article).

The process of thrombin and fibrin formation may also escape control and result in thrombosis, the unwanted and excess formation of a clot (partly) obstructing the normal blood circulation. The process of thrombosis can occur in the venous or arterial circulation, or systemically, in the case of disseminated intravascular coagulation (DIC). We will concisely describe the main differences between these types of thrombosis, as a framework for describing the roles of thrombin formation in the process of thrombosis.

Current views provide a similar scenario for the initiation of thrombosis, irrespective of the vascular bed. Inflammation is the starting point in all cases, which is not surprising assuming that thrombosis is essentially excess clot formation, and hence an excessively triggered response to injury. In all cases of thrombosis, Virchow's triad postulates the interaction of blood, vessel walls, and altered flow. In modern versions of this triad, the blood compartment involves activated blood coagulation, including all circulating cells, the vessel wall, which may show morphological or molecular changes (increased expression of adhesion molecules that may bind leukocytes), and changes in blood flow due to stasis in the case of venous thrombosis, or caused by atherosclerotic vessel wall damage in arterial thrombosis.

Venous thrombosis is thought to occur by interaction of inflammatory mediators, including endothelial-cell-expressed adhesion molecules, circulating neutrophils releasing neutrophil extracellular traps (NETs), and microparticles carrying TF (reviewed in [21]). Proinflammatory mediators upregulate TF on cells and microparticles, initiating thrombin formation. The rate of thrombin formation is also determined by systemic factors that cause "hypercoagulability" (also referred to as "thrombophilia"). These thrombophilia factors are particularly important catalysts in venous thromboembolism and comprise a spectrum of congenital and acquired risk factors. Primary vessel wall damage may also be involved in venous thrombosis (like in orthopedic surgery, where mechanical manipulation of the proximal femoral vein may cause damage), but in most cases that combination of inflammation and hypercoagulability triggers

thrombosis at vulnerable sites in the venous circulation. Venous valves in the proximal leg, as one of the predilection areas vulnerable to hypoxemia when flow is impaired, are such sites.

Arterial thrombosis usually occurs in the context of atherosclerosis (see further). The process of thrombosis appears to occur in recurring episodes of plaque damage, thrombosis, and plaque reorganization as shown in histological studies [22]. Hence, not all thrombi occurring on plaques are symptomatic, but when the blood vessel occludes, symptoms of ischemia occur. Damage to the atherosclerotic plaque involves erosion or rupture. In both cases, the exposure of TF in the plaque, as well as collagen and other proteins from the subendothelial matrix, triggers blood coagulation. This process triggers both platelet adhesion and TF-mediated coagulation, starting a classic hemostatic response to injury [3]. Whereas in normal vessels this response would be limited, in pathologically altered vessels the stimulus may be protracted, and hypercoagulability factors contribute to the occurrence of an occlusive thrombus. Although in atherothrombosis the influence of platelets dominates the effect of hypercoagulability-associated thrombin formation, the effect of thrombin generation does contribute [23]. Both congenital and acquired increased levels of thrombin formation (including a contribution of the factor V Leiden and prothrombin 20210 variants) increase the risk of symptomatic coronary artery disease [24]. Increased levels of thrombin and fibrin formation are also indicative of a risk of recurrent myocardial ischemia and infarction. The vascular endothelium may play an important role in modifying the net effect of thrombin. Although, physiologically, thrombin may be captured by thrombomodulin, facilitating APC formation, in progressive atherosclerosis the level of endothelial thrombomodulin (and EPCR) diminishes, such that more procoagulant thrombin remains [25]. This vascular degeneration may also explain why thrombosis is more frequent with ageing and with more progressive atherosclerosis.

A distinct type of arterial thromboembolism occurs in the context of atrial fibrillation. Here, a combination of local and systemic factors, probably hypercoagulability as well, contributes to the initiation of thrombus formation in the left atrium (and appendage) [26].

Lastly, DIC is the formation of microvascular thrombi that may contribute to multiorgan failure in the setting of acute inflammatory syndromes, including sepsis [27]. Inflammation is central in this process, triggering coagulation through TF expression, impaired natural anticoagulant mechanisms, and impaired fibrinolysis.

Thrombin and Nonhemostatic Properties: The role of PARs

Although the primary function of hemostasis, preventing unwanted blood loss, is an essential function, the blood coagulation cascade appears to be important in immunity as well. Highly conserved animals such as the horseshoe crab (*Limulus polyphemus*) combine hemostasis with immune reactions, such that invading bacteria are walled off by a fibrin-like compound generated at the surface of a leukocyte/platelet type of blood cell, aided by a primitive coagulation cascade [28]. This prototypic immune reaction is observed in humans under conditions of inflammation, which drives a number of procoagulant pathways through proinflammatory cytokines. An extreme example is acute DIC, in which all relevant blood coagulation mechanisms are engaged to the extent of depletion of cells and proteins owing to excess consumption in the clotting process [29, 30]. The interactions between coagulation proteases and immune cells and mediators involve the downregulation of protective cellular receptors such as thrombomodulin [31] and EPCR [12], but also the close interaction between procoagulant enzymes such as factor VIIa, factor Xa, and thrombin with PARs on cells within the vasculature [1]. In inflammatory conditions, the capillary network is most relevant in modifying inflammation–coagulation interactions, since it constitutes by far the largest endothelial surface in the vasculature. Occlusion of capillaries by fibrin, as well as increased leakage of capillaries by endothelial barrier rupture, contributes to organ failure in sepsis/systemic inflammatory response syndrome [32].

On top of hemostatic functions, blood coagulation elements are involved in an array of nonhemostatic functions meant to support the immune system and to maintain vascular integrity. Although, physiologically, thrombin generation protects against inflammation and clotting by mediating protein C activation through the endothelial surface receptors thrombomodulin and EPCR, these protective systems fail when perturbed by inflammatory mediators. Suppressed synthesis as well as shedding of proteins cleaved by neutrophil elastase and other proteolytic enzymes (matrix metalloproteinases) diminishes the vascular anticoagulant reserve. This has been demonstrated in systemic inflammation (sepsis), but also in the context of chronic vascular inflammation, including atherosclerosis. Thus, toxins such as oxidized LDL also suppress thrombomodulin gene expression [33, 34]. Also posttranslational effects that suppress the functional activity of thrombomodulin activity, including oxidation of a methionine in the EGF-like repeat [35], supposedly occurring during neutrophil activation during inflammation, may play a role. Interestingly, some of these phenomena may be reversible, with thrombomodulin and EPCR activity reemerging after cessation of illness [36], or after removal of the atherogenic stimulus, in primates [37]. Pathophysiologically, the reduced capture of thrombin by thrombomodulin, coupled with increased production of thrombin, may increase the amount of thrombin available for interacting with PARs at the surface of many different types of cells, including endothelial cells. The shift from physiology to pathophysiology is associated with a shift in the production of ligands (thrombin versus APC), the presence of PARs versus thrombomodulin/EPCR, the

organization and localization of the enzyme–receptor complex at the cell surface, and the resulting signaling cascade engaged. An example of the complex manner in which this is regulated is provided by PAR-1 in its interaction with either thrombin or APC, both ligands for the same receptor. Thrombin activates PAR-1 on platelets at very low concentrations, leading to aggregation, but it also stimulates an array of proinflammatory effects in other types of cells, including endothelial cells [38]. In contrast, APC in complex with EPCR downregulates several proinflammatory pathways through the same PAR-1 [39]. In a number of elegant experiments, Rezaie [38] showed that binding of the Gla domain of protein C/APC to EPCR determined the type of PAR-1 response rather than the type of protease that cleaved the receptor. Under these conditions, thrombin activation of PAR-1 also elicits protective effects.

Another factor explaining differences in protease-mediated cell signaling involves the rate of internalization of PAR-1 on binding to thrombin, which may be higher than the rate of internalization induced by APC binding to PAR-1 [40]. The localization of EPCR and PAR-1 in association with caveolin-1 in cholesterol-rich lipid rafts in human umbilical vein endothelial cells is an important factor, since ligand occupancy of EPCR determines the migration of PAR-1 from the caveolar compartment. This process also determines the net effect of APC- or thrombin-mediated actions (protective or not). It remains uncertain how EPCR occupancy directs protease-dependent PAR-1 signaling. Different G-coupled proteins are potentially involved, each linked to different signaling pathways. Posttranslational modifications of PARs regulate their function in time and space [41]. Taken together, this complex network of PAR signaling through different G-protein-coupled cellular pathways may explain the flexibility of the human system to handle different proteases depending on the underlying condition. Its complexity may also explain why "simple" one-molecule interventions in complex diseases such as sepsis, e.g., the administration of recombinant APC in sepsis, eventually failed in studies with very heterogenous patient populations [42].

Innate Immunity, Extracellular Nucleic Acids, and Thrombosis

Immunothrombosis is an evolutionarily conserved intravascular host-defense mechanism, designed to limit pathogen dissemination and tissue injury during infection, and operating via the activation of both the innate immune system and blood coagulation [43]. Persistent systemic inflammation and cell death are inextricably linked processes [44]. A continuous inflammatory insult may ultimately disrupt the protective nature of immunothrombosis, thus triggering excessive fibrin generation/deposition and pronounced leukocyte/platelet recruitment [45]. Both neutrophils and proinflammatory monocytes are among the first cell types to respond to tissue injury [46]. As a result of tissue damage, DNA, nucleosomes (chromatin fragments/histone–DNA complexes), RNA, and other cell-death-associated molecules are released locally and into the blood circulation. Neutrophils and monocytes can also release chromatin via a distinct cell death pathway, named extracellular trap formation (ETosis) [47, 48]. NETs bind to von Willebrand factor, fibrinogen, and fibronectin, providing a scaffold for adhesion of platelets and red blood cells [49•]. In fact, NETs play a crucial role in the pathogenesis of deep vein thrombosis in mice [50, 51]. A recent clinical study revealed that increased levels of both circulating nucleosomes and elastase–α_1-antitrypsin complexes were associated with a threefold increased risk of deep vein thrombosis [52]. There is evidence indicating that NETs provide a surface which facilitates the binding to TF and factor XII, and activation of TF and contact pathways of coagulation, respectively [53]. NETs are rich in the serine proteases cathepsin G and neutrophil elastase. These neutrophil lysosomal enzymes mediate the inactivation of the natural plasma anticoagulant protein TF pathway inhibitor, and thus promote coagulation [54•]. In addition, histones induce thrombin generation either by impairing the thrombomodulin-dependent protein C activation or via platelet Toll-like receptor 2 and Toll-like receptor 4 dependent mechanisms [55, 56].

Extracellular RNA in blood is predominantly bound to plasma lipids and proteins, or circulates as part of microparticles and exosomes [57]. Extracellular RNA exerts procoagulant activities through binding to the blood coagulation factors factor XI and factor XII and activating the contact pathway [58]. Furthermore, RNA is a natural cofactor of the procoagulant factor VII activating protease [59]. Several studies have demonstrated that targeting extracellular DNA, chromatin, and RNA may be a new antithrombotic therapeutic strategy. In fact, both DNase I and RNase I were effective in preventing thrombosis in mice [50, 58, 60]. Experimental reports further indicate that infusion of nucleic acid binding polymers prevents thrombosis in mice undergoing $FeCl_3$-induced carotid artery injury or collagen/epinephrine-induced pulmonary thromboembolism without increasing the risk of bleeding [61]. Given the involvement of peptidylarginine deiminase 4 in the process of chromatin decondensation during NETosis, one should also consider selective peptidylarginine deiminase 4 inhibitors as a promising therapeutic tool in the future.

Thrombin and Atherosclerosis

On aging, the vasculature shows several signs of functional decline. In arteries, the process of atherosclerosis is accelerated by several cardiovascular risk factors in concert with a suitable genetic background. Since atherosclerosis is in essence a condition of repeated damage and repair of the vessel wall, repeated activation of coagulation is also involved in an attempt by the

body to heal the vascular lesions. This leads to the presence of fibrin in plaques [62], providing a matrix for cell growth and proliferation. The ongoing inflammatory pressure, characteristic of atherosclerosis, continues to drive coagulation activation. This is illustrated by abundant TF on various cells within plaques, providing a basis for a full coagulation cascade [63]. Virtually all coagulation proteins are detectable in atherosclerotic lesions, and the thrombin potential is associated with the phenotype of such lesions [64]. Thus, early lesions are characterized by relatively large amounts of thrombin formed, linked to a stable plaque phenotype. With progress of atherosclerosis, inflammatory activity linked to plaque instability gets the upper hand [65, 66]. This phase of advanced atherosclerosis is associated with reduced amounts of thrombin, suggesting that, initially, coagulation responses serve to protect by stabilizing plaques, whereas the loss of procoagulant proteins (shedding and or proteolysis) is linked to instability with risk of plaque rupture. The role of thrombin formation in plaques is still unknown. The cell-specific localization of coagulation proteins, including TF, factor VII, factor X, and factor XII suggests involvement in regulation of cell function [64]. In part this may be mediated through TF–factor VIIa and PARs, as indicated earlier. Extensive experimental work supports proinflammatory and cell migration effects by factor Xa- and thrombin-mediated cell signaling [67]. Experimental work by us and many other groups has shown that hypercoagulability, in general, stimulates the development of atherosclerosis in susceptible mice, such as apolipoprotein E deficient (apoE$^{-/-}$) mice [68–71]. This is associated with alterations in plaque phenotype towards more stability or even instability, probably depending on the age and duration of exposure to hypercoagulability ([68] versus [72•]). In apoE$^{-/-}$ mice with increased clotting tendency (imposed by crossing apoE$^{-/-}$ mice with mice carrying a mutation in the thrombomodulin gene, markedly reducing APC generation and increasing thrombin formation), we observed marked inflammatory changes due to neutrophil infiltration and activity, coupled with diminished smooth muscle cell proliferation [72•]. This inflammatory type of atherosclerosis could be completely prevented by concurrent treatment with the specific thrombin inhibitor dabigatran [72•, 73, 74]. In addition, the administration of APC also attenuated the progression towards atherosclerosis [72•]. Thus, under experimental conditions, the formation of thrombin (and the absence of APC, or both) is an important mediator of atherosclerosis development.

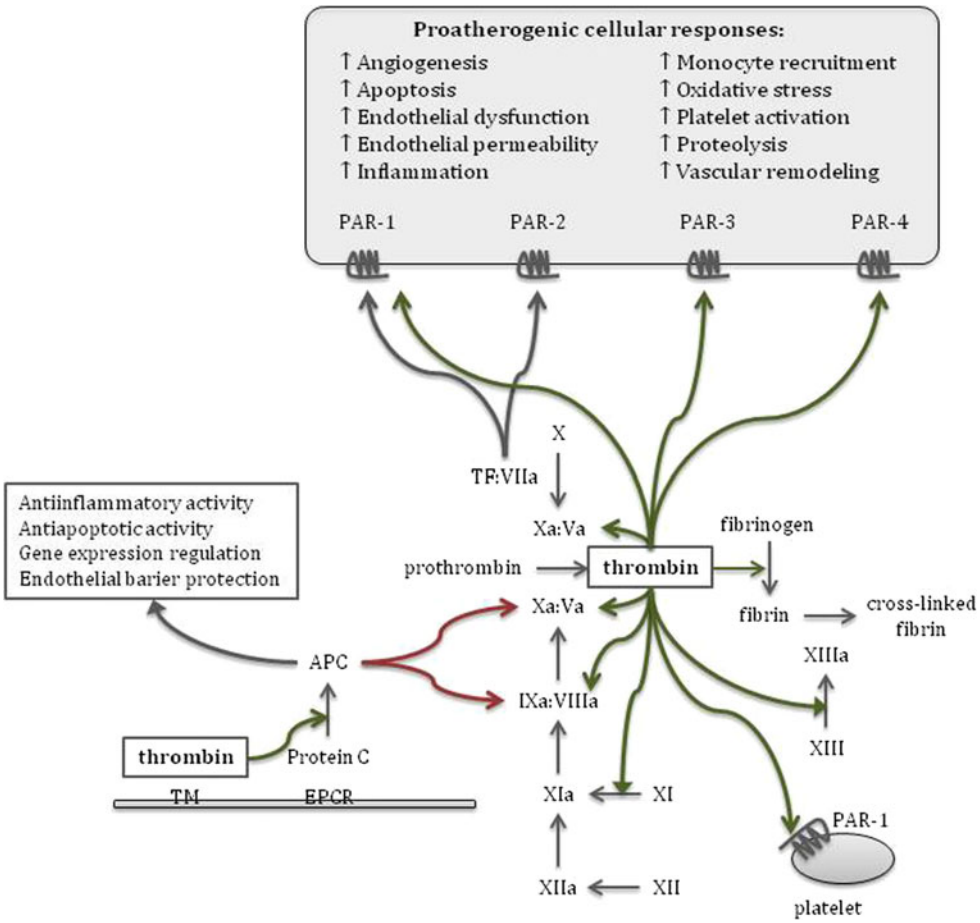

Fig. 1 The central role of thrombin in coagulation and its proatherogenic effects. Thrombin generation is initiated by activation of factor VII on binding to the cellular tissue factor (*TF*). The TF–factor VIIa complex activates factor X to factor Xa through the extrinsic route of coagulation. Once formed, thrombin stimulates its own generation through activation of factor XI in the intrinsic route as well as by activation of the cofactors factor V and factor VIII. Thrombin also attenuates its own formation through activation of protein C to activated protein C (*APC*). Besides a multiple role in coagulation, thrombin can activate protease-activated receptors (*PAR*) 1, 3, and 4, resulting in various proatherogenic responses. *EPCR* endothelial protein C receptor, *TM* thrombomodulin

Critics argue that there is not much evidence from human studies in favor of a role of coagulation in atherosclerosis [75]. For example, in hemophilia patients, there is little evidence of protection against atherosclerosis and its complications such as myocardial infarction [23]. However, hemophilia patients are frequently treated with factor replacement therapy, and hence their coagulation status is at least restored to some extent to maintain a certain level of thrombin generation. Also, the effect size may depend on the genetic background. In hemophilic mice, protection against atherosclerosis was observed against an apoE$^{-/-}$ background [76], but not against an LDL-null background [77]. At the same time, clinical studies also show that markers of thrombin generation are associated with the degree of atherosclerosis scored as coronary calcification [78], whereas the aforementioned studies show that markers of thrombin, as well as congenital thrombophilia, are linked to atherosclerosis. Overall, the contributory effect of alterations in coagulation activity on atherosclerosis may be small, escaping detection in specific populations such as hemophilia patients, but may be relevant in the long term.

Interesting novel data point to close links between hypercoagulability (thrombin formed) and inflammatory activity, showing that high plasma nucleosome levels were independently associated with an increased risk of severe coronary stenosis [79]. Markers of NETs, including myeloperoxidase–DNA complexes, independently predicted the number of atherosclerotic coronary vessels, the presence of a hypercoagulable state, and the occurrence of major adverse cardiac events. These data strengthen a scenario, also derived from our mouse work, whereby neutrophils play an important role in maintaining a hypercoagulable state in vivo, enhancing the risk of atherosclerosis instability as well as thrombotic outcomes.

Thrombin Formation as a Therapeutic Target: Conclusion

From the experimental and human observational data, it is evident that thrombin formation is an important gatekeeper of vascular integrity, and under pathophysiological conditions the excess activity of thrombin is harmful. Several assumptions underlie this argumentation, some of which may still be merely hypothetical. Importantly, the shift in balance from anticoagulant to procoagulant activity of thrombin in the course of atherosclerosis assumes (at least in one aspect) that this is linked to the diminished presence and activity of the cell surface receptors thrombomodulin and EPCR. Although several studies have corroborated this viewpoint histologically, the ultimate proof would be diminished production of APC in vivo. The main practical limitation is the availability of suitable methods to monitor APC in vivo. Given the short half-life of APC, only minute amounts can be detected. Some studies have indeed shown a negative association between a reduced level of APC and the severity of coronary sclerosis [80, 81]. Studies using determination of the APC–protein C inhibitor (PCI) complex have, however, not demonstrated consistent effects. In fact, in patients with systemic atherosclerosis or aortic aneurysms, elevated levels of the APC–PCI complex were found as compared with the levels in nondiseased controls [82, 83]. In aneurysms, however, the association between APC–PCI and disease may be related to the inflammatory process of aneurysm formation rather than to underlying atherosclerosis [84].

It should be stressed that systemic levels of APC or thrombin merely reflect systemic potentials that may be only in part related to local disease. Indeed, the generation of APC may be markedly dependent on microvascular endothelial thrombomodulin and EPCR, such that diminished concentrations of such cell receptors do not contribute much to systemic levels of APC. Thus, it remains to be shown that an actual shift in production of thrombin vis-à-vis APC is an important determinant of progression of atherosclerosis in humans.

In clinical practice, coagulation is inhibited by administration of anticoagulants in patients at risk of thromboembolism. This may be an ideal setting for studying (long-term) effects on the vasculature. In patients receiving long-term treatment with vitamin K antagonists (VKA), vascular side effects have indeed been noted. Owing to inhibition of extrahepatic vascular vitamin K dependent proteins such as matrix Gla protein, increased calcification has been observed in several patient studies on prolonged VKA exposure [85, 86]. This type of calcification may not be comparable to atherosclerosis and is related to this type of medication. Experimental studies addressing the effects of VKA on atherosclerosis are scarce and have produced conflicting evidence. Recent data do show that in apoE$^{-/-}$ mice early atherosclerosis is indeed inhibited by VKA, but this protective effect is lost on ageing and advancing atherosclerosis (M. Chatrou et al., unpublished).

There are no data on the effects of novel selective oral anticoagulants on the human vasculature, owing to the limited follow-up time and the lack of specific studies of this issue in humans. Experimental work shows a clear inhibitory potential of rivaroxaban and dabigatran in mouse studies (all apoE$^{-/-}$ mice) [72•, 87, 88]. It can be expected that on prolonged treatment of patients, such vascular effects may also emerge. Therefore, we argue that studies should address these vascular side effects of novel selective oral anticoagulants using sensitive methods to detect such effects. Figure 1.

Conflict of Interest Henri M.H. Spronk and Julian I. Borissoff declare that they have no conflict of interest.

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Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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