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The Two Faces of Thrombosis: Coagulation Cascade and Platelet Aggregation. Are Platelets the Main Therapeutic Target?

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Abstract

Acute thrombus formation is the pathophysiological substrate underlying several clinical conditions, such as acute coronary syndrome (ACS) and stroke. Activation of coagulation cascade is a key step of the thrombotic process: vessel injury results in exposure of the glycoprotein tissue factor (TF) to the flowing blood. Once exposed, TF binds factor VII/VIIa (FVII/FVIIa) and in presence of calcium ions, it forms a tertiary complex able to activate FX to FXa, FIX to FIXa, and FVIIa itself. The final step is thrombin formation at the site of vessel injury with subsequent platelet activation, fibrinogen to fibrin conversion and ultimately thrombus formation.

Platelets are the key cells in primary hemostasis. For years they have been considered only as cell fragments participating to primary hemostasis and onto which coagulation factors are assembled in the process of thrombus formation. However, recent advances in platelets pathophysiology have shown that these cells are able to regulate their gene and protein expression, make de novo protein synthesis, and release different mediators with paracrine effects that may interfere with different cell functions.

Pharmacological modulation of both side of thrombosis, coagulation cascade and platelet activation, is of great clinical importance. Several clinical trials have clearly shown the efficacy of anticoagulation and/or anti platelet aggregation in different thrombotic disorders. This article aims at reviewing the recent advancements on the two faces of thrombosis focusing on the emerging role of platelets not only as clot-forming components, but highlighting their involvement in the inflammatory-immune system, as well as in modulation of different cell functions.

Keywords: Coagulation cascade; Tissue factor; Platelets; Aggregation

Introduction

Hemostasis is a multistep physiological phenomenon with the primary function being to prevent blood losses [1]. Platelets, on one side, and the coagulation cascade, on the other, are the main components of hemostasis. During the last decade, our knowledge on the pathophysiology of coagulation cascade and platelet activation has progressively increased [1], with one of the most important advances made being the discovery of new roles for platelets beyond their traditional involvement in primary hemostasis [2].

In this review we will discuss the new discoveries that have been made regarding coagulation and platelets and how they are changing the therapeutic scenario.

Coagulation cascade: Something new?

The classic view of hemostasis defines Tissue Factor (TF) as the main initiator of the coagulation cascade [3,4]. Schematically, vessel damage leads to TF exposure, generating trace amounts of thrombin that may exert multiple effects on coagulation factors and platelets [4]. Once activated, platelets adhere to the site of injury and aggregate to form the haemostatic plug. The stability of the platelet aggregate is guaranteed by large amounts of fibrin [5].

The first model of coagulation has been proposed in 1960 in which activation of each clotting factor led to activation of another, thus introducing the "cascade" and "waterfall" concept [4]. Two different initiator pathways were linked to this model: 1) the "intrinsic pathway," in which all the clotting factors were present in blood starting from the conversion of FXII to FXIIa induced by the contact to polyanions secreted by activated platelets, and 2) the "extrinsic pathway", in which the subendothelial thrombogenic material, such as TF, was required in addition to circulating components. Both of them resulted in activation of FX and the formation of a fibrin-rich clot through a common pathway [6].

However, current evidence indicates that the intrinsic pathway is not an alternative way to activate FX, but indeed it increases thrombin generation primarily initiated by the extrinsic pathway [1].

In the current schematic view, coagulation may be divided indifferent separate phases: 1) an initiation phase, classically referred to as the extrinsic pathway of coagulation, characterized by the generation of low amounts of active pro-coagulant factors; 2) an amplification phase, in which the levels of active coagulation factors increase; 3) a propagation phase, in which the generated active coagulation factors, such as thrombin, bind phospholipids, mainly present on the surface of activated platelets, and 4) a stabilization phase, in which thrombin also guarantees strength and stability to the growing clot via activation of factor XIII and of thrombin activatable fibrinolysis inhibitor (TAFI). The first acts covalently linking fibrin

polymers, thus providing stability to fibrin incorporated in platelet plug, the latter protects the clot from fibrinolysis [7].

The extrinsic pathway includes exposure of subendothelial TF to flowing blood following vascular damage. Consequently, circulating FVII/FVIIa is bound with high affinity (Figure 1). Factor VIIa is generated after a cleavage of a single peptide in the protease domain of FVII from a different coagulation enzymes, such as FXa, FIXa, and FVIIa itself. However, FXa seems to be the main activator of FVIIa [6]. It has been shown that the binding of FVII to TF increases its activity by several folds. Moreover, addition of circulating calcium ions leads to the formation of a tertiary complex (TF/FVIIa/Ca2) that induces activation of both FX and FIX [4,8]. Once generated, the "extrinsic" FXa binds to platelet surface, and induces formation of the intermediate FIX providing the first of the two cleavages required for full activation. The intermediate FIX becomes a substrate for the TF/FVIIa complex that provides the second cleavage, thus completing FIX activation. On the other hand, the "extrinsic" membrane-bound FXa induces conversion of small amounts of prothrombin to thrombin that are essential for the acceleration phase as it serves for platelets activation and conversion of FV and FVIII to FVa and FVIIIa, respectively [8]. The complex FIXa/FVIIIa on platelets surface forms the "intrinsic" factor Xase (Figure 1) that is the main generator of FXa. Membrane surface of activated platelets provides the "lipid" substrate essential for combination of FXa with FVa, thus forming the "prothrombinase" complex (Figure 1) that is 3x105-fold more active than FXa alone in the conversion of prothrombin to thrombin [8].

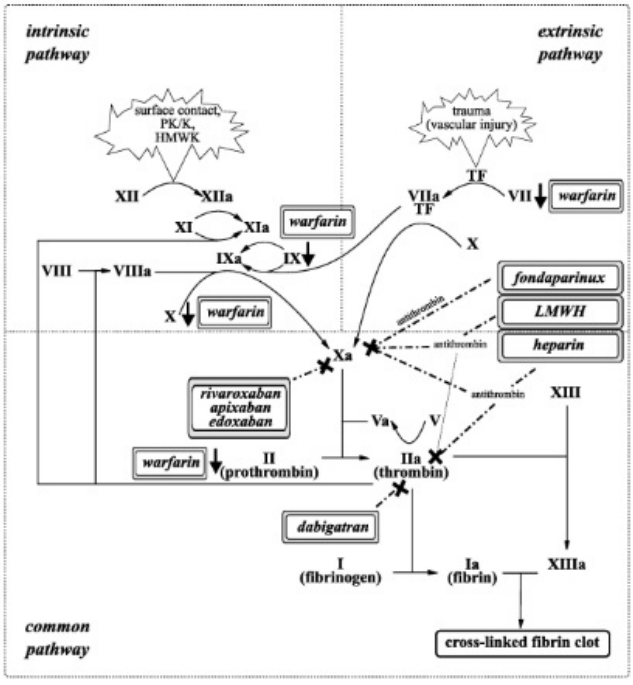

Figure 1: Schematic view of coagulation cascade. Intrinsic and extrinsic pathways are shown. The central role of FXa is indicated and all the current pharmacological strategies are highlighted.

Recently, different reports have indicated that new factors, such as FXII [9], tissue factor-positive microparticles (MPs) [10] and neutrophil extracellular traps (NETs) [11], may contribute to thrombosis but not to hemostasis. Based on the available studies, FXII seems to plays a primary role in thrombosis without implication in hemostasis [9,12,13]. As mentioned above, negatively-charged polyphosphates may activate FXII in vivo. These include inorganic polyphosphates (PolyPs) [14], released following cell damage and infection, but also extracellular RNA and DNA, as recently demonstrated [11]. The role of procoagulant MPs has been highly investigated in different disorders [10,15]. PolyPs are anionic linear polymers ATP-derived, mainly secreted following platelets activation [14]. Beyond the activation of FXII, PolyPs affect blood coagulation by favouring the conversion of FV to FVa, increasing the activity of thrombin-activated fibrinolysis inhibitor (TAFI), inhibiting tissue factor pathway inhibitor (TFPI),and enhancing stability of fibrin clot, thus inducing thrombus formation and stabilization [14].

Neutrophil extracellular traps (NETs) are small combination of nucleic acids, histones and other proteins involved in entrapment and killing of both bacteria and fungi in the innate immunity, and experimental thrombosis [16]. Multiple mechanisms have been proposed for these functions. First, they provide a negatively-charged surface substrate, essential for the activation of FXII [17]; second, histones may induce platelets activation and aggregation [18]; third, NETs inactivate TFPI [19]; finally, they bind both platelets and red blood cells, mainly involved in venous thrombosis [20]. As final effect, coagulation is promoted in animals model [17,20] as well as in humans [21,22].

The traditional role of platelets in the haemostasis process

Platelets are anucleate small cell-fragments derived from megakaryocytes and free circulating in the blood in a resting phenotype [5]. Upon stimulation, platelets rapidly change their structure for a substantial rearrangement of their cytoskeleton characterized by lamellipodia and pseudopodia, thus switching to an activated phenotype with pro-aggregation properties [23]. Several receptors may be indentified on the platelet surface, able to respond to a several stimuli (serotonin, shear stress, ADP, thrombin and others). The final event of the activator-receptor ligand is shape change, receptor clustering, granule secretion, synthesis of thromboxane, exposure of glycoprotein IIb/IIIa and then aggregation [5].

Platelet receptors include integrins, leucine-rich repeats receptors, selectins, tetraspanins, transmembrane receptors, prostaglandin receptors, lipid receptors, immunoglobulin superfamily receptors, tyrosine kinase receptors and miscellaneous platelet receptors [24]. We will briefly described some of these receptors, mainly the ones involved in the pharmacological modulation.

Integrins: transmembrane proteins with adhesion and signalling function. Platelets have three types of integrins divided in β1, β2 and β3. In β1 family, α2β1 receptor or GPIa-IIa is the platelet receptor for collagen. The β2 type includes only αLβ2 (CD102), also known as intercellular adhesion molecule 2 (ICAM-2). Its primary function is platelet adhesion to neutrophils and platelet-leukocyte interaction. The last group, β3 family, includes a surface glycoprotein, only expressed on platelets, αIIbβ3 (CD41/CD61), also known as GPIIb-IIIa complex. In resting state, platelets present about 8-10 × 10^4 molecules of αIIbβ3 on their surface and 2-4 × 10^4 molecules distributed in the α-granules, dense granules and on the membranes of open canalicular system. Following platelets activation, these receptors become translocated to the cell-membrane and are converted to a high affinity state to facilitate binding to fibrinogen, fibrin, von Willebrand Factor (vWF) and fibronectin, vitronectin and thrombospondin, thus promoting platelet aggregation [24-26].

Leucine-rich repeats receptors: proteins with a repeated structural motif rich in leucine (LRR) essential for protein-protein interactions. This group includes GPIb-IX-V complex, toll-like receptors (TLR) and matrix metalloproteinases (MMP) [24].

Selectins: group of receptors involved in the adhesion step. Members of this family are P-selectin, C-type lectin-like receptor-2 (CLEC-2) and CD72 [24].

Tetraspanins: transmembrane proteins containing four membrane-spanning domains with the function of signal transducers on cell surface. The most representative protein of this family is CD63, also known as lysosomal membrane-associated glycoprotein-3 (LAMP-3), mainly expressed following platelet activation [24].

Transmembrane receptors: This is the most important family,which includes the main receptors for platelet activation and aggregation, such as ADP and thrombin receptors, the primary target of the current antiplatelet therapy. On platelet membrane there are two types of purinergic receptors: the P2Y group, that is a guanosine triphosphate coupled protein receptor and the P2X1 that is an ion channel receptor mediating extracellular calcium influx leading to change in platelet shape [5,27].

The P2Y group includes P2Y1 and P2Y12. ADP exerts several functions in platelets, such as calcium ions mobilization, shape changes, secretion, production of thromboxane A2 (TXA2), GPIIb-IIIa activation and platelet aggregation facilitated by P2Y1 receptor binding. These multiple functions may explain why ADP receptor has been considered the main target for pharmacological modulation [27-30]. Thrombin generated during activation of the coagulation cascade, may act on platelets via protease-activated receptors (PARs), which couple to Gq, G12/G13 and, in some cases, also to the Gi family of heterotrimeric G proteins [31,32]. Four types of protease-activated receptors have been identified; of these, only PAR1 and PA R4 are present on human platelets [33].

Prostaglandin receptors: Platelets present different receptors for prostaglandins, such as thromboxane receptors, prostacyclin (PGI2), PGD2 and PGE2 receptors. Thromboxane receptor activates phospholipase A2 and phospholipase C. Its primary function is the amplification of platelet activation by an autocrine mechanism. The PGI2 receptorshave inhibitory effects to keep platelets in the resting state. PGE2 receptors have a dual effect based on the concentrations of ADP and collagen. Low levels of agonists induce platelets activation while higher concentrations of these agonists exert inhibitory effects [24].

Lipid receptors: this group include the platelet activating factor (PAF) receptor involved in inflammation, anaphylaxis, platelet aggregation [34] and degranulation and the lysophosphatidic acid receptor (LPL-R) expressed on activated platelets, which, with an autocrine mechanisms facilitates shape changes, secretion, and aggregation [24].

Immunoglobulin superfamily receptors: this is a large group of surface receptors participating in recognition, binding and adhesion to cells. Members of this family are 1) GPVI, the major receptor for collagen, 2) FcγRIIA (CD32), a single-chain sialoglycoprotein, involves in calcium signalling and cytoskeletal rearrangement, platelets secretion and activation, 3) FcεRI receptors (CD23), the "low-affinity" receptor for IgE, which seems to act as a bridge between aggregation and immunity [35,36].

Tyrosine kinase receptors: this is a family of trans-membrane proteins that binds cytokines, growth factors, hormones and other signalling molecules. It includes the leptin receptor, insulin receptor, platelet-derived growth factor receptors (PDGF) [24].

Other receptors: this is a miscellaneous group that includes 1) serotonin receptors, the binding of which to 5-hydroxytryptamin initiates calcium signalling and enhances aggregation induced by other agonists, such as ADP and thrombin. Moreover, serotonin induces vasoconstriction of the damaged vessels and promotes thrombus formation [37-39]. 2) CD40 ligand, a transmembrane glycoprotein stored in the granules of resting platelets and rapidly expressed on cell surface upon activation that induces release of fragment (CD40L) free circulating in the blood, thus serving as marker for in vivo platelet activation [5,24,40].

Beyond aggregation: New insight in platelet physiology and function

For years, platelets have been considered to be important only for primary hemostasis [2]. However, during the last decade, other functions beyond aggregation have been proposed [41,42]. Although anucleate, platelets contain mRNAs and are capable of de novo protein synthesis [43]. Considering that platelet lifespan is around 8-10 days and that protein half-life is around 48 hours, it is reasonable to assume that each platelet must maintain its transcriptome in a functional state to preserve its proteome. However, how platelets modulate their proteome is still poorly understood. An important mechanism in regulating protein synthesis depends on microRNAs (miRNAs). miRNAs are small non-coding RNAs that can negatively regulate gene expression at post-transcriptional level. In 2009 the existence of miRNAs in human platelets has been reported [44]. The complex machinery involved in miRNAs function has been also identified [45,46].

Since the first report, several studies have been published in this field, showing that the detected miRNAs in human platelets may be involved in several functions: nuclear import, cell cycle control, transmembrane receptor protein serine/threonine kinase signalling pathway, regulation of kinase activity, pathways in cancer and leukocyte differentiation [45]. However, the main question about platelet miRNAs concerns their possible role in regulating platelet reactivity. It has been reported that platelets contain more mRNAs that encode proteins that inhibit platelet activation to maintain platelets in a resting state [47]. Regarding activation, platelets miRNAs may be differentially expressed, thus some of them may predict platelet activation/reactivity [47]. In light of the published data, we have tried to elucidate the molecular changes induced in activated platelets, looking at miRNome and proteome reorganization upon activation [46]. We have reported that the platelet transcriptome is only slightly affected by activating stimuli, showing quantitative changes mainly consistent with RNA loss. Interestingly, we found that in most cases changes in protein levels occurred in the absence of modulation of the corresponding mRNA, suggesting that activation stimuli promote a rapid and extensive reorganisation of the platelet proteome that probably derives from both changes in protein stability or secretion and in the rate of protein synthesis. Quiescent or activated platelets express core components of the miRNA processing pathway, including the most representative protein: the Argonaute proteins (Ago 1, 2 and 3) and Dicer [46]. Moreover, we found that among the miRNAs expressed to a detectable level in platelets, some were found differentially expressed according the agonist, while others were

regulated in the same way, although to a different extent, independently of the agonists used [46].

Interesting findings of our report came out also from the functional analysis of protein modulated during platelet activation, showing that beyond the expected pathway, such as platelet degranulation, hemostasis, blood coagulation, wound healing, a consistent amount of proteins were involved in immune and inflammatory response, confirming previous data regarding the role of platelet in immunity and inflammation [41].

Platelets are recruited at the site of vascular damage as mediators of primary hemostasis. However, vessel injury represents a risk for infection, thus recruited platelets may activate leukocytes via direct cell-cell contacts and indirectly via cytokines and/or platelet-derived microvesicles. Interaction between activated platelets and leucocytes occurs by the binding of P-selectin with P-selectin glycoprotein ligand1 (PSGL-1) and is enhanced by interaction with other cofactors and local cytokine release, thus modulating immune responses and local inflammation [41,48]. Platelets store a variety of mediators in the α-granule such as cytokines, chemokines and growth factors. Once activated, platelets degranulate, releasing the stored mediators and modulating function of different cell types such as 1) monocytes, promoting their extravasation and regulating their activation, polarisation and differentiation, 2) leukocytes, enhancing transmigration by endothelial cell activation via serotonin (5-HT) release, 3) neutrophils, stimulating phagocytosis and NETosis as well as extravasation [41].

As mentioned above, once vessel injury occurs, platelets are activated and express multiple adhesion molecules to bind the subendothelium [16]. These adhesion molecules also support in loco eukocyte recruitment and retention, as part of the immunological response to the damage [48]. Thanks to the soluble mediators secreted following activation, platelets may induce transendothelial leukocytes migration. However, it is unknown whether these mediators recruit specific leukocyte subtypes. Moreover, recent studies indicate that platelets may also recognize microbial pathogens through TLRs, thus actively participating to the immune response [41,42,49].

The Parmacological Modulation of the Two Sides of Thrombosis: Anticoagulant and Antiplatelet Agents

Modulation of the coagulation cascade as well as platelets aggregation is now the cornerstone in the management of thrombotic disorders, such as atrial fibrillation, pulmonary embolism, acute coronary syndromes and stroke. For decades, warfarin and heparin have been the only agents available for prevention and treatment of thromboembolism, while, on the other hand, aspirin was the first line therapy for thrombotic disorders. However, during the last decade, important progress has been made in the antithrombotic medications at both anticoagulant and antiplatelet drugs [50].

As indicated in Figure 1, anticoagulants act at different levels of the coagulation cascade, thus inhibiting the initiation and progress of coagulation and fibrin-clot formation and propagation. For long-term prevention of thromboembolism, oral anticoagulants are the preferred choice, while during acute treatment parenteral anticoagulants (such as unfractionated heparin, low molecular weight heparins and, recently, parenteral anti-factor Xa drugs [fondaparinux] may be considered). While inhibition at TF level was associated to excessive bleeding risk, targeting FXa or thrombin has shown to be effective with a safety profile.

In Figure 2 are shown the main platelets receptors which may represent targets of pharmacological modulation by current available strategies.

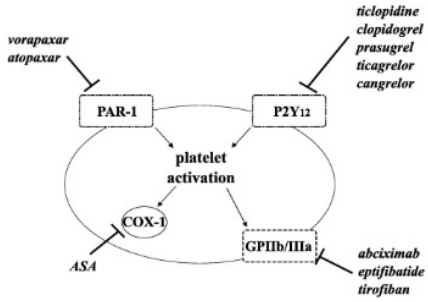

Figure 2: Therapeutic modulation of the main receptors expressed on platelets surface. Class of agents and the main targets are indicated.

We will briefly discuss the most important available anticoagulant and antiplatelet agents

Anticoagulants

Parental administration: 1) Unfractionated heparin and low molecular weight heparin. Heparins act binding and activating antithrombin through a conformational structural change. In the active form, antithrombin accelerates the inactivation of several coagulation factors (IIa, IXa, Xa, XIa, and XIIa). 2) Fondaparinux. It is an inactivator of FXa via selective and irreversible binding of antithrombin III. 3) Direct thrombin inhibitors. This group includes desirudin, argatroban, and bivalirudin. These molecules act binding directly and selectively the active site of thrombin. The main advantage of their use is the reversible binding to thrombin [51].

Oral administration: 1) Vitamin K antagonists. For almost a century, warfarin and acenocoumarol have been the only oral anticoagulants available in the clinical setting. They act interfering with the synthesis of vitamin K-dependent clotting factors. Because of the blocking of vitamin K reductase, a depletion of reduced vitamin K occurs, thus γ-carboxylation and activation of vitamin K-dependent clotting factors II, VII, IX, and X is missing. The main problem related to the use of vitamin K antagonists is the restricted therapeutic range. 2) New oral anticoagulants. In the last 5 years a new class of oral anticoagulants became available (NOACs). It includes the direct thrombin inhibitor, dabigatran, and the direct factor X inhibitors, such as rivaroxaban, apixaban, and edoxaban. These new drugs are not inferior to vitamin K antagonists but do not need frequent monitoring. The dose is related to the renal function, age and body weight. To date, the main issue related to their use is the economic sustainability because of their higher cost [50,52].

On the other hand, new strategies are also in developing for prevention or therapy of thrombosis. Currently, combination therapy of antiplatelet agents (aspirin plus an inhibitor of P2Y12 receptor) or even triple therapy (based on antiplatelet drugs plus anticoagulant agents) may be considered because of high risk of thromboembolism in patient affected by arterial thrombosis (i.e patients affected by atrial fibrillation undergoing coronary stent implantation). In the last decade newer antiplatelet drugs have proven their efficacy in combination

with aspirin in reducing major cardiovascular events in patients presenting with acute coronary syndrome [53,54].

Antiplatelet agents

Oral Antiplatelet drugs: Acetylsalicylic acid (aspirin). This is the fundamental therapy in the treatment and prevention of thrombotic diseases. All the clinical studies designed and published to date have analyzed effects of new agents in association with aspirin. It is a cyclooxygenase (COX) inhibitor that irreversibly inhibits COX1 and, in higher doses, the isoform 2 (COX2). Inhibition of COX1 blocks the formation of prostaglandin H2, thus thromboxane A2, that is the main platelets activator and aggregation inducer. This effect is irreversible, thus aspirin causes inhibition for the lifespan of a platelet [55] P2Y12 receptor antagonists. The discovery of the ADP receptors, such as the P2Y12, and the possibility to block them pharmacologically, has opened new therapeutic scenario. Currently, clopidogrel, prasugrel, and ticagrelor are used in clinical practice for oral administration in combination with aspirin with a significant reduction in MACE [56,57] after acute coronary syndrome. Substantial differences exist between the oral P2Y12 inhibitors that are responsible of the clinical efficacy. Clopidogrel is a prodrug, which needs to be activated to bind irreversibly to P2Y12. An initial loading dose is necessary to get a rapid platelet inhibition. It has been estimated that about 30% of patients treated with clopidogrel do not show adequate platelet inhibition. This may be related to genetic polymorphisms or altered intracellular signal pathways [58]. Prasugrel is an evolution of clopidogrel. It needs to be activated either before to bind irreversibly to P2Y12. Main difference with clopidogrel is a more reliable conversion to the active drug and more rapid onset of action than clopidogrel. Moreover, genetic polymorphisms do not influence efficacy of prasugrel [59]. Ticagrelor is a new generation of reversible inhibitors of the P2Y12 receptor. It is already active, thus its effect on platelet inhibition is faster compared to clopidogrel and prasugrel. The great advantage is the rapid decline of antiplatelets effect after last intake. It has been reported that 24 hours after the last intake, the antiplatelet effect of ticagrelor declines by 50% [60].

Parental antiplatelet drugs: Cangrelor is the latest reversible P2Y12-blocking agent approved for i.v. infusion. No loading dose is recommended and platelet inhibition reaches more 90%. Its effect rapidly declines within one hour after stopping of the infusion. For these pharmacokinetic properties cangrelor may be a promising agent in the clinical setting of ACS [61]. Glycoprotein IIb/IIIa inhibitors prevent interplatelet bridges because they block the adhesion of fibrinogen to the activated platelet. Exposure of GpIIb/IIIa receptor on platelets surface is one of the final steps in platelet activation. This group includes abciximab, a humanized monoclonal mouse antibody, tirofiban, and eptifibatide, synthetic GpIIb/IIIa inhibitors, that are approved for are i.v. use [62].

Other antiplatelet agents: inhibition of the protease-activated receptor-1 has been explored as possible antiplatelets strategy. This class of agents includes vorapaxar and atopaxar, that inhibit thrombin-mediated platelet aggregation. PAR-1 antagonists have shown some reduction in the risk of cardiovascular mortality; however a higher bleeding risk has been reported, thus a very careful selection of patients that may benefit of these drugs without a substantially increased risk of bleeds has to be performed [63,64].

Conclusion

Current data indicate that antithrombotic strategies require a fine balance between anticoagulation and antiplatelets aggregation. A better understanding of the pathophysiology of coagulation cascade as well as platelet activation may lead to a further advancement in the pharmacological modulation.

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