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REVIEW ARTICLE

Pathophysiology of Thrombosis in Peripheral Artery Disease

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Abstract: Under physiological conditions, peripheral arteries release endogenous vascular-protective and anti-thrombotic agents. Endothelial cells actively synthesize vasoactive mediators, which regulate vascular tone and platelet reactivity thus preventing thrombosis. Atherosclerosis disrupts homeostasis and favours thrombosis by triggering pro-thrombotic responses in the vessels, platelet activation, aggregation as well as vasoconstriction, phenomena that ultimately lead to symptomatic lumen restriction or complete occlusion

In the present review, we will discuss the homeostatic role of arterial vessels in releasing vascular-protective agents, such as nitric oxide and prostacyclin, the role of pro- and anti-thrombotic vascular receptors as well as the contribution of circulating platelets and coagulation factors in triggering the pro-thrombotic response(s). We will discuss the pathological consequences of disrupting the protective pathways in the arteries and the pharmacological interventions along these pathways.

Keywords: Endothelium, platelets, anti-thrombotic mechanisms, atherothrombosis.

1. INTRODUCTION

Under physiological conditions, peripheral arteries actively release endogenous, vascular-protective and anti-thrombotic agents. In particular, endothelial cells (EC) release vasoactive mediators important in regulating vascular homeostasis and preventing thrombosis. Atherosclerosis disrupts this homeostasis and favours thrombosis and vasoconstriction, by triggering pro-thrombotic responses in the vasculature, platelet activation and aggregation, phenomena which ultimately lead to symptomatic lumen restriction or complete occlusion.

In the present review, we will discuss the homeostatic role of the arterial vessels in releasing vascular-protective agents, the role of pro- and anti-thrombotic vascular receptors as well as the contribution of circulating platelets and coagulation factors in triggering the pro-thrombotic response(s), with a special focus on nitric oxide (NO), eicosanoids and thrombin-related pathways. We will discuss the pathological consequences of the disruption of the protective pathways in the arteries and the possible pharmacological interventions along these pathways.

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2. VESSEL WALL

EC and vascular smooth muscle cells (VSMC) express a multitude of receptors that respond to mechanical shear forces and influence platelet status. Major endogenous anti-thrombotic products physiologically released by the EC are NO and prostacyclin (PGI_2). Several intracellular signaling pathways lead to the biosynthesis and release of these vasodilators and anti-thrombogenic mediators, as a part of the physiological mechanism controlling vascular reactivity [1, 2]. These mediators are not circulating hormones, but rather short-lived molecules regulating local homeostasis in the place where they are released by neighbouring cells. Fig. **1A** summarizes the major features of these endogenous anti-thrombotic systems.

2.1. Nitric Oxide

L-arginine is a substrate for NO synthases (NOS) and the major source of NO [3]. Distinct genes encode for three different NOSs: the neuronal (NOS-I), the inducible (NOS-II) and the endothelial (NOS-III) NO synthases [4]. Whereas NOS-II is responsible for the acute production of high amounts of NO associated with inflammation and oxidative stress, NOS-I and -III account for the continuous release of basal levels of NO, which is important for different physiological functions including endogenous vasodilation tone, anti-thrombotic protection and neurological signaling [5, 6].

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In the vasculature, the production of NO by the EC was first reported as a regulator of the vascular tone already in the eighties [7]. The regulation of NOS-III in EC constitutes an important mechanism controlling vascular homeostasis, and involves the calcium/calmodulin pathway, the AKT- (AKR mouse thymoma kinase or also known as protein kinase B) dependent phosphorylation and the interaction with caveolin-1 [8].

NOS-III in EC can be activated by shear stress, bradykinin, acetylcholine, and histamine, through the increase in intracellular calcium and/or following the phosphorylation on the serine 1177 residue, or by the dephosphorylation of the inactivating site threonine 495 [9] (Fig. **1A**). The soluble guanylate cyclase in circulating platelets, endothelial and smooth muscle cells is the target for NO [10]. This enzyme promotes the formation of cyclic guanosine monophosphate (cGMP), which results in the inhibition of platelet aggregation and promotes vasodilation, respectively [11] (Fig. **1A**).

Studies in animals have shown that L-arginine supplementation results in the improvement of EC function of small arterial vessels [12, 13]. In addition, the pharmacological inhibition of NOS-III in animals induces hypertension and increases vascular reactivity, thus confirming the importance of NOS in the cardiovascular homeostasis [2]. NOS-III knockout (KO) mice presented with hypertension and insulin resistance [14]. Triple NOS (I, II and III) KO mice fed on high-fat diet showed increased atherosclerosis as compared with single and double knock-out mice, with a parallel increase in blood total cholesterol and low density lipoprotein cholesterol (LDL-C) levels [15]. These studies confirmed the importance of NOS enzymes as endogenous vascular protective mechanisms and the role of NOS dysfunction in promoting atherogenesis [16].

Clinical conditions associated with high cardiovascular risk such as diabetes mellitus, smoking, obesity, severe kidney diseases, and hypertension are all associated with impaired endothelial function and reduced NO bioavailability and/or response [17]. Reduced NO availability likely contributes to the pathogenesis of atherothrombosis in humans by affecting the vascular tone, platelet aggregation and endothelial anti-thrombotic protection, and by promoting smooth muscle cell proliferation and migration [17]. In spite of the above evidence, a protective role for chronic dietary supplementation of L-arginine in patients with cardiovascular diseases or at high cardiovascular risk remains unproven [18]. Acute L-arginine infusion improved endothelial function in small studies of patients with chronic peripheral artery disease (PAD) [19] or with hypertension and hyperhomocysteinaemia [20]. However, large, placebo-controlled trials assessing the incidence of major cardiovascular events in high-risk patients are needed to validate acute or chronic supplementation strategies.

2.2. Vessel-Derived Prostanoids and Prostanoid Receptors

Prostanoids are produced from the arachidonic acid released from phospholipids by the action of phospholipases or from arachidonoyl glycerol by monoacylglycerol lipase [21, 22]. The arachidonic acid is further metabolized into the intermediates prostaglandin (PG) G_2 and H_2 by the cyclooxygenases (COX)-1 and -2 and finally into PGI_2, PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane (TX) A_2 *via* specific prostanoid synthases. Several different prostanoids contribute to the homeostasis of the vascular tone and to platelet reactivity and aggregation (Fig. **1B**).

Both COX-1 and COX-2 enzymes are expressed in EC and VSMC. While COX-1 is constitutive, COX-2 is induced by physiological shear stress in human and murine EC [23, 24]. The relevance of the anti-thrombotic role of COX-2-dependent PGI_2 biosynthesis in EC of human arteries was clearly identified based on the results of phase III trials and upon the marketing of drugs with preferential COX-2 inhibitor capacity (Coxibs). A reduction of PGI_2 biosynthesis *in vivo*, measured as urinary excretion of its major urinary metabolite, was first described in healthy volunteers administered with two different Coxibs (celecoxib and rofecoxib), suggesting COX-2 as the major enzyme accounting for PGI_2 biosynthesis in humans [23]. Then, in large phase III trials of chemoprevention in colorectal cancer patients, the administration of Coxibs was associated with a significant increase in the incidence of myocardial infarction compared with placebo [25].

The role of prostanoids in vessel injury was extensively investigated in animal models [23, 26]. Studies using genetically-modified mice, with global and tissue-specific deletions allowed understanding the mechanisms involved in the protective role of COX-2-dependent PGI_2 biosynthesis in the vasculature. The COX-2 KO mice selectively deleted in the EC, VSMC, or both, showed an increase in blood pressure and a faster occlusion time in a model of carotid injury. This effect was accompanied by a reduction in NOS-III expression and NO release leading to vascular dysfunction [27]. These data also support the knowledge that NO cannot compensate for the pharmacological blockade of the vascular protective effects of COX-2 [28]. In addition to playing a role in limiting thrombosis and lowering blood pressure, vascular COX-2 in mice was shown to be the major source of PGI_2 *in vivo*, owing to the measurement of urinary derivatives of PGI_2, [27], consistent with data in humans [23, 24].

The role of COX-2 has also been investigated in atherosclerosis, owing to the use of tissue-specific deletion of COX-2, and unravelled its contribution in the protection against atherogenesis. Both EC- and VSMC-associated COX-2 showed a protective anti-atherogenic effect in hyperlipidaemic mice as evidenced by the increase of atherosclerotic lesions in mice with selective deletion of COX-2 [29].

The biological effects of prostanoids are mediated by signaling of specific G-protein coupled receptors (GPCR) to TXA_2 (TP), PGI_2 (IP), PGE_2 (EP). The analysis of the m-RNA expression of these receptors was done on different vascular tissues or cells, including EC and VSMC [30]. Functional analysis of the vascular tone in response to prostanoids has been also investigated mainly in deficient mice or using selective receptor antagonists. TP, IP and mainly EP3 and EP4 isoforms are involved in keeping vascular homeostasis and/or promoting cardiovascular events [31, 32] (Fig. **1A** and **B**).

PGI_2 and TXA_2 are the main prostanoids produced by vessels and platelets, respectively. The action of TXA_2 is

mediated through two GPCRs, the TPα and β, coupled to Gαq and $G\alpha_{12/13}$, respectively, and accounts for the increase of intracellular calcium and protein kinase C (PKC) activation. TPs induce vaso-constriction, as indicated by the increased blood pressure response in mice selectively overexpressing the TPβ receptors on the vessel wall [33]. Moreover, in different type of arteries, the vascular response to acetylcholine and angiotensin can be blocked by the selective TP receptor antagonists SQ29548 and Terutroban (S18886), respectively, thus suggesting a role of TP signaling downstream the acetylcholine- and angiotensin-mediated contraction [34, 35]. The selective overexpression of TPα in the mouse vessels, generated a phenotype resembling eclampsia with intra-uterine growth retardation [33], indicating a relevant role for the TXA_2/TP in the vascular hypertensive disorders associated with human pregnancy. Moreover, in hyperlipidaemic mice, double TP-apoE$^{-/-}$ KO showed a significant delay in atherogenesis [36]. The TPs can also be activated by the F_2-isoprostanes, which are derived from arachidonic acid through a non-enzymatic path, following lipid peroxidation [37]. Among this class of compounds, the 8-iso $PGF_{2\alpha}$ can be measured in urine from patients at high cardiovascular risk [38, 39]. Isoprostanes through the TP receptors induce vasoconstriction and platelet activation [40-42] (Fig. **1B**).

The PGI_2 receptor IP is coupled to Gαs and accounts for the increase in cyclic adenosine mono-phosphate (cAMP) [43]. While TXA_2 is vasoconstrictor and platelet pro-aggregant, PGI_2 plays an important role in vascular protection exerting platelet anti-aggregant and vasodilator effects [44]. In fact, the deletion of IP receptor in mice enhanced injury-induced neointima hyperplasia and platelet activation, which involved TP receptor [28] (Fig. **1A**).

Recently, the role of PGE_2 in atherosclerosis has also been investigated. PGE_2 is derived from PGH_2 through different isoforms of PGE_2 synthases (PGES). Among the three PGES isoforms identified, mPGES-2 and cPGES are constitutively expressed, while mPGES-1 is upregulated in response to injury and inflammation [45]. Animal models with global or tissue-selective deletion of mPGES-1 and IP allowed dissecting the role of PGE_2 in vascular injury and atherogenesis. mPGES-1 KO mice showed an increase in PGI_2 levels, associated with a protection against vascular injury and a reduction in atherosclerosis lesions [46, 47] (Fig. **1B**). A re-diversion of PGH_2 to PGI_2 synthesis or a protective role consequent to PGE_2 reduction is possible. Further investigations confirmed a role of myeloid rather than vascular mPGES-1-derived PGE_2 in injury and atherogenesis [48, 49]. Recently, double IP receptor/mPGES-1 knockouts further supported that the anti-atherogenic effect consequent to mPGES-1 invalidation is independent of IP signaling, whereas the anti-thrombogenic effect of mPGES-1 deletion is reduced when IP receptors are deleted [50] (Fig. **1B**).

Beyond the well-known role of PGE_2 in acute inflammation in humans [51], the contribution of PGE_2 in modulating atherogenesis and atherothrombosis in the vessel wall is less known [52]. The effect of PGE_2 on human platelets is discussed in section 3. Based on the major role of PGE_2 in human acute inflammation and pain, a mPGES-1 synthase inhibitor is currently at an early stage of development for human inflammatory disorders only [53]. Therefore, a possible protective contribution of PGE_2 biosynthesis inhibition in human atherothrombosis remains to be established.

2.3. Phosphodiesterase-3

Cyclic nucleotide phosphodiesterases (PD) are a large family of enzymes responsible for the degradation of cAMP and cGMP [54]. PDE3 and PDE4 isoforms predominate in VSMC and in the myocardium, respectively. Elevation of cAMP and cGMP results in the relaxation of VSMC [2, 55]. Thus, the inhibition of PDE3 is associated with vasodilation, inhibition of platelet aggregation and reduced VSMC proliferation in the context of atherogenesis [56].

Cilostazol is a PDE-3 inhibitor approved for intermittent claudication in humans as second-line treatment [57]. The addition of cilostazol to standard dual antiplatelet therapy (mostly low-dose aspirin and clopidogrel) did not show substantial benefit in patients with acute coronary syndrome [60]. A possible role as adjuvant therapy in patients with PAD, including stroke, carotid artery disease and microvessel disease associated with diabetes has been studied or is currently under investigation [59, 60] to explore a possible relevance of this pathway in the pathophysiology of human PAD. However, conclusive clinical evidence is currently lacking.

2.4. Protease-Activated Receptors (PAR) In Vessels

PAR receptors belong to the family of a special GPCR with a unique mode of activation based on the cleavage of their N-terminal end, generating a new N-terminal domain that tethers in the receptor and activates it [61]. PAR receptors are involved in the regulation of vascular tone under physiological conditions where they exert EC-dependent vasodilation [61, 62]. Upon vascular injury or under inflammatory conditions, activation of PARs in the vasculature contributes to an increased vascular permeability, vasoconstriction, integrin expression and increased cytokine and chemokine production. PAR-1, -3 and -4 are expressed in EC and VSMC [61, 63] (Fig. **1A** and **B**). PAR-1 in the EC is activated by thrombin generated during clot formation. Prolonged activation of PAR-1 results in enhanced vascular activation and remodeling, and triggers inflammation. Little is known about the role of PAR-3 in vessels. Recently, it has been shown that PAR-3 heterodimerises with PAR-1 and thus acts as a regulator of its activity [64].

PAR-4 is expressed at low levels in EC and VSMC, whereas its expression is increased during vascular injury and inflammation in response to various ligands including thrombin, sphingosine-1- phosphate, angiotensin, high glucose and oxidative stress products. PAR-4 activation participates in the inflammatory response triggering neutrophil and immune cell recruitment [61, 62, 65, 66]. Therapeutic targeting of PAR-4 in humans might offer an additional or alternative target to PAR-1 in limiting thrombo-inflammation in pathological setting where PAR-4 contribution is substantial [62].

3. PLATELETS AND COAGULATION

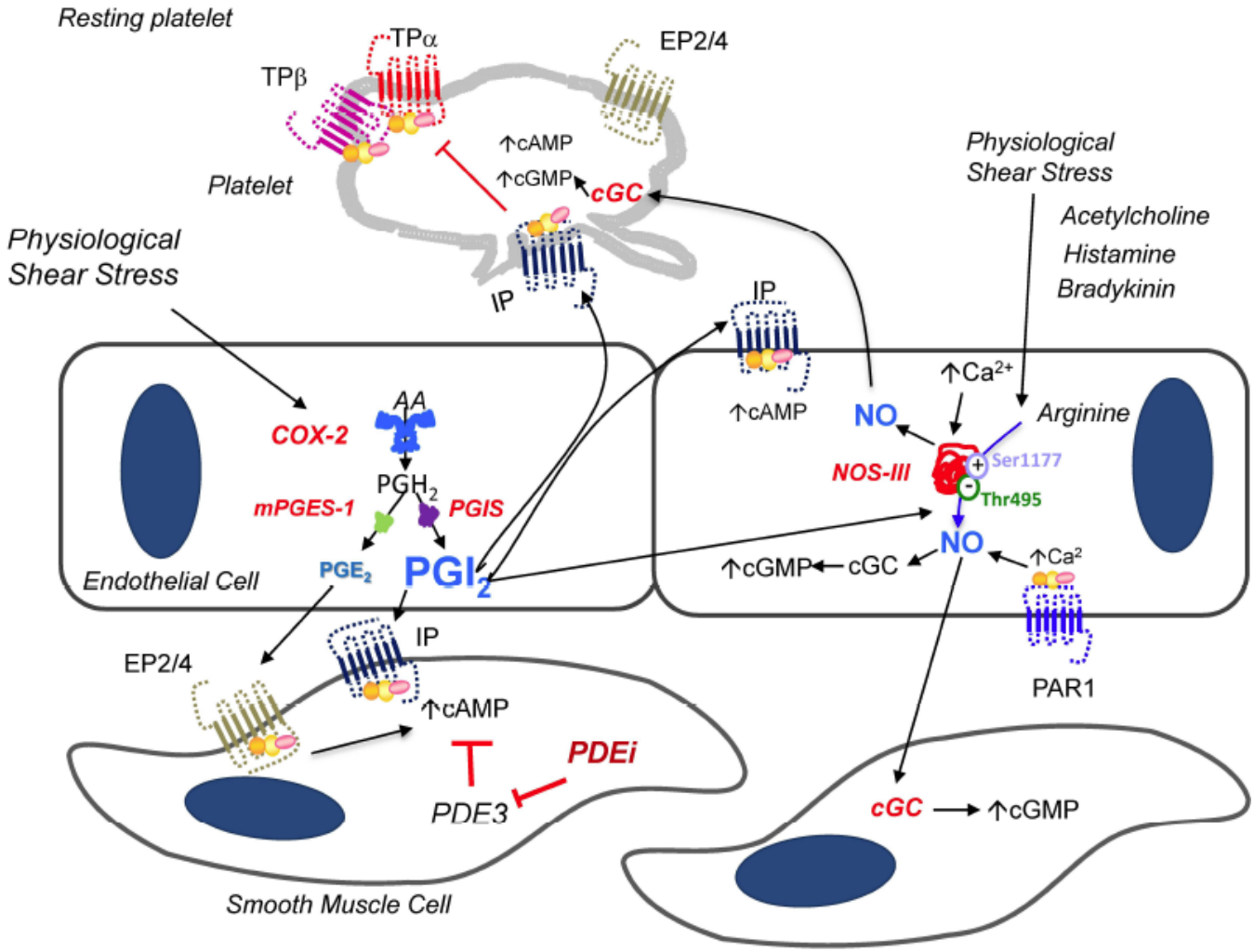
Platelets are major players of thrombus formation in atherothrombotic diseases, [67] including PAD, as shown by

the benefits of the pharmacological blockade of some platelet enzymes or receptors in atherothrombotic patients [68-70] (Fig. **2**).

3.1. Platelet-derived Prostanoids and Prostanoid Receptors

Platelets activated *via* different physical or biochemical signals, activate phospholipases, which releases arachidonic acid from the membrane phospholipids, thus fuelling the activity of COX [70]. Mature peripheral platelets express mostly the COX-1 isoform, while immature platelets and their megakaryocyte precursors express both COX-1 and -2 isozymes [71, 72]. In platelets and megakaryocytes, COX-1 activity is functionally preferentially coupled with TXA_2 generation [71, 72], while PGE_2 is the main end product of COX-2 activity, in both platelets and megakaryocytes. TXA_2 is a short-lived autacoid (32 sec), not a circulating hormone, and a potent mediator of platelet aggregation [73]. Human platelets express mostly the TPα subtype [73, 74] (Fig. **1B** and **2**). Platelet-derived TXA_2 generation contributes to atherogenesis in humans, as indicated by data showing that its biosynthesis *in vivo* is variably increased in overt cardiovascular diseases or in conditions at high cardiovascular risk such as diabetes, hypercholesterolaemia, hypertension, obesity, hyperhomocysteinaemia [30, 67] (Fig. **3**). The relevance of the COX-1 pathway and TXA_2 generation in atherothrombotic disorders is clearly shown by the cardiovascular benefits of irreversibly inhibiting this pathway by low-dose aspirin [70] (Fig. **2**). Terutroban, a TXB_2 antagonist at the TP was able to block platelet activation similarly to aspirin in patients with PAD [75], and showed benefit/risk profile similar to aspirin in stroke patients in the PERFORM trial [76]. However, given the lack of superiority as compared to standard (and cheaper) low-dose aspirin, terutroban has never been approved in cardiovascular disorders.

Among the receptors for PGE_2, EP1 and EP3 appear involved in vascular contraction and platelet activation, while EP2 and EP4 contribute to vasodilation [28, 30, 77, 78] (Fig. **1**). Although PGE_2 is not the main prostanoid produced by platelets, nevertheless platelets can synthesize PGE_2 as well as respond to the PGE_2 produced by neighbouring inflammatory cells, which then acts as a modulator of human platelet activation [79]. Platelet and megakaryocytes express all the EPs. EP3 potentiates platelet aggregation and increases the



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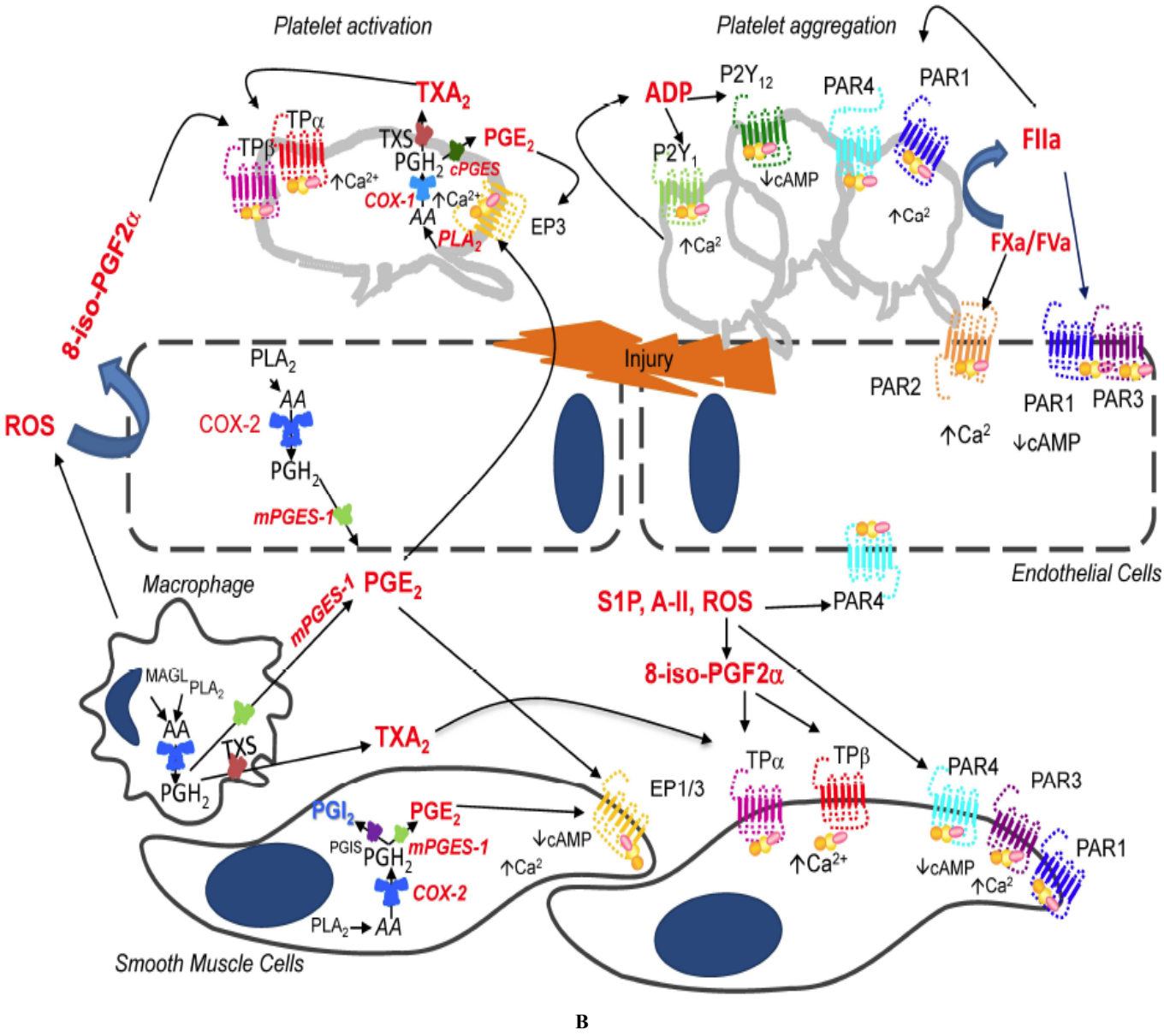

B

Fig. (1). Overview of the major eicosanoid-, PAR- and nitric-oxide-dependent signaling pathways of vessel wall and platelets, in physiological and pathological conditions. A) *Major mechanisms accounting for vascular relaxation and low platelet reactivity/activation under physiological conditions*. Physiological blood shear triggers COX-2-dependent PGI_2 and NO synthesis. PGI_2 interacts with its IP receptors on platelets, endothelial and smooth muscle cells, thus increasing cAMP and is responsible for anti-thrombotic and vasodilator effects. Similarly, NO is formed through the NOS-III enzyme and interacts with the guanylate cyclase in both platelets and smooth muscle cells, increasing cGMP and triggering anti-platelet activities and vasodilation. Moderate degree of activation of PAR-1 in endothelial cells promotes the formation of NO. **B**) *Platelet aggregation and vasoconstrictive pathways under pathological conditions of vascular injury.* TXA_2 derived from platelets, macrophages and vascular cells promotes platelet aggregation and vasoconstriction. PGE_2 and ROS derived from macrophage contribute to thrombosis through the EP3 and TP receptors, respectively. ROS participate in VSMC and EC activation and in isoprostane formation. Isoprostanes, namely 8-iso $PGF_{2\alpha}$, activate both platelets and vascular cells through the TP receptors. Sphingosine-1 phosphate, angiotensin II and ROS induce the expression of PAR4. Activated platelets promote the prothrombinase complex (FXa/FVa), which generates thrombin (FIIa); thrombin triggers platelet aggregation and endothelial activation through PARs, FXa promotes inflammation and endothelial cell activation through PAR2. Abbreviations: AA: arachidonic acid; A-II, angiotensin-II; ADP: adenosine di-phosphate; cAMP: cyclic adenosine monophosphate; cGC, cytosolic guanylate cyclase; cGMP: cyclic guanosine monophosphate; Ca^{2+}: calcium; COX: cyclooxygenase; EC, endothelial cells; EP, Prostaglandin E_2 receptor; PGIS, prostaglandin I syntase, IP, prostacyclin receptor; mPGES-1, microsomal prostaglandin E_2 syntase-1; NO, nitric oxide; NOS, nitric oxide synthase; PAR: protease activated receptor; PDE: phosphodiesterase; PL: phospholipid; PLA_2: phospholipase A_2; PGH_2: prostaglandin H_2; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate; TP: thromboxane receptor; TX: thromboxane; TXS: thromboxane synthase.

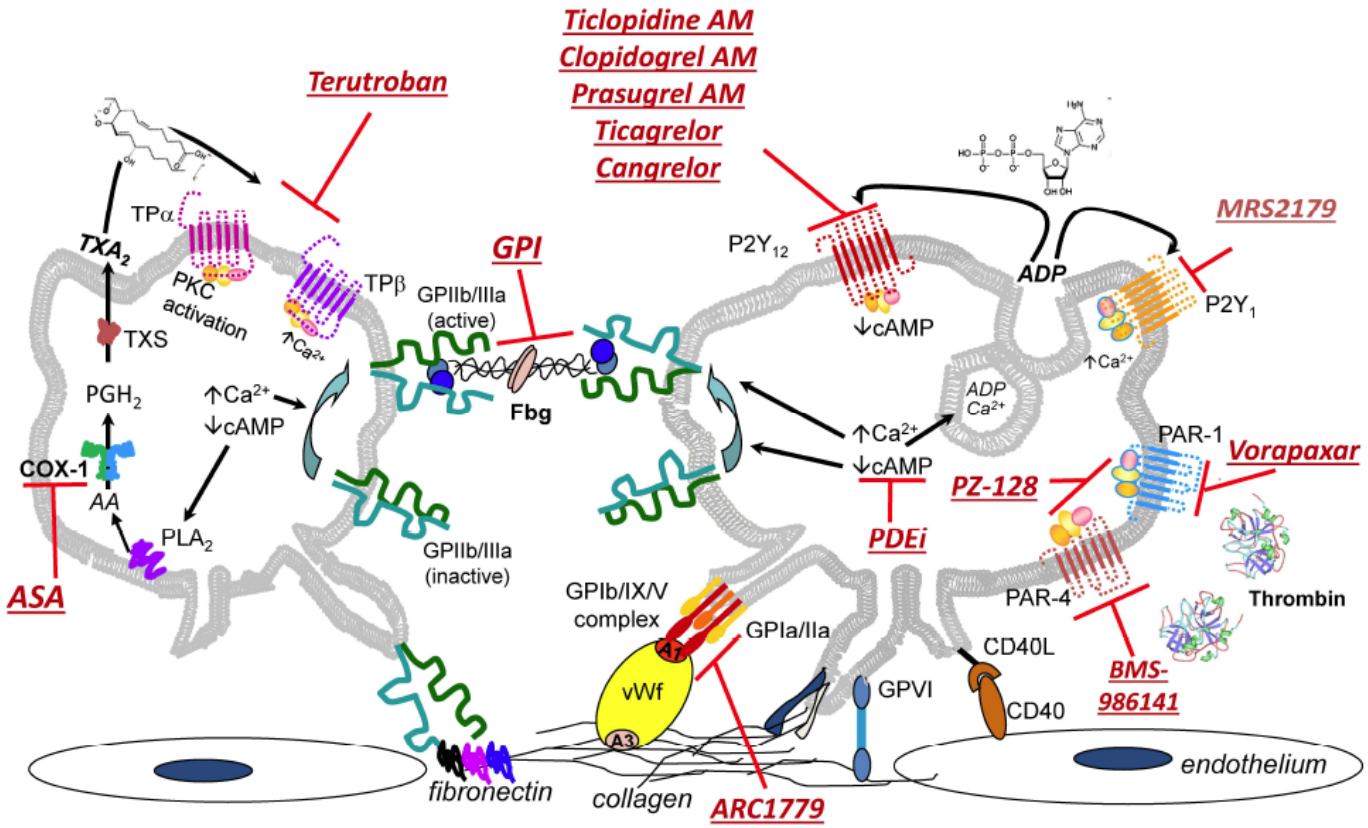

Fig. (2). Platelet activation pathways and targets of current and novel antiplatelet drugs. Platelet activation *via* multiple pathways leads to numerous responses including: secretion of ADP which activates $P2Y_1$ and $P2Y_{12}$ receptors, thrombin generation which activates PAR1 and PAR4 receptors, increased intra-platelet calcium, reduced cAMP concentrations, activation of phospholipases which release of arachidonic acid as substrate for COX-1 and TXA_2 formation, final activation of GPIIb/IIIa leading to fibrinogen binding, platelet aggregation and amplification signals. The antiplatelet drugs already marketed or under development (ARC1779, MRS2179, PZ-128, BMS-986141] are reported in the figure together with their targets. ASA: aspirin; AM: active metabolite; Fbg: fibrinogen; GP: glycoprotein; PDEi: phosphodiesterase inhibitors; vWf: von Willebrand factor. Reproduced modified from [106].

sensitivity of platelets to different agonists, both in animal models [80] and humans [79]. Nanomolar concentrations of PGE_2 appear to act as a positive modulator of human platelets by stabilizing and accelerating aggregation, likely through the EP3. PGE_2 consistently counteracted, at least in part, the inhibition exerted by aspirin, again stabilizing and amplifying aggregation. On the other hand, an EP2-mediated platelet inhibitory effect might prevail at significantly higher concentrations of PGE_2 [79], thus making PGE_2 a fine modulator of platelet responsiveness, depending on the production of PGE_2 from the surrounding microenvironment and EPs exposed. The clinical benefit of selectively blocking the EP3 in humans has been hypothesised, but remains to be established [81].

3.2. Purinergic Receptors

Adenine nucleotides and nucleosides act on purinoceptors. The purinergic P1, $P2Y_1$, $P2Y_{12}$, and $P2X_1$ receptor subtypes are expressed on platelets [82] (Fig. **2**). As compared to the ubiquitous expression of $P2Y_1$, the $P2Y_{12}$ subtype is largely expressed in platelets [83]. The $P2Y_1$ and $P2Y_{12}$ are GPCRs, with $P2Y_1$ associated with Gq and $P2Y_{12}$ with Gi causing a blockade of the adenylate cyclase. Platelet shape change appears mainly mediated by the $P2Y_1$ subtype, while aggregation is preferentially triggered by the $P2Y_{12}$ subtypes [83]. $P2Y_1$ and $P2Y_{12}$ play a role in controlling shape change in human platelets [84, 85]; both receptors bind ADP, which is stored in platelet dense granules and released upon platelet activation, contributing to strengthen and amplify aggregation [86] (Fig. **3**). ADP can also be formed by the extracellular broke down of ATP [85]. The platelet's precursor megakaryocytes can release ATP, and the $P2Y_1$, $P2Y_2$ $P2Y_4$, $P2Y_{12}$, and $P2X_1$ receptors are expressed on megakaryocyte cell lines as well [85]. The $P2Y_{12}$ is the pharmacological target of thienopyridines (clopidogrel and prasugrel), ticagrelor and cangrelor [70]. The active thiolactone metabolites of the thienopyridines bind to the Cys97 residue of the $P2Y_{12}$ [83], irreversibly hampering the ADP binding and signaling. Ticagrelor and cangrelor reversibly compete with the ADP ticagrelor by inducing a transient conformational change of the receptor binding at a site different from the ADP-binding site, while cangrelor directly and reversibly prevents ADP binding [85]. When added to COX-1 inhibition by low-dose aspirin, the $P2Y_{12}$ blockade by clopidogrel adds a further significant 10-20% relative risk reduction of major vascular events in atherothrombotic patients [70]. This additional benefit likely stems from the si-

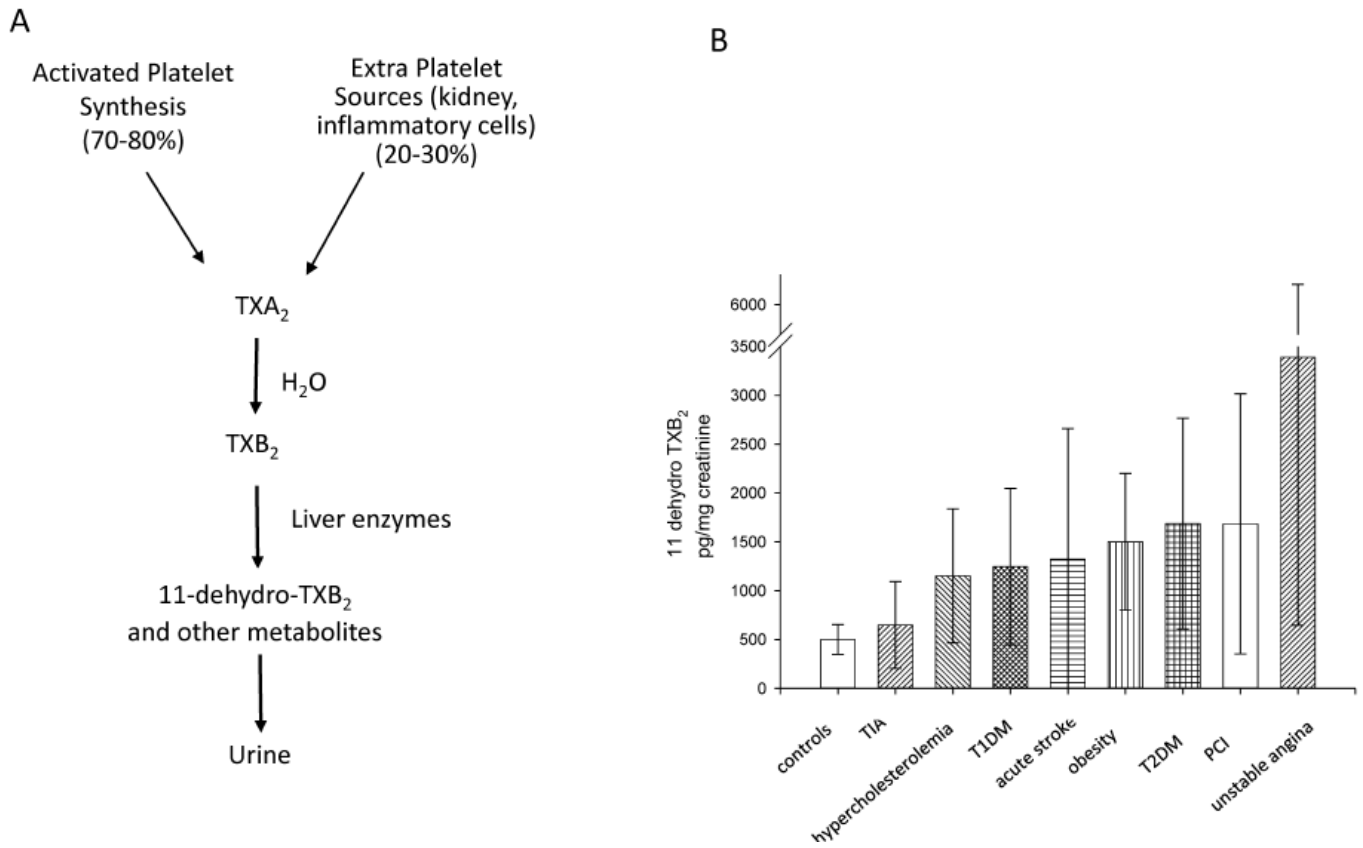

Fig. (3). Pathway and rate of *in vivo* thromboxane A_2 production in healthy subjects and in clinical settings at high cardiovascular risk. A) In humans, the metabolic biotransformation of thromboxane (TX)A_2 *in vivo* leads to the enzymatic final product 11-dehydro-TXB_2, which can be measured in the urine. Approximately 70% of the TXA_2 generated in humans is from platelet origin. **B**) Means ± Standard Deviations of urinary excretion of 11-dehydro-TXB_2 in healthy subjects and in clinical settings characterized by high cardiovascular risk. Abbreviations: TIA, transient ischemic attack; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; PCI, percutaneous coronary intervention. Reproduced modified from [107].

multaneous blockade of the two activation pathways, *i.e*. the $P2Y_{12}$ receptor and COX-1, making unlikely the possibility of a redundancy of the two platelet-signaling pathways. Consistently, a higher $P2Y_{12}$ blockade by prasugrel or ticagrelor, produced an additional benefit as compared with clopidogrel in large phase III trials of patients with acute coronary syndromes [87, 88]. These clinical data support the clinical importance of targeting two independent platelet-signaling pathways. The recent failure of the GLOBAL-LEADERS trial that checked the hypothesis that single P2Y12 blockade (ticagrelor) was superior to dual blockade of COX-1 (aspirin) and P2Y12 (ticagrelor or clopidogrel) [89], confirms that the two pathways are not redundant and should both be targeted in acute coronary syndrome patients. It is unknown whether a simultaneous blockade of both $P2Y_1$ and $P2Y_{12}$ may result in a higher anti-platelet effect and therapeutic benefits in atherothrombotic diseases [85] as compared with $P2Y_{12}$ blockade alone, without increasing the bleeding risk. A $P2Y_1$ antagonist, MRS2179 [90], and a combined $P2Y_1$ and $P2Y_{12}$ blocker [91] are currently at a pre-clinical stage of development.

3.3. Thrombin and PARs

PAR1 and 4 are present on human platelets. PAR1 shows the highest affinity for thrombin (factor IIa) and potentiates the rate of PAR4 cleavage by thrombin through the formation of PAR1-PAR4 heterodimers [86]. Thrombin binds an hirudin-like sequence at the extracellular N-terminus of the PAR-1 cleaving the bond between Arg41 and Ser42, thus exposing the tethered ligand, that binds to the ligand binding site I of the PAR-1 [66]. On the other hand, the interaction between the hirudin-like sequence and the thrombin's exosite I increases the proteolytic efficiency amplifying the prohaemostatic loop [66]. Activated PAR-1 signals mainly through G proteins and ultimately triggers platelet aggregation through calcium mobilization and activation of the glycoprotein IIb/IIIa (Fig. **2**). Activated PAR-4 activated can bind thrombin as well, but with a low affinity and contributes to irreversible platelet aggregation. In addition to thrombin, PAR-1 can be also activated by other proteases such as activated protein C, matrix metalloproteases-1 and -13, kallikrein, although with reduced efficiency and different signaling as compared with thrombin [66]. Vorapaxar in human platelets competes with the tethered ligand of PAR-1 generated by thrombin, disrupting thrombin-triggered signaling. Vorapaxar administered on top of COX-1 and $P2Y_{12}$ inhibition, only added ~10% relative reduction of major vascular events in acute coronary syndromes, which was lower than predicted, but caused a major increase in severe bleeding [92]. Possible explanations might be a certain degree of overlap of the COX-1, $P2Y_{12}$, and PAR-1 pathways, causing

a plateau of the clinical benefit, a possible contribution of PAR-4-mediated activation which is not targeted by vorapaxar, and/or alternative non-canonical proteolytic pathways which are not influenced by vorapaxar [93]. Novel strategies of targeting the PAR1 are pepducins, that bind to the intracellular loops and interfere with G-protein signaling, or bind to the intracellular C-terminus and directly signal through β-arrestin [66]. A compound which blocks the PAR-4, (BMS-986141) is currently tested in a phase II, dose-finding trial to prevent early recurrence of acute ischaemic stroke or transient ischemic attack on top of low-dose aspirin [94]. The underlying hypothesis for PAR-4 blockade would be to provide an additive anti-thrombotic effect on top of current strategies, with minimal bleeding risk.

3.4. Platelet Procoagulant Activity

Platelets not only adhere and aggregate at the site of vascular lesion, but also provide membranes to assemble the prothrombinase complex, which builds up the coagulation process on injury site, thus contributing to local thrombin generation and fibrin clot formation (Fig. **1B**). On the basis of diverse experimental data, it has been hypothesized that 'aggregatory' and 'procoagulant' platelets may represent two different circulating sub-populations in humans, with specific biochemical and morphological phenotypes [95]. Procoagulant platelets seem to have a higher cytosolic calcium increase, which facilitates the exposure of phosphatidylserine (PS), the binding of activated factors X (FXa) and Va (prothrombinase complex) and thrombin generation [95]. GPVI and Gq-coupled signaling, that generate the highest calcium concentration, appear the best triggers of procoagulant platelets [95]. In humans, a high fraction of circulating pro-coagulant platelets and platelet-derived procoagulant microparticles have been described in stroke and coronary artery disease patients [95]. However, more studies are needed to understand the relative contribution of these platelets in arterial thrombosis in humans, their role as bleeding or thrombosis biomarkers, and whether a pharmacological modulation of the procoagulant activity through low-dose acetazolamide or by targeting aquaporin for instance, may have a relevant clinical impact in addition to current antiplatelet therapy [95]. Interestingly, the recent phase 3 COMPASS trial, has shown a superior prevention of major arterial events and death for the combined antiplatelet (low-dose aspirin) and very low-dose anti-FXa (rivaroxaban 2.5 mg bid) strategy as compared to single regimens with aspirin or intermediate dose of rivaroxaban (5 mg bid) alone, in patients with stable atherosclerosis, including PAD [96]. The efficacy of the combined blockade of platelets and coagulation in stable atherosclerosis provides a proof-of-concept for the contribution both primary and secondary haemostasis in arterial thrombosis. Moreover, FXa can also bind and cleave the PAR-2 expressed on human leukocytes, fibroblasts, EC and VSMC [97], promoting inflammation, VSMC proliferation, EC adhesion [97] and von Willebrand factor release from EC [98]. Thus, FXa-blockade may also affect the pro-inflammatory and atherothrombotic phenotype triggered by PAR-2. Consistently, *in vitro* FXa blockade enhances the anti-inflammatory IL-10 and reduces angiogenin from human monocytic cells [99], reduces mitogenesis and inflammatory gene expression in human VSMC [100], inhibits tissue factor-induced platelet aggregation [101] and influences thrombus formation under high shear conditions mimicking arterial flow [102]. FXa blockade dose-dependently inhibits thrombin generated by platelet membranes *in vitro* [103], being thrombin the most relevant crossroad between primary, platelet-dependent and secondary clot-generation haemostasis through fibrin generation [104]. Consistently, Apo-E-deficient mice treated with a Factor Xa blocker showed reduced inflammation and increased plaque stability [105]. In a mouse model of sickle cell anaemia, anti-FXa treatment as well as PAR2 deletion reduced pro-inflammatory IL-6 secretion [106]. However, the contribution in humans of FXa in atherothrombosis, beyond its procoagulant effect remains to be established.

CONCLUSIONS

Atherosclerosis is characterized by the stiffening and hardening of the vessels, by lumen reduction and activation of primary and secondary haemostasis. Glucose and lipid disorders as well as heavy smoking habits accelerate and worsen atherosclerotic processes. In spite of significant pharmacological progresses made in the past decades, nevertheless the presence of a residual risk of recurrence obliges to test new combined strategies. The knowledge of the multiple pathophysiological mechanisms underlying atherosclerosis development and atherothrombotic complications remains the guidance for future research and development.

LIST OF ABBREVIATIONS

cAMP	=	Cyclic Adenosine Mono-Phosphate
cGMP	=	Cyclic Guanosine Monophosphate
COX	=	Cyclooxygenases
EC	=	Endothelial Cells
EP	=	Prostaglandin E2 Receptor
FXa	=	Activated Factor X
GPCR	=	G-protein Coupled Receptors
IP	=	Prostacyclin Receptor
KO	=	Knockout
NO	=	Nitric Oxide
NOS	=	Nitric Oxide Synthases
PAD	=	Peripheral Artery Disease
PAR	=	Protease Activated Receptors
PD	=	Cyclic Nucleotide Phosphodiesterases
PG	=	Prostaglandin
PGES	=	PGE_2 Synthases
PKC	=	Protein Kinase C
TP	=	Thromboxane Receptor
TX	=	Thromboxane
VSMC	=	Vascular Smooth Muscle Cells

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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