



Lipid Metabolism and Signaling in Platelet Function

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Abstract

Modern society has changed its diet composition, transitioning to a higher intake of saturated fat with a 50% increase of cardiovascular risk (CVD). Within the context of increased CVD, there is an induction of a prothrombotic phenotype mainly due to increased platelet reactivity as well as decreased platelet response to inhibitors. Platelets maintain haemostasis through both blood components and endothelial cells that secrete inhibitory or stimulatory molecules to regulate thrombus formation. There exist a correlation between

platelets' polyunsaturated fatty acid (PUFA) and the increase in platelet reactivity. The aim of this chapter is to review the metabolism of the main PUFAs involved in platelet function associated with the role that their enzyme-derived oxidized metabolites exert in platelet function and fate. Finally, how lipid metabolism in the organism affect platelet aggregation and activation and the pharmacological modulation of these processes will also be discussed.

Keywords

Platelets · Arachidonic acid · Eicosanoids · Thrombosis · Dyslipidemias

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7.1 Introduction

With increased industrialization, modern society has changed its diet composition, transitioning to a higher intake of saturated fat [1]. This coupled with an array of genetic mutations, prone humans to develop dyslipidemia, a disorder characterized by high levels of circulating lipids such as low-density lipoprotein (LDL), total cholesterol (TC) or triglycerides and/or decreased levels of high-density lipoprotein (HDL). The last survey conducted by the world health organization (WHO) in 2008 estimated the prevalence of dyslipidemia (characterized by TC > 5 mmol/L) to be 23.1% in

Africa, 53.7% in Europe, with a global prevalence of 38.9% [2]. Such high prevalence developed onto a public health debate, given that a rise in 2 mmol/L in TC has been shown to increase cardiovascular risk (CVD) by 50% [3], while a more recent meta-analysis questioned this assumption [4]. Within the context of increased CVD, it is well established that dyslipidemia induces a prothrombotic phenotype mainly due to increased platelet reactivity as well as decreased platelet response to inhibitors [5–8].

Platelets are fragments of megakaryocytes circulating within the bloodstream to block leakages – an evolutionary adaptation required for mammal survival. These cells maintain haemostasis through both blood components and endothelial cells that secrete inhibitory (e.g. nitric oxide) or stimulatory (e.g. thromboxane) molecules to regulate thrombus formation, which involves the exposure of sub-endothelial collagen and fibrinogen after endothelial injury. Platelets will then bind to both collagen and fibrinogen through glycoprotein (GP) VI and GPIIb-V-IX receptors, respectively culminating in platelet adhesion, secretion of stimulatory molecules such as adenosine diphosphate (ADP) and aggregation to other platelets – a process deemed irreversible through binding to the platelet fibrinogen receptor α IIb β 3 [9–11]. While these processes are important to maintain haemostasis in physiological environments, it has been described an up-regulation of stimulatory signals as well as down-regulation of inhibitory ones in chronic diseases such as dyslipidemia and atherosclerosis [12]. Therefore, increased platelet activity has been perceived as a maladaptation implicated in chronic non-communicable diseases, opening up perspectives of new scientific discoveries – according to Prof Barry Collier, this is the “Golden Age of Platelet Research” [13].

The rise in platelet reactivity is due to the actions of different lipids on platelet function, which will be discussed in the following sections of this chapter. We will review the metabolism of the main polyunsaturated fatty acid (PUFA) involved in platelet function, arachidonic acid (AA), associated with the role that its

enzyme-derived oxidized metabolites exert in platelet function and fate. Finally, how lipid metabolism in the organism affect platelet aggregation and activation and the pharmacological modulation of these processes will also be discussed.

7.2 Overview of Arachidonic Acid Metabolism

Arachidonic acid (AA, all-cis-5, 8, 11, 14-eicosatetraenoic acid) is an omega-6 PUFA. It is an essential constituent of cell membrane, necessary for membrane fluidity, flexibility and function in all cell types. The presence of its cis-four double bonds gives the compound a certain degree of flexibility to interact with proteins or to react against molecular oxygen, forming a range of bioactive oxygenated molecules via enzymatic and non-enzymatic mechanisms [14–18]. Esterified AA is usually localized in the glycerol backbone sn-2 position, constituting an important part of phospholipid-content in the membranes and cytosol of mammalian cells and tissues. In platelets, up to 25% of phospholipid fatty acids are AA [14–18], reaching levels near to 5 mM in resting platelets [19]. Arachidonic acid cellular concentration may influence both normal cellular functions and the development of platelet diseases. More important, it has been proposed that a low dietary intake of AA decrease the production of pro-inflammatory eicosanoids, which contributes to processes resolution in chronic inflammatory diseases [15, 17]. However, our body’s AA needs are higher than the concentration found in human diet. Thus, our tissues depend on endogenous formation of AA from its precursor, linoleic acid (LA 18: 2n-6), in a process regulated by the action of enzymes such as desaturases and elongases. The activity of Δ 6 and Δ 5 desaturases (d-5-d) converts LA to gamma-linolenic acid (GLA, 18: 3), dihomo-GLA (DGLA, 20:3) and AA [15, 17].

Arachidonic acid is released from its esterified form by the action of phospholipase A₂ (PLA₂) complex, which mediates the one-step hydrolysis of the AA present at the sn-2 position

on phospholipid backbone [14, 20–23]. Cytosolic calcium (Ca^{2+})-dependent group IV PLA₂ (cPLA₂ α) catalyzes that hydrolysis to generate a free fatty acid and a lysophospholipid. Secretory PLA₂ (sPLA₂), which is induced by the action of cPLA₂, controls magnitude and duration of free AA release, as well as paracrinely propagates the inflammatory response to neighboring cells [24–26]. Lastly, cytosolic Ca^{2+} -independent PLA₂ plays a role on cellular homeostasis through generation of SPM (specialized pro-resolvins mediators) and reacylation of free AA in membranes as well [16, 27]. Nevertheless, there are other phospholipases equally able to release AA: phospholipase C (PLC) and phospholipase D (PLD). PLC participates in the formation of diacylglycerol (DAG) by the action of diacylglycerol lipase and lipid products containing AA by the action of monoacylglycerol lipases [15, 20, 26, 27]. Similarly, phosphatidic acid or DAG are formed from phosphatidylcholine by PLD. The former can be further catalyzed by phosphatidate phosphohydrolase to form DAG. Then, DAG-lipase hydrolyzes DAG to generate AA. Upon stimulation, Gq protein-coupled receptor activates phospholipase C that cleaves phosphatidylinositol 4,5 bisphosphate (PIP₂) to inositol 1,4,5 triphosphate (IP₃) and diacylglycerol (DAG). The presence of DAG and Ca^{2+} activates protein kinase C and IP₃ opens ER Ca^{2+} channel [28–30]. The increase in intracellular Ca^{2+} ions levels guides the PLA₂ enzyme translocation to the perinuclear membrane where exerts the explained release of AA from the membrane to let the AA-metabolizing enzymes their substrate [14, 20–23].

Free AA can undergo four possible enzymatic pathways: prostaglandin endoperoxide H synthase (PGHS), lipoxygenase (LOX), cytochrome p450 (CYP 450) and anandamide pathway, which create bioactive 20-carbon oxygenated PUFA generally denominated eicosanoids. These bioactive lipids act as local hormones and/or signaling molecules produced both in response to basal metabolism as well as in inflammatory sites upon regulation by normal immune response stimuli (IL-1, TNF- α , lipopolysaccharides, etc.). The metabolic fate subsequent to AA release into the

cytosol depends on characteristics of each tissue type, although on different tissues the same precursor often gives rise to products with antagonistic function, e.g. PGE₂ [14, 17]. Cyclooxygenase (COX), more precisely known as Prostaglandin Endoperoxide H synthase (PGHS), converts AA first into PGG₂, via a COX function and then to PGH₂ following a peroxidase reaction [31]. Two different PGHS isoforms (PGHS-1 and PGHS-2) are involved in the PGHS pathway to generate prostaglandin H₂ (PGH₂), an intermediate hub whose metabolization by downstream enzymes leads to different prostaglandins: PGE₂, PGD₂ and PGF₂ α , prostacyclin (PGI₂), or thromboxane A₂ (TXA₂). LOX pathway consists of AA oxidation by the enzyme isoforms LOX-5, LOX-8, LOX-12, and LOX-15 to generate their products, leukotrienes (LTA₄, LTB₄, LTC₄, LTD₄ and LTE₄), lipoxins (LXA) and 8-, 12- or 15- hydroperoxyeicosatetraenoic (HpETE) acids or their corresponding reduced derivatives hydroxyeicosatetraenoic (HETE) acids. CYP 450 pathway involves two enzymes, CYP450 epoxygenase and CYP450 hydroxylase giving rise to epoxyeicosatrienoic acid (EETs) and 20-HETE, respectively. Finally, anandamide pathway comprises the FAAH (fatty acid amide hydrolase) to produce the endocannabinoid, anandamide [14–17, 20, 32–37].

Cell–cell interaction is important for eicosanoids synthesis since a donor cell has to transfer its unstable intermediate, e.g. PGH₂, to a recipient cell to trigger eicosanoid biosynthesis in the latter. The single donor cell should have all the enzymes necessary to produce the different eicosanoids while the recipient cell does not necessarily have all the required enzymes for AA release. Thus, for inflammation initiation, the complete set of enzymes to initiate eicosanoids production must be present in at least two cells in the injured tissue. The AA intermediate metabolites are lipophilic and require a group of special proteins called fatty acid binding proteins (FABP), specific for each cell type, which are responsible for the increase of AA intermediates export via their stabilization and lengthening their half-life time to allow them to exert their biological activities. The major action of AA

metabolites is promotion of acute inflammatory response, characterized by the production of pro-inflammatory mediators, e.g. PGE₂ and PGI₂, followed by a second phase in which lipid mediators with pro-resolution activities may be generated [14–17, 20, 32–37]. Resolution of inflammation is no more considered a passive process, but rather an active programmed response regulated by mediators with pro-resolving capacity.

7.3 Arachidonic Acid Metabolism in Platelets

The activation or inhibition of platelets can be modulated by many agents with a central role being played by eicosanoids, being TxA₂ and PGI₂ the main eicosanoids affecting platelets' function [38].

Arachidonic acid is metabolized to PGH₂ in the cytosol of platelets and for this, PLA₂ activity is necessary to release AA from platelet membrane [39, 40]. PGH₂ in platelets undergoes further transformations catalyzed by Tx synthase, PGD isomerase or PGE synthase to form TxA₂, PGD₂ or PGE₂ respectively (Fig. 7.1). In addition to the wide variety of eicosanoids formed by the COX pathway [41], 11-HETE and 15(S)-HETE-but not 5-HETE- are produced when AA is inserted at the active site of COX-1 in a different structural arrangement than the one necessary for PGH₂ synthesis [42]. Both products can be formed at similar levels to TxA₂ whose formation is associated with an increase in AA levels, e.g. at platelet hyperactivation where high concentrations of AA are released from platelet membranes. The participation of COX in 11-HETE and 15(S)-HETE formation was also demonstrated by the lack of decrease in 12-HETE when using only aspirin thus LOX is not the enzyme involved in these products formation [42]. Reduction of 12-HETE was only observed when both PGHS and P2Y₁₂ inhibitors were used confirming the former proposal [42].

Platelets mainly express PGHS-1, but traces of PGHS-2 have been detected, possibly carried over from megakaryocytes, the platelet precursor

cells, or as a result of the transcription of residual mRNA into protein [22, 33, 43]. In addition to PGHS, AA can also be oxidized by the non-heme iron-containing enzymes LOXs. Different isoforms of LOXs are found depending on the carbon where the hydroperoxyl (-OOH) group is added. Platelets present 12-LOX which inserts oxygen primarily at C-12 of AA forming the 12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid [12(S)HpETE] [44]. The highly reducing environment present in the platelet rapidly reduce 12(S)-HpETE to its hydroxyl derivative 12-HETE [44]. The biological activity of 12-HETE in platelets *in vivo* remains under discussion; some authors suggest a pro-thrombotic activity by acting at vascular hypertension (Fig. 7.2) while others propose an anti-aggregant and anti-inflammatory action by decreasing the release of AA from the membrane and regulating integrin activation (Fig. 7.3). The pro-inflammatory leukotrienes precursor, being able to affect platelet activation during an inflammatory process, is formed by the 5-LOX present in neutrophils. One important difference between the AA-metabolizing enzymes due to their capacity to be inhibited is that while aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit PGHS activity [45, 46], LOX remain active in their presence being able to oxidize AA to HpETEs.

7.3.1 Role of Distinct Eicosanoids in Platelets

7.3.1.1 Thromboxane A₂ (TxA₂)

The most directly important prostanoid for platelet function is the PGHS-1-generated TxA₂, which is synthesized by activated platelets acting in an autocrine and paracrine manner to induce thrombosis. On platelets, TxA₂ binds to the thromboxane prostanoid (TP) receptor and initiates an amplification loop leading to further platelet activation, aggregation and TxA₂ formation. In the vasculature, TxA₂ induces vasoconstriction and the proliferation of vascular smooth muscle cells (Fig. 7.2) [21, 38, 47, 48].

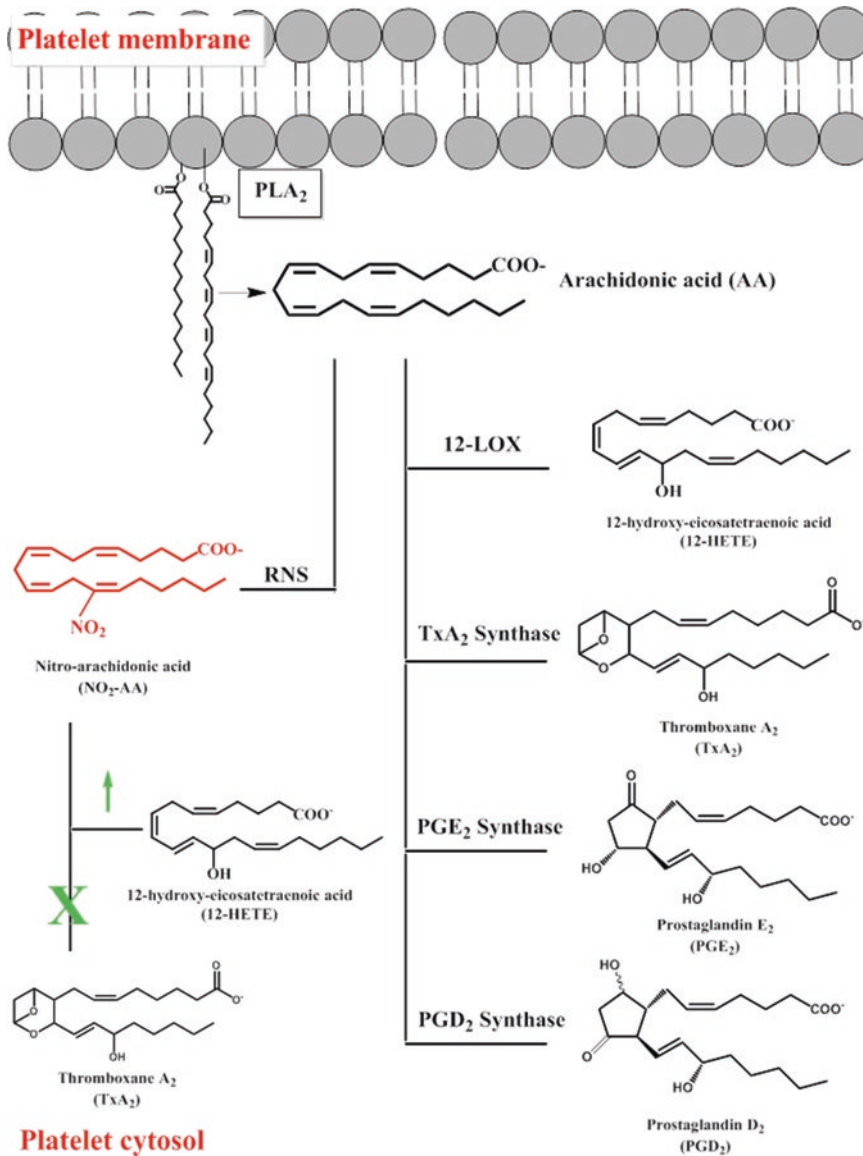


Fig. 7.1 Arachidonic acid metabolism in platelets. Arachidonic acid is esterified at the platelet membrane and released to the cytosol due to the activity of PLA₂. Then, AA is the substrate for PGHS-1 forming PGH₂ which then is the substrate for the subsequent formation of TxA₂, PGE₂ or PGD₂. If AA is oxidized by LOX, then 12-HETE is formed. When an increase of vasculature

RNS formation or the appearance of an inflammatory process occur, AA is nitrated to NO₂-AA which inhibit PGHS-1 thus decreasing the pro-aggregant TxA₂ formation [40]. The increase of non-oxidized AA in the cytosol led to a subsequent oxidation of the fatty acid by the 12-LOX thus increasing the levels of 12-HETE [47]

7.3.1.2 12-Hydroxy-Eicosatetraenoic Acid (12-HETE)

12-HETE is the major 12-LOX-catalysed metabolite and the most abundant eicosanoid produced by platelets upon stimulation [41, 42], although

its effects on platelet function are not completely understood. There are studies reporting that inhibition of 12-LOX led to decreased platelet aggregation correlated with a significant reduction of 12-HETE levels in response to collagen [49].

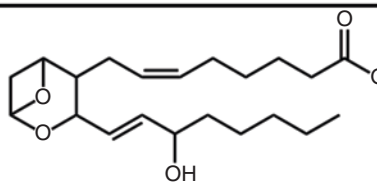
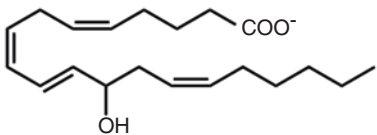
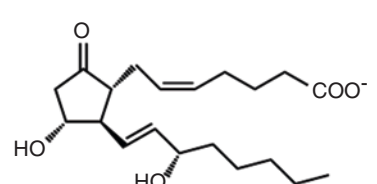
Pro-aggregant biomolecules formed in platelets	References	
 <p style="text-align: center;">Thromboxane A₂ (TxA₂)</p>	<ul style="list-style-type: none"> - Binds to the thromboxane prostanoid (TP) receptor - Induce thrombosis - Amplification loop leading to further platelet activation 	<p>Holinstat <i>et al.</i> [21]</p> <p>Jennings [38]</p> <p>Rouzer and Marnett [48]</p>
 <p style="text-align: center;">12-hydroxy-eicosatetraenoic acid (12-HETE)</p>	<ul style="list-style-type: none"> -Pro-aggregant effects depend on: i) concentration, ii) estereospecificity; iii) co-incubation with different agonists 	<p>Porro <i>et al.</i> [50]</p>
 <p style="text-align: center;">Prostaglandin E₂ (PGE₂)</p>	<ul style="list-style-type: none"> -At 0.1–10 μM, binds to EP3 Gi-coupled receptors to stimulate platelet aggregation by decreasing cAMP - After plaque rupture, PGE₂ enhances thrombus formation 	<p>Petrucci <i>et al.</i> [56]</p> <p>Glenn <i>et al.</i> [57]</p> <p>Friedman <i>et al.</i> [2015]</p>

Fig. 7.2 Structure and biological pro-aggregant activities of bioactive lipid derivatives of AA formed inside platelets

A recent review concluded that 12-HETE can exert both pro- and anti-aggregant effects (Figs. 7.2 and 7.3) on platelets, depending on 12-HETE concentration, stereospecificity and co-incubation with different agonists [50].

7.3.1.3 Prostacyclin (PGI₂)

Endothelium-produced PGI₂ binds to the Gs-coupled PGI₂ receptor (IP) on platelets and generally reduces platelet reactivity, which can be critical to minimizing the risk for atherothrombotic events [51, 52]. Binding of PGI₂ to the IP receptor, results in the activation of adenylyl cyclase and a subsequent rise in cAMP levels in platelets [51, 52]. This stimulates phosphorylation of PKA, which suppresses various signaling pathways involved in platelet function such as adhesion, aggregation and granule secretion.

7.3.1.4 Prostaglandin D₂ (PGD₂)

PGD₂ is well established as a macrophage product but, in lesser amounts, is also synthesized by platelets. By interaction with platelet DP1 receptors, PGD₂ increases adenylyl cyclase activity and so, like PGI₂, inhibits platelet activation [53, 54] (Fig. 7.3).

7.3.1.5 Prostaglandin E₂ (PGE₂)

PGE₂ is released by endothelial cells (ECs) and, to some extent, by activated platelets. It acts on a range of prostanoid receptors, EP1 – EP4, that differently modulate second messengers, such as cAMP and free Ca²⁺, within platelets and exert contrasting effects on platelet function [55]. The effects on platelets of PGE₂ acting through EP receptors are concentration dependent. At low concentrations (0.1–10 μM), PGE₂ binds to Gi-coupled receptors (EP3) to enhance

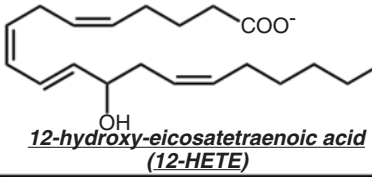
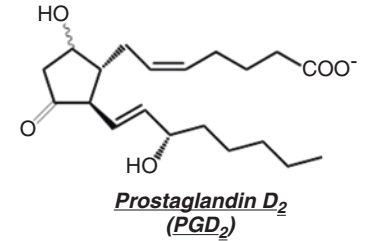
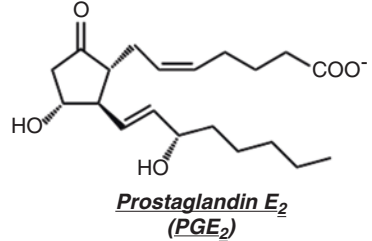
Anti-aggregant biomolecules formed in platelets	References
 <p data-bbox="524 256 870 324">-In response to collagen, 12-HETE decreased platelet aggregation</p> <p data-bbox="166 369 505 421">12-hydroxy-eicosatetraenoic acid (12-HETE)</p>	Maskrey <i>et al.</i> [49]
 <p data-bbox="524 473 897 575">-Activating DPI receptors, increases adenylyl cyclase activity and inhibits platelet activation</p> <p data-bbox="252 627 427 681">Prostaglandin D₂ (PGD₂)</p>	Gimenez-Bastida <i>et al.</i> [53] Schuligoj <i>et al.</i> [53]
 <p data-bbox="524 743 884 884">-At concentrations of 100 μM, PGE₂ activates EP2 and EP4 Gs-coupled receptors that inhibit platelet aggregation</p> <p data-bbox="252 888 427 942">Prostaglandin E₂ (PGE₂)</p>	Petrucci <i>et al.</i> [56] Glenn <i>et al.</i> [57] Friedman <i>et al.</i> [58]

Fig. 7.3 Structure and biological anti-aggregant activities of bioactive lipid derivatives of AA formed inside platelets

aggregation (Fig. 7.2), whereas at higher concentrations (100 μM), it activates Gs-coupled receptors (EP2, EP4) to inhibit aggregation [56–58] (Fig. 7.3). Stimulation of EP3 receptors by PGE₂ decreases cAMP levels, thus favoring platelet aggregation, but the full effect is only seen in the presence of another platelet agonist [58]. On the other hand, the increased cAMP levels which accompany EP4 receptor activation correlate with suppressed platelet aggregation [57]. In addition to PGE₂, PGF₂α and PGD₂ can also bind to EP3 and EP4 receptors but with lower affinity and reversible effects [57, 58]. As well as the well-characterized effects of PGE₂ mediated through EP3 and EP4 receptors, EP1 receptors are also expressed on platelets [56, 59]. Although the signal transduction pathway is not clear, studies in several cell lines expressing EP1 receptors suggest that its activation increases Ca²⁺ influx and might thereby stimulate platelet aggregation

[60]. While PGE₂ seems to both inhibit and potentiate platelet aggregation *in vitro*, a study by Gross *et al.* has elegantly shown that, *in vivo*, PGE₂ is produced by the vessel wall or after the rupture of a plaque. Under these conditions, PGE₂ activates the EP3 receptors on platelets and clearly enhances, rather than reduces, thrombus formation in the arterial vessel wall [61].

7.4 Diversion of Normal AA Metabolism in Platelets: Formation of NO₂-AA

Regarding AA metabolism in platelets and its oxidation by PGHS and/or LOXs, the participation of nitric oxide (•NO) and reactive nitrogen species (RNS) during platelet activation has been widely demonstrated. PGHS is a target for •NO and peroxynitrite, modulating prostaglandins as

well as TxA_2 synthesis [62, 63]. Inflammatory processes and PUFAs are linked by eicosanoids, which represent mediators and regulators of inflammatory processes. Many of the reported protective, anti-aggregant and signaling actions of $\bullet\text{NO}$ are due to its interaction with iron-containing enzymes. In fact, the heme-containing enzyme PGHS is a target for $\bullet\text{NO}$, modulating prostaglandin synthesis [62]. Nitric oxide can act as a reducing co-substrate of the peroxidase (POX) activity of the enzyme favoring the catalytic cycle of POX [39] or as a precursor of peroxynitrite which may also be a POX substrate or an inhibitor of the enzyme by tyrosine nitration at the enzyme active site. Indeed, $\bullet\text{NO}$ enhanced COX inactivation by peroxynitrite [62]. During COX catalysis, AA is oxidized being formed arachidonyl radicals which are potential targets for $\bullet\text{NO}$ or RNS reactivity thus influencing enzyme activity. Our reports suggest that during COX catalysis, those AA-derived radicals can be “sequestered” by RNS to form nitrogen-containing AA products decreasing enzyme substrates thus diminishing enzyme activity [62].

We have extensively described the synthesis of the nitrated derivative of AA, which we propose that besides being formed by acidic gastric nitration, can be a byproduct of COX activity in the presence of RNS. Nitroarachidonic acid ($\text{NO}_2\text{-AA}$) can be formed in biological membranes from AA, as explained above the most abundant fatty acid present in the 2-carbon position of phospholipids, to exert biological effects upon PLA_2 cleavage. One of the currently accepted mechanisms for RNS (e.g. $\bullet\text{NO}_2$)-mediated oxidation and nitration for AA involve different routes including hydrogen atom abstraction and addition reactions [64]. Reaction of $\bullet\text{NO}_2$ with PUFAs leads to the generation of isomerized, oxidized and/or nitro-allylic, nitroalkene, dinitro, or nitro-hydroxy lipid derivatives. An arachidonyl carbon-centered lipid radical and nitrous acid (HONO) are formed, and under anaerobic conditions or when O_2 tension is low, a second molecule of $\bullet\text{NO}_2$ reacts with the carbon-centered radical generating a nitroalkane (the $-\text{NO}_2$ moiety is bound to a double bond) [64, 65].

As AA is the substrate for PGHS activity, we evaluated if nitration of the carbon chain of AA can exert any effects on PGHS normal activity of oxidizing AA. When analyzed *in vitro*, $\text{NO}_2\text{-AA}$ inhibited both POX and COX activities of PGHS-1 while only affected POX in PGHS-2 [40], while nitration of other fatty acids was unable to modify PGHS activity. The mechanism of inhibition involves the release of heme as a result of $\text{NO}_2\text{-AA}$ reaction with the protein [40]. Not only *in vitro* the effects of $\text{NO}_2\text{-AA}$ on PGHS were analyzed. The capacity of $\text{NO}_2\text{-AA}$ to modulate PGHS-1 activity was also evaluated in human platelets [40, 47] being a potent inhibitor of thrombin-mediated platelet aggregation with an inhibitory concentration 50 (IC_{50}) of $1.3 \mu\text{M}$ (refs. [40, 47]). Inhibition of PGHS in platelets lead to a decrease in TxA_2 synthesis with a concomitant diversion of AA to the LOX pathway, thus AA is mainly oxidized by LOX forming 12-HETE [40, 47]. Importantly, platelet aggregation and activation was modulated by $\text{NO}_2\text{-AA}$ in response to several membrane receptors activation, *i.e.* protease activated receptors -PARs- for thrombin, P2Y receptors for ADP and TxA_2 receptors for AA, by either TxA_2 positive retroalimentation after its synthesis or through direct stimulation of protein kinase C (PKC), indicating that $\text{NO}_2\text{-AA}$ acts downstream membrane receptors to exert its antiplatelet effects [47].

The PKC family is centrally involved in platelet activation and aggregation. As explained previously, stimulation of PLC led to the generation of IP3 and DAG where the latter activates at the platelet membrane the cytosolic PKC isoforms (cPKC), which act in a synergistic manner with Ca^{2+} . While $\text{NO}_2\text{-AA}$ did not mobilize Ca^{2+} , it was able to inhibit α -granule secretion [47]. The effect of the nitroalkene on PKC α was analyzed in non-activated and thrombin activated platelets. PKC α was diffusively distributed in the cytosol of untreated platelets and migrated to the plasma membrane after thrombin-stimulation in a process that was abolished by $\text{NO}_2\text{-AA}$ in addition to a prevention of platelet shape change and cytoskeletal reorganization, characteristics of platelet activation [47]. It has been reported that the bioactive signaling activities of nitroalkenes

are due to their capacity to perform Michael-addition reactions with nucleophilic Cys of His residues. cPKC isoforms contain Cys rich motifs that are duplicated as a tandem domain which are critical for its interaction with membrane phospholipids, suggesting that PKC inhibition could be mediated by electrophilic modifications of the enzyme. In addition, NO₂-AA modulation of PKC route has been shown by its capacity to inhibit platelet responses downstream to PKC activation (α -granule secretion, Erk2 phosphorylation, PKC translocation to the membrane) while not affecting upstream responses (*e.g.* Ca²⁺ mobilization). Moreover, when platelet activation is directly activated by the PKC activator PMA, NO₂-AA inhibited platelet aggregation. These observations provide a possible novel mechanism for platelet regulation under conditions where AA acts as a mild agonist for hemostasis, but adopts potent anti-platelet properties at inflammatory environments associated with increased •NO and RNS production when transformed into NO₂-AA [47].

7.5 How Lipoproteins Modulate Platelet Function

7.5.1 Native and Oxidized LDL in Cardiovascular Disease

High levels of LDL are more prone to be altered by reactive oxygen species (ROS), which are normally present in physiological processes acting as second messengers, but also induce oxidative stress in conditions such as ageing, smoking, diabetes mellitus, metabolic syndrome and dyslipidemia. ROS act on PUFAs (free or ester-bound) on the LDL particle, leading to hydroperoxides formation, which in turn, breaks down to form reactive particles as malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE). MDA and 4-HNE react with lysine residues at the Apo lipoprotein B in LDL, together with amine groups in some phospholipids such as phosphatidylethanolamine and phosphatidylserine, forming Schiff-bases, generating neopeptides. These processes produce oxidized LDL (OxLDL) particles, which are

highly immunogenic. In fact, IgG autoantibodies against OxLDL have been found, although their role in atherogenesis and atherothrombosis remains to be elucidated [66].

Oxidized LDL has been associated to atheromatous plaque formation through four mechanisms that complement each other: endothelial dysfunction, foam cell formation, smooth muscle cell migration and proliferation, and induction of platelet adhesion and aggregation. Endothelial dysfunction occurs when its anticoagulant and vascular regulatory properties are compromised and a pro-inflammatory phenotype is produced. OxLDL can be formed and retained in subendothelial space, where it can induce adhesion molecules as ICAM-1 and VCAM-1 and the secretion of chemotactic protein MCP-1 and mCSF, favoring monocyte recruitment. Also, OxLDL is associated with a decrease in •NO production, leading to endothelial dysfunction, facilitating the atheromatous plaque formation [67]. One of the main mechanisms is that OxLDL up-regulates arginase I expression in the vascular wall without altering eNOS expression, which contributes to endothelial dysfunction by reducing l-arginine availability to eNOS for •NO production [68].

The oxidation of LDL particles increases their uptake by macrophages, so OxLDL is considered highly atherogenic by inducing foam cells formation in the first steps of the atherosclerotic lesion. Indeed, macrophages of the sub-endothelial space express scavenger receptors, which have a high affinity for OxLDL (in contrast to normal LDL) and they are not downregulated, leading to a massive fat accumulation. In turn, the foam cells present pro-inflammatory and pro-oxidative features that promote more LDL oxidation in the sub-endothelial space, and the recruitment of more monocyte to form macrophages and then, foam cells. Finally, the excessive intracellular accumulation of oxLDL induces the apoptosis or necrosis of foam cells, forming an important amount of cell debris that constitute the core of atherosclerotic plaque [67].

The vascular smooth muscle cells migrate from tunica to intima and proliferate in sub-endothelial space, as part of the process of ath-

eromatous plaque formation. OxLDL can favor this, due to its capacity to induce platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) release from endothelial cells and macrophages. Also, OxLDL induces LOX-1 expression, a scavenger receptor also present in macrophages, allowing the entry of OxLDL in smooth muscle cells, forming new foam cells, this time derived from smooth muscle [69].

7.5.2 Native and Oxidized LDL in Platelet Function

Relevant to dyslipidemia, several groups have described an increase in platelet activation after platelet exposure to native (nLDL) and oxLDL [5, 70, 71]. Given the pro-oxidant environment that develops in dyslipidemia and associated metabolic diseases, the accepted hypothesis is that LDL is partly oxidized into oxLDL, which is the main molecule responsible for potentiating platelet response. It has been shown the importance of platelets in atheromatous plaque formation, and OxLDL also have a role in it. As mentioned above, OxLDL induces an endothelial dysfunction, which in turn favors platelet activation. But also, OxLDL have direct effects on platelets that lead to activation and thrombus formation in the atherothrombosis context. We will review this last aspect with more detail next.

Studies demonstrated that specific oxidized phospholipids that accumulate in plasma in dyslipidemia promote platelet activation by interacting with different scavenger receptors: CD36, lectin-like oxidized LDL receptor-1, scavenger receptor class B type I (SR-BI), Toll-like receptor 2 (TLR2), scavenger receptor that binds phosphatidylserine and oxidized lipoprotein/chemokine (C-X-C motif) ligand [71–75]. Although it was known for some time that platelets possessed CD36, a class B scavenger receptor that binds to several unrelated molecules, such as thrombospondin-1 and -2, [76, 77] as well as oxLDL [78], it was only on the last decade that it was shown that CD36 is the main receptor for oxLDL in platelets. Upon binding to CD36, oxLDL

enhances oxidative stress and pro-thrombotic phenotype. In fact, Podrez et al. [7] were the firsts to describe the importance of CD36 in dyslipidemia, showing that genetic deletion of CD36 in murine models of dyslipidemia restored arteriole and venule occlusion times back to control levels.

Less than a year later, the first signalling pathway for oxLDL-CD36 was described. Chen et al. [79] demonstrated for the first time that mitogen-activated protein kinase kinase 4 (MKK4) and downstream c-Jun N-terminal kinase (JNK)2 were phosphorylated in oxLDL-exposed platelets. Interestingly, src family kinases Fyn and Lyn, which are also important for collagen receptor GPVI signalling, were recruited to the cytoplasmic tail of CD36 after exposure to oxLDL. Also, immobilized oxLDL increased protein tyrosine kinase Syk activation and intracellular Ca^{2+} while enhancing thrombus formation under flow in a collagen-coated surface – an effect highly dependent on CD36 signalling [80]. Platelet-bound oxLDL was increased in patients with acute coronary syndromes, while incubation of oxLDL with platelets from healthy individuals was able to enhance platelet adhesion to collagen, as well as activate endothelial cells [81]. Overall, it seems CD36 and collagen signalling pathways are intrinsically connected in the context of dyslipidemia and other metabolic diseases, prompting the discovery of novel treatments targeting CD36 and its pathway components.

In addition to the strong body of evidence showing how oxLDL induce platelet activation, platelets may induce LDL oxidation per se, prompting a positive feedback loop. For instance, supernatant collected from collagen-activated platelets was able to enhance copper-induced LDL oxidation [82]. More direct evidence showed that AA-stimulated but not resting platelets were able to oxidize LDL and HDL in a concentration-dependent manner. Moreover, the authors ascribed the oxidative stress induced by platelets to be due to secretory PLA_2 , produced by activated platelets, that would then induce adverse modifications in lipoproteins [83]. Another group proposed that activated platelets

oxidize LDL in an NADPH oxidase (Nox)2-dependent manner, by showing that oxLDL had no effect on platelet activation in Nox-2-deficient patients and that the addition of a Nox-2 inhibitor abrogated the production of oxLDL induced by activated platelets [84]. Finally, platelets contribute to the deleterious actions of oxLDL in other cells, such as monocytes, inducing oxLDL-dependant monocyte migration and thus contributing to vascular oxidative stress and atherosclerotic plaque instability [85]. Overall, it seems there is an intricate positive feedback loop in which platelets oxidize LDL that will then activate platelets in a continuum that also involves other blood cells and possibly the endothelium.

Besides increased oxLDL levels, patients with dyslipidemia and associated metabolic syndrome tend to produce more microparticles, derived from platelets (PMP), endothelial cells (EMP) as well as other blood components [86, 87]. Curiously, EMPs were found to bind to platelet surface CD36, since the inhibition of CD36 with monoclonal antibodies or the addition of competitive agonist oxLDL prevented EMP binding [88]. However, the majority of microparticles in blood is derived from platelets [89], which also induce a pro-thrombotic and pro-coagulant state due to PMP-induced platelet activation and the presence of negatively-charged phospholipids, like phosphatidylserine (PS) [86, 90, 91]. Hypothesizing that PS might be a ligand for CD36, Wang et al. [92] demonstrated that not only oxLDL leads to increased production of PMPs, but PMPs per se activate platelet through binding of CD36. Therefore, CD36 is a receptor for PMP binding and consequent pro-thrombotic outcome, while both contribute to the positive feedback installed in metabolic syndrome.

7.5.3 Native and Oxidized HDL in Platelet Function

Although LDL and its oxidized form oxLDL display pro-thrombotic effects through binding to CD36, HDL and oxidized HDL (oxHDL) have been shown to exert the opposite effect binding to

a similar receptor. Population studies have shown that HDL is inversely correlated to thrombosis [93], whereas this observation was corroborated *in vitro* by showing that HDL reduces platelet aggregation in response to a variety of agonists, such as collagen and ADP [71]. Although HDL was firstly described to bind to platelet through fibrinogen receptor $\alpha_{IIb}\beta_3$ [94, 95], later reports refuted these findings using more refined techniques [96], proposing scavenger receptor B1 (SR-B1) as the binding site of HDL in platelets [97]. It is curious to notice that the initial data on SR-B1 knockout mice showed increased platelet activation, with elevated P-selectin binding at resting and increased adherence to immobilized fibrinogen, due to dyslipidemia and platelet cholesterol overload [73]. However, in ApoE $^{-/-}$ dyslipidemic mice, bone marrow transfusion from SR-B1 $^{-/-}$ mice resulted in resistance to platelet hyperactivity as well as protected the pro-thrombotic phenotype present in these dyslipidemic animals [72]. It is now widely accepted that SR-B1 is the HDL receptor in platelets and that there is an inverse relationship between levels of this receptor and platelet response to ADP [98, 99].

Interestingly, oxHDL has been shown to inhibit platelet function more potently than native HDL [71]. Valiyaveetil [71] showed that oxHDL was able to inhibit platelet aggregation induced by collagen, ADP and thrombin – an effect that was dependent on platelet SR-B1. Another study using HDL from diabetic patients have found that glycoxidized HDL was also able to inhibit platelet aggregation induced by collagen [100]. However, others have shown that the oxidation of HDL by hypochlorite produces a different form of oxHDL that is seemingly more oxidized and able to potently activate platelets through binding to CD36 [101, 102]. Hence, it seems that the degree of HDL oxidation determines whether these lipoproteins will exert an anti- or pro-thrombotic effect [103]. It is still elusive which form of oxHDL is predominant in dyslipidemia and whether or not oxHDL functions as a negative loop for the already established oxLDL-platelet positive feedback system.

7.6 Dyslipidemias and Platelet Activation

There is evidence that distinct dyslipidemias (See Box 2 for dyslipidemias definition, classification and diagnosis) are associated with increased platelet activity. Hyperactive platelets with increased platelet cholesterol may contribute to accelerated atherogenesis associated with coronary artery disease. Plasma cholesterol levels appear to have a critical role in modulating platelet activity because hypercholesterolemia increases platelet activation (GPIIb/IIIa, P-selectin and phosphatidylserine expression), platelet FXa generation and platelet tissue factor activity, more potently than hypertriglyceridemia. Thus, plasma membrane cholesterol accumulation in platelets could potentially alter the membrane structure and affect signaling via surface receptors [104–108]. As compared with 26 normal subjects, platelets from patients with the Type II hyperlipoproteinemia aggregated in response to 1/25 the mean concentration of epinephrine, one third the concentration of collagen, and one third the concentration of ADP. In contrast, platelets from type IV hyperlipoproteinemia showed normal sensitivity to ADP and collagen, and normal secretion. This suggests that platelet activation is associated with the thrombotic complications of type II hyperlipoproteinemia [6].

Low-density lipoprotein particles sensitize platelets via binding of apoB-100 to a receptor on the platelet membrane and via transfer of lipids to the platelet membrane. Oxidative modifications of LDL (oxLDL) generate a platelet-activating particle, and this interaction might contribute to the development of the atherosclerotic plaque. Preincubation of isolated platelets with oxLDL, but not with native LDL, resulted in enhanced platelet adhesion to collagen and activated endothelial cells. The oxLDL has been shown to promote platelet activation by interact with different scavenger receptors: class A scavenger receptor, CD36, lectin-like oxidized LDL receptor-1, scavenger receptor class B type I (SR-BI), Toll-like receptor 2

(TLR2), scavenger receptor that binds phosphatidylserine and oxidized lipoprotein/chemokine (C-X-C motif) ligand. Also, oxidized choline glycerophospholipids are markedly increased in plasma of hyperlipidemic mice and in plasma of subjects with low HDL level, and promote platelet activation and hyperreactivity [7, 81, 109, 110].

7.6.1 Dyslipidemias: Definition, Classification and Diagnosis

Dyslipidemias are a group of abnormalities in the lipid metabolism that leads to altered blood levels of triglycerides, lipoproteins or phospholipids. More frequently, dyslipidemias are associated with high levels of blood lipids, although an altered lipoprotein pattern is also important and not only the total lipids levels in blood. Dyslipidemias can be classified regarding their causes: Genetic (familial) or Acquired (secondary). Most of dyslipidemias are hyperlipidemic, and between them, most are caused by a combination of genetic polymorphism and dietary and lifestyle factors. An iconic example of this is the apolipoprotein *E2/E2* polymorphism, which is present in 1/200–1/500 frequency, but its dyslipidemia associated is present only in 1/5000, because it needs the association with certain lifestyles (sedentary, hypercaloric diets) and other disorders, such as obesity, diabetes and/or hypothyroidism. On another hand, only a 2% of hyperlipidemic disorders can be associated with identifiable mutations (as a strong genetic cause) and they are the most difficult to treat and with a high cardiovascular risk. Acquired dyslipidemias are hyperlipemic also, and they commonly associate with diabetes mellitus, hypothyroidism, nephrotic syndrome and use of drugs (thiazide diuretics, beta adrenergic blockers, glucocorticoids, etc.) [111].

Other important classification is based on the type of lipid and lipoprotein elevated in plasma, and it is applied to familial dyslipidemias (Table 7.1) [112]. A third classification (Table 7.2) can be made based on clinical phe-

Table 7.1 Frederickson classification of familial dyslipidemias

Dyslipidemia	Frederickson classification	Lipid elevated	Lipoprotein altered	Genetic cause
Familial hyperchylomicronemia	Type I	TG	Chylomicrons elevated	Autosomal recessive: <i>LPL</i> , <i>ApoC2</i> , <i>Apo A-V</i> , <i>LMF-1</i> or <i>GPIIIBP1</i> mutated
Familial hypercholesterolemia	Type IIA	TC	LDL elevated	Autosomal codominant in heterozygous form: <i>LDL receptor</i> , <i>ApoB</i> or <i>PCSK9</i> mutated in one allele. Homozygous form: two alleles mutated of these genes.
Familial combined Hyperlipoproteinemia	Type IIB	TC and TG	VLDL and LDL elevated	Polygenic
Dysbetalipoproteinemia	Type III	TC and TG	IDL elevated	Homozygous for <i>Apo E2/E2</i> Heterozygous for mutations in <i>APO E</i>
Primary hypertriglyceridemia	Type IV	TG	VLDL elevated	No determined
Mixed hypertriglyceridemia	Type V	TC and TG	VLDL and chylomicrons elevated	Polygenic

TG Triglycerides, TC Total Cholesterol

Table 7.2 Clinical classification of dyslipidemias

Dyslipidemia	Genetic	Secondary or associated to	Environmental factors associated
Hypercholesterolemia (High TC)	Familial hypercholesterolemia	Hypothyroidism	Diet rich in saturated fats and cholesterol
	Familial combined hypercholesterolemia	Cholestasis	Drugs: androgens, progestogens, anabolic
	Polygenic hypercholesterolemia	Nephrotic syndrome	
Hypertriglyceridemia (High TG)	Familial combined	Obesity	Diet rich in high glycemic index carbs
	Hypercholesterolemia	Diabetes mellitus	High alcohol consume
	Lipoprotein lipase deficiency	Renal insufficiency	Drugs: estrogens, beta blockers, diuretics
	Primary hypertriglyceridemia	Metabolic syndrome	
		Nephrotic syndrome	
HDL-C deficiency	Apo A deficiency	Obesity	Diet rich in saturated or trans fatty acids
	<i>ABCA1</i> mutations	Diabetes mellitus	Diet rich in high glycemic index carbs
			Sedentary lifestyle
			Smoking
		Drugs: beta blockers, diuretics	
Mixed hyperlipidemia (high TG and TC)	Polygenic	Hypothyroidism	Diet rich in saturated fats and cholesterol
		Cholestasis	Drugs: androgens, progestogens, anabolic
		Nephrotic syndrome	Diet rich in high glycemic index carbs
		Obesity	High alcohol consume
		Diabetes mellitus	Drugs: estrogens, beta blockers, diuretics
		Renal insufficiency	
		Metabolic syndrome	

TG Triglycerides, TC Total Cholesterol

notype independently of causes, and includes the most frequently dyslipidemia manifestation: the genetic and environmental factors combination. In general, this classification takes into account not only elevations in LDL, VLDL or Chylomicrons, but also HDL decrease, and some of them can be associated to other pathologies [113].

The diagnosis is based mainly on clinical tests for a lipid profile, searching for abnormal levels of triglycerides, LDLc (LDL cholesterol), VLDLc (VLDL cholesterol), or HDLc (HDL cholesterol). Usually, low levels of LDLc and/or triglycerides are not considered of clinical importance by themselves. A decrease below normal levels of LDLc and triglycerides are usually considered as secondary to other disorders as liver disease, malabsorption syndromes, hyperthyroidism, etc. On the other hand, high levels of HDLc as an abnormal condition, constitutes a very rare genetic disorder with not well elucidated cardiovascular risk. For these reasons, we will focus on hyperlipidemic disorders [114]. The only clinical manifestation that helps to think specifically in hyperlipidemia is the presence of xanthomas, but they are observed mainly in genetic dyslipidemias with low frequency. In these patients, hepatosplenomegaly, *lipemia retinalis* and family history of pancreatitis (when triglycerides are elevated) can be present. Independently of the dyslipidemic origin, of special concern in all of them is the cardiovascular risk associated, and its determination and management is one of the main goals in dyslipidemia treatment [114]. To evaluate the cardiovascular risk in a dyslipidemic patient is important to consider: clinical manifestations of atherosclerosis (coronary, cerebral or peripheral), age (>45 years in males, >55 in females), family background with atherosclerosis in young, smoking, hypertension, diabetes mellitus and HDLc <40 mg/dl. Of course, the blood levels of triglycerides and total cholesterol are important, but of special relevance for cardiovascular risk, are the LDLc levels. A large amount of evidence support

the idea that high levels of LDL in blood, in concert with an elevated oxidative stress in the organism, leads to a high risk to the development of atherosclerotic and atherotrombotic events [115].

7.7 Closing Remarks and Perspectives

Platelets are central workers in the cardiovascular function and hemodynamics, acting as watchers of changes within the bloodstream. As shown in this chapter, lipids play an essential role on platelet metabolism and reactivity, modulating its response in accordance to the serum lipid profile. Although the focus has been placed on the circulating levels of distinct lipoproteins, with LDL particles augmenting and HDL particles decreasing platelet reactivity (Fig. 7.4), the immunological essence of platelet lipid metabolism increases its importance in disorders characterized by low-grade inflammation. Of importance, metabolic syndrome allies pro-thrombotic and pro-inflammatory statuses with dyslipidemia, whereas constitutes itself an independent risk factor for cardiovascular disease [116, 117]. We have previously revised the relationship between platelet hyperactivity and metabolic syndrome, suggesting the redox-sensing protein disulfide isomerase as a central actor [118]. Notwithstanding, the role of lipids in platelet biology has been recently widened with the application of lipidomic approaches [119]. A study just released by Peng, Geue [120] showed how changes in the platelet lipidome may modulate platelet function, uncovering novel therapeutic targets and agents. On this matter, the NO₂-AA might be additionally proposed as a potential target/agent in metabolic syndrome-derived platelet hyperactivity, since its formation depends on both redox and inflammatory processes. Thus, the tale of lipid role on platelet function and dysfunction seems to be an open novel with many interesting chapters to be written.

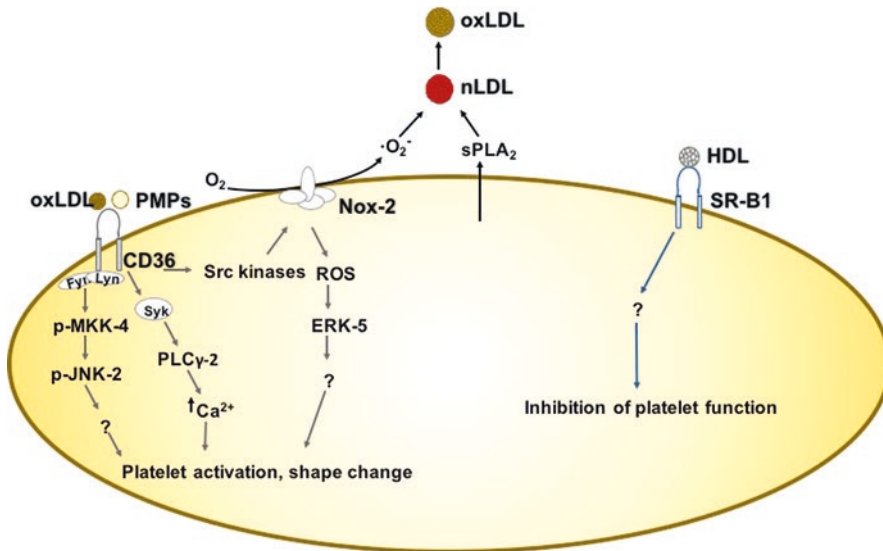


Fig. 7.4 How lipoproteins affect platelet function. Oxidized low-density lipoprotein (oxLDL) or platelet-derived microparticles (PMPs) bind to CD36 on platelets, generating three concomitant responses. On the left, active fyn and lyn will activate mitogen-activated protein kinase kinase 4 (MKK-4) and downstream c-Jun N-terminal kinase (JNK-2). Upon activation, CD36 will also stimulate platelets through a Src kinase-dependent activation of Syk and downstream phospholipase C (PLC γ -2), which will then increase intracellular Ca²⁺. The third signaling pathway of CD36 involves activation of

NADPH oxidase (Nox-2), which will generate reactive oxygen species (ROS) that will act as second messenger, inducing extracellular-signal-regulated kinase (ERK-5) phosphorylation. All of the CD36 pathways culminate in platelet activation, which produces oxidant molecules on the extracellular environment that will oxidize native LDL (nLDL) into oxLDL and perpetrate the positive feedback loop. To counter balance, high-density lipoprotein (HDL) binds to its own receptor, scavenger receptor B1 (SR-B1) to inhibit platelet function. The mechanisms of such inhibition are still poorly understood

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