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Abstract.
The oxygenation metabolism of arachidonic acid (ArA) has been early described in blood platelets, in particular with its conversion into the potent labile thromboxane A_2 that induces platelet aggregation and vascular smooth muscle cells contraction. In addition, the primary prostaglandins D_2 and E_2 have been mainly reported as inhibitors of platelet function. The platelet 12-lipoxygenase (12-LOX) product, i.e. the hydroperoxide 12-HpETE, appears to stimulate platelet ArA metabolism at the level of its release from membrane phospholipids through phospholipase A_2 ($cPLA_2$) and cyclooxygenase (COX-1) activities, the first enzymes in prostanoid production cascade. Also, 12-HpETE may regulate the oxygenation of other polyunsaturated fatty acids (PUFA) by platelets, especially that of eicosapentaenoic acid (EPA). On the other hand, the reduced product of 12-HpETE, 12-HETE, is able to antagonize TxA_2 action. This is even more obvious for the 12-LOX end-products from docosahexaenoic acid (DHA), 11- and 14-HDoHE. In addition, 12-HpETE plays a key role in platelet oxidative stress as observed in pathophysiological conditions, but may be regulated by DHA with a bimodal way according to its concentration. Other oxygenated products of PUFA, especially omega-3 PUFA, produced outside platelets may affect platelet functions as well.

Highlights.
- The arachidonic acid oxygenated product, thromboxane A_2, is central in blood platelet function.
- Thromboxane A_2 production is stimulated by 12-HpETE, the hydroperoxide product from arachidonic acid through platelet 12-lipoxygenase.
- 12-HpETE stimulates the platelet oxygenation of other polyunsaturated fatty acids as well.
- 12-HETE, the reduced product of 12-HpETE, as well as its homolog 14-HDoHE from DHA, antagonize TxA_2-induced platelet aggregation.
- w-3 FAs (especially DHA) ingested at low dosage lower the peroxide tone in oxidative stress-linked pathophysiological states.

Keywords: Platelet aggregation Aging Diabetes Peroxidation Eicosanoids Docosanoids Octadecanoids

Introduction.

Blood platelets, the second most numerous blood cells after erythrocytes, are required for the initial phase of hemostasis, as their aggregation leads to what is called white thrombus, to stop the bleeding before a more consolidated thrombus made with fibrin [1]. Beyond this physiological activity, a deficit of platelet aggregation is characteristic of a thrombocytopathia, such as in Glanzmann's thrombasthenia [2], whereas increased platelet aggregation has long been associated with thrombosis [3]. However, platelet-related thrombotic states are far more frequent than excess of bleeding. This means that increased platelet aggregation or activation in response to physiological agonists is relatively frequent, as involved in cardiovascular diseases.

The first polyunsaturated fatty acid (PUFA) to be associated with platelet functions is arachidonic acid (ArA) or 20:4n-6, with early reports on its conversion by platelet cyclooxygenase (COX) into prostaglandin (PG) endoperoxides, PGG_2/H_2 [4], previously called PGR_2 [5], to induce platelet aggregation. The requirement of this cyclooxygenase activity for normal platelet activation by specific agonists was then assessed in patients with hemostatic deficiency [6, 7]. In contrast, an increased prostaglandin endoperoxide formation in response to the platelet activator collagen in post-operative thrombosis has been reported [8].

Beyond these early reports on the crucial role of ArA oxygenated metabolism in platelet physiology and pathophysiology, other polyunsaturated fatty acids (PUFA), especially from the omega-3 family, have been considered to affect platelet function. A recent review [9] gives an overall view on possible/putative activities of all the known PUFA oxygenated products on platelet function. However, only few of the oxylipins reported to act on platelets are produced by them. The current review aims to focus on the functional relationship between PUFA oxygenation and oxidative stress within platelets.

Oxygenation of ArA in human blood platelets.

As mentioned in the introduction, the specific oxygenation of ArA by COX has been first reported more than 40 years ago. Platelets being anucleated cells, this occurs through the constitutive isoform COX-1. Once ArA is oxygenated into PGG_2, the 15-hydroperoxide of this resulting prostaglandin endoperoxide is reduced into PGH_2, the 15-hydroxylated form, by the peroxidase activity associated with the COX-1 protein. A specificity of platelets is to further convert PGH_2 into thromboxane (Tx) A_2 by the enzyme discovered in platelets and named thromboxane synthase [10]. This quite unstable derivative (half-life estimated of 30 s) is a potent activator of platelet aggregation, as well as a vaso-constricting agent [reviewed in 11], and it is assumed to be the active product of ArA to aggregate platelets. TxA_2 is quickly hydrolyzed into TxB_2, a stable and inactive metabolite which is used as a marker of the ArA oxygenation in blood platelets. However, TxB_2 is easily metabolized into 11-dehydro-TxB_2, by dehydrogenation of the secondary alcohol next to the oxane moiety, and further to the 15-oxo derivatives due to 15-PG-dehydrogenase, as well as to dinor- and tetranor- beta-oxidized products by peroxisomal beta-oxidation, like the other prostanoids [12].

In addition to production of TxB_2, a major metabolite, PGH_2 is converted into primary prostaglandins PGD_2, E_2 and $F_{2\alpha}$, and to 12-hydroxy-heptadeca-trienoic acid (12-HHT) and malondialdehyde (MDA) by cleavage of the pentagonal ring of PGH_2 [13]. Only primary prostaglandins are biologically active, with PGD_2 being a strong inhibitor of platelet aggregation through the activation of adenylyl cyclase, and PGE_2 having a bimodal effect on aggregation, according to its concentration, also through either adenylyl cyclase inhibition (low concentrations of PGE_2) or activation (high concentrations) [14]. The biological relevance of platelet $PGF_{2\alpha}$, has not been reported, except in stroke where it could act as a constrictor of cerebral arteries, together with TxA_2 and the released serotonin from platelet dense granules [15]. Although MDA is often used as a marker of global lipid peroxidation [16], it remains that it may also represent ArA cyclooxygenation. 12-HHT has long been considered as an inactive product, but more recent work reveals it may be a natural ligand for a leukotriene B_4 receptor subtype [17].

On the other hand, a specific platelet lipoxygenase, so-called 12-LOX, oxygenates ArA into its 12(S)-hydroperoxide derivative (12(S)-HpETE), further reduced into 12(S)-HETE by cytosolic glutathione peroxidase-1 (GPx-1), and represents a prominent pathway [18, 19]. This pathway plays an important role for the regulation of the peroxide tone in the cell (see further on), and oxygenates many more PUFA than COX.

Oxygenation of other PUFA than ArA in human blood platelets.

In addition to ArA as the reference PUFA and the most abundant in blood, two other C_{20} PUFA have been described as substrates of COX-1 in platelets. There are di-homo-gamma-linolenic (DGLA) or 20:3n-6, and eicosapentaenoic (EPA) or 20:5n-3 acids. DGLA is converted into PG_{1s} [20] and EPA into PG_{3s} [21]. But the proportions of the PGs from each of the three substrates are different. An important feature is the relative substrate specificity of thromboxane synthase for PGH_2. This leads to few TxB_1 and TxB_3 from DGLA and EPA, respectively, which facilitates diverting PGH_1 and PGH_3 towards primary prostanoids [21, 22].

Adrenic acid (AdA) or 22:4n-6 is known to be oxygenated as well through the COX-1/Tx synthase pathway to provide dihomo-TxB_2, but five times less than TxB_2 from the same concentrations of ArA. Also, AdA dose-dependently inhibits the conversion of ArA into TxB_2 and 12-HHT, suggesting a competition at the COX level [23].

In contrast to the COX pathway, the 12-LOX one does not show much specificity towards the three other PG precursors, making 12-OOH-20:3n-6, 12-OOH-20:5 and 14-OOH-22:4, respectively, further reduced into 12-OH-20:3n-6, 12-OH-20:5 and 14-OH-22:4 by GPx-1 [20, 22]. However, complex interactions may occur between the three substrates. As a matter of fact, EPA lipoxygenation is markedly stimulated by ArA through 12-HpETE [21, 24].

Beyond the COX substrates, some other C_{20} PUFA may be converted by the 12-LOX pathway. 20:3n-9, the marker of n-6 PUFA deficiency is a good substrate of platelet 12-LOX, as it just lacks the double bond at carbon 14 [25], and its end-product 12-OH-20:3n-9 may potentiate platelet aggregation at low concentrations, and inhibits it at high concentrations, as does PGE_2 [26]. This might be of biological relevance when 20:3n-9 accumulates in platelets in response to a saturated fat diet [27]. Another non-PG precursor eicosanoic acid, 20:4n-3, appears to be a good substrate of platelet 12-LOX, the product exerting bimodal effects on ArA oxygenation through the inhibition of COX-1 and stimulation of ArA lipoxygenation [28].

Docosanoic acids, especially docosahexaenoic acid (DHA) or 22:6n-3, have been considered as substrates of platelet 12-LOX. DHA has been first reported as being converted into two end-products, namely 11-OH-22:6 and 14-OH-22:6 [29]. The main metabolite, 14-OH-22:6, has been clearly shown of interest for being the most potent to antagonize the pro-aggregatory and vaso-constricting effects of TxA_2 on platelets and smooth muscle cells, respectively [30, 31]. In addition, it has been reported that some maresins from DHA (14S,22-diHDoHA (maresin-L1) and 14R,22-diHDoHA (maresin-L2)) are produced by both platelets and leukocytes, and partly responsible for reparative functions of these cells in wounds [32].

Docosapentaenoic acids, 22:5n-3 and 22:5n-6 are also good substrates of platelet 12-LOX, and the former behaves like DHA with its conversion into 11- and 14-OH derivatives, whereas 22:5n-6 is only converted into 14-OH-22:5 [33]. Both metabolites from 22:5n-3 were shown to inhibit the ArA-induced platelet aggregation [34]. Although 22:5n-3 is generally considered as a minor omega-3 PUFA in marine sources, compared to DHA and EPA, it may increase *in vivo* as a result of EPA elongation. However, 22:5n-3 accumulation in platelets, in response to marine PUFA intake, remains lower than that of DHA and EPA. Yet its biological relevance must be taken into consideration [35]. In addition, intravenous administration of n-3 DPA products or resolvin D1 (RvD1) led to a significant decreased plasma PGE_2 and TxB_2 [36], the TxB_2 reduction suggesting an effect on platelet ArA oxygenation through COX-1/Tx synthase.

Similarly, AdA was shown to be converted into 14-OH-22:4 through the platelet 12-LOX, but as shown for dihomo-TxB_2, the amount of 14-OH-22:4 produced was around five times lower than 12-HETE from ArA [23]. Figure 1 summarizes the main oxygenation of PUFA relevant to platelet function.

Octadecanoic acids, 18:2n-6 and 18:3n-3, have the potential to be oxygenated by dioxygenases, but their oxygenation by platelets is not clear. 18:2n-6 seems to be oxygenated by both platelet COX and LOX into 9-OH-18:2 (9-HODE) and 13-HODE, respectively, with a majority of 13-HODE [37, 38]. 13-HODE has been shown to inhibit platelet adhesion to endothelial cells [39, 40]. 18:3n-3 has not been described as a platelet dioxygenases substrate, although its position isomer 18:3n-6 (γ-linolenic acid) was historically used to suggest that human platelets have a lipoxygenase activity [41]. However, double lipoxygenation end-products (*via* soybean 15-LOX) from 18:3n-3 have been described as inhibitors of platelet aggregation through COX inhibition [42], and named linotrins [43] as they belong to the poxytrin family based on the report of the same platelet inhibition by protectin DX, the double lipoxygenation end-product of DHA [44].

Platelet receptors to oxygenated PUFA and transduction mechanisms.

The pro-aggregating ArA metabolite, TxA_2, acts through specific receptors coupled with the G-protein Gq. TxA_2 being a very short-lived molecule, its receptor has been studied using stable agonists such as U46619, in which the oxygen atom at carbon 9 of the 9,11-endoperoxide in PGH_2 is replaced by CH_2 [45]. By cloning, the human platelet receptor has been characterized as a seven trans-membrane (7TM) protein [46], coupled with Gq protein [47]. TxA_2-induced platelet aggregation then results from phosphoinositide-phospholipase C activation [48].

In contrast, PGD_2 is a potent inhibitor of platelet activation through another 7TM protein coupled with Gs protein that activates adenylyl cyclase [49], whereas PGE_2 exerts bimodal effects through Gs and Gi, depending on its concentration. Gs, associated with a rise of cyclic AMP in platelets, is coupled with the PGE_2 receptor subtype EP_2 which responds to high concentrations of the agonist, whereas Gi, leading to a decreased cyclic AMP level for potentiating platelet activation, is coupled with EP_3 in response to low concentrations of PGE_2 [reviewed in 50]. PGD_3, that is as potent as PGD_2 to inhibit platelet aggregation, seems to act through DP common receptors, further through activation of adenylyl cyclase [51].

Several limitations for PGD_2 and E_2 action could however be considered. First, *in vivo*, these PGs can be made available outside platelets for deactivation by 15-PG-dehydrogenase to produce 15-oxo-PGD_2/E_2 and further modifications [reviewed in 52]. Second, they can be esterified within platelet phospholipids, which may limit interactions with their specific receptors [53].

Although the lipoxygenase end-products are clearly inhibitors of TxA_2-induced platelet aggregation, especially the mono-hydroxy derivatives 12-HETE and 14-HDoHE, their interactions with TxA_2 receptor is only putative [54, 55]. However, the observation that the (R) stereoisomers appear more potent than the (S) ones, e.g. 13(R)-HODE *vs* 13(S)-HODE, 12(R)-HETE *vs* 12(S)-HETE, 12(R)-HHT *vs* 12(S)-HHT [47], 9(R),16(S)-diOH-18:3 *vs* 9(S),16(S)-diOH-18:3 [36], and 10(R),17(S)-diOH-22:6 *vs* the 10(S),17(S)-diOH isomer named PDX [44, 56], suggests that the 3D conformation of these hydroxylated derivatives might be important for antagonizing the TxA_2 receptor. As the (R) stereoisomers can be made by aspirin-treated COX-2 [57], this reinforces the protective potential of aspirin that also acts by inhibiting TxA_2 production at the platelet COX-1 level.

Production and action of oxygenated PUFA derivatives in oxidative stress situations.

In contrast to the inhibition of platelet aggregation by the lipoxygenase end-product 12-HETE, its hydroperoxide precursor increases platelet function. This is associated with the fact that 12-HpETE, is converted into 12-HETE by a peroxidase that requires the reduced form of glutathione (GPx-1) [58], and it is well-accepted that oxidative stress leads to conversion of reduced glutathione (GSH) into its oxidized form GS-SG [reviewed in 59], which slows down

the GPx-1 activity. Subsequently, an increased life span of endogenously formed 12-HpETE is expected [60]. Early report has shown that 12-HpETE stimulates its own formation [61], suggesting an overall increased metabolism of ArA in oxidative stress. Indeed, moderate concentrations of 12-HpETE added exogenously to human platelets activate the oxygenation of various PUFA by COX and LOX [62], and specifically stimulate COX activity [63]. The activation of platelet dioxygenases by 12-HpETE has clearly been involved in the oxygenation of EPA whereas it appears as a poor substrate in the absence of such an oxidative stress [21, 24].

Upstream to dioxygenases, the release of endogenous ArA by cytosolic phospholipase A_2 ($cPLA_2$) may be stimulated by oxidative stress, and 12-HpETE in particular. Such a stimulation, through stress kinase-induced phosphorylation, has been shown in response to hydrogen peroxide [64], and further to ~~in platelets by~~ 12-HpETE [65]. Figure 2 points out the pivotal role of 12-HpETE in TxA_2 generation which results in platelet aggregation. The activation of platelet $cPLA_2$ is not specific of these hydroperoxides ~~but~~ and may be observed in response to various oxidative stress conditions, as shown with highly reactive isoprostane aldehydes [66]. This suggests that a general oxidative stress may activate platelets through both the increased release of endogenous ArA from phospholipids and its oxygenation into pro-aggregatory prostanoids. Other mechanisms of action for increased platelet aggregation in oxidative stress might be involved as shown by adding hydroxyl-alkenals which derive from the cleavage of PUFA hydroperoxides from omega-6 and omega-3 fatty acids, such as 4-hydroxy-nonenal (4-HNE) and 4-hydroxy-hexenal (4-HHE), respectively. Those hydroxyl-alkenals are produced in higher amounts in oxidative stress situations, and make covalent adducts with platelet phosphatidylethanolamine (PE). These adducts at low concentrations are able to potentiate collagen-induced platelet aggregation [67]. Beyond these observations on platelets, we may hypothesize that the overall generation of endocannabinoids of the acyl-N-ethanolamine family would be blocked by such PE covalent modification, as acyl-N-PE are required intermediates in production of this kind of endocannabinoids [68].

All these data are of pathophysiological relevance in the vascular risk involving increased platelet activation. As a matter of fact, enhanced platelet function in type-2 diabetes may be attributed to an increased oxidative stress and thromboxane production [69]. This might involve oxidized and/or glycoxidized low-density lipoproteins (LDL) of those diabetic subjects [70]. Similarly, an increased ArA cascade has been reported in platelets incubated with LDL isolated from patients having a metabolic syndrome [71]. In addition, recent studies in humans have shown that the 12-LOX pathway generating 12-HETE, but not thromboxane generation, was associated with heparin-induced platelet activation during carotid endarterectomy [72]. This reinforces the notion that the 12-HETE significance might have been previously neglected [73].

On the opposite of increased platelet activation, moderate concentrations of long-chain omega-3 PUFA (EPA and DHA) may lower such activation, although these PUFA are usually considered as highly sensitive to peroxidation [74]. Indeed, low intake of EPA by elderly people, who show elevated oxidative stress, including in platelets, significantly decreased platelet aggregation and thromboxane production [75], likely through platelet vitamin E protection, resulting in its elevation [76]. This could be reproduced with normal platelets *in vitro* [77]. Using DHA instead of EPA *in vitro* allowed to demonstrate a bimodal effect of low and high concentrations on normal human platelets [78]. Most interesting was to reproduce this bimodal effect of DHA in healthy volunteers taking DHA. In particular, the urinary excretion of the main ArA-derived isoprostane 8-epi-$PGF_{2\alpha}$, a classical marker of the overall lipid peroxidation [79], was either decreased or increased in response to low or high DHA intake, respectively [80]. The mechanism of this bimodal effect according to DHA concentrations is not clear, except that it inversely correlated with platelet vitamin E content.

However, by measuring the 4-HHE concentration in plasma, a constant rise was observed in healthy volunteers ingesting increasing doses of DHA [81]. This putatively suggests that DHA might have been used as a trap for being oxidized, then sparing vitamin E, with an accelerated oxidation for the upper dosage of DHA resulting in vitamin E depletion and increased oxidative stress. Whatever the mechanism involved, the decreased oxidative stress by low intake of DHA is especially of interest in pathophysiological situations, such as type-2 diabetes in which platelet activation is associated with an increase of this stress. Further studies with diabetic subjects taking moderate daily DHA supplementation (400 mg) have provided results in agreement with those observed in healthy subjects [82]. This protective effect of moderate DHA supplement could also be observed in cystic fibrosis patients [83], where an oxidative stress has clearly been considered as an additional disorder [reviewed in 84]. Figure 3 schematizes the unbalanced platelet situation in presence of protective lipids and those which induce platelet activation.
The relationship between oxidative stress and platelet activation is obviously not limited to PUFA oxygenation. A typical example is given with the involvement of nitric oxide, well-known as a potent vasodilating agent [85], which has been more recently described as a possible accompanying agent of, and may be responsible for, platelet activation [86, 87].

Conclusion.
Based on studies related to the oxygenation of ArA as a reference PUFA, there is no doubt about the role of dioxygenase pathways in blood platelet activation. Other PUFA of nutritional interest such as EPA and DHA interfere with ArA oxygenation, either in competing with TxA_2 generation from ArA (EPA) and/or generating potent antagonists of TxA_2 at its receptor level (DHA). The platelet dioxygenases may also be regulated by the 12-LOX product of ArA, 12-HpETE, which appears as a key player in platelet oxidative stress. Its enhancing effect on platelet activation refers to the endogenous ArA cascade, including its release from phospholipids by $cPLA_2$ and the activation of COX-1. In contrast to the activating effects of 12-HpETE, EPA and DHA at low concentrations, both *in vitro* and *in vivo*, may oppose the oxidative stress, which is of relevance in pathophysiological situations such as aging, diabetes/metabolic syndrome and cystic fibrosis.

Acknowledgements.
The authors thank the financial support of Inserm and the Ministry of Research all along their investigations.

Figure legends

Figure 1. Summary of specific oxygenated products from several polyunsaturated fatty acids (PUFA) through platelet cyclooxygenase-1 (COX-1), with further conversion by thromboxane (Tx) synthase from some PUFA only, and 12-lipoxygenase (12-LOX). In the latter case, glutathione peroxidase-1 (GPx-1) is required to convert the 12-LOX products into their OH-derivatives (e.g. 11-, 12- and 14-OH-PUFA).

Figure 2. This figure shows resting platelets evolving to platelet aggregates, by pseudopod emission and subsequent aggregation each other, in response to the potent prostanoid thromboxane A_2 (TxA_2). 12-HpETE, the 12-lipoxygenase product from arachidonic acid (ArA), stimulates (+ above dotted purple arrows) the release of ArA from membrane ArA-containing phospholipids (PL-ArA) by phospholipase A_2 ($cPLA_2$), COX-1 and subsequently

Tx synthase leading to TxA_2 production. As a result, the increased (black arrow) TxA_2 leads to platelet aggregation (red arrow).

Figure 3. Imbalance between non-activated (blue) and activated (red) platelets in response to pivotal players. Low concentrations of the omega-3 fatty acids DHA and EPA (in blue) protect platelets from activation by lowering their peroxide tone. Also, 12-HETE and 14-HDoHE, the end-products of platelet 12-LOX from ArA and DHA antagonize the TxA_2-induced platelet aggregation. In contrast, high concentrations of DHA and EPA (in red) promote oxidative stress. In addition, 12-HpETE within platelets and oxidized LDL in their plasma environment, increase TxA_2 production which is the key player in platelet aggregation.

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	COX-1 + Tx synthase	12-LOX + GPx-1
20:3n-6	PG_{1S}	12-OH-20:3n-6
20:3n-9	none	12-OH-20:3n-9
20:4n-6	PG_{2S} + TxB_2	12-HETE
20:5n-3	PG_{3S}	12-OH-20:5
22:4n-6	di-homo-PG_{2S} + di-homo-TXB_2	14-OH-22:4
22:5n-6	none	14-OH-22:5n-6
22:5n-3	none	11- and 14-OH-22:5n-3
22:6n-3	none	11- and 14-OH-22:6

Figure 1

Figure 2

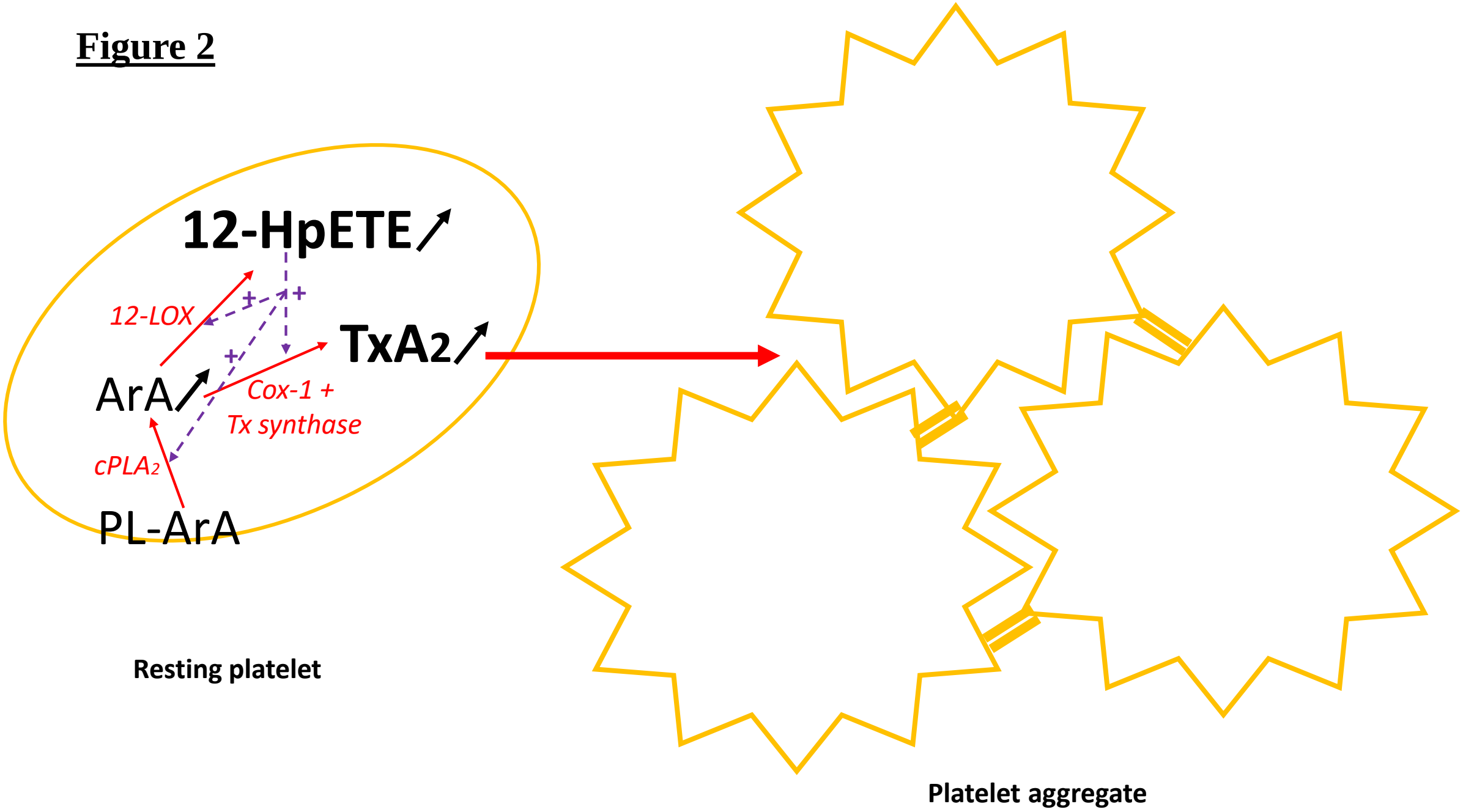
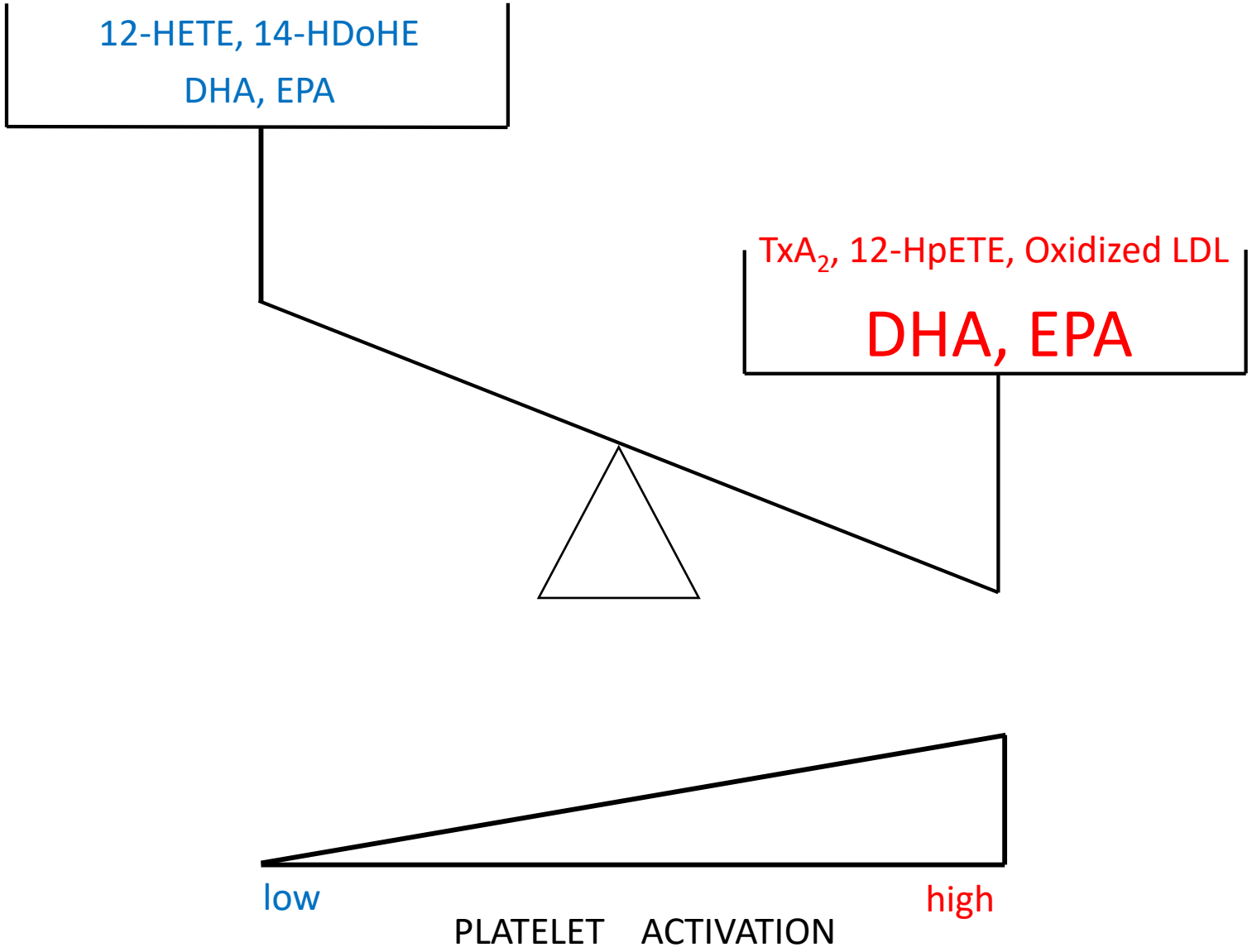

12-HpETE
12-LOX
TxA2
ArA
Cox-1 +
Tx synthase
cPLA2
PL-ArA
Resting platelet
Platelet aggregate

Figure 3


12-HETE, 14-HDoHE
DHA, EPA
TxA2, 12-HpETE, Oxidized LDL
DHA, EPA
low
high
PLATELET ACTIVATION