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Omega-3 polyunsaturated fatty acids and oxygenated metabolism in atherothrombosis

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Abstract

Numerous epidemiological studies and clinical trials have reported the health benefits of omega-3 polyunsaturated fatty acids (PUFA), including a lower risk of coronary heart diseases. This review mainly focuses on the effects of alpha-linolenic (ALA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids on some risk factors associated with atherothrombogenesis, including platelet activation, plasma lipid concentrations and oxidative modification of low-density lipoproteins (LDL). Special focus is given to the effects of marine PUFA on the formation of eicosanoids and docosanoids, and to the bioactive properties of some oxygenated metabolites of omega-3 PUFA produced by cyclooxygenases and lipoxygenases. The antioxidant effects of marine omega-3 PUFA at low concentrations and the pro-oxidant effects of DHA at high concentrations on the redox status of platelets and LDL are highlighted. Non enzymatic peroxidation end-products deriving from omega-3 PUFA such as hydroxy-hexenals, neuroketals and EPA-derived isoprostanes are also considered in relation to atherosclerosis.

1. Introduction

Atherothrombosis and its complications are a major cause of morbidity. Indeed, in 2010, one out of four deaths worldwide was due to ischemic heart diseases and stroke [1], and the prevalence could be growing. This pathophysiology is complex, involving many risk factors (such as inflammation, hyperlipidemia, diabetes mellitus, hypertension, smoking, age…) and interactions between vascular and blood cells. The relationship between coronary heart diseases and fatty acids has been known since the sixties when epidemiologic studies showed a strong association between the high incidence of such diseases and high intake of saturated fatty acids [2]. At the opposite, about a decade later, some evidence from studies of Bang and Dyerberg [3,4] indicated that n-3 or omega (ω)-3 polyunsaturated fatty acids (PUFA) consumption could protect against cardiovascular diseases (CVD). Numerous epidemiological and clinical trials have reported the cardioprotective effects of omega-3 PUFA [5-8]. However, other contemporary clinical trials show controversial data regarding the association of omega-3 PUFA and cardiovascular points [9,10,11,12,13,14]. Neutral results of recent intervention trials could be due to heterogeneity of patients (age of the patients, time enrollment in the clinical trial after myocardial infarctions, level of omega-3 PUFA erythrocyte at baseline: omega-3 index [15]) but also be due to differences in the omega-3 PUFA supplements (EPA, DHA alone or in combination, or fish oil), their dose and duration of the supplementation. In addition, increased optimal medical therapy (including statins) [14] could induce an improvement in cardioprotection. Thus, all these discrepancies could explain the differences between intervention trials and epidemiological studies since the Multi-Ethnic study published in 2013 [16] suggest that increased consumption of omega-3 PUFA may prevent CVD.

The omega-3 PUFA family is one of the biologically relevant fatty acid family which mainly includes the precursor alpha-linolenic acid (ALA; 18:3ω3), stearidonic acid (18:4ω3), and the long-chain eicosapentaenoic (EPA; 20:5ω3), and docosahexaenoic (DHA; 22:6ω3) acids. The other relevant family is the omega-6 one (Fig. 1) including linoleic acid (18:2ω6) and arachidonic acid (ARA, 20:4ω6).

To date, most studies concern the cardioprotective effects of ALA, EPA and DHA which would be the main focus of this review.

2. Non-marine omega-3 fatty acids and cardiovascular risk

Compared to long-chain PUFA, there is less compelling evidence that plant-derived ALA may reduce the cardiovascular risk although meta-analyses of dietary and biomarker studies concluded that increased consumption of ALA reduces the risk of fatal coronary heart disease [17] and confers cardiovascular benefits [18]. Dietary ALA may display anti-thrombotic effects, independently of its conversion to EPA, by indexinhibiting arterial thrombus formation and decreasing collagen- and thrombin-induced platelet aggregation [19]. Supplementation with ALA may also decrease some inflammatory markers in dyslipidemic patients [20], resulting in anti-inflammatory effects. Studies on the effects of dietary ALA on plasma lipid concentrations are contradictory. Altogether, dietary ALA had no effect, moderately decreased or even increased plasma triacylglycerols (TAG) concentrations [21]. No changes were generally observed in plasma, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) cholesterol although lipoproteins were enriched in ALA and EPA following ALA supplementation [22]. It should be added that ALA is poorly converted into longer chain PUFA and mainly partitioned toward beta-oxidation [23,24]. Recent evidence indicates that ALA is also metabolized into mono- and di-hydroxylated compounds by 15-/ω6-lipoxygenase, with the latter which might account for part of ALA anti-inflammatory and anti-thrombotic effects [25].

Besides ALA, a few studies focused on stearidonic acid (SDA, 18:4ω3). SDA is the direct product of ALA via the Δ6-desaturase action. Because Δ6-desaturase is a rate-limiting enzyme in humans [26], SDA could represent a sustainable alternative to ALA [27,28]. In vitro studies showed that the addition of SDA, pre-coated onto albumin to isolated human platelets inhibits agonist-induced aggregation to the same extent as EPA [29]. Human studies have shown that the consumption of SDA, provided as an ethyl ester, leads to an enrichment of plasma phospholipids, red blood cells [30] and peripheral blood mononuclear cells [31] into EPA and DPA, but not into DHA. Neither SDA nor 8,11,14,17-eicosatetraenoic acid (ETA, 20:4ω3) increased in response to dietary SDA. Although SDA did not significantly modify plasma cholesterol concentrations in healthy subjects [32,33], dietary echium oil (containing 14% SDA as total fatty acids) decreased plasma TAG in

hypertriglyceridemic patients [30]. Further studies are required to determine the effects of SDA on other cardiovascular risk factors and to uncover oxygenated derivatives of SDA putatively displaying biological activities.

Much less is known on the putative effects of ETA, an intermediate fatty acid between SDA and EPA. It has been described to be as potent as EPA in inhibiting prostaglandin H synthase (PGHS) activity [34].

3. Marine PUFA and some factors associated with atherothrombosis

3.1 Marine PUFA and plasma lipids

Hypertriglyceridemia is recognized as a risk factor for cardiovascular disease. There is strong evidence that the administration of high doses (2-4 g/day) of omega-3 PUFA (mainly EPA and DHA) to hypertriglyceridemic patients leads to a significant reduction of plasma TAG [35,36], notably in chylomicrons and very low-density lipoproteins (VLDL) particles, thus improving cardiovascular health. The hypo-triglyceridemic effects of omega-3 PUFA are dependent on the background dietary fat intake, the dose administered, and the baseline plasma TAG concentrations [37]. Regarding the mechanism of action, the effects are partly due to a decreased hepatic synthesis of TAG and to an increased clearance of chylomicrons [38,39] and VLDL from the circulation. Prescription of omega-3 PUFA in combination with cardiovascular drugs has also to be taken into account since adding statins to omega-3 PUFA may lower TAG levels to a greater extent than statins alone in hypertriglyceridemic patients [40]. Administration of fish oils to hypertensive patients treated with an angiotensin converting enzyme (ACE) inhibitor may further decrease plasma TAG [41].

Considering plasma total cholesterol levels, fish oils or omega-3 PUFA concentrates have generally no significant effect on total cholesterol. Ingestion of fish oils or DHA may lead to very modest increases in LDL and HDL cholesterol which are likely to be related to lower TAG concentrations and/or genetic factors [42]. Some studies showed indeed that supplementation with fish oils may raise LDL cholesterol and total cholesterol concentrations in individuals with an apoE4 allele [43,44]. In

addition, some studies showed indeed that supplementations wiHowever, some studies showed that omega-3 PUFA may impact cholesterol levels in lipoprotein subclasses. Supplementation with DHA [45] or omega-3 PUFA [46] may increase LDL particle size and consequently large LDL particles rather than small and dense LDL ones, shifting the lipoprotein profile to a less atherogenic situation [47]. The intake of fish oils [48] or high doses of DHA (4g/d for 6 weeks), but not of EPA [45], may result in increased concentrations of HDL cholesterol, especially HDL2. The mechanism of action of DHA could be related to decreased lipid transfer protein activity [49].

3.2 Effects of marine PUFA on platelet function

Numerous studies have been conducted with EPA and DHA because these fatty acids are present in the diet of Greenland Inuits (mainly in the form of marine oils and seafood) [50], and found in their plasma lipids [51]. The role of platelets in hemostasis and thrombosis is known for a long time and well defined but more recently, a new concept has emerged stating that platelets play a central role in the atherothrombotic process [52,53]. Because of the pivotal role of platelets, some studies focused on the effect of omega-3 long chain PUFA on platelet function. The first one was reported by Dyerberg and Bang [54] showing that Inuits had attenuated platelet reactivity. Since then, platelet aggregation was found to be diminished [55-59] after omega-3 fatty acids intake and even after ingestion of low doses of omega-3 PUFA [60,61]. The heterogeneity of observed results [62] is probably due to great variability of numerous factors including target populations, quantities of omega-3 PUFA, sources of these fatty acids, duration of supplementation. However, a meta-analysis conducted by Gao *et al*. [63] has demonstrated that omega-3 PUFA are associated with a significant reduction of platelet aggregation. Considering that most experimental feeding studies have been performed with an EPA and DHA mixture, it is difficult to determine the own effect of each PUFA. Pure sources of EPA, DPA or DHA provide more information. After supplementation with EPA, von Schacky and Weber [7] found a decreased platelet reactivity and an increased docosapentaenoic acid (DPA; 22:5ω3), an intermediate between EPA and DHA (Fig. 1). In addition, low intake of EPA may reduce platelet aggregation, without changing the fatty acid

platelet composition [64,65]. In the same way, following supplementation with DHA [7], platelet function was reduced and a retroconversion of DHA in DPA and EPA was evidenced. Such a retroconversion occurred with normal dietary amounts of DHA, and the rate, calculated by Brossard *et al.* [66] after ingestion of trace amounts of [^{13}C]DHA-TAG, was relatively low (1.4%). More recently, we performed a dose-response study with middle-aged healthy volunteers ingesting increasing amounts of DHA (200, 400, 800 and 1600 mg/day) for two weeks each dosage. We found that DHA supplementation led to a dose-dependent increase of DHA in platelet phospholipids, an increase of EPA after 1600 mg DHA/day while DPA decreased [67], in agreement with results observed by Conquer and Holub [68]. In addition, platelet reactivity was decreased after 400 and 800 mg DHA/day [67]. They are only a limited number of studies with DPA suggesting it might be more potent than EPA and DHA to inhibit platelet aggregation [69]. Thus, the relationship between EPA, DPA and DHA in the inhibition of platelet function is not clear. It is difficult to precisely attribute the effects to each PUFA, and their beneficial and complementary effects could well be linked to formation or diminution of specific eicosanoids and/or docosanoids.

4. Marine PUFA and enzymatic oxidized products

4.1 Effects of marine PUFA on oxygenated metabolites (eicosanoids and docosanoids)

The beneficial effects of marine oils have been largely discussed in terms of effects on arachidonic acid (ARA) oxygenated metabolites. Dietary omega-3 PUFA compete with ARA for acylation into cellular phospholipids [57] and one of the proposed explanations for the prevention of atherosclerosis by omega-3 PUFA may be due to changes in eicosanoid formation. Eicosanoids are signaling lipid metabolites from 20-carbon fatty acids, including prostaglandins, prostacyclin, leukotrienes, hydroxy-derivatives and epoxy-derivatives. When platelet activation occurs, ARA is released from phospholipids and oxygenated to give pro-aggregatory prostanoids, mainly thromboxane A_2 (TxA_2). ARA is also oxygenated by 12-lipoxygenase to provide 12-hydroperoxyeicosatetraenoic acid (12-HpETE) [70] (Fig. 2). In presence of EPA, the ARA-derived eicosanoids shift to EPA-derived metabolites [71,72] which are less

vaso-constrictive and have less potent effect on platelet aggregation [73], which leads to overall antithrombotic activities. Indeed, TxA_2, formed by PGH synthase (COX-1)/Tx synthase from ARA, is a pro-aggregatory and vaso-constricting substance [64] while TxA_3, formed from EPA, is weakly pro-aggregatory [72]. Also, the conversion of EPA to TxA_3 is much lower than ARA to TxA_2 [75]. In addition, incorporation of EPA and DHA to membrane phospholipids leads to an inhibition of TxA_2/PGH_2 receptor binding as shown by the reduction of platelet sensitivity to a thromboxane receptor agonist [76]. Accordingly, we showed that supplementation with low doses of EPA and DHA, or DHA alone, decreased platelet basal TxB_2 concentration [60,64,67].

For more than 50 years, aspirin is used as an antiplatelet medication. The acetylation of Ser 529 in human COX-1 by aspirin [77] inhibits the production of TxA_2. Some studies have reported the action of aspirin in combination with omega-3 PUFA. In healthy subjects, omega-3 PUFA can enhance the anti-thrombotic effects of aspirin [78]. Moreover, adding omega-3 PUFA may improve the response to aspirin in aspirin-resistant patients [79]. In the same way, the administration of omega-3 PUFA in combination with aspirin and clopidogrel (an anti-aggregatory drug) decrease platelet aggregation in patients after percutaneous coronary intervention (The OMEGA-PCI) [80].

Omega-3 PUFA may also modify the blood levels of several coagulation factors but the results are conflicting [81]. However in the OMEGA-PCI intervention, the authors have shown that adding omega-3 PUFA to aspirin and clopidogrel prescription in coronary artery disease patients decreases thrombin formation and favorably alters fibrin clot properties. Their findings indicate novel antithrombotic effects induced by omega-3 PUFA [82].

There is some evidence that DPA also inhibits the cyclooxygenase activity but enhances the formation of 12-hydroxyeicosatetraenoic acid (12-HETE) [83]. Moreover, anti-thrombotic effects of these PUFA might also be endothelium-dependent. The 3-series prostacyclin (Fig. 2), synthesized from EPA by endothelial cells, had the same anti-aggregatory effects as the 2-series prostacyclin from ARA [84]. DPA has been shown to inhibit prostacyclin formation but its effect may be attributed to retroconversion of DPA to EPA [85].

In addition, cytochrome P450-dependent metabolites of EPA and DHA may also function as mediators of the vaso-dilatory and cardio-protective effects of omega-3 PUFA [86]. Dietary EPA/DHA supplementation results in a tissue-specific accumulation of epoxides derived from EPA and DHA, that are 17,18-epoxy-eicosatetraenoic and 19,20-epoxy-docosapentaenoic acids. These omega-3-derived epoxides display anti-arrhythmic actions at nanomolar concentrations [87].

As with ARA, EPA and DHA are substrates of lipoxygenases (LOX). Numerous end-products mono-, di-and tri-hydroxy derivatives can be formed. Such oxygenated lipid mediators can be produced enzymatically or non-enzymatically.

4.2 Effect of enzymatic-derived lipid mediators from omega-3 fatty acids

The role of chronic inflammation in atherosclerosis which involves leukocytes infiltration in the arterial intima is well established and its resolution offers novel approaches. Lipoxygenase pathways play an important role since they are involved in the recruitment of leukocytes and also in the formation of lipid mediators deriving from omega-6 and omega-3 PUFA [88].

Long chain omega-3 PUFA such as EPA and DHA are also precursors of potent anti-inflammatory compounds. Among them, resolvin E1 (RvE1) from EPA [89,90] exhibits an inhibition of neutrophil activation associated with a decrease of their adhesion to the endothelium as well as a decrease of oxidative stress as assessed by lower ROS formation. Interestingly, its formation is enhanced by aspirin [91] which is also known to inhibit cyclooxygenases. RvE1 is an inhibitor of both ADP-induced and thromboxane receptor agonist U46619-stimulated aggregation in platelet-rich plasma in a concentration-dependent manner [92].

EPA can also be metabolized by cyclooxygenase into PGH_3 which is then converted to the alternative 3-series PGs, PGD_3, PGI_3, PGE_3 and $PGF_{3\alpha}$ [93-95]. PGD_3 is also converted to the cyclopentenone products, 15d-PGD_3, PGJ_3, and 15d-PGJ_3 [96,97] (Fig. 3). Prostanoids derived from EPA have less inflammatory activities compared with those produced from ARA [98-100]. PGD_3 was described as a potential circulating antithrombotic agent [98]. PGD_3 also antagonizes the migration of neutrophils across endothelial cells mediated by PGD_2 [101]. PGE_3 is less pro-inflammatory than PGE_2 [102]. 15d-PGJ_3 has been shown to increase the secretion

of adiponectin (a plasma protein adipocytokine showing anti-atherogenic, insulin-sensitizing, and anti-inflammatory properties) by adipocytes partly via a peroxisome proliferator-activated receptor-gamma-dependent mechanism [97].

EPA and DHA are substrates of platelet 12-lipoxygenase to produce monohydroxy-derivatives including 12 hydroxy-eicosapentaenoic acid (12-HEPE) and 14-hydroxy-docosahexaenoic acid (14-HDoHE), which inhibit thromboxane-induced platelet aggregation and aortic contraction [103] and may antagonize the thromboxane action by interfering with their receptor sites [104]. DHA also leads to potent anti-inflammatory resolvin D1 (RvD1) and D2 (RvD2). RvD1 enhances both nitric oxide and prostacyclin production in endothelial cells, decreases adhesion receptors and the formation of ROS and pro-inflammatory cytokines. RvD2 is mainly produced by dendritic cells. It inhibits the PMN infiltration into inflamed tissues, decreases PMN activation and promotes phagocytosis and clearance of apoptotic cells. On the other hand, DHA is also converted into protectin D1 (PD1) which is a potent anti-inflammatory agent. PD1 exerts potent agonist actions on macrophages and vascular endothelial cells that can control the magnitude of the local inflammatory response involved in atherosclerosis [105]. However, PD1 had no effect on agonist-induced platelet aggregation at the tested concentrations (1nM to 100nM) [92]. Moreover, protectin DX (PDX), a stereo and geometric isomer of PD1, produced from DHA via a dioxygenation mechanism by 15-LOX [106], exhibits anti-aggregatory properties. PDX inhibits platelet aggregation induced by collagen, ARA and U-46619 at concentrations as low as 300nM. PDX inhibits cyclooxygenase-1 (COX-1) and also competes with the receptor of thromboxane A_2 [107]. These properties are linked to the E,Z,E configuration of the conjugated double bonds/triene motif [107]. PDX also exhibits anti-inflammatory properties in reducing the production of ROS by inhibiting NADPH oxidase and cyclooxygenase-2 (COX-2) activities [108]. It is noteworthy to state that the weak structural difference between these geometric and stereo-isomers (PD1 & PDX) has important consequences on their biological activities, as it has recently been pointed out [109]. Other PUFA can also be converted by 15-LOX into dihydroxylated fatty acids having the same E,Z,E motif (Fig. 4), collectively named poxytrins and sharing the anti-aggregatory properties. Recent investigations reveal that poxytrins synthesized from ALA, called linotrins (Fig. 4), display anti-aggregatory

properties as well, and one of them inhibits the 5-lipoxygenase activity in human neutrophils [25].

Another metabolite derived from DHA has been recently discovered in activated macrophages which converts DHA into 14S,21-diHDHA and 14R,21-diHDHA via 12-lipoxygenase and cytochrome P450 oxygenase. These metabolites promote wound healing and microvasculature formation and may partially represent the molecular mechanisms for macrophage pro-healing function [110].

Besides this enzymatic peroxidation giving bioactive oxygenated metabolites, PUFA are obviously susceptible to the non-enzymatic peroxidation.

5. Marine PUFA and non enzymatic peroxidation

5.1 Effects of omega-3 PUFA upon non enzymatic lipid peroxidation

An excess of ROS, resulting from either their overproduction and/or insufficient activity of antioxidant defense systems, disturbs the physiological balance between pro- and anti-oxidant systems leading to injurious consequences. Lipid peroxides by-products namely malondialdehyde (MDA), 4-hydroxy-alkenals, isoketals/neuroketals and isoprostanes/neuroprostanes are formed and are implicated in the etiology of a number of diseases, including cardiovascular ones [111]. Amongst PUFA, marine PUFA are highly oxidizable owing to the presence of 5 or 6 double bonds in the fatty acyl chain. Clinical studies using large doses of omega-3 PUFA indeed reported an increased lipid peroxidation in plasma [112,113] and lipoproteins [114,115] Moreover, *in vitro*, we have found that large amounts of EPA and/or DHA induce lipid peroxidation in platelets [116,117] whereas low DHA concentrations decrease it. Our results clearly evidenced a bimodal effect of DHA with antioxidant and pro-oxidant effects at low and high concentrations, respectively [118]. *Ex vivo*, we first showed that the ingestion of 100 mg EPA per day for two months by elderly people increased platelet vitamin E concentration while decreasing thrombin-induced platelet aggregation [65]. We next reported that the intake of 150 mg EPA and 30 mg DHA for 6 weeks resulted in increased vitamin E and decreased MDA concentrations in platelets from elderly people [60]. More recently, we found that supplementation of healthy men with 200 mg DHA per day for two weeks increased significantly platelet

vitamin E while urinary isoprostane (IsoP) formation decreased [67]. At the opposite, the supplementation of these same healthy men with 1600 mg per day for two weeks increased urinary IsoP formation [67]. In addition, we showed that supplementation with doses of DHA ranging from 200 to 800 mg DHA/day increased alpha-tocopherol concentrations, decreased MDA concentrations and lowered the susceptibility of LDL to oxidation [119]. In addition, 4-hydroxy-hexenal (4-HHE), a major aldehyde product of omega-3 fatty acids, was increased following supplementation with 800 and 1600 mg DHA/day while 4-hydroxy-nonenal (4-HNE) concentrations did not vary throughout the DHA supplementation periods. The antioxidant effects of DHA might have been due to its concomitant peroxidation, reflected by the production of 4-HHE. DHA might be a target for free radicals and then might spare the endogenous vitamin E at low supplementation levels. Once oxidation reached a threshold level, the free radical chain reaction of lipid peroxidation might have exceeded the protection supplied by DHA, leading to pro-oxidant effects. In agreement with our results on 4-HHE, Ishikado *et al.* [120] recently reported that supplementation with dietary fish oil led to increased HHE aortic concentrations and increased the expression of the antioxidant enzyme heme oxygenase-1, and endothelium-dependent vasodilatation. As reviewed very recently [121], potential molecular mechanisms to explain the antioxidant effects of omega-3 fatty acids would be the quenching of ROS as well as direct inhibition of NADPH oxidase 4 (Nox-4) by DHA [122]. Altogether, our results obtained in healthy subjects [67,119,123] reinforce the antioxidant potential of low omega-3 PUFA intake as originally described in elderly subjects displaying increased oxidative stress [60,65] and in dyslipidemic diabetic patients [124].

5.2 Effect of non-enzymatic lipid mediators deriving from omega-3 fatty acids

Actives aldehydes, including 4-HHE (Fig. 5), can readily react with free primary amine and thiol groups, such as histidine and lysine, and cysteine residues, to generate Schiff base and Michael adducts [125-128]. They are being considered as possible causal agents of different diseases such as chronic inflammation and atherogenesis. Moreover, 4-oxo-nonenal (Fig. 5) which is also a major product of lipid peroxidation [129,130] is more reactive than 4-HNE towards amino acid residues [131]. Similarly, 4-oxo-hexenal (Fig. 5) is more reactive than 4-HHE. Hydroxy-

alkenals interact with LDL lysine residues of apoB [132,133]. They are recognized by scavenger receptors [134,135] through specific epitopes [136]. Such oxidized LDL are toxic toward endothelial cells by inhibiting their proliferation. 4-HHE, 4-HNE and 4-hydroxy-dodecadienal (4-HDDE) can also form adducts with phosphatidylethanolamine [137-139], and 4-HHE-phosphatidylethanolamine adducts were the main ones detected in retina of streptozotocin-induced diabetic rats [139]. Also, hydroxy-alkenal adducts are present in atherosclerotic lesions [136,140,141]. Monocyte/macrophage CD36 has been shown to play a critical role in the development of atherosclerotic lesions by its capacity to bind and endocytosize oxidized low-density lipoproteins (OxLDL), and is implicated in the formation of foam cells [142,143].

Neuroketals (NKs) are isoketal (IsoK)-like compounds formed via the neuroprostane pathway of DHA peroxidation [144-146] (Fig. 5). Five docosahexaenoyl radicals are initially formed to generate eight D4-NK and eight E4-NK regioisomers. Each regioisomer is theoretically composed of eight racemic diastereoisomers for a total of 128 D4-NKs and 128 E4-NKs. NK isomers are designated by the location of the hydroxyl group. The designation “D” and “E” is a carryover from the established prostaglandin nomenclature for PGD and PGE and levuglandins E and D to indicate the location of the ketone moiety. When the keto/oxo group is located in the higher numbered carbon relative to the aldehyde, NK is designed D4 and when the ketone moiety is located in the lower numbered carbon, NKs is designed E4.

These γ–ketoaldehydes are some of the most reactive products of lipid peroxidation that covalently modify the lysine residues of proteins at a rate that far exceeds that of 4-HNE [144]. NKs initially form a reversible Schiff base adduct, which then proceeds through a pyrrole to stable lactam and hydroxylactam adducts. Increased levels of NKs-protein adducts are associated with oxidant injury. Moreover, NKs exhibit a remarkable proclivity to form protein cross-links. These γ–ketoaldehydes also covalently modify aminophospholipids, forming pyrrole and Schiff base adducts with phosphatidylethanolamines [147-150]. Under oxidizing conditions, the pyrrole adduct generally evolves to form NK-phosphatidylethanolamine lactam and hydroxylactam adducts.

Because of their capacity to covalently modify proteins, phospholipids and DNA, reactive aldehydes derived from lipid peroxidation have been suggested to be key mediators of oxidant injury including atherosclerotic cardiovascular diseases. IsoKs have been shown to potentiate aggregation of human platelets via p38 MAP kinase, a phenomenon that plays an important role in the pathogenesis of atherosclerosis and its thrombotic complications [151]. IsoKs-protein adducts have also been detected in oxidized LDL [144] and the levels of these adducts increase in blood plasma from patients with atherosclerosis compared with healthy volunteers [152]. Relevant to these pathological states is the activation of the endoplasmic reticulum stress signaling pathways and endothelial activation by IsoKs-phosphatidylethanolamine adducts [147], as well as studies showing that IsoKs facilitate mitochondrial calcium dysfunction [153]. It is reasonable to speculate that NKs would have the same effects.

EPA- and DHA-derived IsoPs could also contribute to the beneficial biological effects of fish oil supplementation. For example, the EPA-derived IsoP, 15-F3t-IsoP, unlike 15-F2t-IsoP, does not increase human platelet shape change or aggregation [154]. Moreover, IsoPs generated from the oxidation of EPA are more abundant than those of F2-IsoPs generated from ARA, perhaps because EPA contains more double bonds and is therefore more easily oxidizable [155]. In addition, EPA supplementation markedly reduced levels of arachidonate-derived F2-IsoPs in mouse heart tissues. Such data are especially relevant since F2-IsoPs are generally considered to be pro-inflammatory molecules. Supplementation with EPA decreases F2-IsoP generation and should prevent some diseases associated with increased levels of F2-IsoPs [156].

Recently, targeted lipidomics analyses revealed that both the profiles of EPA and DHA and their corresponding oxygenated metabolites were substantially modulated in plasma and liver. The hepatic F4-neuroprostanes (NPs) level was strongly correlated with the hepatic DHA level. Among all measured metabolites, hepatic F4-NPs were mostly negatively correlated with the plaque extent, indicating that F4-NPs are important positive predictors of atherosclerosis prevention [157,158].

6. Specific analytical issues

Lipid mediators, generally present in fluids in small amount, are usually extracted by solid phase extraction on C18 or anionic and C18 cartridges. This procedure can be applied directly to biological fluids, and blood cells after their lysis.

Lipid mediators such as mono-, di- and tri-hydroxylated fatty acids and prostaglandins can be measured together by LC-MS/MS using ESI MRM mode [159-161]. The specificity is achieved by their specific retention times and their daughter ions. Their corresponding deuterated internal standards are used for quantification.

The position of the hydroxyl group on the chain may be determined by GC-MS analysis after hydrogenation and derivatization into methyl esters and trimethysilylethers. Such a derivatization allows a specific cleavage to both sides on the carbon which carries the hydroxyl group.

It is of interest to determine the stereochemical configuration of the hydroxy metabolites in order to determine whether the products originate from an enzymatic or non-enzymatic lipid peroxidation. The configuration of the hydroxyl group is achieved by HPLC using chiral columns. The R or S configuration is obtained by comparison of retention times with chemically-synthesized standards.

The geometry of the double bounds in oxygenated products of PUFA is crucial to be determined as the function of the metabolite may depend on it. The gold standard method is NMR, which requires substantial amounts of highly purified products.

The mechanism of oxidation is solved by using $^{18}O_2$. Indeed, for a double lipoxygenation process, two ^{18}O atoms are present in the final hydroxyl derivatives. In contrast, only one ^{18}O atom is present if the mechanism involves an epoxidation mechanism followed by epoxide opening subsequently to hydration of the intermediate. Such a method has been successfully applied to differentiate the formation of PDX through a double oxygenation mechanism [106] from the formation of PD1 which requires a transient epoxidation after the lipoxygenation, followed by the water-dependent epoxide opening.

4-hydroxy-alkenals can be quantified directly through HPLC with UV detection at 220 nm [162-164] or after derivatization with 2,4dinitrophenylhydrazine (DNPH) at 340 nm

[165]. They can also be detected with a higher sensitivity by NICI GC-MS after derivatization using O-pentafluorobenzyl hydroxylamine hydrochloride for the carbonyl group, and N,O-bis trimethylsilyltrifluoroacetamide for the hydroxyl group [166-172].Their corresponding deuterated hydroxyalkenals are used for quantitation (Fig. 6). NKs adducts are detected and quantified by LC/ESI/MS/MS in the positive ion mode as described [145,148,149,150,173]. The basis of the method for quantifying NK-proteins adducts involves enzymatic digestion of proteins to individual amino acids followed by analysis of lysyl-NK adducts.

7. Conclusion

Omega-3 PUFA might play an important role in preventing atherothrombosis by themselves and through their oxygenated metabolites. However, it is obvious that the molecular mechanism explaining these effects on cardiovascular risk factors remain partially unclear, and further research needs to be launched. It seems important to look at the effects of other omega-3 PUFA than the main ones (ALA, EPA and DHA), such as docosapentaenoic acid and the very long-chain omega-3 PUFA (C24 and above), to search for new bioactive oxygenated metabolites.

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Abbreviations

ARA, arachidonic acid; ALA, alpha-linolenic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ETA, 8,11,14,17-eicosatetraenoic acid; HDDE, 4-hydroxy-dodecadienal; HDL, high-

density lipoproteins; 12-HETE, 12-hydroxy-eicosatetraenoic acid; HHE, hydroxy-hexenal; HNE, hydroxy-nonenal; 12-HpETE, 12-hydroperoxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; HPLC, high-performance liquid chromatography; IsoK, isoketal; IsoP, isoprostane; LDL, low-density lipoproteins; LOX, lipoxygenase; MDA, malondialdehyde; NK, neuroketal; NP, neuroprostane; PD, protectin D; PG, prostaglandins; PGHS, prostaglandin H synthase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; RvD, resolvin D; RvE, resolvin E; SDA, stearidonic acid; TAG, triacylglycerols; Tx, thromboxane; VLDL, very low-density lipoproteins.

Figure legends

Figure 1: Biogenesis of long-chain polyunsaturated fatty acids (PUFA) from the essential fatty acids in mammals, linoleic (18:2ω6) and alpha-linolenic (18:3ω3) acids. The whole sequence mainly occurs in the endoplasmic reticulum, except for shortening the 24-carbon intermediates by beta-oxidation (β-ox) which occurs in peroxisomes.

Δ and E represent desaturases and elongases, respectively.

Figure 2: Selected oxygenation pathways related to the three main omega-3 PUFA (ALA, EPA and DHA) discussed in the review, with some pathways regarding ARA as a reference PUFA. Apart from the cyclooxygenase (COX) activities involved in the conversion of EPA into prostanoids of the three series (as largely described for ARA that is converted into prostanoids of the two series), the three omega-3 PUFA are mainly oxygenated by lipoxygenases (LOX). “LOX” means that several LOX and/or LOX plus other oxygenation pathways may be involved.

COX: cyclooxygenase; Epox/Hydr: epoxidase + hydrolase; GPx: glutathione peroxidase.

Hp/HEPE: hydroperoxy/hydroxy-eicosapentaenoate; Hp/HETE: hydroperoxy/hydroxy-eicosatetraenoate; Hp/HDoHE: hydroperoxy/hydroxy-

docosahexaenoate; Hp/HOTE: hydroperoxy/hydroxy-octadecatrienoate; LT: leukotriene; PG: prostaglandin; Tx: thromboxane.

anti-aggregatory:

pro-aggregatory :

Figure 3: Dehydration of PGD_2 and D_3 into PGJ_2 and J_3, then into $15dPJ_2$ and J_3. The former are activator of adenylyl cyclase, and the latter are ligands of peroxisome proliferator-activating receptor gamma.

Figure 4: Three poxytrins (Pufa OXYgenated TRIenes), characterized by an E,Z,E conjugated triene. Linotrin, LTBX and PDX derive from the double lipoxygenation of ALA, ARA and DHA, respectively. LTBX and PDX are stereochemical and geometric isomers of LTB_4 [5(S),12(R)-dihydroxy-6Z,8E,10E-20:4] and PD1 [10(R),17(S)-dihydroxy-11E,13Z,15E-22:6], respectively.

Figure 5: Structures of the two hydroxy-alkenals 4-HNE and 4-HHE from omega-6 and -3 PUFA, respectively, and their dehydrogenated oxo derivatives.

The structure of a major neuroketal from DHA is also shown.

Figure 6: NICI gas-chromatography/mass spectrometry tracing of 150 pg of derivatized 4-HHE and 4-HNE. Each of them show the syn and anti isomers.

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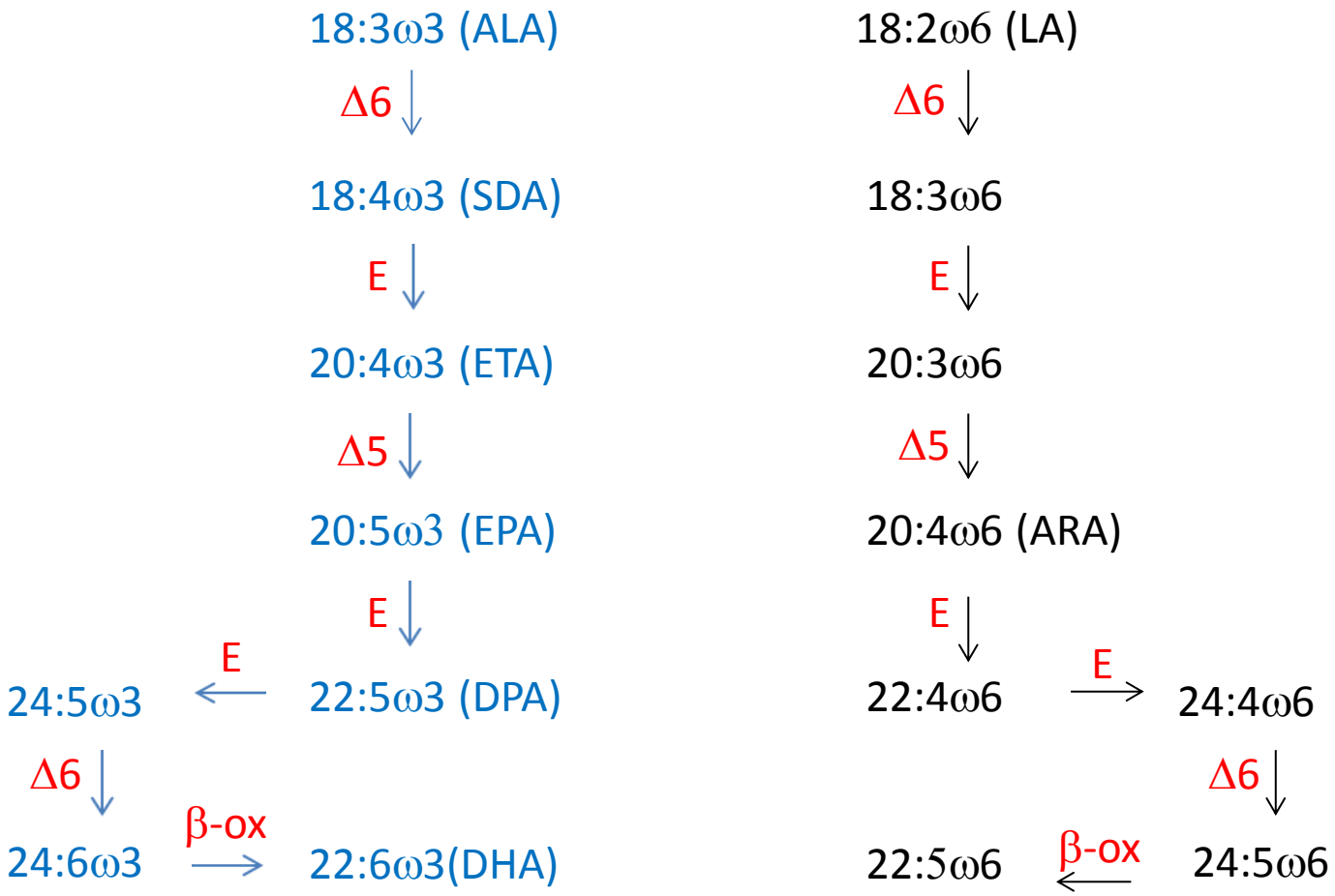

18:3ω3 (ALA)
Δ6
18:4ω3 (SDA)
E
20:4ω3 (ETA)
Δ5
20:5ω3 (EPA)
E
22:5ω3 (DPA)
E
24:5ω3
Δ6
24:6ω3
β-ox
22:6ω3(DHA)
18:2ω6 (LA)
Δ6
18:3ω6
E
20:3ω6
Δ5
20:4ω6 (ARA)
E
22:4ω6
E
24:4ω6
Δ6
24:5ω6
β-ox
22:5ω6

Fig. 1

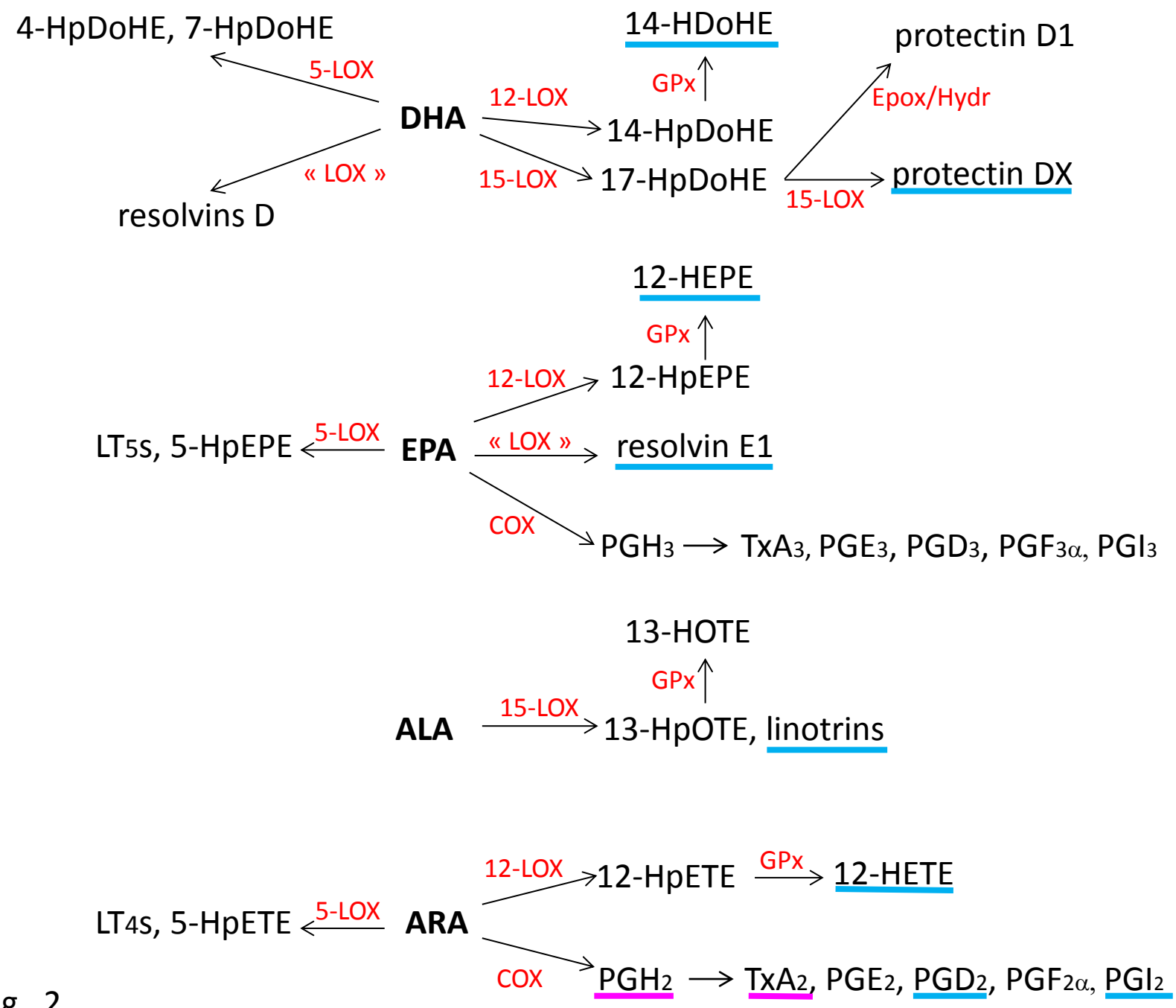
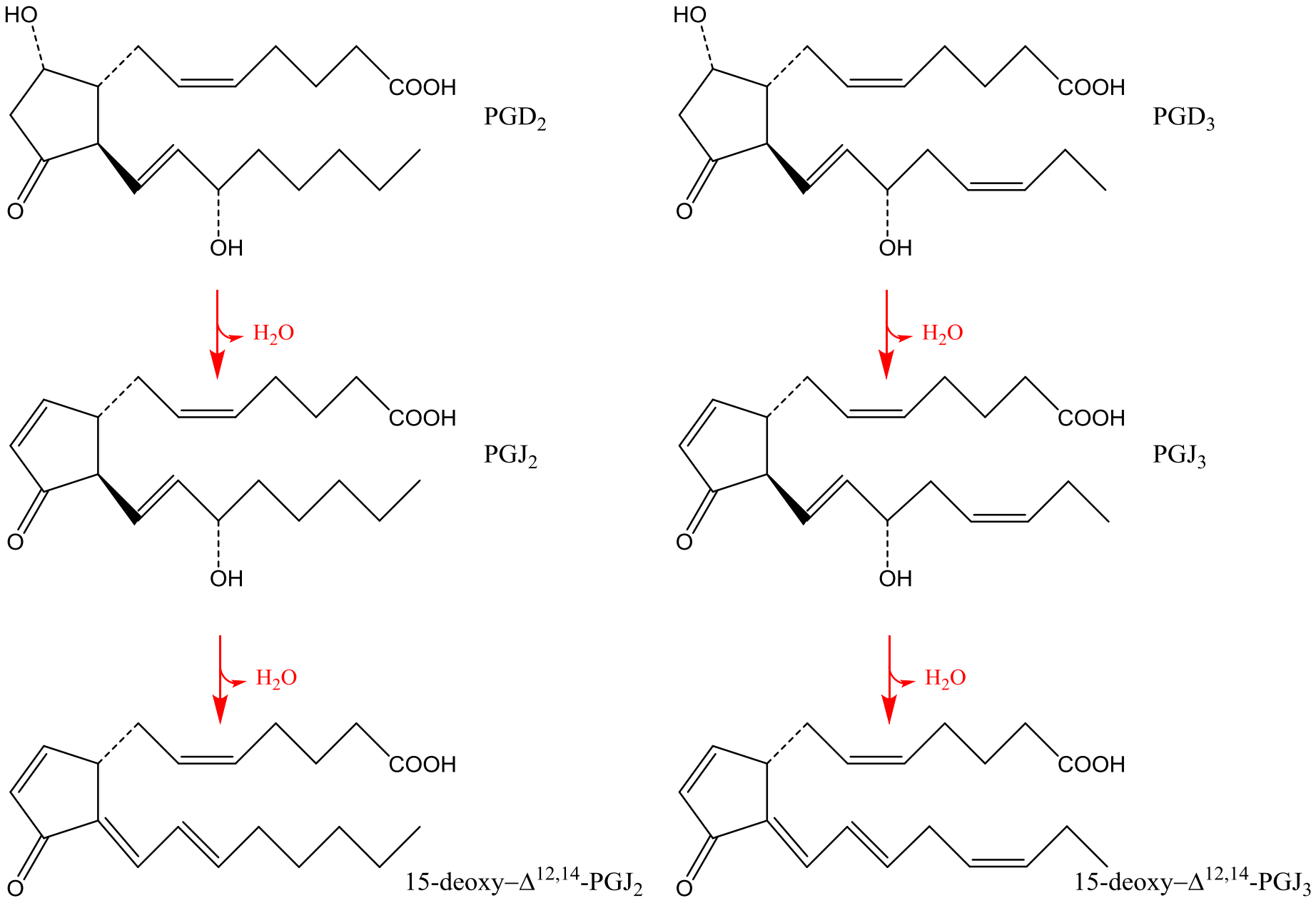

4-HpDoHE, 7-HpDoHE
5-LOX
DHA
« LOX »
resolvins D
12-LOX
14-HDoHE
GPx
14-HpDoHE
15-LOX
17-HpDoHE
Epox/Hydr
protectin D1
protectin DX
15-LOX
12-HEPE
GPx
12-HpEPE
12-LOX
LT5s, 5-HpEPE
5-LOX
EPA
« LOX »
resolvin E1
COX
PGH3 ⟶ TxA3, PGE3, PGD3, PGF3α, PGI3
13-HOTE
GPx
ALA
15-LOX
13-HpOTE, linotrins
12-LOX
12-HpETE
GPx
12-HETE
LT4s, 5-HpETE
5-LOX
ARA
COX
PGH2 ⟶ TxA2, PGE2, PGD2, PGF2α, PGI2

Fig. 2


HO
COOH
O
OH
PGD2
H2O
COOH
O
OH
PGJ2
H2O
COOH
O
15-deoxy–Δ12,14-PGJ2
HO
COOH
O
OH
PGD3
H2O
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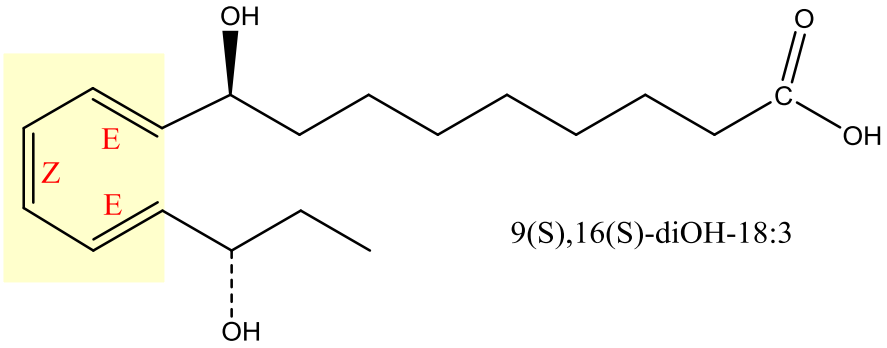
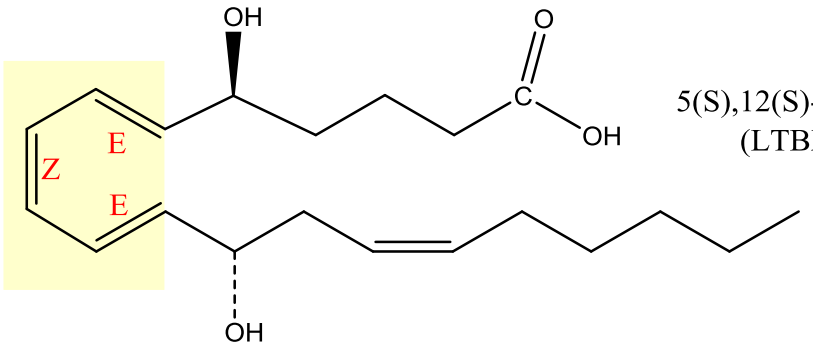
O
OH
PGJ3
H2O
COOH
O
15-deoxy–Δ12,14-PGJ3

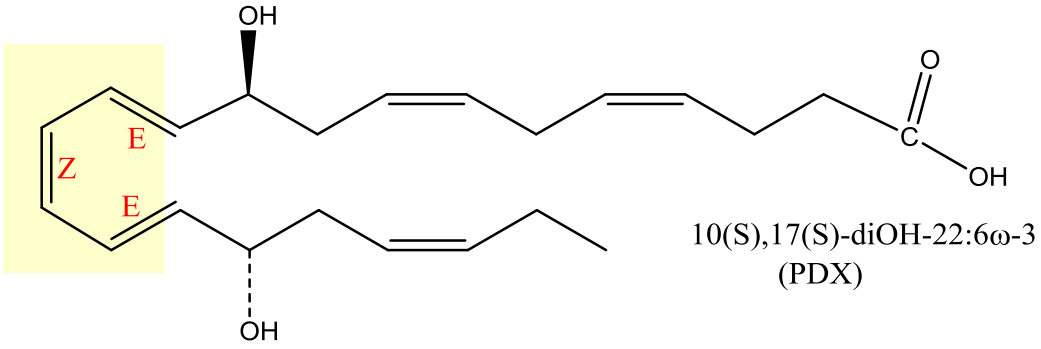
Fig .3



9(S),16(S)-diOH-18:3



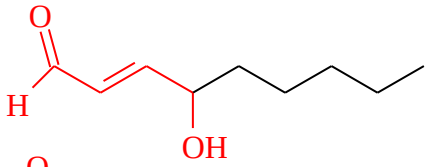
5(S),12(S)-diOH-20:4ω-6
(LTBX)



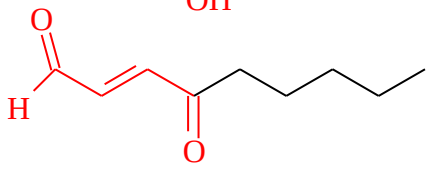
10(S),17(S)-diOH-22:6ω-3
(PDX)

Fig. 4

ω-6 fatty acids

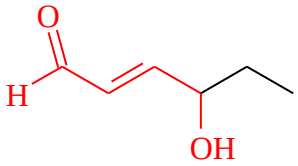


4-hydroxy-nonenal

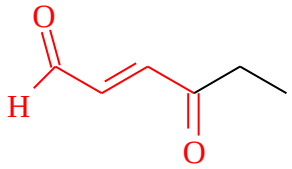


4-oxo-nonenal

ω-3 fatty acids

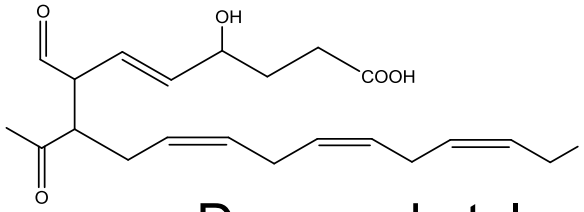


4-hydroxy-hexenal



4-oxo-hexenal

Docosahexaenoic acid



D_4-neuroketal

other neuroketal regioisomers

Fig. 5

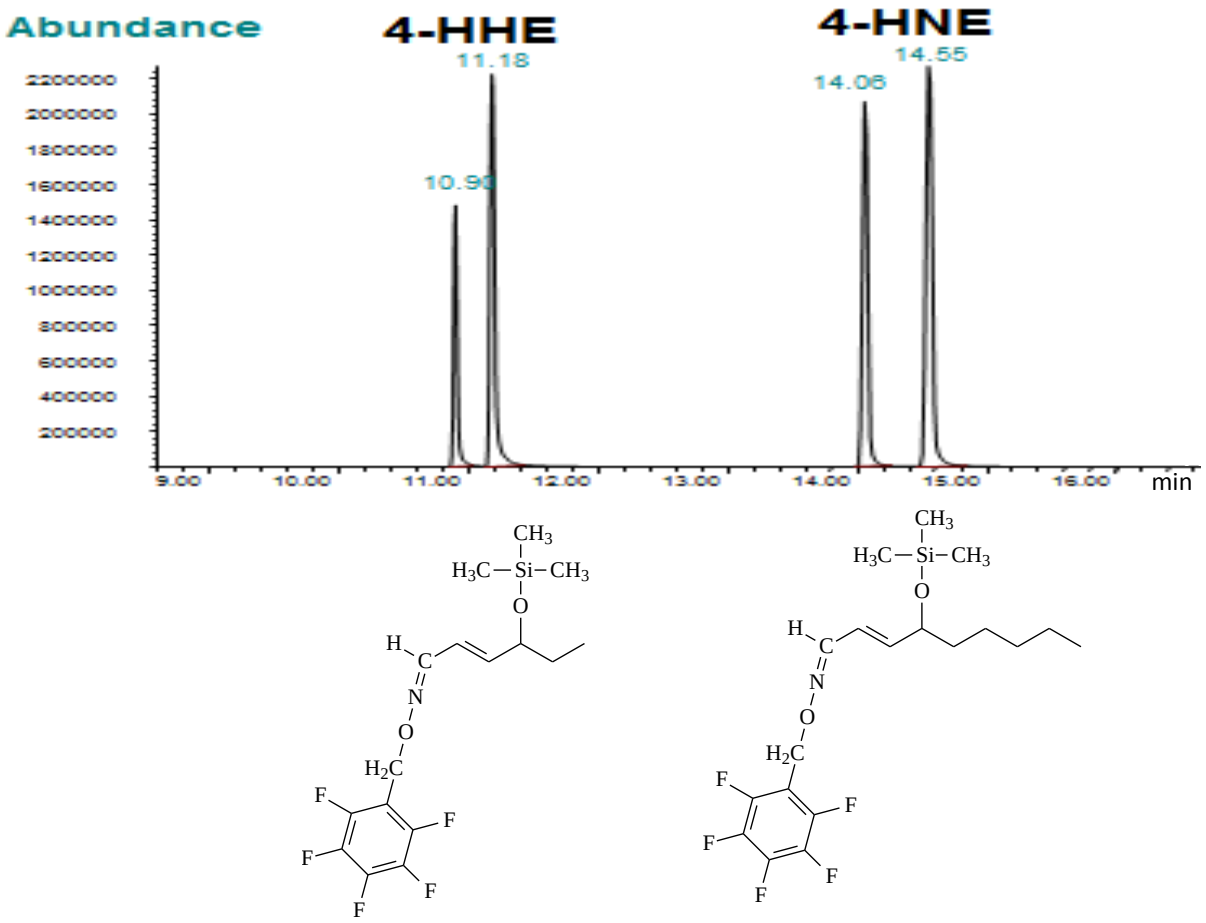

Abundance
4-HHE
11.18
10.90
4-HNE
14.55
14.06
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
min

Fig. 6