

F3-Isoprostanes and F4-Neuroprostanes: Non-enzymatic Cyclic Oxygenated Metabolites of Omega-3 Polyunsaturated Fatty Acids: Biomarkers and Bioactive Lipids

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Abstract:

The isoprostanooids are non-enzymatic oxygenated metabolites derived from polyunsaturated fatty acids (PUFA) formed *in vivo* by free radical mechanism. Those cyclic oxygenated metabolites named isoprostanes (IsoPs) were originally discovered from arachidonic acid (AA, C20:4 n-6) in 1990 and since then best known as biomarkers for assessing endogenous *in vivo* oxidative stress (OS) in humans and animals. During the last twenty-five years, a few chemist groups have successfully synthesized these cyclic oxygenated metabolites derived from omega-3 (n-3) PUFA such as F₃-IsoPs from eicosapentaenoic acid (EPA, 20:5 n-3), and F₄-neuroprostanes (F₄-NeuroPs) from docosahexaenoic acid (DHA, 22:6 n-3), and their availability allowed a better understanding of their potential roles as bioactive compounds but also extended their use as more specific biomarkers of OS. Accordingly, we will discuss the impact of F₃-IsoPs and F₄-NeuroPs generated from EPA and DHA in this review.

1. Introduction

The understanding of the role of n-3-PUFA peroxidation in the pathogenesis of various diseases is continuously increasing but the biological activity and the biochemical role of the myriad of metabolites generated have been largely undetermined by investigators and remain unexplored for most of them. The reasons for the small number of investigations could be due to the false idea that the rate of non-enzymatic PUFA oxidation *in vivo* is negligible, and/or to the previously held idea that any oxygenated metabolites derived from lipid peroxidation are undesirable and toxic. Moreover, not all of these metabolites are commercially available and need to be custom synthesized.

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the major n-3 polyunsaturated fatty acids (PUFA) of marine fish oil. Evidence from epidemiological studies, clinical trials, animal and cellular experiments showed fish oil, and specifically n-3 PUFA, having beneficial effects in numerous diseases [1]. Due to the number of double bonds in the structure of EPA and DHA, they are prone to free radical attack and can undergo non-enzymatic peroxidation to generate cyclic oxygenated metabolites, termed isoprostanes (IsoPs) and neuroprostanes (NeuroPs) [2-5]. The comprehension of the effect of PUFA and their non-enzymatic metabolites has been reported in a number of recent prominent reviews [6-12].

The isoprostanes can be generated from different PUFAs; for example, arachidonic acid (AA) generates 64 isomers of F₂-isoprostanes (F₂-IsoPs) [3], EPA generates 192 isomers of F₃-isoprostanes (F₃-IsoPs) and DHA generates 256 isomers of neuroprostanes (NeuroPs) [4, 5]. We will focus on F₃-IsoPs and F₄-NeuroPs in this review.

Previously, quantification of lipid metabolites 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) was the main assessment for oxidative stress measurement (OS) in biological systems. However, they appear to be not as robust biomarkers when compared to F₂-IsoPs measurement and correlated to OS. Since the discovery of Morrow and Roberts, F₂-IsoPs have become a “gold standard” for assessing endogenous OS in humans, animal models and in biological fluids [13]. These lipids are oxidized *in situ* on the phospholipid membranes and hydrolyzed via phospholipase A₂ (PLA₂) and platelet activating factor acetylhydrolase into the free form, and finally released in tissues and systemic circulation. Among these metabolites, some have been commonly, and in some cases routinely, measured as OS biomarkers related to vascular systems and neurodegeneration [8, 9, 11].

The discovery and study of isoprostanooids have provided a major step forward in the field of free radical research. The quantification of these oxygenated lipids has opened up new areas of investigation regarding the role of free radicals in human physiology and pathology, and appears to be the most useful tool currently available to explore the role of endogenous lipid peroxidation in human diseases. However, as explained below such lipids are not only reliable biomarkers but also exert bioactive properties.

So far, evidence in favor of the bioactive role of isoprostanooids from n-6 PUFA were shown in various biological systems [6, 7, 11]. 15-F_{2t}-IsoP will be not developed in this review (for references, see recent reviews) [6, 7, 11, 14, 15].

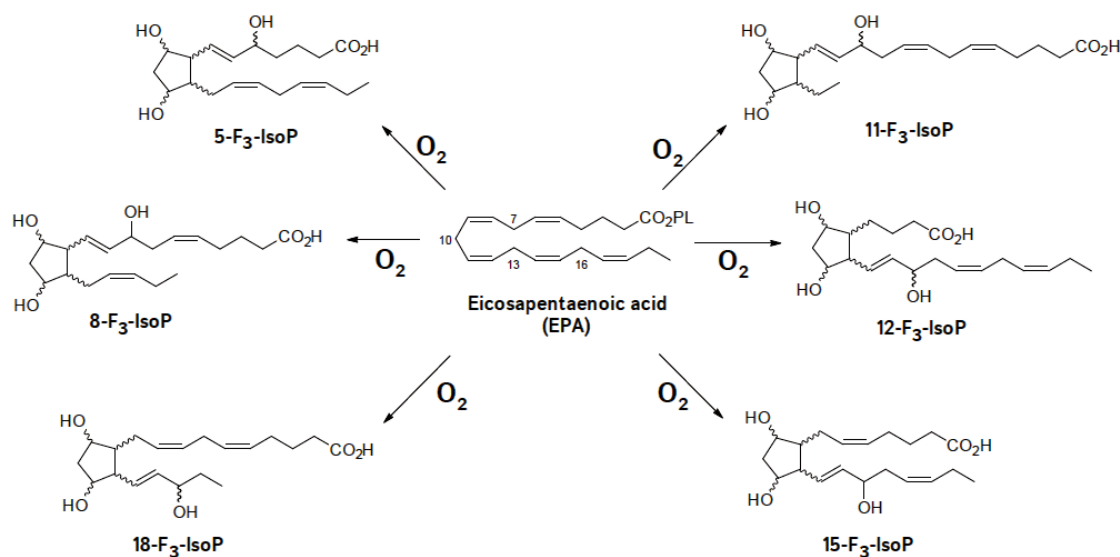


Figure 1: Biosynthesis of F_3 -IsoP derived from EPA

2. Formation, nomenclature and quantitation of F_3 -IsoPs, F_4 -NeuroPs derived from EPA and DHA, and isofurans.

Morrow et al. discovered in 1990 novel prostaglandin (PG)-like isomers, which were termed isoprostanes (IsoPs) [3]. In contrary to PG initiated by cyclooxygenases, their mechanism of formation proceeds via a non-enzymatic free radical peroxidation of AA bound to phospholipids and not from free AA [3]. The main structural characteristics com-

pared to PGs are the *cis*-relationship of the side chains, the absolute number of potential isomers and the racemic generation of the metabolites. Once formed in the membranes, the IsoPs can then be released by phospholipases in the circulating fluids [16]. Later, it was discovered that other PUFAs such as EPA and DHA can undergo a similar oxidation process leading to F_3 -IsoPs and F_4 -NeuroPs respectively [4] [5] (Figures 1 and 2).

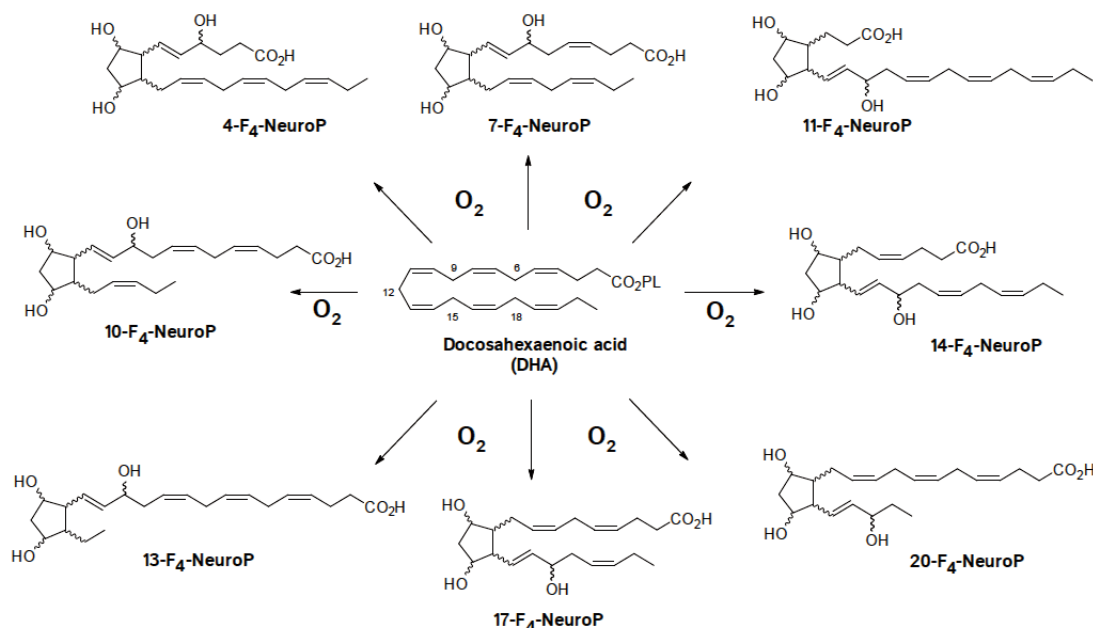


Figure 2: Biosynthesis of F_4 -NeuroP derived from DHA

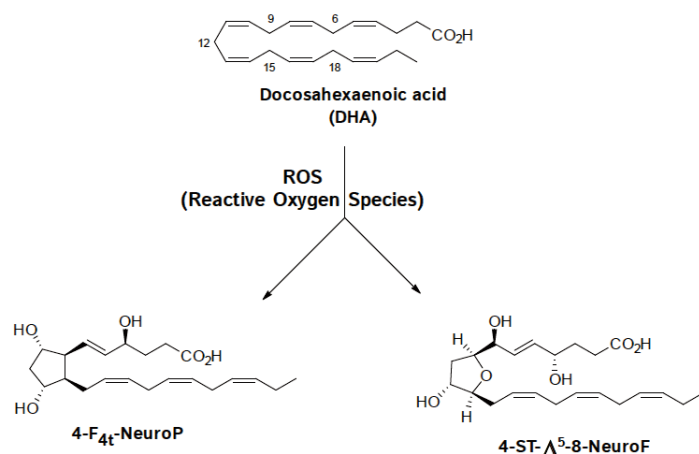


Figure 3: Formation of the 4-ST- Δ^5 -8-NeuroF and 4-F_{4t}-NeuroP from DHA

In 2002, new oxygenated metabolites were discovered with a furan core as the main feature. Their formation follows the initial same free radical cascade pathway of IsoPs, but a competition between the five-membered ring formation (IsoPs) and attack of further diradical oxygen lead to the competitive generation of isofurans (IsoFs) from AA [17]. It is now well described that both types of metabolites are present in most lipid matrices. Similarly, other PUFAs can also generate isofuranoid derivatives, [6] including neurofurans (NeuroFs) (**Figure 3**) [17, 18] and dihomio-isofurans [19] (dihomo-IsoFs) from DHA and adrenic acid (AdA, 22:4 n-6) respectively.

There are two nomenclatures proposed by Taber [20] and Rokach [21] to name such metabolites. Taber nomenclature was approved by IUPAC and will be used throughout this review. To avoid confusion, the structure of 4-F_{4t}-NeuroP is presented in **Figure 4** and will be described briefly.

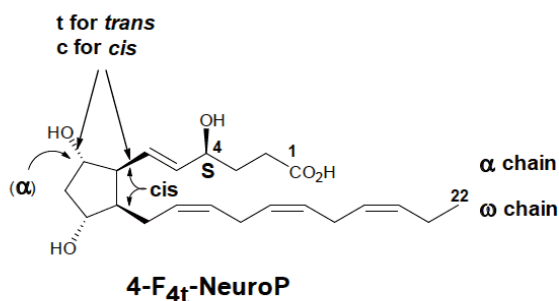


Figure 4: Structure of 4-F_{4t}-NeuroP from DHA

The ring substitution pattern with A-H, J and L is based on the common PG nomenclature (F in this case with two hydroxyls). The number as a subscript gave the number of double bonds on the two side chains (4 in this case). The t or c as a subscript determines the relative orientation of the α chain with respect to the adjacent α OH in the cyclopentane ring (t in this case). The family name NeuroP, IsoP, and PhytoP is based on the PUFAs (NeuroP from DHA in this case).

Refined extraction methods, robust analysis and elucidation of chemical structures have improved the sensitivity of detection in biological tissues and fluids. The first reliable instrumentation for measurement was gas chromatography-mass spectrometry (GC-MS), and nowadays the use of liquid chromatography tandem mass spectrometry (LC-MS/MS) is gaining much attention [see recent prominent reviews, [8-10, 22, 23].

3. Chemical syntheses.

A number of talented chemists have developed, all around the world, different chemical strategies to reach these IsoPs and NeuroPs (see few reviews [6, 7, 11], for the general understanding of their *in vivo* formation and biological functions, but also for diagnostic applications. We will mention briefly in this review only the four groups which performed the total syntheses of F₃-, A₃-IsoPs derived from EPA and F₄-, A₄-NeuroPs derived from DHA.

Our group has developed the first synthesis of F₄-NeuroP, 4(RS)-F₄-NeuroP, in 2000, using a radical carbocyclization strategy [24]. Later, in 2010, by using a more flexible strategy using our bicyclo[3.3.0]octene key intermediate [25], we have reached other series of F₃- and F₄-NeuroPs and deuterated analogues [26]. Rokach and co-workers reported the synthesis of the major metabolites of EPA, 5-F_{3c}-IsoPs and 5-F_{3t}-IsoPs [27]. Cha and co-workers in 2002 reported a total synthesis of 17-F_{4c}-NeuroP using a double cyclization step, with Pd(OAc)₂ [28]. Taber and co-workers described in 2008 an interesting approach towards the synthesis of the four diastereomers of 13-F_{4t}-NeuroP, using a thermal diastereoselective en cyclization of 1,6-dienes to the 1,2-*cis*-cyclopentane skeleton [29]. It is worthy to mention that Zanoni, Vidari and co-workers are the only chemists who have developed a strategy to reach A₃-IsoPs and A₄-NeuroPs [30].

4. Biomarkers

4.1. Neuroprostane and Isofuran

OS may contribute to the pathogenesis of pre-eclampsia, a life-threatening disorder of pregnancy that adversely affects the mother and the baby [31]. In a recent study, Bardeen et al. quantified F₂-IsoP, IsoF and F₄-NeuroP in maternal

plasma and cord blood of women with pre-eclampsia and normal pregnancies [32]. Women with pre-eclampsia had significantly elevated maternal IsoF and F_4 -NeuroP, but no F_2 -IsoP. Interestingly, cord blood IsoF were approximately 5-fold higher than those found in maternal plasma. This could reflect the oxidative challenge presented at birth, when there is transition from a relatively low intra-uterine oxygen environment to a significantly higher extra uterine oxygen environment.

The brain is vulnerable to oxidative insult because of high oxygen requirements for its metabolism and high PUFA composition, in particular DHA, hence F_4 -NeuroP was considered to be a specific marker of brain OS. Aneurysmal subarachnoid hemorrhage (aSAH) and traumatic brain injury (TBI) are associated with devastating central nervous system (CNS) injury. We and others have shown a significant increase in cerebrospinal fluid (CSF) IsoF in aSAH and TBI patients compared with their respective age- and gender-matched controls. aSAH patients also had significantly increased levels of CSF F_4 -NeuroP and F_2 -IsoP. Patients with TBI had significantly increased CSF F_4 -NeuroP, but F_2 -IsoP levels were similar to control [33]. These data confirm that CNS injury, in case of aSAH or TBI, results in increased OS and as DHA is the brain major PUFA, F_4 -NeuroP levels in CSF could be a much more specific indicator of neurological dysfunction than F_2 -IsoP. Hsieh et al. have shown that increased F_4 -NeuroP in CSF of patients with aSAH correlated with poor neurological outcome [34]. They suggested that F_4 -NeuroP might be more useful than F_2 -IsoP in CSF to predict outcome and interpret the role of hemorrhage in aSAH.

The anti-atherogenic effects of omega 3 fatty acids EPA and DHA are well recognized but the impact of dietary intake on bioactive lipid mediator profiles remains unclear. Gladine et al. studied the impact of DHA supplementation on the profiles of PUFA oxygenated metabolites and their contribution to atherosclerosis prevention [35]. A special emphasis was given to the non-enzymatic metabolites knowing the high susceptibility of DHA to free radical-mediated peroxidation and the increased OS associated with plaque formation. Targeted lipidomic analyses revealed that both the profiles of EPA and DHA and their corresponding oxygenated metabolites were substantially modulated in plasma and liver. Notably, the hepatic level of F_4 -NeuroP was strongly correlated with the hepatic DHA level. Moreover, unbiased statistical analysis revealed that the hepatic level of F_4 -NeuroP was the variable most negatively correlated with the plaque extent ($p < 0.001$) and an important mathematical positive predictor of atherosclerosis prevention.

4.2. Dihomo-Isoprostane

F_2 -Dihomo-IsoPs belong to the family of IsoPs deriving from the non-enzymatic oxidation of adrenic acid (C22:4 n-6, AdA), a polyunsaturated fatty acid distributed in the body, but also a specific component of myelin in the brain of primates [36, 37]. Rett syndrome (RTT) is a pervasive abnormality of development affecting almost exclusively females, which is included among the autism spectrum disorders. RTT is caused, in up to 95% of cases, by mutations in the X-linked methyl-CpG binding protein 2 (MeCP2) genes [38]. The disease shows a wide phenotypical heterogeneity, with at least four distinct major clinical presentations, i.e., typical, preserved speech, early seizure variant, and congenital variant. Clinical evidence indicates that F_2 -IsoP and F_4 -NeuroP are involved in the intimate pathogenetic mechanisms of RTT. Plasma levels of free F_2 -IsoP are significantly higher in the early stages of RTT, as compared with the late natural progression of typical RTT. Until recently it was thought that the predominant central nervous system damage in RTT occurred in gray matter. However, the relative abundance in myelin of the precursor AdA and the increased level of F_2 -dihomo-IsoP, strongly confirm an early and severe damage to the brain white matter as suggested by previous brain MRI evidence. Thus F_2 -dihomo-IsoP can be considered early markers of lipid peroxidation in RTT [39]. F_4 -NeuroPs also appear to be important biomarkers in RTT. Plasma F_4 -NeuroPs levels correlate with disease severity in RTT and are significantly related to neurological symptoms severity, mutation type and clinical presentation. Therefore, F_4 -NeuroP may play a major role along the biochemical pathway from MeCP2 gene mutation to clinical evidence, proving that a DHA oxidation process occurs.

4.3. Dihomo-Isosfuran / Neurofuran

Neurofurans (NeuroF) and dihomom-isofurans (dihomo-IsoF) are produced *in vivo* by non-enzymatic free radical pathways from DHA and AdA, respectively. As these metabolites are produced in minute amounts, their analyses in biological samples remain challenging. We performed syntheses of NeuroF and dihomom-IsoF, thanks to an enantiomerically enriched intermediate, which allowed, for the first time, access to both families: the alkenyl (4(RS)-ST- Δ^5 -8-NeuroF) (**Figure 3**) and enediol (7(RS)-ST- Δ^8 -11-dihomom-IsoF) [19, 40] and their quantitation in rat brain and heart tissues. It is also the first report to show concentration of known NeuroP and dihomom-IsoP in the heart tissue. These DHA and AdA metabolites are presently in testing for various pathological models as OS biomarkers and bioactive compounds.

5. Biological activities

The biological roles of oxygenated metabolites from the per-

oxidation of n-3-PUFA mainly emphasize their formation by enzymatic pathways, especially with respect to anti-inflammatory activities and the reduction of pro-inflammatory eicosanoids stemming from AA [7, 41]. For example, lipoxygenases mediate formation of metabolites such as resolvins, protectins and maresins [42, 43] that have shown a large range of potent anti-inflammatory activities in diseases. Nevertheless, recent studies showed that isoprostanoids *per se* derived from n-3-PUFA (mainly from EPA, DHA and α -linolenic acid) are new actors to be considered [5, 15, 41], suggesting that bioactive roles of oxygenated n-3-PUFA are not limited to those released through enzymatic pathways.

Metabolites of EPA

Over a decade ago, one study highlighted that unlike the 15-F_{2t}-IsoP derived from AA, 15-F_{3t}-IsoP from EPA does not activate platelet aggregation [44]. This notable difference of activity between cyclic oxygenated products derived from n-6-PUFA and n-3-PUFA suggests a very subtle structure-activity relationship [7]. More recently, Jamil *et al.* [45] investigated the ability of 5-F₃-IsoPs to regulate glutamatergic neurotransmission. Hence, 5-F₃-IsoPs could have important pharmacological implications in neurology since EPA, its precursor, is rich in the brain and retina. Glutamate serves as the primary excitatory neurotransmitter in several vertebrate retinal cells, including ganglion cells. The group also investigated the modulatory role of 5-*epi*-5-F_{3t}-IsoP on K⁺-induced glutamate release in isolated bovine retina. They found that 5-*epi*-5-F_{3t}-IsoP attenuates K⁺-induced [³H] D-aspartate release in a concentration-dependent manner and indicated that the mechanism involved is due to, in part, pre-junctional prostanoid EP1-receptors activation. This result displays the beneficial role of 5-*epi*-5-F_{3t}-IsoP by reducing excitatory neurotransmitter release, thereby retarding the progression of ocular neuropathic disease.

A₃/J₃-IsoPs, the EPA-derived cyclopentenone isoprostanooids, were also identified for their biological qualities *in vivo* under an OS environment. Two studies observed that these EPA-cyclopentenone derivatives possess anti-inflammatory activities and have antioxidant properties. Indeed, the 15-A_{3t}-IsoP inhibited in a concentration-dependent manner the expression and the activity of iNOS and COX-2 in mouse macrophages when pre-treated for 30 minutes with 15-A_{3t}-IsoP. It is postulated that 15-A_{3t}-IsoP exerts an anti-inflammatory activity via the inhibition of the nuclear factor kappa B (NF κ B) by blocking the degradation of inhibitory subunit I κ B α [46]. The second study was performed in hepatocarcinoma cells and found that the compound J₃-IsoP, isomers of A₃-IsoP from EPA, induced the nuclear related factor 2 (Nrf2)-based antioxidant re-

sponse through the inhibition of Keap-1, a negative regulator of Nrf2 [47].

Metabolites of DHA

The group of Morrow and Roberts, which pioneered the *in vivo* identification of NeuroPs, also demonstrated the biological effects of A₄/J₄-NeuroPs, cyclopentenones derived from DHA, to be mainly anti-inflammatory mediators in murine macrophage cell line [48]. Notably, they reported that in particular 14-A₄-NeuroP suppressed the effect of pro-inflammatory mediators such as lipopolysaccharide in macrophages, and confirmed the inhibition of the NF κ B pathway as the major mechanism of action of DHA as well as EPA peroxidized metabolites.

The most recent study on the biological effects of a DHA metabolite, 4(*RS*)-4-F_{4t}-NeuroP (**Figure 3**) specifically was performed in the cardiovascular system [49]. It is proposed that the oxidation of DHA and generation of 4(*RS*)-4-F_{4t}-NeuroP is necessary to prevent ischemia-induced arrhythmias in mice with myocardial infarction [50]. As previously observed in different oxidative conditions [51], we proposed that in oxidative stress conditions such as ischemic diseases, non-enzymatic cyclic oxygenated metabolites of DHA formed by peroxidation in cardiac membrane lipids, namely 4(*RS*)-4-F_{4t}-NeuroP, are responsible for the anti-arrhythmic properties of DHA by counteracting the cellular stress by ROS. Importantly, it appears that non-enzymatically released cyclic oxygenated metabolites of n-3 PUFA regulate cell communication, exert a physiological role and potentially act as a therapeutic agent [51]. Besides 4(*RS*)-4-F_{4t}-NeuroP, Le Guennec's group associated with our group evaluated the *in vitro* anti-arrhythmic properties of other oxygenated metabolites of EPA and DHA on single cardiac cells isolated from mice hearts. They observed that several cyclic oxygenated metabolites showed anti-arrhythmic (4(*RS*)-4-F_{4t}-NeuroP, and 15-F_{3t}-IsoP from EPA) or pro-arrhythmic (5(*RS*)-5-F_{3t}-IsoP and 8-F_{3t}-IsoP from EPA) properties, opening the way for likely biological roles; in depth validation is required to further substantiate the bioactive roles. Finally, Le Guennec *et al* reported in a recent patent that 4(*RS*)-4-F_{4t}-NeuroP is suitable for the treatment of acute myocardial infarction [52, 53]. Finally, a recent study by Gladine and co-workers reported that A₄- and F₄-neuroprostanes possess anti-inflammatory activities similar or even more pronounced than neuroprotectins (PD1, PDX), supporting that neuroprostanes should be considered as important contributors to the anti-inflammatory effects of DHA [54].

6. Conclusion

The experimental evidence outlined here supports the notion

that several of the biological activities of n-3 PUFAs in OS conditions could be explained by the action of non-enzymatic peroxidation products. In general, it appears that non-enzymatically cyclic oxygenated metabolites of n-3 PUFA could exert a physiological role. It highlights that in diseases which involve ROS production, some non-enzymatic oxygenated metabolites of n-3 PUFAs could be produced and prevent deleterious consequences of diseases, such as arrhythmias.

Care should be taken by scientists not to overlook the production of non-enzymatic metabolites of n-3 PUFAs, which could have traits equally or more active than the well-known enzymatic metabolites. Their production is very sensitive to the environment (diet and oxidative status) and may play advantageous or disadvantageous roles in many diseases, where oxidative status is highly related to the severity of the diseases such as cardiovascular, neurodegenerative, pulmonary, developmental, and metabolic disease and cancer.

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