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Commentary

Dietary $n-6$ and $n-3$ polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention

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ABSTRACT

Linoleic acid (LA) and alpha linolenic acid (ALA) belong to the $n-6$ (omega-6) and $n-3$ (omega-3) series of polyunsaturated fatty acids (PUFA), respectively. They are defined "essential" fatty acids since they are not synthesized in the human body and are mostly obtained from the diet. Food sources of ALA and LA are most vegetable oils, cereals and walnuts. This review critically revises the most significant epidemiological and interventional studies on the cardioprotective activity of PUFAs, linking their biological functions to biochemistry and metabolism. In fact, a complex series of desaturation and elongation reactions acting in concert transform LA and ALA to their higher unsaturated derivatives: arachidonic acid (AA) from LA, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) from ALA. EPA and DHA are abundantly present in fish and fish oil. AA and EPA are precursors of different classes of pro-inflammatory or anti-inflammatory eicosanoids, respectively, whose biological activities have been evoked to justify risks and benefits of PUFA consumption. The controversial origin and clinical role of the $n-6/n-3$ ratio as a potential risk factor in cardiovascular diseases is also examined. This review highlights the important cardioprotective effect of $n-3$ in the secondary prevention of sudden cardiac death due to arrhythmias, but suggests caution to recommend dietary supplementation of PUFAs to the general population, without considering, at the individual level, the intake of total energy and fats.

1. Introduction

Current nutrition recommendations are directed to prevent degenerative pathologies, such as cardiovascular diseases (CVD) and cancer. In fact, inhibition or promotion of atherogenesis can be influenced by a specific dietary pattern [1], and, similarly, factors such as food and nutrition may reduce the incidence of different types of cancers [2]. In this

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Abbreviations: AA, arachidonic acid (20:4$n-6$); AI, adequate intake; ALA, alpha-linolenic acid (18:3$n-3$, omega-3); CHD, coronary heart disease; COX, cyclooxygenase; CVD, cardiovascular diseases; d-6-d and d-5-d, delta-6- and delta-5- desaturases; DHA, docosahexaenoic acid (22:6$n-3$); EFA, essential fatty acids; OA, oleic acid (18:1$n-9$); EPA, eicosapentaenoic acid (20:5$n-3$); FADS2, delta-6 fatty acid desaturase; LA, linoleic acid (18:2$n-6$, omega-6); LDL, low-density lipoprotein; LOX, lipoxygenase; LXR, liver X receptors; MI, myocardium infarction; PPAR, peroxisome proliferator-activated receptor; PUFA and MUFA, polyunsaturated and monounsaturated fatty acids respectively; SREBP-1c, sterol regulatory element-binding protein 1c; TCR, T-cell receptor; VLDL, very low-density lipoproteins; UL, upper intake limit.

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context, a strong interest is devoted to the adequate intake (AI), from a quantitative and qualitative point of view, of dietary lipids.

The current guidelines formulated by the most authoritative nutritional organizations invite the population worldwide to consume no more than 7–10% of calories from saturated fatty acids; less than 300 mg/day of cholesterol; keep *trans* fatty acids consumption as low as possible. In Western countries, the total fat intake should be in the range of 25–35% of total daily calories, with most fats coming from sources heavily endowed with monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively), such as fish, nuts, and vegetable oils [3] (Table 1). The net effect of the changes occurring in the Western diet during the last 20 years led to a decrease in the use of animal fats and an increase in the consumption of vegetable fats, relatively high in linoleic acid (LA, 18:2*n* – 6, omega-6) and alpha-linolenic acid (ALA, 18:3*n* – 3, omega-3) (Table 1, Fig. 1). As an example, in Europe, during the last two decades, the consumption of LA increased by about 50%, passing from 10 to 15 g/day, while the consumption of ALA almost doubled, moving from 1 to 1.9 g/day [4].

LA and ALA are members of two well-known classes of PUFA, namely *n* – 6 (omega-6) and *n* – 3 (omega-3) series. From a biochemical point of view, both have 18 carbon atoms in their acyl chain presenting two (LA) or three (ALA) C=C double bonds. The position of the first unsaturation counting from the methyl end of the fatty acid, the so-called omega-C, generated the name of the two different classes (Fig. 1). From a nutritional point of view, LA and ALA are commonly considered as ''essential'' fatty acids (EFA), since they are not synthesized in the human body and are mostly obtained from the diet. Unsaturated fatty acids include also the *n* – 9 series, derived from oleic acid (OA, 18:1) and the *n* – 7 series, derived from palmitoleic acid (16:1), which are not essential [5,6]. Dietary sources of *n* – 6 FAs are abundantly present in liquid vegetable oils, including soybean, corn, sunflower, safflower oil, cotton seed oils, while linseed and canola oils are rich in *n* – 3 FAs (Table 1). Eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) belong to the *n* – 3 series of FAs and are abundantly present in fish and shellfish. Fish such as salmon, trout and herring are higher in EPA and DHA than others (e.g., cod, haddock and catfish); in fact, fish-oil supplements typically contain 30–50% of *n* – 3 FAs (Table 1). However, quantities vary among species and within a species according to environmental variables such as diet and whether fish are wild or farm-raised. As an example, farm-raised catfish tend to have less EPA/DHA than do wild catfish, whereas salmon and trout contain similar amounts in the two different growing processes [7]. It is worthwhile to note that the limited amounts of *n* – 3 FAs present in meats became nutritionally important considering the large quantities of beef, pork, poultry consumed in Western diets [8].

Table 1 – Dietary sources of selected EFAs/PUFAs[a,b,c].

Product	LA	ALA	AA	EPA+DHA
n – 6 FA rich foods				
Corn oil	50000	900		
Cotton seed oil	47800	1000		
Peanut oil	23900			
Soybean oil	53400	7600		
Sunflower oil	60200	500		
Safflower oil	74000	470		
Margarine	17600	1900		
Lard	8600	1000	1070	
Chicken egg	3800	220		
Bacon	6080	250	250	
Ham	2480	160	130	
Soya bean	8650	1000		
Maize	1630	40		
Almond	9860	260		
Brazil nut	24900			
Peanut	13900	530		
Walnut	34100	6800	590	
n – 3 FA rich foods				
Canola oil	19100	8600		
Linseed oil	13400	55300		
Herring	150	61.66	36.66	1700
Salmon	440	550	300	1200
Trout	74		30	500
Tuna	260	270	280	400
Cod	4	2	3	300

[a] Data reported as mg/100 g.
[b] Data elaborated from [16,36].
[c] Content of *n* – 6, *n* – 3 FAs may slightly vary according to species, sources and analytical methods.

Although the terms ''PUFA'' and ''EFA'' are not synonymous (only LA and ALA are essential from a biochemical and nutritional point of view), they are often used interchangeably since many biological functions of EFAs are exerted by EFA-derived PUFAs, such as arachidonic acid (AA, 20:4*n* – 6), DHA, EPA [5]. Deficits in *n* – 6 EFAs/PUFAs were correlated with the severity of atopic dermatitis by affecting skin barrier function and cutaneous inflammation [9], which may be ameliorated by diets with evening primrose or borage oil (vegetable oils that contain gamma-linolenic acid (GLA)) [10]. It is still debated which of the different biological functions of *n* – 6 PUFAs are predominant in this pathology. Essentiality of ALA and its metabolites are still a matter of opinion. In many cases, *n* – 3 and *n* – 6 FAs can compensate each others function in ameliorating pathological conditions, such as growth retardation. In other situations, the biological activity of the *n* – 3 series is more specific. DHA, in fact, is required in the nervous system for optimal neuronal and retinal function and influences signalling events which are vital for neuronal survival and differentiation [11]. Whether EFAs/PUFAs are essential for cell viability remains elusive. In fact, a recent work demonstrated that deletion of FADS2 (Fatty acid desaturase) gene in mouse, abolished the expression of delta-6-desaturase (d-6-d), a key enzyme in the enzymatic cascade of EFA/PUFA biosynthesis (see below). However, lack of PUFAs did not impair the normal viability and lifespan of male and female FADS2$^{-/-}$ mice [12].

The last two decades have seen a proliferation of studies on the cardioprotective effects of EFA/PUFA, especially the *n* – 3 series which represents the focus of this review together with the importance of the *n* – 6/*n* – 3 FA ratio in a healthy diet.

Indeed, this field is already too expansive for a comprehensive, single review; thus I apologize in advance for the many omissions, hoping that this commentary may help to predict the future developments in the field.

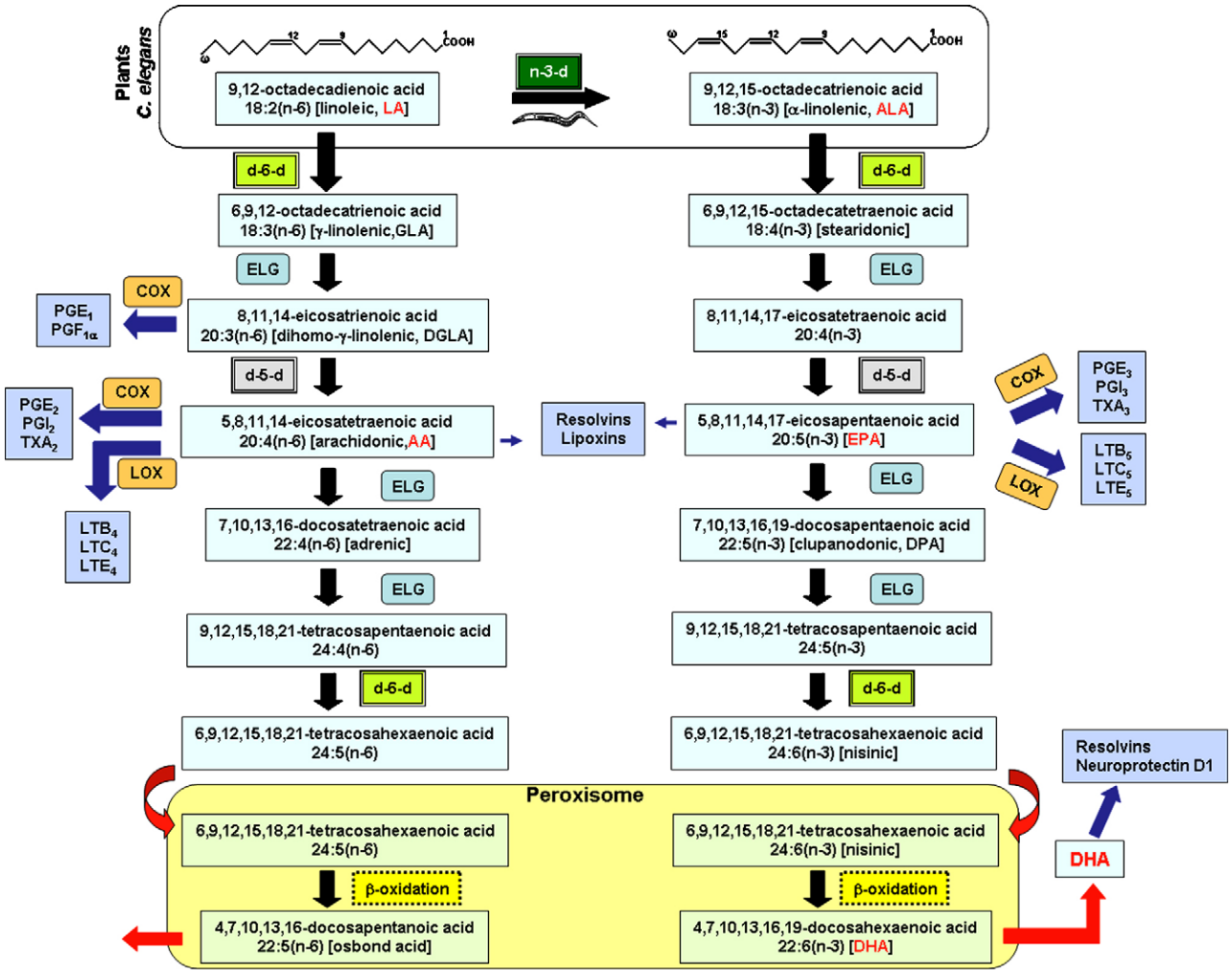

Fig. 1 – PUFA biosynthesis. The IUPAC names (*All-cis*) and the common names (in square brackets) with abbreviations are reported. ELG indicates elongase, while d-6-d and d-5-d indicate delta desaturases. In plants and *C. elegans* a *n* − 3 desaturase (*n* − 3-d) converts LA to ALA. *C. elegans* also possesses all of the *n* − 3-d activities required to produce 18:4*n* − 3, 20:4*n* − 3 and EPA from 18:3*n* − 6, 20:3*n* − 6 and AA, respectively (not shown), but is unable to elongate C20 PUFAs further. Mammals convert LA and ALA to long chain fatty acids using a series of desaturation and elongation reactions in the ER. However the synthesis of DHA from 24:6*n* − 3 and osbond acid (22:5*n* − 6) from 24:5*n* − 6 requires the synthesis of 24:6*n* − 3 and 24:5*n* − 6 in the ER, their passage into the peroxisome where they undergo one cycle of beta-oxidation to produce DHA and osbond acid which move back to the ER (red arrows). The synthesis of eicosanoids from AA, EPA and DGLA by COX and LOX enzymes is also reported (blue arrows; see text for details). Formation of resolvins and protectins from DHA is also shown. Data elaborated from references [6,11,48].

2. *The biochemistry and metabolism of EFAs/PUFAs*

Non-esterified fatty acids present in the diet are rapidly and efficiently absorbed (>96% according to [13]) and enter cells via FA transporters, they are rapidly converted to FA acyl-CoA thioesters, before undergoing three main metabolic fates. First, they contribute to ATP production by the classical beta-oxidation pathway. In men, following ingestion of [^{13}C]-labelled PUFAs of different length and degree of unsaturation, the recovery of $^{13}CO_2$ in breath ranges between 20–30% of the proportion ingested, in the following order: about 20% for *n* − 9, 25% for *n* − 6 and 30% for *n* − 3 series [13]. Fatty acyl-CoA is also substrate for the synthesis of neutral (triglycerides, cholesterol esters) and polar (phospholipids, sphingolipids, etc.) lipids. In adipose tissues in men, it has been calculated that the percentage content of LA is about 11.0% versus 0.7% of ALA [13,14]. Finally, FA acyl-CoA is subjected to a complex series of elongation/desaturation reactions to generate long chain PUFAs.

Fig. 1 shows the most significant steps transforming LA and ALA to their higher unsaturated derivatives (AA, EPA, DHA) by the activities of consecutive desaturation and elongation reactions. One of the key enzymes in the metabolism of EFA/PUFA is d-6-d which recognizes and metabolizes LA and ALA producing GLA, and octadecatetraenoic (stearidonic) acids, respectively. The affinity of d-6-d for EFAs is different; in fact, the concentration of ALA required to inhibit GLA formation by

50% is about 10 times the concentration of the substrate (LA) [15], suggesting that in the presence of higher concentration of LA, such as occurs in a living system, the pathway leading to AA is preferred [15–17]. After desaturation by d-6-d, a cycle of elongation and desaturation by d-5-d (delta-5-desaturase) generates AA (20:4$n-6$) and EPA (20:5$n-3$) starting from LA and ALA, respectively (Fig. 1). The obvious formation of the 22:5$n-6$ and 22:6$n-3$ series by a further step of elongation and desaturation by a hypothetical d-4-d (delta-4-desaturase) has been a matter of controversy, since this enzyme has been only identified in microalgae [18]. In mammals, two cycles of elongations and one of desaturation by d-6-d form tetracosahexaenoic acid (24:6$n-3$; Fig. 1) and tetracosapentaenoic acid (24:5$n-6$). These two PUFAs are transferred from the endoplasmic reticulum (ER) to peroxisomes (the so-called Sprecher's shunt), where they undergo beta-oxidation to generate DHA (22:6$n-3$; Fig. 1) and docosapentaenoic acid (22:5$n-6$, also called osbond acid) which both return to the ER [19]. ALA deficiency reduced DHA and enhanced osbond acid levels in tissue membranes; therefore, it is considered a functional indicator of DHA status [20]. Mammals can also convert DHA into EPA, but human bodies struggle to make this conversion which is not a very efficient process. Retroconversion of supplemental DHA to EPA was significantly greater in an EFA-deficient cell line (EPC-EFAD) [21].

Since biosynthesis of FAs and phospholipids occurs in ER, the intermediates of the PUFA metabolism can either be incorporated into phospholipids, or become the substrate for a further elongation/desaturation reaction. When in the membranes, PUFAs contribute to their fluidity that is an important determinant for the correct hormone-receptor binding, which is a function of the fluidity of cell membranes. A good example is represented by insulin receptors. In fact, insulin resistance might be associated to a rigid membrane which limits the number of insulin receptors. On the opposite, increasing cell membrane fluidity by increasing PUFA concentration might result in an enhanced number of insulin receptors, an increased affinity of insulin to its receptors and a reduced insulin resistance [5]. The exact mechanism leading to decrease insulin resistance by PUFAs is not known. Insulin might enhance the activity of d-6-d and d-5-d desaturases augmenting the formation of PUFAs that, in turn, enhance the action of insulin by increasing the number and the affinity of its receptors on the cell membrane [22].

Cell membrane fluidity by EFAs/PUFAs also interferes with T-cell receptor (TCR). TCR stimulation activates Lck and Fyn, two members of the Src family, leading to the activation of ERK cascade of signal transduction. Treatment of Jurkat T cells with EPA and AA results in marked incorporation of PUFAs into phosphatidylethanolamine. This event leads to displacement of palmitate-labeled Lck and Fyn from the lipid rafts and down-regulation of ERK signalling [23,24]. Fyn is also differently acylated by saturated, monounsaturated and polyunsaturated FA probably by the same promiscuous enzyme. Replacement of palmitoyl group by AA or EPA results in Fyn loss of function since the kinase reduces its interaction with rafts [24]. It is worthwhile to note that $n-6$ (AA) and $n-3$ (EPA) FAs are interchangeable in the regulation Lck and Fyn functions, suggesting that, at least in this case, PUFAs affect raft composition and function through similar mechanism(s) independent of other, more specific, metabolic fates.

EFAs/PUFAs and their metabolites exert a second messenger action when intercalated in the cell membrane. In fact, following the binding of growth factors and hormones to membrane receptors, phospholipase A2 is activated and releases DGLA, AA, EPA and DHA from sn-2 position of phospholipids. These molecules become substrates for eicosanoid biosynthesis, depending on the activities of cyclooxygenases (COX-1 a constitutive enzyme, or COX-2 an inducible enzyme), lipoxygenases (5-, 12-, or 15-LOX), or cytochrome P450 monoxygenases. More in details, the activity of COX enzymes and peroxidases on AA leads to the synthesis of 2-series prostanoids (prostaglandins E_2, prostacyclin I_2 and thromboxane A_2), while the activity of 5-LOX on AA generates 5-HPETE (arachidonic acid 5-hydroperoxide), which in turn is used to produce the 4-series leukotrienes (Fig. 1). The synthesis of AA-derived eicosanoids is influenced by the concentration of DGLA. In fact, when in excess, DGLA competes with AA for COX and LOX, inhibiting the production of AA-derived eicosanoids and driving the synthesis of 1-series prostaglandins (PGE_1, DGLA does not give rise to a thromboxane) [25]. EPA and DHA are converted by the same enzymes, e.g., COX and LOX, to 3-series prostanoids (prostaglandins E_3, prostacyclin I_3 and thromboxane A_3), and 5-series leukotrienes, respectively. EPA is a poor substrate for COX and LOX enzymes than AA, suggesting that the biological activities of COX- and LOX-derived EPA eicosanoids are strictly depend upon their intracellular availability, with respect to AA-derived compounds present at higher concentrations [24]. On the opposite, DHA oxygenation by LOX enzymes seems to have a more specific role in the brain as potential biomarkers for oxidative stress in neurodegenerative diseases [11].

Other anti-inflammatory lipid mediators, such as lipoxins and resolvins, derived from $n-6$ and $n-3$ PUFAs, respectively, control inflammation in a variety of experimental models of inflammatory disorders. As an example, $n-6$-derived lipoxins and $n-3$-derived resolvin E1 were shown to protect from experimental colitis in animal models [5,26] (Fig. 1).

EFAs/PUFAs rapidly modulate gene expression in different systems, by regulating transcription factors like peroxisome proliferator receptors (PPARs), liver X receptors (LXRs) and sterol regulatory element-binding protein 1c (SREBP-1c) (extensively reviewed in [24,27]). These nuclear receptors play crucial roles in the regulation of FA metabolism: LXRs activate expression of SREBP-1c, a dominant lipogenic gene regulator, whereas PPAR promotes FA beta-oxidation gene expression. EFAs/PUFAs are potent PPAR activators leading to the increased expression of genes responsible for FA oxidation, such as acyl-CoA oxidase, fatty acyl-CoA synthetase, hydroxymethylglutaryl-CoA synthase [16,17]. In many cases, the effects persist as far as PUFAs are present in the diet. Among those cited, PPAR family (alpha, beta, gamma 1/2 subtypes) represents the most interesting and studied target of hormone-like PUFA activity. Detailed structural analysis indicates that all PPARs are able to bind EPA with a K_d ranging between 1 and 4 μM. DHA must be retroconverted to EPA in order to fit into the PPAR hydrophobic binding pocket, representing one of the few examples of beta-oxidation of

DHA to EPA [28]. PPAR-alpha regulates several genes involved in lipid metabolism, including d-5-d, d-6-d and d-9-d desaturases [29]. PUFAs (both the $n-6$ and $n-3$ series, such as LA, EPA and DHA) also affect transcription of lipogenic genes (e.g., FA synthase, stearoyl-CoA desaturase-1, L-pyruvate kinase) by suppressing SREBP-1c nuclear activation, occurring by proteolysis, or SREBP-1c mRNA transcription [27]. This event might be explained since competitive inhibition of PUFAs reduces oxysterols binding to LXR, resulting in a decreased SREBP-1c expression [30]. Recently, a cross-talk between PPAR-alpha and LXR via SREBP-1c has been reported: competition of PPAR-alpha ligand with LXR ligand can suppress LXR-SREBP-1c pathway through reduction of LXR binding to its activator RXR (retinoid X receptor) [31,32]. EFAs/PUFAs contribute to the triangular relationship PPAR/LXR/SREBP-1c by independent bindings to PPAR-alpha and/or LXR. As reported above for cell membrane fluidity and raft composition, also in regulating gene expression $n-6$ and $n-3$ FAs are often interchangeable with $n-3$ series acting as more potent ligands to nucler receptors.

3. Effects of EFAs/PUFAs in cardiovascular diseases

The cardioprotective effects of $n-3$ FAs have long been recognized. The original observation is dated almost 50 years ago, when Hugh M. Sinclair published his observations on the negative effects of some EFA deficiency on CVD. He strengthened his hypothesis noting the low incidence of mortality rate from CHD (coronary heart disease) in Greenland Eskimos, a population consuming a high fat diet, but rich in $n-3$ FAs [33]. Late in the seventies, Sinclair's group and others confirmed the positive association between the high dietary intake of EPA and DHA of Greenland Inuits and lower rate of death from acute myocardium infarction (MI) compared to a Danish population, although these two groups consumed similar amount of total fat (about 42% of total calories) and showed comparable level of blood cholesterol [34,35]. Similarly, Japanese population eats more fish than North Americans and presents a lower rate of acute myocardial infarction, atherosclerosis and other ischemic pathologies [36,37].

Among the possible mechanisms that may contribute to the cardiovascular benefits of $n-3$ FAs, their ability to decrease triglycerides and VLDL has been reported, with moderate rise in HDL, whereas $n-6$ FAs do not [16,35,38]. On the opposite, the GISSI study showed only a very small decrease in triglyceride concentrations and no clinically significant changes in cholesterol [39]. Overall, $n-3$ FAs do not seem to have a very significant effect neither in lowering blood lipids, nor fibrinolysis and plasminogen activator inhibitor-1 (reviewed in [38]), generating a paradox on their protective effects against CHD. More recent reviews analyzed the past and recent achievements in favor of the cardiovascular benefits of $n-3$ FAs. In these studies (summarized in Table 2 of [40]; Tables 4 and 5 of [35]; Table 1 of [41]), epidemiological and interventional approaches clearly demonstrated that individuals with a diet rich in fish (30–35 g/day, or, alternatively, one fish meal per week), or supplemented with EPA and DHA (up to 665 mg/day) showed a 30–50% reduction in CHD and CHD-related mortality compared to those who ate no fish. Of particular interest are two intervention studies, the DART study [42] and the GISSI Prevention trial [39,43], which strongly support the anti-arrhythmic effects of $n-3$ FAs. Both studies were conducted on post-MI patients who received a fish rich diet (200–400 g/week), or fish oil capsule (500–850 mg/day of EPA/DHA), showing a significant reduction of both cardiac death and total mortality (30–45%) within 3–4 months from the treatment. Despite the existing differences between these two studies, the absence of a significant reduction in the incidence of recurrent non-fatal MI, compared to the high significant reduction of sudden death and recurrent fatal MI indicate a protective mechanism of $n-3$ FAs in preventing fatal arrhythmias, rather than anti-thrombotic or anti-atherosclerotic effects [42,43]. It is worthwhile to note that the administration of ALA or ALA-containing food to replace fish or EPA/DHA for those who dislike or cannot eat fish do not modify the conclusions. In fact, two intervention trials, the Lyon Diet Heart Study [44] and the Indian Diet Heart Study [45], confirmed that an ALA rich diet may improve prognosis in patients with a first episode MI. However, in the former, the consumption of ALA (1.8 g/day) was associated with a Mediterranean style diet, leaving doubt that the reduction in sudden cardiac death could be due to other ingredients present in the diet. In the latter study, the increased consumption of vegetables rich in ALA was part of a low-fat diet (24–28% of the total calories). Also in this case, the interpretation of the result is based on the specific experimental design. Finally, in the Indian Experiment of Infarct Survival [46], fish oil capsules (1.08 g/day EPA plus 0.72 g/day DHA) and mustard seed oil (2.9 g/day ALA) behaved similarly in reducing total cardiac death and risk of cardiac arrhythmias in patients treated for 1 year starting 24 h after a first episode of MI.

The molecular explanation for the anti-arrhythmic effects of $n-3$ FAs are still a matter of opinion and further studies are required to confirm or exclude the different hypothesis formulated. Human data are strongly supported by observational and interventional studies, but lack a mechanistic demonstration from a molecular point of view. Data obtained on animal models and cultured cardiomyocytes suggest that the anti-arrhythmic effects of $n-3$ FAs are due to the ability of ALA and EPA/DHA to influence the activity of myocyte sarcolemma ion channels (sodium and L-type calcium). In fact, these EFAs/PUFAs are able to: (i) increase the threshold of ventricular fibrillation; (ii) increase heart rate variability; (iii) reduce ischemic damage (reviewed in [35,47]). Although simplistic, the final explanation may reside in the synergistic interaction of two or more of these mechanisms.

A remarkable aspect of the $n-3$ FA treatments is that they are well tolerated and have no serious side effects during the trials. Following a prolonged supplementation, bleeding complications may have been expected, which was never reported in the literature [48].

More controversial is the role of $n-6$ FAs in cardiovascular disease prevention. Earlier studies have shown that LA improves lipid profile by lowering total cholesterol and LDL-cholesterol and slightly increasing HDL-cholesterol (reviewed in [40]), whereas others seem to contradict these conclusions

[16,35,38]. More recently, the ability of $n-6$ FAs to increase oxidation susceptibility of lipoproteins (LDL and VLDL) has been evoked as a possible mechanism to explain adverse effects of a diet high in six FAs against CVD [49]. However, $n-3$ FAs contains even more unsaturations. In fact, fish oil FAs adversely raise the susceptibility of LDL to copper-induced and macrophage-mediated oxidation [50]. Perhaps, this paradox diminishes the force of the oxidation argument in establishing the role of EFAs/PUFAs in CVD.

Actually, one of the main concerns regarding the dietary intake of $n-6$ FAs is related to the synthesis of specific eicosanoids (see above). In fact, the 2-series prostaglandins, such as PGE_2, PGI_2 and TXA_2, and the 4-series leukotrienes, such as LTB_4, derived from AA (Fig. 1) exert a more potent pro-inflammatory effect compared to 3-series prostaglandins, such as PGE_3, PGI_3 and TXA_3, and the 5-series leukotrienes, such as LTB_5, derived from EPA (Fig. 1). Theoretically, lowering the concentration in the lipid rafts of AA in favor of EPA may indicate decreased production, from specific cell types (monocytes, neutrophils and eosinophils), of AA-derived mediators of inflammation and a diminished ability for platelets to produce the pro-thrombotic agent TXA_2. Contrarily, increasing EPA intake and, consequently, decreasing the lipid AA/EPA ratio, result in the production of anti-inflammatory (PGE_3), or even less inflammatory compounds and thromboxanes with reduced pro-aggregatory and vasoconstrictive properties (TXA_3). Although this picture is described and sustained by several authors [5,8,16,35,40,48,51], it is prone to criticism. First, GLA is converted to DGLA, which, in a specific dietary situation may accumulate in cells, compete with AA and drive the synthesis of PGE_1, a potent vasodilator and platelet anti-aggregator prostaglandin [38] (Fig. 1). Similarly, AA strongly enhances the endothelial cell synthesis of PGI_3 from EPA, in stimulating COX enzymes rather than prostacyclin synthase [52]. Second, PGI_3, derived from EPA metabolism, possesses similar antiaggregatory and vasodilation effects compared with PGI_2 derived from AA [51]. Third, lipoxins represent a class of AA metabolites that carry potent immunoregulatory and anti-inflammatory properties. Finally, earlier prospective cohort studies support the view that increasing dietary LA intake is protective, or, at least, not detrimental relative to CVD [53–56]. More recently, the analysis of 25 case-control and prospective studies investigating the association between FA composition and CVD risk confirmed the cardioprotective effect(s) of $n-3$ FAs, but also suggested that lower LA content was associated with increased risk for non-fatal events. Only the level of AA in adipocytes, but not in PL-rich tissues, was associated with increased CHD risk [57]. Overall, this and other studies do not reach the conclusion that elevated tissues AA and LA are detrimental in terms of cardiovascular risk [57–59].

4. The $n-6/n-3$ EFA/PUFA ratio

Several authors tended to explain the EFA/PUFA effects in terms of a balance between total $n-6$ and $n-3$ FAs, rather than the absolute amount of each single molecule. The importance of the $n-6/n-3$ ratio has been evoked not only in the pathogenesis of cardiovascular diseases, but also in cancer, inflammatory and autoimmune diseases. In the most simplistic interpretation, a very high $n-6/n-3$ ratio is considered detrimental for human health, while a value as much as possibly close to 1 is considered protective against degenerative pathologies.

In his pivotal study dated 1991, Simopoulos first defined the importance of the $n-6/n-3$ FA ratio [60]. His study also includes anthropological data, since in the Palaeolithic period (40,000 years ago), he noted, the human diet was much lower in saturated fatty acids and contained small but roughly equal amounts of $n-6$ and $n-3$ FAs. Unfortunately, no data are available on the healthy status of our ancient predecessors. A dramatic change in human diet and lifestyle occurred over the past 10,000 years, with the Agricultural revolution which introduced in the diet cereals and grains high in $n-6$ FA. During the last 150 years human population experienced a drastic increase in consumption of vegetable seed oil rich in $n-6$ FAs and a parallel decrease of $n-3$ FAs intake. The result is the actual $n-6/n-3$ FA ratio which in the Western diet ranges between 15:1 and 20:1 [61,62].

Circumstantial evidence has been assembled in favor of the importance of the $n-6/n-3$ FA ratio. As discussed above, the scapegoats remain the AA-derived eicosanoids which, if formed in large amounts, increase the ratio and contribute to the formation of thrombus and atheromas, allergic and inflammatory disorders and abnormal cell proliferation [61]. It is less clear how ''abundant'' should the production be of these biologically active molecules in order to generate their putative negative effects. Several clinical intervention studies support the view that decreasing the $n-6/n-3$ FA ratio results in an increased protection against degenerative diseases (reviewed in [61,62]). As an example, replacing corn oil (high in LA) with olive and canola oil (low in LA) to reach a 4:1 ratio of LA/ALA, a 70% decrease in total mortality was observed [63]. Healthy subjects fed with a typical Swedish diet with a $n-6/n-3$ FA ratio measured in the serum of 4.72:1, showed increased leukocytes, platelets and VEGF when compared to individuals who ate a Mediterranean-style diet (serum $n-6/n-3$ FA ratio of 2.6:1) [64]. A 2.5:1 $n-6/n-3$ FA ratio (but not a 4:1 ratio) obtained by increasing fish oil intake suppressed rectal epithelial cell proliferation and PGE2 synthesis [61,65]. Several cellular and animal models support the negative effects of a high $n-6/n-3$ FA ratio in the diet. Using adenoviral transfer, the cDNA coding for $n-3$ desaturase, the enzyme which converts $n-6$ to $n-3$ series, absent in mammalians but present in worms (*Caenorhabditis elegans*) (Fig. 1), was transferred to rat cardiomyocytes and MCF-7 cells. In the first case, cells were able to convert various $n-6$ FAs to the corresponding $n-3$ series, changing the $n-6/n-3$ ratio from about 15:1 to 1:1. In the case of MCF-7, cancer cells died by apoptosis compared to controls which maintained a higher $n-6/n-3$ FA ratio [66]. The same group also showed that transgenic mice expressing the *C. elegans fat*-1 gene coding for $n-3$ desaturase, resulted in an abundance of $n-3$ and a reduction in $n-6$ FAs in the organs and tissues of these mice who appeared to be normal and healthy for four generations [67].

An opposing view considers that the $n-6/n-3$ FA ratio is of little value from a theoretical and experimental point of view, creating confusion in the field and diluting the main

interest represented by increasing the intake of $n-3$ FAs [56,57,68]. According to these authors, "without knowledge of the absolute value of the numerator and the denominator, the meaning of a given ratio, whether as a biomarker or dietary target, will be impossible to discern" [56]. This conclusion is the result of different arguments, such as: (i) there are several independent and nutritionally unrelated strategies to decrease the $n-6/n-3$ ratio (summarized in Table 1 of [56]); (ii) apparently, the $n-6/n-3$ FA ratio does not distinguish among the different classes of EFAs/PUFAs, putting on the same level the LA:ALA versus the AA:EPA/DHA ratio which is questionable from a functional and biochemical point of view; (iii) evidence suggesting that an increased intake of $n-3$ FAs reduces risk for CVD is strong, while it is still a matter of opinion that the same effect can be obtained by decreasing the levels of $n-6$ FAs. It is worthwhile to note that ethnic differences in the percentage of all deaths from CVD in Europe and United States versus Japan is 45% and 12%, respectively, corresponding to a significant difference in the concentration of EPA in thrombocyte phospholipids (0.5% versus 1.6%), while the percentage of AA is similar in the two groups (26% versus 21%) [61]. In other words, the difference in EPA concentration gives the same indication as the $n-6/n-3$ FA ratio; therefore, there is no apparent need to look at this ratio, but simply to the $n-3$ FA concentration.

Recently, von Schacky and Harris proposed "the omega-3 index" as a new risk factor for sudden cardiac death [68]. It is defined as the percentage of EPA + DHA of total fatty acids in erythrocytes and it should reflect the $n-3$ FA status in a given individual. Although the authors bring convincing evidence for the clinical and preventive efficacy of the omega-3 index (reviewed in [68]), further studies are needed to validate this novel biomarker for cardiovascular risk.

5. Conclusions

From the topics discussed above, light and shade rise on the effects of EFA/PUFA intake on human health. Paradoxically, the abundance of the literature in this field complicates the analyses since it is very easy to meet studies with similar experimental design that reach opposite conclusions. In addition, under the common umbrella of long-chain unsaturated fatty acids, a plethora of molecules resides with different chemical structure, metabolism, bioavailability, biological functions.

Despite these difficulties, several conclusive points emerge from the analysis of the existing data. We agree with the guidelines of the major American and European heart associations which recommend the intake of 1 g/day of EPA and DHA for secondary prevention, treatment of post-MI and prevention of sudden cardiac death and other cardiovascular dysfunctions [69,70], despite the results of a Cochrane analysis reaching the opposite conclusions and stating that "long chain and shorter chain omega-3 fats do not have a clear effect on total mortality, combined cardiovascular events, or cancer" [71]. The section of the study regarding CVD have been formally rejected by the Society for the Study of Fatty Acids and Lipids [68].

The strong conclusions of the GISSI [39,43] and other studies [42,44,46] make difficult to reject the efficacy of $n-3$ FAs in the secondary prevention of cardiovascular events. More difficult is it to extrapolate from these studies the recommendation to supplement also the diet of healthy people with fish oil capsules, including in the potential cardiovascular risk subjects who do not present any evident symptom of cardiac suffering. Certainly, for those who will pursue this preventive strategy, an undoubted advantage is represented by the virtually lack of any deleterious and side effects of $n-3$ FAs and the possibility to find EPA/DHA as oral preparations at low cost in drug store without prescription and at a relatively low cost [48].

If the protective role of $n-3$ FAs in CVD is better defined, more controversial is the interpretation of data regarding $n-6$ FAs, which are more abundantly present in the human diet and, sometime, exceed the recommended AI fixed at 5–10% of total energy.[1] When hypotheses are formulated on the mechanism(s) of action of $n-6$ FAs, it should be kept in mind that, from a quantitative point of view, the largest amount of dietary FAs undergoes two main fates: (i) beta-oxidation to acetyl-CoA to enter the Kreb's cycle generating ATP (20–30%) and *de novo* synthesis of long chain FAs; (ii) storage in adipocytes (15–80%) after travelling in the bloodstream as lipoproteins. The biosynthesis of different classes of eicosanoids, as well as the effects on gene expression, is strictly dependent upon the intracellular concentrations and bioavailability of different EFA/PUFA derivatives. Substantial alteration of their fine regulation is not achievable by small changes in the diet, but only after administration at massive doses of EFAs/PUFAs, as those reported in many experimental studies.

Clinical and nutritional studies largely agree on the delicate balance in a healthy diet between saturated FAs, MUFAs and PUFAs and their role in LDL/HDL composition and oxidation, with MUFA being the most efficacious. In fact, OA in virgin olive oil, when substituted for saturated FAs, lowers LDLs and increases their resistance to oxidation. The mechanism whereby incorporation of PUFAs into LDLs enhances susceptibility of LDL oxidation has been studied extensively. Nonetheless, the hypothesis suggesting that a diet rich in $n-6$ FAs increases the PUFA content of LDL particles and increases their susceptibility to oxidation, which in turn leads to atherosclerosis and CVD, still needs to be substantiated in human studies before measures of oxidation can be used as adequate indicators of chronic disease [72]. Although, the upper intake limit (UL) for $n-6$ and $n-3$ series FAs has not been established, the AI for LA is of 17 g/day for young men and 12 g/day for young women, while for ALA is of 1.6 and 1.1 g/day for men and women, respectively [72]. Recent estimations of PUFA consumption in the food chain in Europe [4] and United States [73] indicate that the ratio of $n-6/n-3$ FAs is still much higher than that recommended. In terms of healthy nutrition, increasing the concentration of PUFAs in the diet over the AI of 5–10% is potentially dangerous for two reasons: (i) increased AI for total fats; (ii) increased amount of $n-6$ FAs potentially prone to LDL oxidation. Although favorable changes in the $n-6/n-3$ FA ratio can be achieved more easily by decreasing total fats and $n-6$ FAs intake, this does not necessarily mean that EPA/DHA will increase

[1] Dietary Reference Intakes report 2002/2005 at www.nap.edu.

accordingly. In fact, chronically increased consumption of ALA results in an increased EPA concentration in plasma and cell pools, but an insufficient conversion to DHA [13].

The view that $n-6$ FAs have a strong tumor-enhancing effect, whereas the $n-3$ series possesses a protective effect is also prone to criticism (reviewed in [16]). Although the present review does not specifically address the association between cancer risk and EFA/PUFA intake, many studies comfortably conclude that the epidemiological evidence largely contradicts the studies on cell lines and animal models. At least to date, no association between $n-6$ and $n-3$ FAs and risk of several types of cancer has been detected [74]. Some studies showed a correlation between total fat intake and increased risk, while others failed to demonstrate this relationship. These contradictory results also remained in interventional studies performed to address the beneficial effects of fish and fish oil supplementation (Table 4 in [48]).

In conclusion, the most significant effect of EFAs/PUFAs in terms of human health is the $n-3$ FA protection in the secondary prevention of sudden cardiac death due to arrhythmias. Whether this indication can justify the application of a preventive program of dietary supplementation of $n-3$ FAs to the general population is still debatable. The dietary recommendations to increase the consumption of fish or $n-3$ FA rich vegetables, for those who dislike fish, remain. Regardless, it is important to know the type, the size (larger fish can be subjected to methyl mercury contamination) and from where the fish have been procured. Dietary recommendations should distinguish between ALA and EPA/DHA and would be preferred to be made on a mass basis (g/day) of $n-3$ FAs to be consumed, strongly considering, at individual level, the intake of total energy, total fats and $n-6$ FA intake.

The apparent absence of deleterious side effects of EFA/PUFA treatments, accordingly to the Hippocratic aphorism: *primum nil nocere*, is neither a conclusive demonstration of their efficacy, nor a suggestion for an indiscriminate supplementation. It is necessary to intensify the scientific efforts in order to clarify many controversial aspects of EFA/PUFA consumption in order to make human diet healthier.

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