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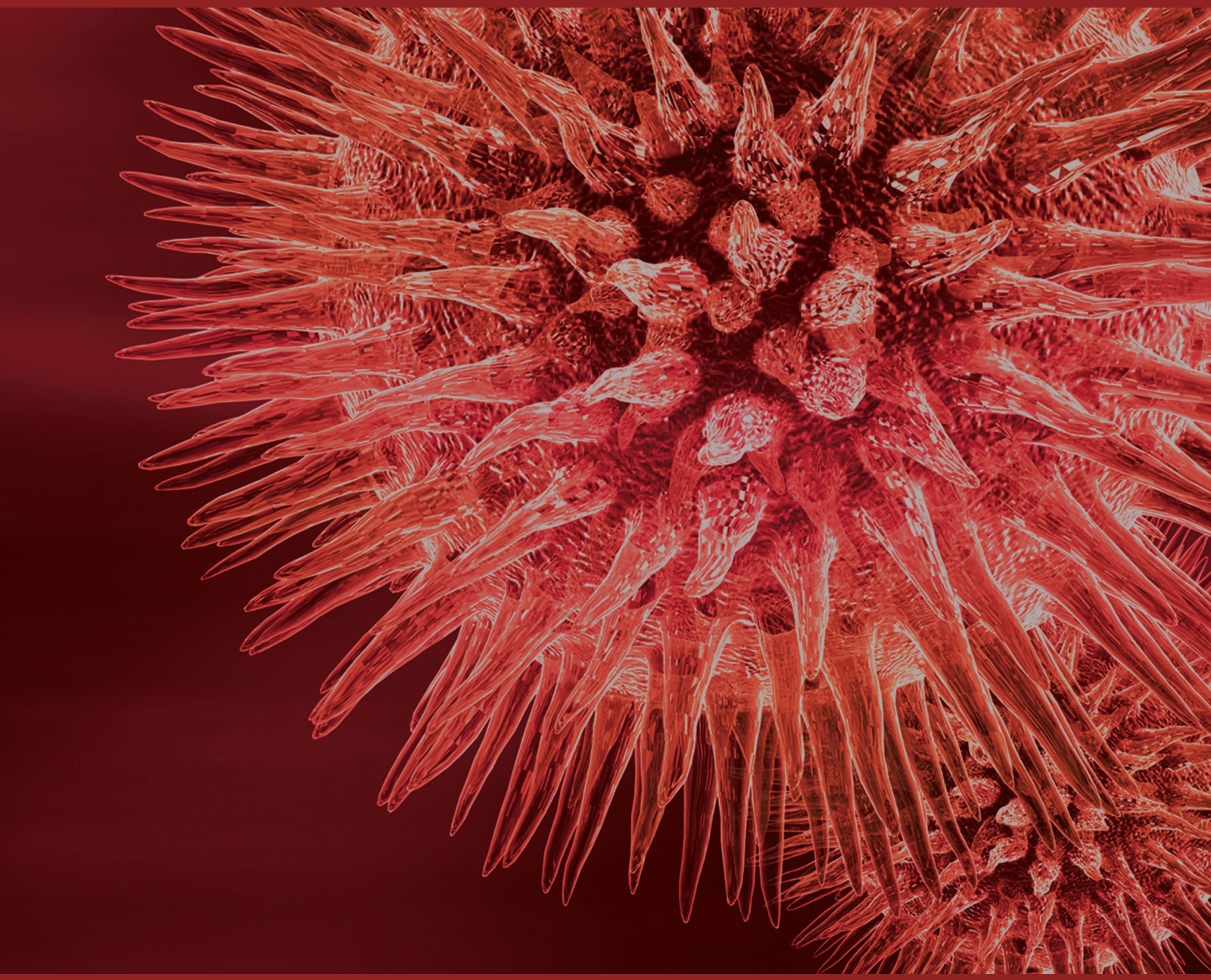
ω-3 PUFAs in the Prevention and Cure of Inflammatory, Degenerative, and Neoplastic Diseases 2014

Guest Editors: Achille Cittadini, Gabriella Calviello, Hui-Min Su, Karsten Weylandt, Yong Q. Chen, and Kyu Lim



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Editorial

ω-3 PUFAs in the Prevention and Cure of Inflammatory, Degenerative, and Neoplastic Diseases 2014

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While still a controversial topic, the omega-3 fatty acids as subject of research have come of age, particularly in recent years. They have made their way from being simple nutrition components, through possibly some exaggerations nominating them as universal tool to improve human health, to being the object of research in a wide variety of preclinical basic-research contexts as well as in smaller and larger clinical studies with mixed results regarding their potential health effects.

This is reflected in the wide variety of organ effects described in this special issue. The papers presented here range from the assessment of biological effects of omega-3 PUFA in vascular disease (M. Zanetti et al.; C. S. Bürgin-Maunder et al.) to their potential role in the prevention of Alzheimer's disease (T. Köhnke et al.), as dietary approach in the regulation of Helicobacter pylori-associated gastric diseases (J.-M. Park et al.), as combination treatment with anti-estrogens for breast cancer prevention (A. Manni et al.), and to modulate apoptosis and proliferation in the placenta (E. Wietrak et al.). A particular focus of papers in this special issue is the lung, both regarding the acute respiratory distress syndrome (P. Cotogni et al.; M. Garcia de Acilu et al.) and regarding potential antitumor effects mediated by DHA effects on the mTOR/AMPK/PI3K-Akt pathway (N. Kim et al.).

This brings us to some of the most important issues in omega-3 PUFA research right now. This special issue presents several papers regarding mechanisms behind the biological effects of omega-3 PUFAs, in comparison to resveratrol in the context of inflammation, through their resolvin metabolites in the context of pain (J. Y. Lim et al.), as modulators of arachidonic acid lipid mediator cascade reactions (B. Lands), and through generation of biological active omega-3 PUFA derived electrophilic derivatives (C. Cipollina). It is probably the most important contribution of omega-3 PUFA research in recent years that a field of newly discovered and characterized metabolites from omega-3 PUFA has led our focus on metabolic products derived from omega-3 PUFA which are valid biologically active compounds by themselves.

Notwithstanding all these findings, there is also the role of the omega-3 PUFA as metabolic modulators and fuel for beta-oxidation. Both aspects, lipid metabolites modulation inflammation and metabolic effects of the fatty acids, are reflected in the context of obesity and hypercaloric diets and discussed in this special issue with regard to inflammation (Y. Wang and F. Huang) and mitochondrial function in the context of high fat diets (A. Ferramosca et al.). This focus on mitochondria then leads directly to the role of omega-3 PUFAs in energy metabolism, glycolysis, and the Warburg effect in cancer (L. Manzi et al.).

Finally, two papers address issues of omega-3 PUFA supply and administration, exploring approaches for heterologous reconstitution of these PUFAs in *Arabidopsis* (S. H. Kim et al.) and the pharmacokinetic profile of administering eicosapentaenoic acid (EPA) as free fatty acid in a stomach-protective capsule formulation (E. Scaioli et al.).

Given all these lines of research, the concluding review in this special issue then aims at the outline of a way forward for human studies with omega-3 PUFAs, in order to address and subsequently eliminate the uncertainties that are currently present in this exciting field of biomedical research (K. Weylandt et al.).

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Review Article

Combination of Antiestrogens and Omega-3 Fatty Acids for Breast Cancer Prevention

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The molecular and biological heterogeneity of human breast cancer emphasizes the importance of a multitargeted approach for effective chemoprevention. Targeting the estrogen receptor pathway alone with the antiestrogens, Tamoxifen and Raloxifene reduces the incidence of estrogen receptor positive tumors but is ineffective against the development of hormone independent cancers. Our preclinical data indicate that the administration of omega-3 fatty acids potentiates the antitumor effects of Tamoxifen by inhibiting multiple proliferative and antiapoptotic pathways, several of which interact with estrogen receptor signaling. The complementarity in the mechanism of antitumor action of Tamoxifen and omega-3 fatty acids is well supported by our signaling, genomic, and proteomic studies. Furthermore, administration of omega-3 fatty acids allows the use of lower and, hence, likely less toxic doses of Tamoxifen. If these findings are supported in the clinical setting, the combination of omega-3 fatty acids and anteistrogens may emerge as a promising, effective, and safe chemopreventive strategy to be tested in a large multi-institutional trial using breast cancer incidence as the primary endpoint.

1. Efficacy and Limitations of Antiestrogens as Chemopreventive Breast Cancer Agents

Prevention represents the optimal method to reduce breast cancer morbidity and mortality. The two selective estrogen receptor modulators, Tamoxifen and Raloxifene, have been shown to be effective chemopreventive agents by reducing the incidence of estrogen receptor positive breast cancer by 50% and 38%, respectively [1, 2]. However, the wide applicability of these interventions to the population of women at large is limited by toxicity such as thromboembolic events as well as endometrial cancer in the case of Tamoxifen which, though rare, are significant when considering that these drugs are given to healthy women for prevention. The acceptance of Tamoxifen and Raloxifene for reducing breast cancer risk has indeed been shown to be poor [3]. Of the approximately 2 million U.S. women who could potentially benefit from treatment with Tamoxifen, only 4% of those at increased risk for breast cancer and only 0.08% of all U.S. women 40–79 years of age have accepted the use of this drug for chemoprevention [4–6]. A recent survey conducted in high-risk women indicates that they perceive that antiestrogens do not lower their risk of breast cancer sufficiently to justify the use of potentially toxic drugs [3]. The steroidal aromatase inhibitor exemestane has been shown to reduce the annual incidence of invasive breast cancer by 65% after a median follow-up period of three years [7]. Whether this drug will be more acceptable to the general public remains to be determined.

An additional limitation of Tamoxifen and Raloxifene is that neither drug reduces the incidence of estrogen receptor

negative tumors [1, 2]. This deficiency is likely to be explained by the fact that multiple cellular pathways, in addition to the estrogen receptor, contribute to breast cancer development. Therefore, in order to optimally inhibit mammary carcinogenesis, a multitargeted approach is needed employing interventions with complementary mechanisms of action leading to increased chemopreventive efficacy and reduced toxicity. As discussed in this chapter, we believe that the addition of omega-3 fatty acids (n-3FA) to antiestrogens will increase the spectrum of molecular subtypes of breast cancer which can be prevented. In addition, we believe that this combined approach will be more acceptable in view of the perceived health benefits derived from n-3FA ingestion and the possibility of using lower and, hence, less toxic doses of antiestrogens as a result of their expected synergism with n-3FA in reducing mammary carcinogenesis.

2. Omega-3 Fatty Acids and Mammary Carcinogenesis

2.1. Epidemiological Studies. The influence of diet on breast cancer development remains controversial. The contribution to mammary carcinogenesis of the specific fatty acid composition of the diet has received considerable attention in the literature. Among the fatty acids, n-3FA and n-6FA have been suggested to decrease and increase breast cancer risk respectively [8]. Despite the perception that n-3FA protect against breast cancer, epidemiological studies have yielded inconsistent results [9, 10]. While some studies have shown an association between n-3FA intake and reduction in breast cancer risk, others have not shown this association and one has actually reported an increased risk of breast cancer with high n-3FA intake [10]. However, a recent meta-analysis of data from 21 independent prospective cohort studies revealed that dietary intake of marine n-3FA was associated with a 14% reduction in breast cancer risk [11]. Importantly, a dose-response effect was noted with a 5% lower risk of breast cancer per 0.1 gm per day increment of n-3FA intake [11].

2.2. Preclinical Studies. In experiments conducted both in a prepubertal [12] and postpubertal model [13] of MNU-induced rat mammary carcinogenesis, we observed that administration of fish oil providing clinically achievable ratios of n-3FA : n-6FA (up to 2.3) had a marginal antitumor action of its own and modestly influenced a variety of host and tissue biomarkers potentially involved in mammary carcinogenesis [14]. When similar clinically relevant ratios of n-3FA : n-6FA were tested in transgenic models of mammary carcinogenesis, we observed a protective effect in the HER-2neu model, a well-established model of estrogen receptor negative breast cancer (unpublished observations) in agreement with a previous report [15] but no protection in polyoma middle T transgenic mice [16]. These results suggest that gene/diet interactions play a critical role in the development of breast cancer.

These variable results prompted us to perform a critical review of the preclinical data on the role of n-3FA in mammary carcinogenesis. Our review of the literature, stemming over 30 years of investigation, produced similarly mixed results [17]. We found that the quality of the experiments varied so markedly that it was difficult to compare results across studies. In our review, a series of recommendations was made concerning the experimental approaches that would serve to guide the design of experiments with the potential of resolving the fish oil-breast cancer conundrum. These included (1) use of translationally relevant diets with 30% of calories from fat with equal distribution between monounsaturated, polyunsaturated, and saturated fats (e.g., 10% each); (2) experimental verification of FA composition of the diets in view of the multifactorial variability of n-3FA sources and bioavailability; (3) analysis of FA in the plasma and within target tissues for better comparison of results across studies; (4) consideration of variability in FA metabolism due to genetic polymorphism of related enzymes. Mindful of these issues, we decided to formulate a series of purified diets modeled after the AIN-93G formulation but with the major exception that the level of dietary fat was modified to reflect that currently recommended in the U.S. dietary guidelines. Thus, diets were formulated to provide 30% of dietary calories from fat and an equal amount of those calories from saturated, monounsaturated, and polyunsaturated fatty acids. Within the polyunsaturated fatty acids, we sought to vary the ratio of n-3FA : n-6FA from 25 : 1 to 1 : 25 to provide a robust evaluation of the role of this ratio in affecting the postinitiation phase of chemically induced mammary carcinogenesis [18]. In these experiments, at 21 days of age, female Sprague-Dawley rats were injected with 50 mg N-methyl-N-nitrosourea/kg body weight intraperitoneally. Seven days following carcinogen injection, all rats were randomized to the different diets ($n = 30$ rats/group). We observed that a calculated n-3FA : n-6FA dietary ratio of at least 10 : 1 was necessary to obtain a significant chemopreventive effect on MNU-induced mammary carcinogenesis. We also observed that increasing levels of dietary n-3FA resulted in a progressive reduction of mammary gland density ($R = -0.477$, $P = 0.038$) which was predictive of the carcinogenic response (Figures 1(a) and 1(b)) [18]. Increasing dietary amounts of n-3FA caused a significant decrease in plasma leptin and IGF-I while adiponectin levels increased [18]. However, neither cytokine was predictive of mammary gland density. In contrast, we observed a significant relationship between plasma IGF-I concentration and mammary gland density ($R = 0.362$, $P < 0.005$; Figure 1(c)) [18]. In the aggregate, these results provide evidence for the first time that breast density, a validated biomarker of breast cancer risk in women [19, 20], is a valuable screening tool for chemopreventive studies in preclinical models of breast cancer. The data also point to the importance of the IGF-I pathway in mediating the antitumor action of n-3FA. Following these observations, we performed an extensive analysis of the molecular signature underlying inhibition of mammary carcinoma by dietary n-3FA [21]. In these experiments, we analyzed tumors obtained from rats which were fed diets in which the ratio of n-3FA : n-6FA was either 0.7 (low n-3FA, control) or 14.6 (high n-3FA). We observed that cell proliferation assessed by Ki67 immunostaining was reduced by 60% in carcinomas from

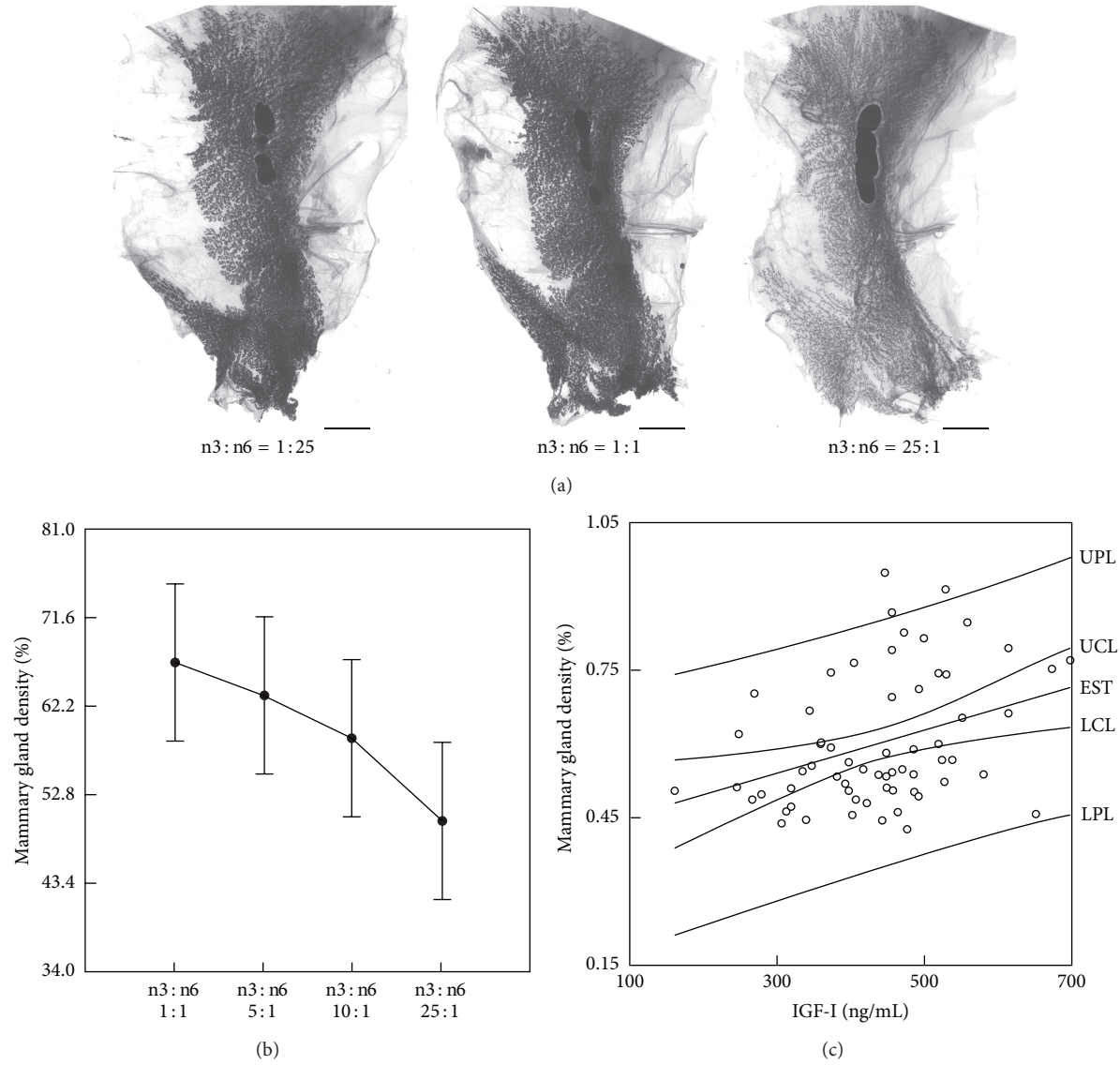

Figure 1: (a) Carmine-stained abdominal-inguinal mammary gland whole mounts depicting the effect of increasing dietary n-3FA : n-6FA on breast histology, bars = 0.5 cm. (b) Mammary gland density analysis shows a decreasing trend in density as n-3FA : n-6FA increases. The methodology for measurement of mammary gland density is described in detail in our publication [18]. Briefly, whole mounts of the abdominal-inguinal mammary gland chains were photographed and the images obtained were digitized. Digital images of the whole mounts were captured using a semiautomated image acquisition system. Images were evaluated for total area of the mammary fat pad occupied by mammary epithelium as well as total area of the fat pad encompassed by the mammary ductal tree. Area occupied by the mammary epithelium divided by the total area encompassed by the mammary ductal tree was calculated. (c) Linear regression of mammary gland density and IGF-I upper prediction limit (UPL); upper confidence limit (UCL); estimate (EST); lower confidence limit (LCL); lower prediction limit (LPL). Reproduced with permission from [18].

the high n-3FA : n-6FA (14.6 ratio) treatment group and was associated with a reduction in the levels of cyclin-D1 and phospho-Rb as well as an increase in the levels of two cyclin dependent kinase inhibitors, p21 and p27, as determined by Western blotting and densitometric analysis. These changes are consistent with a block at the G1-S transition induced by the high n-3FA diet. The apoptotic index (computed as the number of apoptotic cells divided by the total number of cells counted) was significantly increased by 29% in carcinomas from the high n-3FA : n-6FA group. Relative to apoptosis and consistent with the elevated apoptotic index observed in the high n-3FA diet group, the level of cleaved PARP (PARP89/116 ratio) was elevated as were levels of Bax and Apaf-1, whereas the level of Bcl-2 was not significantly affected. These changes are indicative of the induction of apoptosis via the intrinsic pathway. The suppressive effect of n-3FA on proliferation was

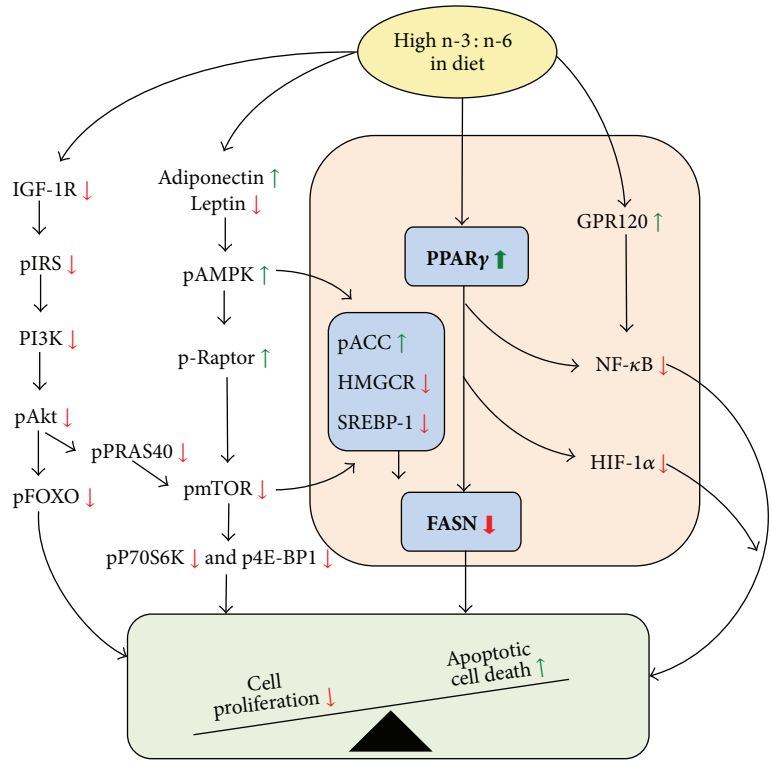

Figure 2: Cellular processes regulating transcription factors, insulin signaling, and lipid synthesis that are likely to account for the effects on cell proliferation and apoptosis in mammary carcinomas of rats fed high versus low (control) dietary ratio of n-3 : n-6 fatty acids. Diameter of red (decreased expression) and green arrows (increased expression) indicates magnitude of effect and font size of stated proteins indicates relative importance as determined by Orthogonal Projections to Latent Structures for Discriminant Analysis (OPLS-DA) (Refer to [21] for detailed description of the method). Peroxisome proliferator-activated receptor gamma (PPARγ) and to a lesser extent, G-coupled protein receptor 120 (GPR-120) attenuate inflammation via direct or indirect effects on nuclear factor kappa B (NFκB) and hypoxia-inducible factor-1α (HIF1α). PPARγ affects multiple targets in lipid metabolism including fatty acid synthase (FASN). In addition, high dietary n3 : n6 is accompanied by reduced activity of the mammalian target of rapamycin (mTOR) as reflected in the reduced phosphorylation of its downstream targets including 70-kDa ribosomal protein S6 kinase (P70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which in turn exert effects on cell proliferation and cell survival. Mechanisms by which mTOR activity is downregulated include (1) downregulation of insulin growth factor 1 receptor (IGF-1R), phosphorylated insulin receptor substrate 1 (pIRS1), phosphoinositide 3-kinase (PI3K), phosphorylated Akt, phosphorylated Forkhead box O, and phosphorylated 40-kDa proline-rich protein (PRAS40); and (2) upregulation of phosphorylated adenosine monophosphate-activated protein kinase (pAMPK) by increased adiponectin and decreased leptin, phosphorylated acetyl-CoA carboxylase (ACC), and phosphorylated regulatory associated protein of mTOR (Raptor). Decreased phosphorylated mTOR and increased pAMPK further attenuate fatty acids synthesis via reduction of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and of sterol regulatory element-binding protein 1 (SREBP1) that results in decrease of FASN. The overall consequence of these changes in cell signaling is a decrease in cell proliferation and an increase in cell death by apoptosis. Reproduced with permission from [21].

dominant over its effect on induction of apoptosis. Using Western blotting followed by densitometry, we performed an extensive analysis of transcription factors, growth factor-related molecules and proteins involved in lipid metabolism in the attempt to identify the cellular mechanisms by which a high n-3FA diet leads to inhibition of proliferation and induction of apoptosis. The results are summarized in Figure 2 [21]. As described in detail in the figure legend, the predominant effect of high n-3FA diet was PPARγ activation resulting in suppression of lipogenesis primarily through downregulation of fatty acid synthase (FASN). In addition, high n-3FA diet suppressed mTOR pathway (well established to be critical in carcinogenesis) both by suppressing IGF-I signaling and upregulating pAMPK as a result of the reduction in leptin and the increase in adiponectin. Furthermore, the activation of pAMPK also contributed to the inhibition of lipogenesis through its effect on key regulators of lipid synthesis (pACC, HMGCR, and SREBP-1), thus potentiating the effect of PPARγ activation on this critical metabolic parameter. The fact that high ratios of n-3FA : n-6FA were required to achieve profound antitumor effects not only indicates that these biological activities are not likely to be

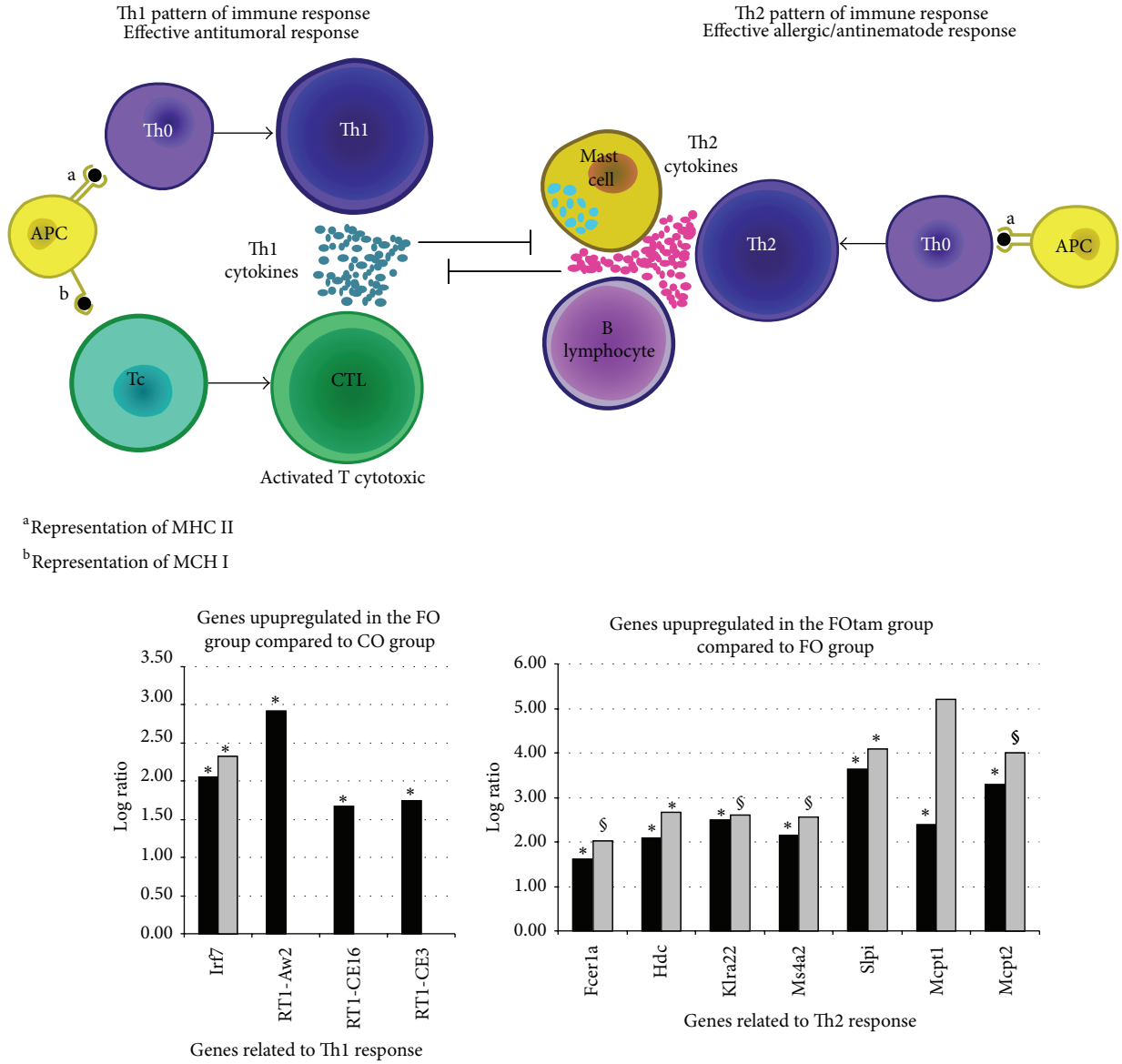

FIGURE 3: Diagram depicting the patterns of immune responses found represented among the genes upregulated by fish oil (FO) and Tamoxifen in a FO rich diet (FOtam). Graphs show side-by-side log 2 values of gene expression in microarray (black bars) and real time PCR (grey bars) of genes related to the immune response. $^{*}P < 0.05$; § $0.05 < P < 0.20$, with fold change >3.0 (log 2 > 1.58). Figure modified from [25].

achieved by dietary consumption of fish oil but also that there are specific metabolites of n-3FA that account for these effects and that are likely to be endogenously synthesized. The identification of these metabolites is currently under active investigation in our laboratories.

3. Combination of Omega-3 Fatty Acids and Antiestrogens

A major focus of research in our laboratories has been to test the antitumor efficacy and safety of the combination of n-3FA and antiestrogens for breast cancer prevention. The rationale behind this approach is based on the multiplicity of signaling pathways affected by omega-3 fatty acids (Figure 2), several of which are well known to interact with the estrogen receptor pathway [21]. For instance, there is a well-documented crosstalk between the estrogen receptor and the PPARγ receptor [22, 23], the latter being a major mediator of n-3FA effects in breast cancer cells. There is experimental evidence that inhibition of estrogen receptors with antiestrogens and activation of PPARγ synergistically downregulates the PI-3 kinase/AKT pathway and inhibits breast cancer cell proliferation [22]. Because of the complementarity of their antitumor action and the known antiproliferative effects of n-3FA in estrogen receptor negative breast cancer cell lines [15, 16,

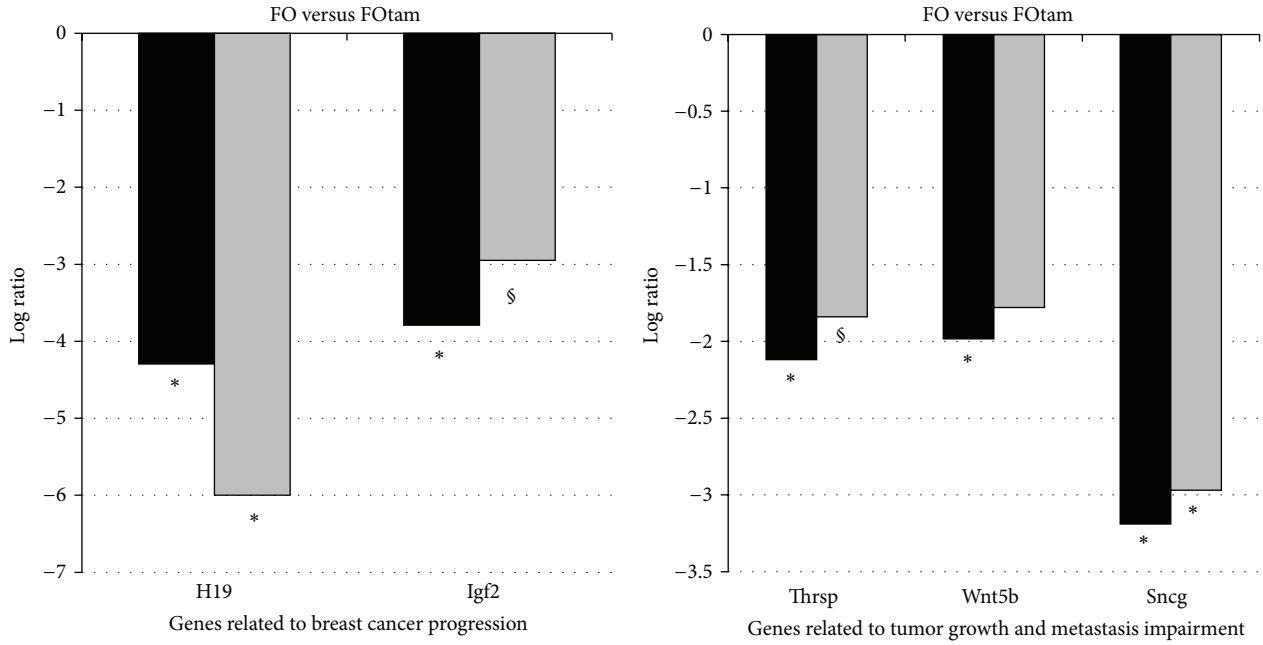

Figure 4: Side-by-side log 2 values of gene expression in microarray (black bars) and real time PCR (grey bars) of genes related to tumor profile. $^{*}P < 0.05$; § $0.05 < P < 0.20$, with fold change >3.0 ($\log 2 > 1.58$). Figure adapted from [25].

24], we believe that the chemopreventive effect of the combination of n-3FA and antiestrogens will not be restricted to ER positive tumors but will extend to ER negative tumors which are more aggressive and associated with shorter survival.

3.1. Preclinical Studies. In experiments conducted both in a prepubertal and postpubertal model of MNU-induced rat mammary carcinogenesis, we observed that administration of fish oil providing clinically achievable ratios of n-3FA : n-6FA (up to 2.3) potentiated the chemopreventive effects of Tamoxifen [12, 13]. The potential superiority of the combination was particularly evident at a suboptimal dose of Tamoxifen which, by itself, was unable to significantly decrease tumor development [12]. We recently reported a detailed time course study of the individual and combined chemopreventive effects of Tamoxifen and a high fish oil diet on multiple histologic parameters of MNU-induced mammary carcinogenesis [13]. In these experiments, groups of female Sprague-Dawley rats were injected ip with MNU at 50 days of age and randomized to either a control diet (20% corn oil) or a fish oil (FO) rich diet (10% FO + 10% CO) with or without the addition of Tamoxifen (Tam) in the diet (0.6 ppm). Rats (18/group) were sacrificed at weeks 4 (before palpable tumors), 8, and 12 (when ~90% of control rats had palpable tumors). In addition to removing palpable tumors, abdominal-inguinal mammary fat pads were excised for full histologic analysis of preneoplastic lesions classified as mild hyperplasia, modest hyperplasia, and florid hyperplasia as well as ductal carcinomas *in situ* and invasive adenocarcinomas. Our results indicated that the FO rich diet enhanced the antitumor action of Tam on all histologic parameters of carcinogenesis. Importantly, we observed that the combination treatment was the only intervention that not only inhibited the development of preneoplastic lesions but also induced regression of established ones. This effect is of particular translational relevance since it is likely that when a chemopreventive intervention is applied to women, preneoplastic lesions are already present.

To gain insight into the potential mechanisms underlying the superior chemopreventive efficacy of the combination, we have performed transcriptomic analysis (microarray followed by real time PCR validation of select genes of interest) in the tumors of control rats and Tamoxifen-treated rats each fed either a corn oil or fish oil rich diet [25]. We used gene ontology analysis and analysis of the relation of each differentially expressed gene with cancer related processes. We identified alterations in genes directly related to the biologic features of breast cancer (such as tumor differentiation and progression) as well as genes related to the immune response. Gene ontology enrichment analysis showed that administration of a fish oil rich diet resulted in the differential expression of several genes that promote a more efficient immune response against tumor development (Figure 3). In addition, tumors of fish oil fed animals that received Tamoxifen showed decreased mRNA for genes directly related to tumor growth and metastasis (Figure 4), thus indicating that Tamoxifen treatment was more efficient in a fish oil rather than corn oil diet background. On the other hand, we observed that the expression of genes associated with immunity in animals in the fish oil + Tamoxifen group indicated a shift to the Th2 pattern of immune response which may favor tumor escape (Figure 3) [25]. In conclusion, a FO rich diet resulted in the

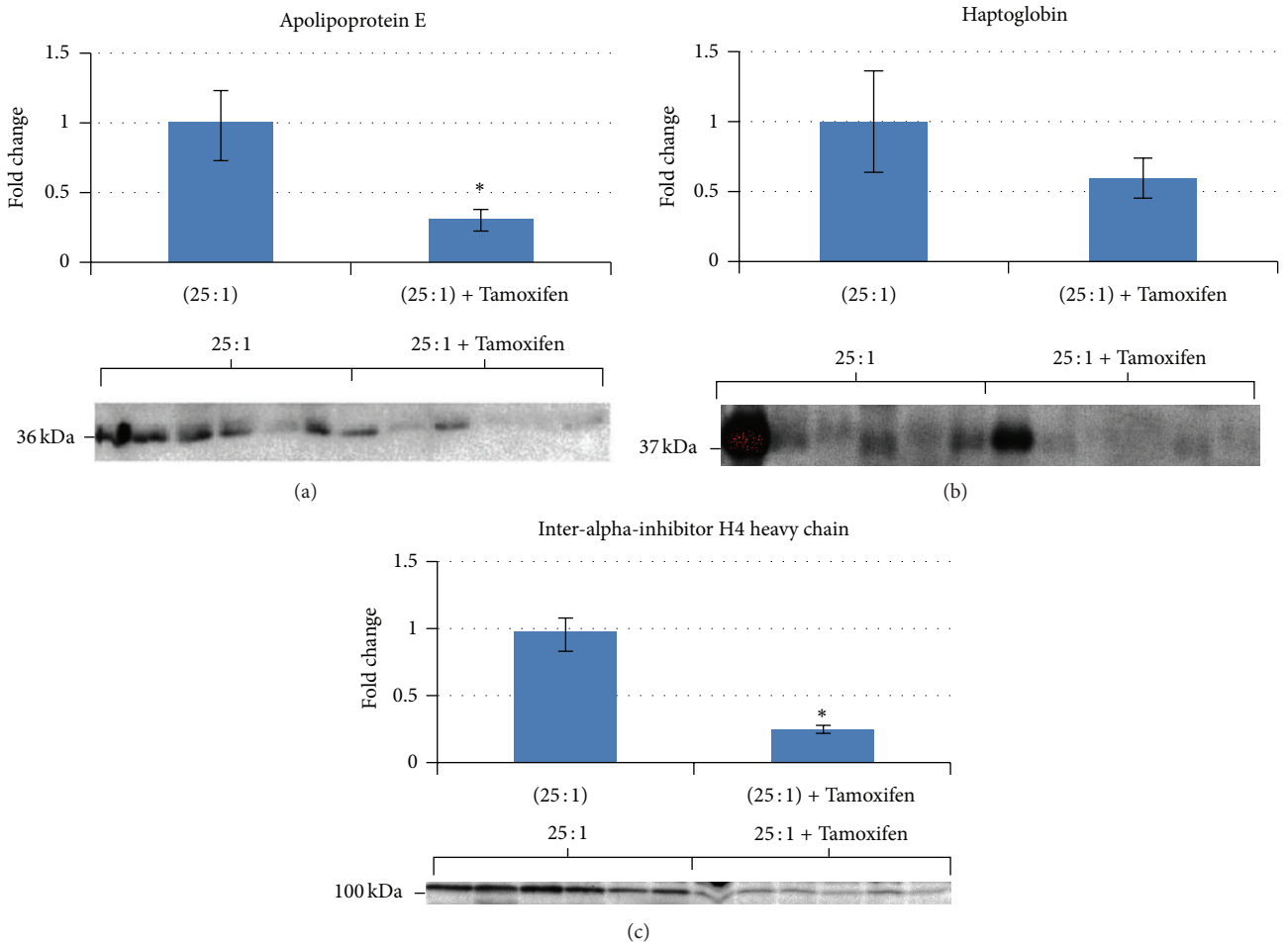

FIGURE 5: Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 25 : 1 n-3 : n-6 with 25 : 1 n-3 : n-6 plus Tamoxifen). (a) Lipoprotein E expression; (b) haptoglobin expression; (c) inter-α-inhibitor H4 heavy chain expression; $^{*}P \leq 0.05$. Reproduced with permission from [26].

differential expression of several mRNAs that encode genes that promote more differentiated tumors and a more efficient immune response against tumorigenesis compared to a CO rich diet. While genes related to tumor growth and metastasis were downregulated by Tamoxifen in FO fed rats, our data also point to a potential immunologic mechanism of tumor escape from the combined intervention.

We have also used a proteomic approach to gain insights into the mechanism of protection at the protein level by n-3FA in the absence and in the presence of Tamoxifen [26]. Using the isobaric tags for relative and absolute quantitation (iTRAQ) followed by confirmation by western blots, we found that increasing ratios of n-3FA : n-6FA in the diet induced dose-dependent changes in the plasma level of several proteins in a manner consistent with chemoprevention. Those included an increase in gelsolin and vitamin D binding protein, both shown to have tumor protective properties [27, 28]. A high ratio of n-3FA : n-6FA also increased the expression of 14-3-3 sigma, a well-known tumor suppressor gene [29]. In contrast, alpha-1β-glycoprotein, shown to be increased in a variety of cancers [30–32] was reduced by a high n-3FA diet. We also observed that the combined administration of Tamoxifen with a high ratio of n-3FA : n-6FA altered additional proteins also in a manner consistent with chemoprevention (Figure 5) [26]. These changes included a reduction in apolipoprotein E, haptoglobin, and inter-alpha inhibitor H4 heavy chain all shown to have tumor promoting properties [33–35]. Measurement of these differentially regulated proteins could be useful for monitoring the efficacy of n-3FA and Tamoxifen as chemopreventive agents in clinical trials.

3.2. Clinical Studies. While basic mechanisms of cooperativity between n-3FA and antiestrogens are under investigation using preclinical models of mammary carcinogenesis, we are testing concomitantly the clinical relevance of this approach in postmenopausal women using a reduction in breast density, a well-established risk factor for breast cancer [19, 20], as our primary endpoint. We have just completed a clinical trial involving 266 healthy, postmenopausal women at increased risk for breast cancer based on a breast density ≥25%, detected at their annual screening mammogram. They were randomly

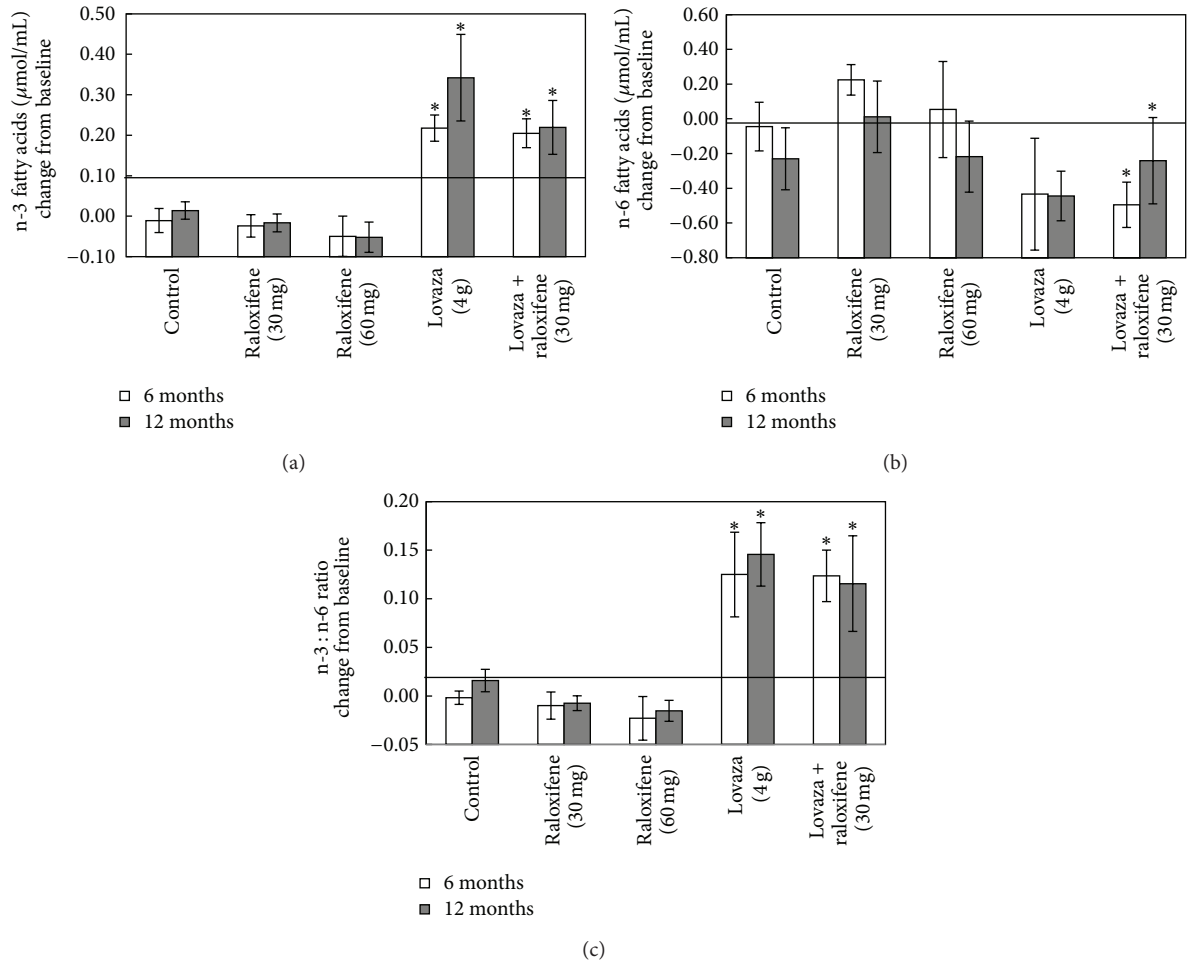

Figure 6: Treatment effects on serum n-3FA (a), n-6FA (b), and their ratio n3FA/n-6FA (c). The number of subjects was 8 in the control, 11 in the Lovaza 4 g group, and 8 in the Lovaza 4 g + Ral 30 mg group. Data represent mean values ± s.e.m. $^{*}P < 0.05$ versus the other groups. Reproduced with permission from [36].

assigned to one of the following groups: (1) no intervention; (2) Raloxifene 60 mg; (3) Raloxifene 30 mg; (4) omega-3 fatty acids (Lovaza) 4 gm; and (5) Lovaza 4 gm + Raloxifene 30 mg for two years (NCT00723398). While the final analysis of the effects of our interventions on breast density is in progress, we have published a preliminary report demonstrating the feasibility and acceptability of this approach with excellent compliance (96 ± 1% overall) by pill count [36]. In addition, we found that Lovaza administration increased the plasma levels of n-3FA by about 3-fold (Figure 6); such levels are quite comparable to those we have reported to exert chemopreventive effects in rats fed fish oil diet [12, 14], thus supporting the translational relevance of our animal studies described above.

4. Concluding Remarks

Despite the efficacy of antiestrogens in breast cancer prevention, there is an urgent need to develop safer and more effective chemopreventive strategies which could also inhibit the development of estrogen receptor negative tumors which are more aggressive and associated with a shorter survival. We judge that a combination approach targeting several cellular pathways involved in mammary carcinogenesis is needed for optimal antitumor efficacy. Our preliminary data strongly suggest that the combination of n-3FA and antiestrogens is superior to the individual interventions in reducing the incidence and multiplicity of chemically induced mammary tumors in rats. Our signaling, genomic, and proteomic studies suggest complementarity in the mechanism of antitumor action of antiestrogens and n-3FA which allows the use of lower and hence less toxic doses of antiestrogens without losing chemopreventive efficacy. The clinical relevance of these observations will soon be revealed in the final analysis of our recently completed clinical trial where we use a reduction in breast density, a well-established biomarker of breast cancer risk, in assessing the antitumor effects of our intervention. If positive, the results of our study would provide the rationale for launching a large multi-institutional trial testing the chemopreventive efficacy of n-3FA and antiestrogens using breast cancer incidence as the primary endpoint.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Omega-3 Polyunsaturated Fatty Acids Intake to Regulate *Helicobacter pylori*-Associated Gastric Diseases as Nonantimicrobial Dietary Approach

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Omega-3 polyunsaturated fatty acids (n-3 PUFAs), commonly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been acknowledged as essential long-chain fatty acids imposing either optimal health promotion or the rescuing from chronic inflammatory diseases such as atherosclerosis, fatty liver, and various inflammatory gastrointestinal diseases. Recent studies dealing with EPA and DHA have sparked highest interests because detailed molecular mechanisms had been documented with the identification of its receptor, G protein coupled receptor, and GPR120. In this review article, we have described clear evidences showing that n-3 PUFAs could reduce various *Helicobacter pylori*- (*H. pylori*-) associated gastric diseases and extended to play even cancer preventive outcomes including *H. pylori*-associated gastric cancer by influencing multiple targets, including proliferation, survival, angiogenesis, inflammation, and metastasis. Since our previous studies strongly concluded that nonantimicrobial dietary approach for reducing inflammation, for instance, application of phytoceuticals, probiotics, natural products including Korean red ginseng, and walnut plentiful of n-3 PUFAs, might be prerequisite step for preventing *H. pylori*-associated gastric cancer as well as facilitating the rejuvenation of precancerous atrophic gastritis, these beneficial lipids can restore or modify inflammation-associated lipid distortion and correction of altered lipid rafts to send right signaling to maintain healthy stomach even after chronic *H. pylori* infection.

1. Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of mortality worldwide. Almost two-thirds of affected individuals will die from their disease. Gastric carcinogenesis has a multifactorial etiology. *Helicobacter pylori* (*H. pylori*) infection is the most important risk factor for both gastritis and gastric carcinoma [1]. Host genotype, bacterial virulence factors, and environmental conditions increase the risk of gastric cancer [2]. Gastric cancer develops due to *H. pylori* infection and chronic inflammation in a slow pace. It begins by an *H. pylori* infection that progresses to chronic active gastritis. *H. pylori* is adapted to survive in the acidic gastric environment. Bacterial adhesion to gastric epithelial cells induces inflammation that results in the recruitment of neutrophils followed by B and T lymphocytes, macrophages, and plasma cells. As a result, reactive oxygen and nitrogen species are produced, which are involved in gastric epithelial cell damage and carcinogenesis. *H. pylori* is considered to be the initiator of a chronic inflammatory response that summons bone marrow-derived cells to the gastric mucosa, thereby directly contributing to development of gastric cancer. Main virulence factors of *H. pylori* for the induction of mucosal inflammation are cytotoxin-associated gene (cag) pathogenicity island- (PAI-) encoded virulence factors, such as cytotoxin-associated antigen (CagA) protein, vacuolating toxin-A (VacA), blood group antigen-binding adhesion (BabA), and outer inflammatory protein (OipA). Under the influence of various environmental and host factors, chronic active gastritis may in turn evolve into atrophic gastritis and intestinal metaplasia. Metaplasia undergoes further genomic and phenotypic changes,

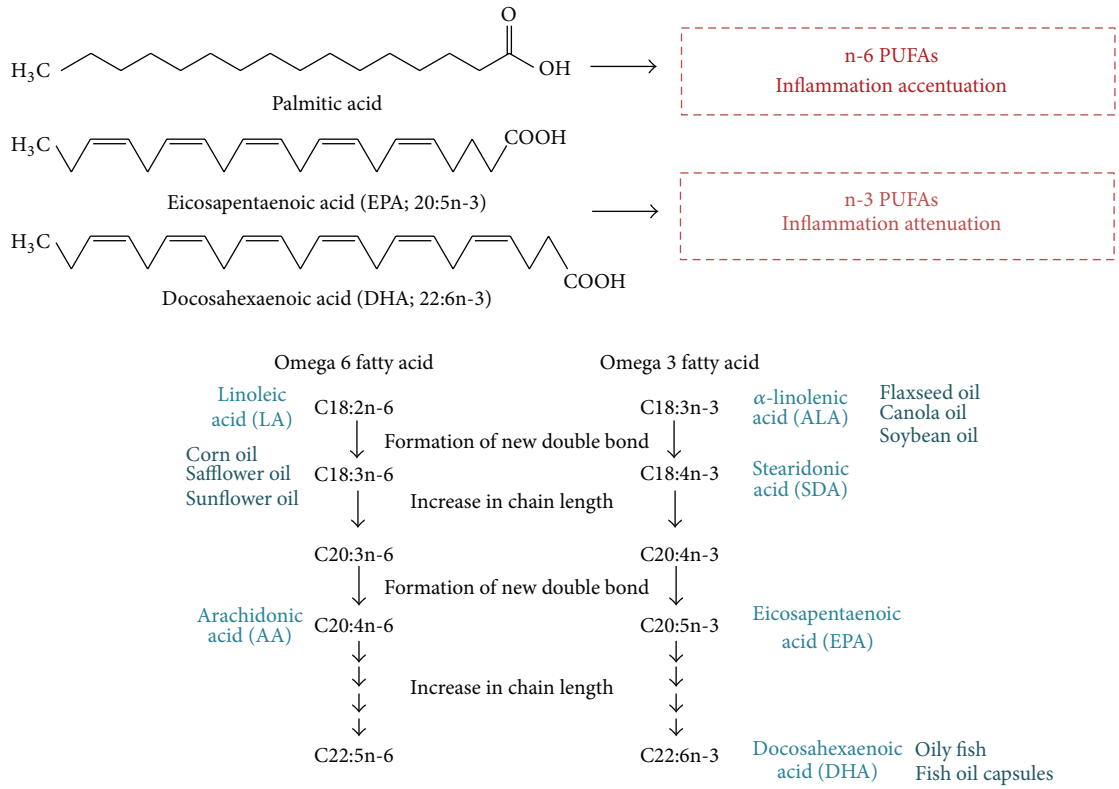

Figure 1: Essential n-3 PUFAs contributed to anti-inflammatory action based on their unsaturated bonds.

resulting in gastric dysplasia and finally adenocarcinoma [3].

Omega-3 (n-3) polyunsaturated fatty acids [n-3 PUFAs, eicosapentaenoic acid (EPA 20:5n-3), and docosahexaenoic acid (DHA 22:6n-3)] are the long-chain PUFAs, which are essential fatty acids as they can be synthesized by mammals from other dietary precursors containing n-3 PUFAs. They are sufficiently found in fish. Fatty acids are key nutrients affecting early growth and development and preventing chronic disease in later life [4]. PUFAs that contain more than one carbon double bond are divided into two major classes, namely, n-6 and n-3 (Figure 1). Several lipid metabolites can be made from these PUFAs. Linoleic acid (LA 18:2n-6) is a representative n-6 PUFA, which is the precursor of arachidonic acid (AA 20:4n-6) that is involved in inflammation, inducing cardiovascular diseases, diabetes, cancer, and age-related diseases [4]. α-Linolenic acid (ALA), EPA, and DHA are important n-3 PUFAs that are required for human to remain healthy. Several studies suggest that n-3 PUFAs are capable of preventing diseases due to their antioxidant and anti-inflammatory characteristics. Particularly, n-3 PUFAs are shown to have protective effects against chronic inflammatory diseases such as cardiovascular diseases [5, 6], rheumatoid arthritis [7], diabetes [8], other autoimmune diseases [9, 10], and cancer [11, 12]. However, scant evidence exists for their role against *H. pylori* infection despite their known favorable effects.

Increased western-style fat consumption in the Eastern world (high in n-6 to n-3 PUFA ratio) is associated with the development of esophageal, breast, gastric, colon, and pancreatic and prostate cancers. Yet, n-3 PUFAs have multiple beneficial antitumor functions that are shown to change the malignant growth in a number of studies [13]. Studies on the fatty acid level in patients with bladder, pancreatic, lung, and esophageal cancer have shown low concentrations of plasma n-3 PUFAs, ranging from 55 to 88%, in comparison with healthy individuals [14–16]. Epidemiologic studies suggest that a high n-3 to n-6 PUFA ratio may be the optimal strategy to decrease cancer risk [17]. A solid epidemiologic study added the evidence that consumption of n-3 PUFAs appears to protect against the development of hepatocellular carcinoma even among patients with HBV and/or HCV infection [18]. A possible protective effect of dietary n-3 PUFAs against prostate cancer has been reported [19]. Fasano et al. [20] reviewed many *in vivo* and *in vitro* studies and found that n-3 PUFAs have antitumor properties against lung cancer. Then, how about the role of n-3 PUFAs against *H. pylori* infection?

2. Omega-3 Polyunsaturated Fatty Acids (n-3 PUFAs) and Inflammation

n-3 PUFAs are essential for health. They are widely studied for their roles in human health and disease [21]. Recently, they are shown to be effective in treating and preventing various diseases [22]. n-3 PUFAs have a therapeutic role against inflammatory diseases such as rheumatoid

arthritis (RA), inflammatory bowel disease (IBD), asthma, and cardiovascular and neurodegenerative diseases [23–25]. A number of animal and human studies have provided convincing evidence for the anti-inflammatory effects of n-3 PUFAs. n-3 PUFAs are beneficial as a dietary supplement in RA by reducing the level of AA-derived eicosanoids and inflammatory cytokines, which include interleukin-1, interleukin-2, interleukin-6, and interleukin-8, as well as TNF-α and LTB_4, promoting anti-inflammatory activities [26, 27]. Moreover, n-3 PUFAs have an NSAID-sparing effect in RA by decreasing pain and gastroduodenal damage [28, 29]. Dietary n-3 PUFA intake is associated with a decreased risk of developing Crohn's disease (CD) and ulcerative colitis (UC) by modulation of inflammatory events such as reducing LTB_4, PGE_2, and thromboxane B_2 production [30–32]. n-3 PUFA administration has been shown to exert beneficial effects on animal model with induced lung inflammation or acute injury through decreasing the eosinophilic infiltration into the lung and improving the lung function [22, 33]. *Fat-1* transgenic mice with elevated n-3 PUFAs levels have lower concentrations of proinflammatory cytokines and higher concentrations of protectin D1 and resolvin E1 in their lungs [34]. Fish oil with n-3 PUFAs decreases the incidence of atherosclerotic lesions and frequency of cardiac arrest contributing to a reduction in overall mortality in patients at risk of cardiovascular diseases [35–37]. n-3 PUFAs prevented the functional and anatomical changes in diabetic neuropathy, reduced oxidative damage, prevented learning disability in brain injury, and reduced the accumulation of β-amyloid in Alzheimer's disease [38–40].

Overall, supplementation of n-3 PUFAs directly reduces the production of inflammatory eicosanoids by replacing AA as an eicosanoid substrate and inhibiting AA metabolism by changing the cell membrane phospholipid fatty acid composition. n-3 PUFAs indirectly reduce the production of cytokines, reactive oxygen species (ROS), and adhesion molecules by altering the expression of inflammatory genes. This is achieved by intervening in the inflammatory signaling cascade, which include disruption of lipid rafts, activation of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor γ, and binding to the G protein-coupled receptor GPR120 [23, 41]. Moreover, n-3 PUFAs can partially inhibit the inflammatory processes that involve leukocyte chemotaxis and T-helper 1 lymphocyte reactivity [41]. These properties suggest that n-3 PUFAs could have a therapeutic role in inflammatory diseases.

3. The Effect of n-3 PUFAs on Inflammation-Based Gastric Cancers

Most GI cancers including esophageal, stomach, and colorectal cancers have a natural history of multistep transition from precursor lesions to malignant lesions, over which inflammation, adenoma formation, and dysplastic changes prevail [42]. Therefore, GI cancers usually go through a premalignant lesion before development of the invasive cancer. Examples include Barrett's esophagus before esophageal cancer, chronic atrophic gastritis, and intestinal metaplasia before gastric cancer and adenoma or dysplasia in the setting of chronic ulcerative colitis before colon cancer. Since western diet contains disproportionally high amounts of n-6 PUFAs and low amounts of n-3 PUFAs (high n-6 to n-3 PUFA ratio), n-3 PUFAs-rich diets could become cancer preventive measures by affecting several stages of GI cancer development. Here, we introduce more carcinogenic detail regarding the interplay of inflammation and n-3 PUFAs intake.

Gastric cancer is the fourth most common cancer worldwide and almost two-thirds of affected individuals will die of their disease. Despite national efforts, 20 out of 100,000 Koreans die from gastric cancer. A number of studies investigating the association between n-3 PUFAs and gastric diseases suggest a protective effect for n-3 PUFAs in gastric cancer. Recently, Correia et al. [43] showed that DHA inhibits *H. pylori* growth *in vitro* and mice gastric mucosa colonization. It has been proposed that PUFAs hold an inhibitory effect on bacterial growth via disruption of cell membrane leading to bacteria lysis [44]. Mohammed et al. [45] showed that n-3 PUFAs reduce iodoacetamide-induced gastritis in rats by decreasing malondialdehyde (MDA), gastrin and nitric oxide (NO), and normalizing mucosal glutathione. Erythrocyte composition of DHA is found to be negatively associated with the risk of well-differentiated gastric adenocarcinoma [46]. n-3 PUFAs-rich diet delayed tumor growth in a mouse xenograft model of gastric cancer [47]. Another *in vitro* study showed that n-3 PUFAs inhibit macrophage-enhanced gastric cancer cell migration and attenuate matrix metalloproteinase- (MMP-) 10 expression through ERK and STAT3 phosphorylation [48] and inhibit the growth of human gastric carcinoma via apoptosis [49]. Moreover, n-3 PUFAs are beneficial for preventing oxidative stress-induced apoptosis by inhibiting apoptotic gene expression and DNA fragmentation of gastric epithelial cells [50]. DHA induces apoptosis of gastric cancer cells by inducing the expression of apoptotic genes [51]. Although a large body of literature spanning numerous cohorts from many countries with different demographic characteristics does not provide evidence to suggest a significant association between n-3 PUFAs and the incidence of stomach cancer [52], precise studies are required to investigate the antitumor properties of n-3 PUFAs in the stomach.

4. Molecular Mechanisms of n-3 PUFAs Effect against *H. pylori* Infection

4.1. Attenuation of H. pylori-Associated Inflammation. *H. pylori* is clever as it changes n-6 PUFAs metabolism to foster gastric environment or to enhance gastric inflammation. Nakaya et al. [53] studied the formation of n-6 PUFAs from LA in rat gastric mucosal cells after *H. pylori* infection. They found that addition of LA leads to an increase in the composition of AA, LA, and PGE_2. Cyclooxygenase-2 (COX-2) expression is induced in *RGM-1* cells by addition of LA. *H. pylori* culture broth filtrates had decreased LA and increased AA compositions. Moreover, after incubation with *H. pylori* culture filtrates, PGE_2 concentrations were higher than controls. Thus, *H. pylori* infection can enhance PGE_2

synthesis and accelerate n-6 PUFAs metabolism in gastric mucosal cells, which can make the gastric mucosal barrier more fragile. On the other hand, since essential dietary fatty acids bestow gastroduodenal mucosal protection [54], n-3 PUFAs can provide efficient gastroprotection against *H. pylori*, drug-induced and stress-induced gastric mucosal damages [55]. These effects were first observed in 1987 when the link between low levels of PUFAs and *H. pylori*-associated duodenal ulcer was shown in an interventional dietary study [56]. Recent studies have shown anti-inflammation potential propertied for n-3 PUFAs [57, 58]. n-3 PUFAs suppressed the activation of EGFR, PKC δ, MAPK, NF-κB, and AP-1 in *H. pylori*-infected gastric epithelial *AGS* cells. n-3 PUFAs are beneficial for prevention of *H. pylori*-associated gastric inflammation by inhibiting proinflammatory IL-8 expression [57]. *AGS* cells that were infected with DHA pretreated *H. pylori* showed a 3-fold reduction in IL-8 production and a decrease in COX-2 and inducible nitric oxide synthase (iNOS) [58]. However, the other side of the n-3 PUFAs coin is shown in gastrointestinal inflammations, where worse effects are reported with n-PUFAs despite the above-mentioned positive effects. For example, Woodworth et al. [59] and Butler and Yu [60] studied the effect of dietary fish oil enriched with DHA on reduction of experimentally induced colitis and colon cancer risk. In their experiments, they infected *Smad*3 null mice with *H. hepaticus* to induce colitis. They observed mild colitis 4 weeks after infection, but paradoxically, mice fed with isocaloric diets modified to include corn oil, sunflower oil, or DHA (as the fatty acid source) developed severe colitis and adenocarcinoma with DHA after 8 weeks. They concluded that DHA-fed mice may be less equipped to mount a successful response to *H. hepaticus* infection (increasing colon cancer risk), supporting the need to establish a tolerable upper limit for DHA intake particularly in the setting of chronic inflammation. In another study, DHA-fed *Smad*3 knockout mice had significantly higher levels of plasma IL-5, IL-13, and IL-9 (Th2-biasing cytokines) and cecal IgA compared with control, leading to the emerging concept that fish oil might enhance B cell function *in vivo* to aggravate an existing inflammatory response [61]. A proposed molecular mechanism by which DHA exerts its effects is modification of lipid raft organization [62]. Regarding the discrepancy in the efficacy of n-3 PUFAs, Rockett et al. [63] demonstrated that a physiologically relevant dose of n-3 PUFAs increases the size of B cell rafts, GM1 surface expression, and membrane molecular order upon cross-linking. Increasing lipid raft size with fish oil was accompanied by changes in innate and adaptive function. Other factors should also be exploited to use n-3 PUFAs as a safer rescue agent against *H. pylori* infection in future.

4.2. Antimicrobial Activity against H. pylori: A Direct Effect on the Bacteria. Eradication of *H. pylori* with a proton pump inhibitor-based triple therapy is currently the treatment of choice for *H. pylori* infection. However, it has a success rate of 80–90%. Treatment failure and contraindications in some patients are common. Furthermore, rapidly emerging drug resistance in *H. pylori* strains during treatment with different antibiotics is a major obstacle for successful eradication. Due to the prevalence of antibiotic-resistant *H. pylori* strains, there is an increasing effort in seeking other safe and effective compounds that inhibit *H. pylori* growth. Recent studies have shown that n-3 PUFAs have anti-*H. pylori* potentials. PUFAs significantly inhibit *H. pylori*'s growth [44]. A very recent *in vitro* and *in vivo* investigation [43] clearly showed that n-3 PUFAs inhibit *H. pylori* growth *in vitro* and its colonization in mice gastric mucosa *in vivo*. This study is implicated in therapy and alleviation of *H. pylori*-induced inflammation. Since drug-resistant *H. pylori* strains and noncompliance to therapy are the major causes of *H. pylori* eradication failure, n-3 PUFAs could be used to lower the recurrence rate of infection in combination with standard triple therapy. This can prohibit *H. pylori*'s ability to colonize the mouse stomach. DHA has also been shown to decrease *H. pylori*'s growth and associated inflammation. Anti-*H. pylori* effects of DHA are associated with changes in bacterial morphology, metabolism, and alteration of the composition of outer membrane proteins, which ultimately reduces the adhesion of bacteria and the burden of *H. pylori*-related inflammation [58]. In contrast to the *in vitro* bacteriostatic and bactericidal efficacy of n-3 PUFAs, Meier et al. [64] showed that fish oil is less effective than metronidazole, in combination with pantoprazole and clarithromycin, for *H. pylori* eradication.

4.3. Imposing Restorative Mechanism and Reducing H. pylori-Induced Cytotoxicity. Regeneration-promoting role of n-3 PUFAs is well acknowledged in several medical fields, namely, liver regeneration [65], wound healing [66], bone preservation [67, 68], burn [69], endothelial regeneration [70], cellular resolution of inflammation [71], recovery from nerve injury [72], and regeneration from previous intestine surgery [73]. n-3 PUFAs are shown to prevent acute liver failure and promote liver regeneration in rats by protecting the structure of the sinusoidal endothelial cells (SEC) in the acute phase after hepatectomy along with phosphorylation of the STAT3 and Akt [74]. A large number of animal and human studies have reported a positive n-3 PUFAs effect on increased bone formation, reduced bone resorption, and protection from osteoporosis [68]. Improvement in bone health is achieved by attenuation of mediators of osteoclastogenesis, namely, PGE2, COX-2, IL1-β, TNFα, and NF-κB, in particular via the E (EPA derived) and D (DHA derived) series of resolvins. Eicosapentaenoic acid-derived resolvin E1 (RvE1) enhances resolution of inflammation, prevents bone loss, and induces bone regeneration [67]. RvE1 modulates osteoclast differentiation and bone remodeling by direct actions on bone. It rescues osteoprotegerin (OPG) production and restores a favorable NF-κB ligand/OPG receptor-activator ratio along with known anti-inflammatory and proresolving actions. RvD2 effectively prevents thrombosis of the deep dermal vascular network (DDVN) in early phases of burn injury. Therefore, it reduces secondary tissue damage and promotes long-term survival of deep dermal components after the initial thermal insult [69]. Alexander et al. have shown improved protein synthesis after burn injury in rats receiving n-3 PUFAs supplementation [75]. Interestingly, in

a model of corneal nerve regeneration after surgery, it has been shown that DHA has strong proregenerative effects in combination with pigment epithelial growth factor. The authors hypothesized that this may involve formation of neuroprotectin D1 (a DHA metabolite) [76]. Dietary supplementation with n-3 PUFAs improved colonic anastomoses healing [73]. n-3 PUFAs enhance the colonic wound healing in a rat model. Finally, n-3 PUFAs may prompt faster resolution of inflammation within the wound microenvironment, which leads to facilitated regeneration and reepithelialization. A small randomized controlled trial evaluated a formula supplemented with fish oil in patients with pressure ulcers and noted decreased progression of pressure ulcers in those receiving fish oil supplementation [77].

There is growing evidence that the diverse biological roles of n-3 PUFAs contribute to their regenerative actions against chronic inflammatory disease. This could effectively help resolve the inflammation and promote a transition from the inflammatory to the proliferative and remodeling phases of wound healing [78]. n-3 PUFAs can be incorporated into membrane phospholipids, which causes reduced membrane fluidity. It could be associated with lipid raft assembly and function. Lipid rafts are cholesterol-rich microdomains at the host cell surface and are required for NF-κB-dependent responses to *H. pylori*. Recently, several studies have suggested that n-3 PUFAs can be converted into bioactive mediators, including resolvins, which exert inflammation-resolving properties via counterregulation of lipid mediators including proinflammatory leukotriene (LTs) and prostaglandin (PGs). Thus, our group investigated long-term treatment of n-3 PUFAs in an *H. pylori*-infected animal model and found that n-3 PUFAs administration ameliorated *H. pylori*-induced gastric inflammation and atrophic gastritis. It also reduced the incidence of *H. pylori*-associated gastric carcinogenesis (unpublished data). We could be the first group to document the rejuvenating action of n-3 PUFAs on *H. pylori*-associated atrophic changes in stomach. As the use of n-3 PUFAs for treatment of *H. pylori*-induced GI disorders is rapidly moving into clinical settings with more studies explaining the mechanism of action, detailed randomized controlled trials are required to obtain strong evidence for the incorporation n-3 PUFAs into the therapeutic armamentarium in near future.

4.4. H. pylori-Induced Oxidative Stress and the Scavenging Action of n-3 PUFAs. Although limited reports are available on the antioxidative action of n-3 PUFAs against *H. pylori* infection, this antioxidative feature is well known in cardiovascular and neurological fields. A very recent high impact evidence came from Lluís et al. [79] showing that a diet of 1 : 1 ratio of EPA/DHA improves many oxidative stress parameters like superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes, plasma antioxidant capacity, and cardiovascular risk factors (glycated hemoglobin) relative to the other diets. Avramovic et al. [80] showed beneficial effects of n-3 PUFAs on the oxidative stress in brain tissue, improving neurogenesis and neuroplasticity [81]. Regarding antioxidative effects of n-3 PUFAs against *H. pylori* infection, we investigated the direct scavenging action of EPA using electron spin resonance and found significant antioxidative actions against hydroxyl and superoxide radicals.

4.5. Proposed Mechanisms of n-3 PUFAs Cancer Prevention against H. pylori-Associated Carcinogenesis. n-3 PUFAs can suppress the anti-inflammatory-related function of T lymphocytes and antigen presenting cells (APCs). The mechanism by which DHA modifies lymphocyte function is changing the organization of sphingolipid/cholesterol lipid raft membrane domains. Two contradictory models have been proposed to explain how DHA exerts its effects by changing the raft organization. One is the cellular model, which proposed that DHA is directly incorporated into the lipid rafts and changes the protein activity to suppress the lymphocytic functions. The other suggests modification of lymphocytic function by formation of nanometer scale DHA-rich domains, which disrupts the optimal raft-dependent clustering of proteins necessary for initial signaling. A biophysical DHA-containing phospholipid membrane model showed that unique nonraft membrane domains are formed that are organizationally distinct from lipid rafts. This alters the conformation and/or lateral organization of lymphocyte proteins [82]. Based on n-3 PUFAs effects on lymphocytes and modulation of inflammation, n-3 PUFAs, especially EPA or DHA, are shown to have multiple antigastric-tumor actions. Our current knowledge of the antitumor activity of n-3 PUFAs has been comprehensively reviewed elsewhere [12, 19, 83]. Recently, Stephenson et al. [13] have reviewed more recent underlying mechanisms of antitumor properties of n-3 PUFAs. n-3 PUFAs inhibit growth signal transduction. They appear to downregulate epidermal growth factor receptor (EGFR), protein kinase C (PKC), *Ras*, NF-κB, and insulin-like growth factor (IGF), which are important cell-signaling mediators often found to be elevated in cancer. n-3 PUFAs also induce cancer cell apoptosis via modulation of peroxisome proliferator-activated receptors (PPARs), Bcl-2 family, and NF-κB cell signaling. They decrease sprouting angiogenesis by suppressing vascular endothelial growth factor- (VEGF-) and platelet derived growth factor- (PDGF-) stimulated endothelial cell proliferation, migration and tube formation through inhibition of MMPs via NO production, and NF-κB and β-catenin cell signaling. Moreover, n-3 PUFAs decrease cell-cell adhesion via downregulation of Rho-GTPase (inhibits cytoskeleton reorganization) and reduction in intercellular adhesion molecule- (ICAM-) 1 and vascular cell adhesion molecule- (VCAM-) 1 expression.

Cockbain et al. [12] have proposed four main antitumor actions for n-3 PUFAs, namely, (1) modulation of COX activity, (2) alteration of membrane dynamics and cell surface receptor function, (3) increase in cellular oxidative stress leading to cytotoxicity, and (4) enhancement of anti-inflammatory lipid mediators. In mechanistic details, n-3 PUFAs can act as alternative substrates for COX-2 (instead of AA) leading to a reduction in formation of protumorigenic "2-series" PGs (PGE_2) in several cell types. They also bind the substrate channel of COX-2 and inhibit COX-2 activity. In addition, incorporation of n-3 PUFAs into the cell membrane alters the fluidity, structure, and/or

function of lipid rafts or caveolae. Localization of cell surface receptors such as G protein-coupled receptors (GPCRs), toll-like receptors (TLRs), and epidermal growth factor receptor (EGFR) in lipid rafts is believed to be crucial for downstream receptor signaling, which controls proliferation and apoptosis.

Rockett et al. [84] showed that membrane raft organization is more sensitive to disruption by n-3 PUFAs than the nonraft organization. n-3 PUFAs can also have an antitumor effect via alteration of the cellular redox state. They can increase ROS due to being highly peroxidizable. Therefore, n-3 PUFAs can induce cancer cell apoptosis via elevation of intracellular ROS levels. Lastly, n-3 PUFAs can be metabolized into novel anti-inflammatory lipid mediators including resolvins, protectins, and maresins. Resolvins exhibit anti-neoplastic activity via anti-inflammatory and inflammation resolution features in animal models of acute inflammation. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive master regulatory transcriptional factor that plays an important protective role in cells by regulating cellular redox balance [85]. Recently, it was reported that n-3 PUFAs can activate Nrf2 and induced Nrf2-directed gene expression [86, 87]. They can suppress lipopolysaccharide-induced inflammation through induction of Nrf2 expression [88]. Last but not least, n-3 PUFAs significantly reduce oxidative stress-induced endothelial cell Ca^{++} influx. This effect might be associated, at least in part, with altered lipid composition of membrane lipid rafts [89].

5. Dietary Walnuts Intake as Source of n-3 PUFAs to Rejuvenate *Helicobacter pylori*-Associated Atrophic Gastritis as well as to Augment *H. pylori* Eradication Rate

It has been reported that Asian dietary pattern and *H. pylori* infection are typically associated with increased risk of gastric cancer. *H. pylori*, a Gram-negative bacterial pathogen that infects approximately 50% of the world's population, provokes chronic gastric inflammation, which is considered to be a major risk factor for development of gastric and duodenal ulcers, mucosa-associated lymphoid tissue lymphoma (MALToma), and gastric cancer [2]. Despite the existing debates, there is enough evidence that *H. pylori* infection is strongly associated with gastric cancer, for which it is defined as a class I carcinogen by IARC [90–92]. Mechanistically, gastric epithelial cells respond to *H. pylori* infection by upregulating the expression of proinflammatory genes, which includes the upregulation of COX-2, iNOS, and IL-8 [93, 94]. With perpetuating gastric inflammation and oxidative stress, *H. pylori* infection gives rise to significant DNA damage, apoptosis, and cell cycle dysregulation, all of which are closely associated with significant oncogenic insults on infected mucosa [95, 96]. Since gastric cancer is a multistep and multifactorial disease and not every individual infected with *H. pylori* develops gastric cancer, population-wide eradication strategies are not generally considered as the preventive measures for gastric cancer [97].

H. pylori-related gastric diseases are now considered as infectious diseases and are treated with antibiotic regimens, which are often composed of two antibiotics (amoxicillin and clarithromycin) and a proton pump inhibitor [97, 98]. However, this treatment can fail for several reasons, which include insufficient antibiotic concentration due to mutation-acquired resistance, limited antibiotic efficacy in a low gastric pH, and insufficient antibiotic concentration due to a high bacterial load [99]. In addition, existence of viable bacteria in dormant forms that are not accessible to antibiotics and antibiotic-related damage to the mucosal immunity can also be observed. Seeking alternatives to solve the problems of the expense, lower compliance and antibiotic resistance has stretched to nonantimicrobial approaches, which include supplementation with probiotics and other nutrients.

Fresh antioxidant fruits, vegetables, and certain micronutrients such as selenium and vitamin C can reduce the risk of infection. A food that can inhibit *H. pylori* viability, colonization, and infection can also reduce the risk of cancer. Korean red ginseng, licorice extracts, S-allyl cysteine (SAC) from garlic, probiotics like *L. plantarum* or *Bifidobacillus,* and n-3 PUFAs are shown to be effective antibacterials and antimutagenics along with rejuvenating activities in *H. pylori* infection. Our group extended the supplementation of effective phytoceuticals or phytonutrients to achieve higher eradication rates and possibly prevent the *H. pylori*-associated gastric cancer [43, 44, 100–102].

Mere eradication yielded limited benefit in some clinical diseases but supplementation with natural products or phytoceuticals rendered higher eradication rates. It is also associated with fewer side effects compared to the triple regimen, higher levels of anti-inflammatory cytokines, and some restorative actions. Our studies showed that SAC supplementation could lead to efficient reduction in inflammation due to potent demethylating mechanism of HDAC inhibition. It can also enforce the regenerative activities along with its antimutagenic action.

Natural phytochemicals with antioxidant, anti-inflammatory, and anticarcinogenic properties that regulate or target specific molecules in gastric carcinogenesis can increase the efficacy of *H. pylori* eradication as an effort to prevent gastric cancer. Nuts and seeds, particularly walnut, are nutritionally dense and rich in vitamin E and n-3 PUFAs. They are an ample source of dietary fiber, B vitamin, and essential mineral such as magnesium, copper, manganese, calcium, and potassium as well as monounsaturated and polyunsaturated fats, which can potentially lower the LDL cholesterol [103]. Potential health benefits, which have not been scientifically validated, include improved complexion and decreased risk of cancer [104, 105].

The fundamental basis of walnuts treatment in *H. pylori* infection is suppression of inflammation and/or inhibition of pathogenic bacteria. There is the possibility that the enhancement of negative regulators of inflammation-associated cytokine by walnuts could also contribute to *H. pylori* eradication. Sliced walnuts contain biotin, vitamin R, manganese, copper, vitamin B12, phosphorus, magnesium, molybdenum, and fiber. Walnut is also high in monounsaturated fats, the same type of health-promoting fat found in

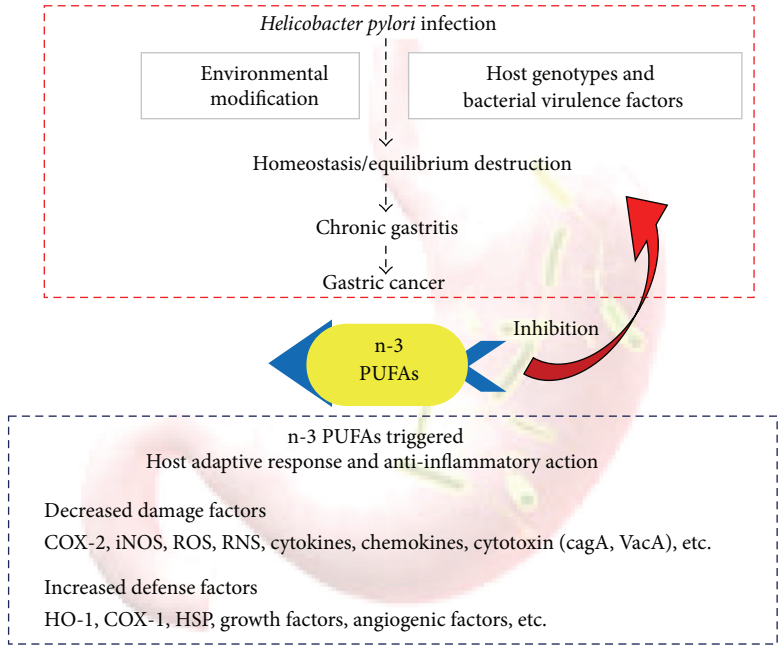

Figure 2: Host adoptive and antimutagenic response enhanced with n-3 PUFAs in *H. pylori* infection.

olive oil, which have been associated with reduced risk of heart disease. In addition to the healthy fats and vitamin E, a quarter-cup of walnuts contains 62 mg of magnesium and 162 mg of potassium. These nutritional facts can shed light on the possible beneficial effects of walnuts in *H. pylori* treatment.

We postulate that high walnut's n-3 PUFAs and vitamin E contents help the treatment and eradication of *H. pylori* infection. *H. pylori* creates a microenvironment to protect itself from gastric acid and host defense systems. It increases the oxidative stress in its colonized areas. It is shown that ROS levels are increased in patients with *H. pylori* infection and that ROS levels are decreased following *H. pylori* eradication. Vitamin E impairs lipid preoxidation pathways and exerts an antioxidant effect. It is also shown that vitamin E concentration is low in the gastric fluid and mucosa of *H. pylori*-infected patients and high after *H. pylori* eradication. These facts suggest a possible benefit from vitamin E supplementation in *H. pylori* infection.

In animal models, short-term therapy with vitamin E is shown to have restorative effects on *H. pylori* gastritis. It is also shown to have protective effects on *H. pylori*-induced mucosal injury by preventing the accumulation of activated neutrophils. In conclusion, therapeutic doses of vitamin E decrease the intensity of *H. pylori* infection and neutrophil activity in the antrum. These effects on the bacterial microenvironment could be the reason for the supplementation with antibiotics to increase the eradication rate [106]. We have solid evidence that n-3 PUFAs ameliorated *H. pylori*-induced gastritis and significantly prevented the gastric cancer. However, some studies showed that magnesium affects the outcome.

6. Conclusion

Although *H. pylori* is defined as a class 1 carcinogen by IARC, debate exists on whether the eradication can prevent the associated gastric cancer. Furthermore, host genotype, bacterial virulence factors, and environmental factors can affect the outcome after *H. pylori* infection. Host adaptive responses can modify the carcinogenic influence of *H. pylori* infection (Figure 2). n-3 PUFAs are known to be antioxidant, anti-inflammation, and anticancer. Recently, several investigators have examined the anti-inflammatory, antimicrobial, and rejuvenating effects of n-3 PUFAs on *H. pylori*-induced gastric diseases (Figure 2). There is accumulating evidence that suggests higher consumption of dietary n-3 PUFAs is associated with lower risk of GI cancer development in animal models and human. Recent studies suggest that endogenous n-3 PUFAs delay the progression of stomach cancer and elevating n-3 PUFAs could be an important strategy to delay/prevent gastrointestinal cancer in high-risk patients. This effect is bestowed via several mechanisms mediating cancer prevention such as anti-inflammatory and rejuvenating effects of n-3 PUFAs against carcinogenic *H. pylori* infection. Despite the orchestrated mechanisms of action, maintenance of optimal cellular levels of n-3 PUFAs can be the royalty gatekeeper of *H. pylori*-associated gastric inflammation and cancer. Supplementation with n-3 PUFAs in combination with other antitumor agents can possibly improve the efficacy of GI cancer prevention. Large-scale translational and comprehensive mechanism studies are required to demonstrate the preventive or therapeutic effects of n-3 PUFAs against *H. pylori*-induced gastric diseases. Clinical trials are prerequisites of the decision on the role of chemopreventive agents in *H. pylori*-associated gastric

cancer. A long-term study that shows the preventive effect of n-3 PUFAs against gastritis and *H. pylori*-induced tumorigenesis will be a prime example of cancer prevention by food.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Heterologous Reconstitution of Omega-3 Polyunsaturated Fatty Acids in *Arabidopsis*

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Reconstitution of nonnative, very-long-chain polyunsaturated fatty acid (VLC-PUFA) biosynthetic pathways in *Arabidopsis thaliana* was undertaken. The introduction of three primary biosynthetic activities to cells requires the stable coexpression of multiple proteins within the same cell. Herein, we report that C_{22} VLC-PUFAs were synthesized from C_{18} precursors by reactions catalyzed by Δ^6-desaturase, an ELOVL5-like enzyme involved in VLC-PUFA elongation, and Δ^5-desaturase. Coexpression of the corresponding genes (*McD6DES*, *AsELOVL5*, and *PtD5DES*) under the control of the seed-specific vicilin promoter resulted in production of docosapentaenoic acid (22:5 n-3) and docosatetraenoic acid (22:4 n-6) as well as eicosapentaenoic acid (20:5 n-3) and arachidonic acid (20:4 n-6) in *Arabidopsis* seeds. The contributions of the transgenic enzymes and endogenous fatty acid metabolism were determined. Specifically, the reasonable synthesis of omega-3 stearidonic acid (18:4 n-3) could be a useful tool to obtain a sustainable system for the production of omega-3 fatty acids in seeds of a transgenic T3 line 63-1. The results indicated that coexpression of the three proteins was stable. Therefore, this study suggests that metabolic engineering of oilseed crops to produce VLC-PUFAs is feasible.

1. Introduction

In both plants and animals, polyunsaturated fatty acids (PUFAs) are important membrane components that serve as universal cellular regulators, playing key roles in many cellular events. Very-long-chain (VLC) PUFAs are ≥20 carbons long (C_{20}–C_{22}) and have three or more methylene-interrupted *cis* double bonds in omega-3 (*ω*-3 or n-3) or omega-6 (*ω*-6 or n-6) arrangements (Table 1). Typically, VLC-PUFAs such as eicosapentaenoic acid (EPA; 20:5 n-3), arachidonic acid (ARA; 20:4 n-6), and docosahexaenoic acid (DHA; 22:6 n-3) are important nutritionally and as components of membrane phospholipids in specific tissues or as precursors for the synthesis of different groups of eicosanoid effectors. VLC-PUFAs are not only required for the development of the fetal nervous system but also contribute via a multiplicity of roles to the maintenance of health with increasing development and age, particularly by reducing the incidence of cardiovascular diseases [1, 2]. These fatty acids are either directly available as components of the diet or produced from the two essential fatty acids: *α*-linolenic acid (ALA; 18:3 n-3) and linoleic acid (LA; 18:2 n-6). Since mammalians and human cells are unable to synthesize ALA and LA from precursors, they are defined as essential PUFA. In humans, conversion of ALA and LA into their metabolites is extremely low and several factors, including age, gender, immune state, alcohol, and smoking, may influence LA and ALA metabolism. Thus, the major source of VLC-PUFAs is diet. Reliable dietary sources of VLC-PUFAs are fish oils, whereas ALA and LA are found predominantly in green vegetables and some plant oils, which do not contain VLC-PUFAs. Fish stocks are decreasing throughout the world, raising questions regarding the sustainability of this source of VLC-PUFAs, and this has resulted in strong interest in

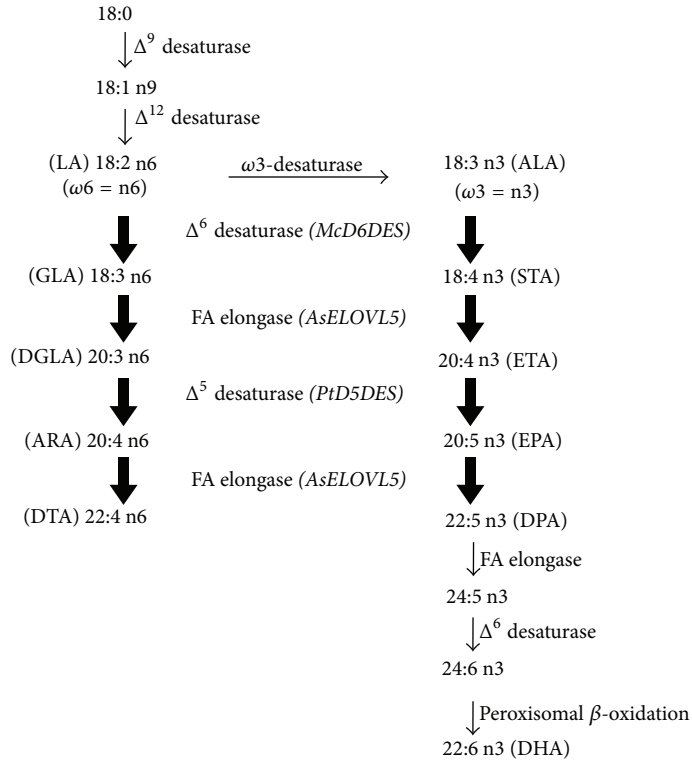

Figure 1: Metabolism of the two series of polyunsaturated fatty acids (PUFAs). Eicosapentaenoic acid (EPA, 20:5 n-3) is the main n-3 VLC-PUFA derived from the essential precursor α-linolenic acid (ALA, 18:3 n-3), and arachidonic acid (ARA, 20:4 n-6) is the main n-6 VLC-PUFA derived from the essential precursor linoleic acid (LA, 18:2 n-6). VLC-PUFAs are synthesized by successive elongations and desaturations. The key enzymes involved in this work are indicated in the pathway with large bold arrows.

Table 1: Abbreviations of PUFAs nomenclature used in this study.

n-3 PUFA	n-6 PUFA
ALA (α-linolenic acid; $C_{18:3}$ $\Delta^{9,12,15}$)	LA (linoleic acid; $C_{18:2}$ $\Delta^{9,12}$)
STA (stearidonic acid; $C_{18:4}$ $\Delta^{6,9,12,15}$)	GLA (γ-linolenic acid; $C_{18:3}$ $\Delta^{6,9,12}$)
ETA (eicosatetraenoic acid; $C_{20:4}$ $\Delta^{8,11,14,17}$)	DGLA (dihomo-γ-linolenic acid; $C_{20:3}$ $\Delta^{8,11,14}$)
EPA (eicosapentaenoic acid; $C_{20:5}$ $\Delta^{5,8,11,14,17}$)	ARA (arachidonic acid; $C_{20:4}$ $\Delta^{5,8,11,14}$)
DPA (docosapentaenoic acid; $C_{22:5}$ $\Delta^{7,10,13,16,19}$)	DTA (docosatetraenoic acid; $C_{22:4}$ $\Delta^{7,10,13,16}$)

the production of long-chain PUFAs in plants [3]. This goal may be realized by the introduction of VLC-PUFA biosynthetic pathways into annual oilseed crops.

The conversion of C_{18} PUFAs into EPA or ARA requires three consecutive enzymatic steps: Δ^6-desaturation, PUFA elongation, and Δ^5-desaturation (Figure 1). ALA and LA are desaturated by Δ^6-desaturase to form stearidonic acid (STA; 18:4 n-3) and γ-linolenic acid (GLA; 18:3 n-6), respectively [4–7]. Next, STA and GLA are converted to eicosatetraenoic acid (ETA; 20:4 n-3) and dihomo-γ-linolenic acid (DGLA; 20:3 n-6), respectively, by an ELOVL5-like fatty acid elongase [8–11]. Finally, Δ^5-desaturase converts ETA and DGLA into EPA and ARA, respectively [12–14]. Additionally, VLC-PUFAs such as EPA and ARA are further converted to docosapentaenoic acid (DPA; 22:5 n-3) and docosatetraenoic acid (DTA; 22:4 n-6) by ELOVL5 [10, 11, 15–19] or an ELOVL2-like fatty acid elongase [20] and then desaturated by Δ^4-desaturase to give DPA and DHA [21, 22].

Indeed over recent years researchers have endeavored to reconstruct the VLC-PUFA biosynthetic pathways in plants [23–28]. In our previous study, we reconstituted the EPA and ARA biosynthetic pathways in yeast [11]. Here, we report the production of C_{22} VLC-PUFAs as well as EPA and ARA in seeds of transgenic *Arabidopsis* plants expressing appropriate heterologous enzymes under the control of seed-specific promoters. Our study reports a new gene set required to direct the efficient synthesis of these fatty acids in transgenic seeds.

2. Materials and Methods

2.1. Plant Material and Growth Conditions. Wild-type and transgenic plants were raised simultaneously from seeds in pots with soil. *Arabidopsis thaliana*, Columbia (Col-0) ecotype, was grown in a controlled environment chamber under the following conditions: 22°C day/18°C night, 70% humidity, and a 16 h photoperiod (250 μmol m^{-2} s^{-1}).

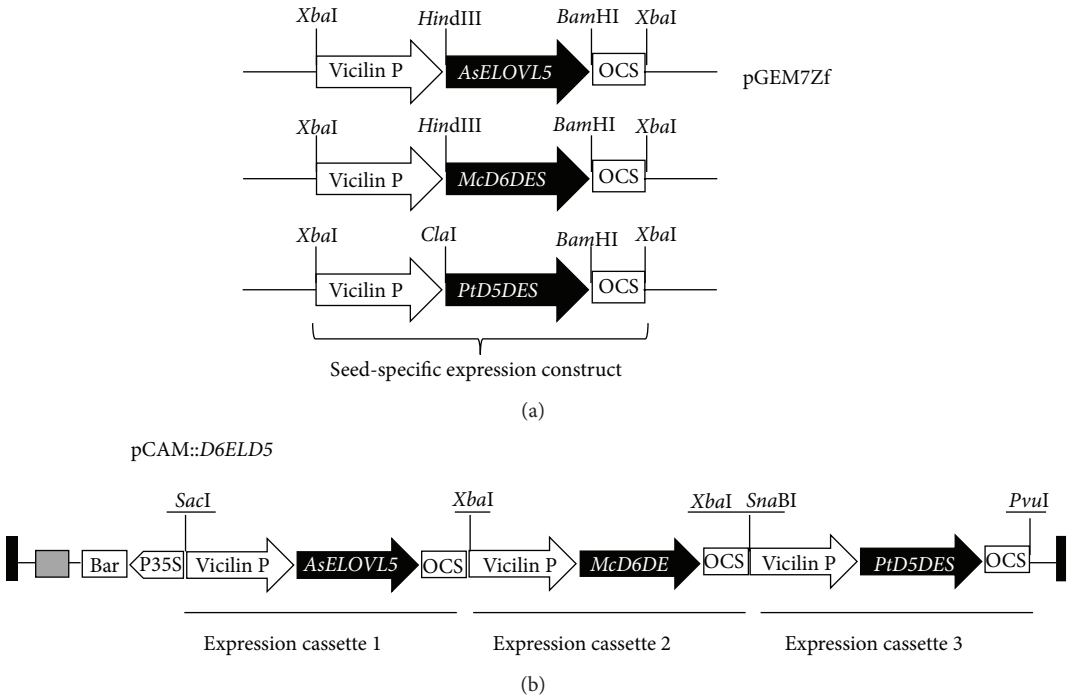

Figure 2: Schematic of constructs used for coexpression of the genes *McD6DES*, *AsELOVL5*, and *PtD5DES* in seeds of *Arabidopsis*. (a) The intermediate vector pGEM7Zf, used for individual seed-specific expression constructs. (b) The seed-specific expression vector *D6ELD5*, designed for easy coexpression of multiple genes. Vicilin P: vicilin promoter; OCS: *octopine synthase* terminator; bar, used to confer Basta resistance to plants under the control of a CaMV 35S promoter (P35S).

2.2. Construction of the Expression Vector. To produce VLC-PUFAs from ALA and LA through the n-3 and n-6 pathways, we needed a Δ^6-desaturase, a fatty acid elongase, and a Δ^5-desaturase. We identified genes encoding these enzymes in a variety of VLC-PUFA-producing organisms such as fish and algae in previous research [6, 7, 10]. We engineered a series of plant transformation constructs containing a Δ^6-desaturase gene (*D6DES*) from the pike eel, *Muraenesox cinereus* [7]; a polyunsaturated fatty acid elongase gene (*ELOVL5*) from blackhead seabream, *Acanthopagrus schlegelii* [10]; and a Δ^5-desaturase gene (*D5DES*) from a microalga, *Phaeodactylum tricornutum* strain KMCC B-128 (GenBank accession number GQ352540), all under the control of seed-specific promoters. The vector pGEM7Zf was used for construction of intermediate expression vectors. The primer sequences and other PCR-related information are summarized in Table 2.

For the construction of expression vectors containing *McD6DES*, *AsELOVL5*, or *PtD5DES* under a seed-specific vicilin promoter, an expression construct containing a vicilin promoter, multiple cloning site, and *octopine synthase* (*OCS*) terminator from a vicilin cloning cassette was incorporated into pGEM7Zf. The individual *McD6DES*, *AsELOVL5*, and *PtD5DES* coding sequences were inserted into the *Hin*dIII and *Bam*HI or *Cla*I and *Bam*HI sites of the modified pGEM7Zf vector (Figure 2(a); Table 2 for primer set II). The fragment containing the vicilin promoter, *McD6DES*, and *OCS* terminator was isolated from the modified pGEM7Zf vector using a unique *Xba*I site and then ligated into the plant transformation vector pCAMBIA3300 (Cambia, Canberra, Australia) to form the seed-specific expression vector pCAM::*McD6DES*.

For the easy coexpression of the three genes in a single transformation step, one recombinant plasmid containing all three expression cassettes was constructed using an In-Fusion Advantage PCR Cloning Kit (Clontech, Mountain View, USA) according to the manufacturer's instructions. The vector was linearized with restriction enzymes. To amplify a target gene expression cassette, the 5′ end of the primer was designed to contain a 15 bp sequence homologous to the sequence at one end of the linearized vector. The 3′ end of the primer contained a sequence specific to the target gene (see primer set III in Table 2). The PCR product P_{vic}AsELOVL5OCS was subcloned into pCAM::*McD6DES* linearized at the unique *Sac*I site to yield the plasmid pCAM::*McD6DES–AsELOVL5*. Finally, the recombinant plasmid pCAM::*D6ELD5* was generated by inserting the P_{vic}PtD5DESOCS fragment into pCAM::*McD6DES–AsELOVL5* linearized at the unique *Pvu*I site introduced into P_{vic}AsELOVL5OCS (Figure 2(b)).

2.3. Plant Transformation. The engineered expression construct was transformed into *Agrobacterium tumefaciens* strain GV3101 [29] by the freeze-thaw method and cultured at 28°C on a rotary shaker to the appropriate growth phase. *A. thaliana* ecotype Columbia was transformed with the pCAM::*D6ELD5* construct by the floral dipping method [30, 31]. Briefly, freshly opened flower buds were dipped in *A. tumefaciens* solution for 15 s, wrapped in plastic film, and

Table 2: Primers used for this study. The data include sequences and annealing temperatures (T_a) for the primer pairs.

Primer set	Primer name	Sequence (5′ → 3′)	T_a
I	McD6D-106F	TGG TTG GTA ATC GAC AGA AAG GTG T	65°C
	McD6D-730R	TCT TTC CTA GCA CAA AGG TGT GGA G	
	AsELO-ORF-F	ATGGAGACCTTCAATCACAAACTGAACGTTTAC	
	AsELO-ORF-R	TCAATCCACTCTCAGTTTCTTGTGTGCAGTGT	
	PtD5D-ORF-F	ATGGCTCCGGATGCGGATAAGCTT	
	PtD5D-ORF-R	TTACGCCCGTCCGGTCAAGGGATTTT	
II	Hind-McD6D-F	CCC AAG CTT ATG GGA GGT GGA GGC CAG	62°C
	BamH-McD6D-R	CGG GAT CCT CAT TTA TGG AGA TAA GCA TCC	
	Hind-AsELO-F	CCC AAG CTT ATG GAG ACC TTC AAT CAC	
	BamH-AsELO-R	CGG GAT CCT CAA TCC ACT CTC AGT TT	
	Cla-PtD5D-F	CTT TAT TCA TCG ATA TGG CTC CGG ATG CGG AT	
	BamH-PtD5D-R	GCA GGA CTC TAG GAT CCT TAC GCC CGT CCG GTC AA	
III	Infu-AsELOVL5-F	GAT TAC GAA TTC GAG CTC GCG GCC GCT AAT ATT TTG CAA AAA	68°C
	Infu-AsELOVL5-R	CCC CGG GTA CCG AGC TCG TCC TGC TGA GCC TCG A	
	Infu-PtD5D-F	GCG AAG AGG CCC GCA CTA CGT ATA ATA TTT TGC AAA AAG A	
	Infu-PtD5D-R	CAA CTG TTG GGA AGG GCC AAT TGG TCC TGC TGA GCC TCG A	
qPCR	AtACT1 537F	TCT TGA TCT TGC TGG TCG TG	60°C
	AtACT1 707R	GAG CTG GTT TTG GCT GTC TC	
	McD6D 223F	TTC CAT CCA GAG CCT GAC TT	
	McD6D 371R	CCC TCC CTC TCT ACC TCC TC	
	AsELO 720F	CAC GCT AAT TTT CCT GTT CTC A	
	AsELO 871R	GTT TCT TGT GTG CAG TGT GCT	
	PtD5D 782F	GAT ACT GGT TGT CCG CTG TCT	
	PtD5D 935R	ATC ACG TTC ACC GCA ATG TA	

Restriction enzyme sites are underlined.

left overnight in the dark at 22°C, after which the plastic was removed.

2.4. Generation of Transgenic Plants. Transgenic *Arabidopsis* lines were generated by 0.3% Basta (glufosinate) selection. Selection of transgenics was performed by spraying seedlings with 0.3% Basta upon emergence and twice afterwards at 3-day intervals. Transgenic plants displayed tolerance to Basta, whereas the untransformed control plants were severely damaged and died following Basta treatment. Basta-resistant plants were transferred to pots and grown to maturity. Plants were observed during growth for the presence of visible phenotypes. Copy number of the T-DNA insertion was not determined. In all cases, no phenotypic alterations of the plants were observed upon modification of the seed oil composition.

2.5. Verification of pCAM::D6ELD5 in Transgenic Arabidopsis. To confirm the presence of all three transgenes in transgenic *Arabidopsis* plants, we performed PCR using primer set I (see Table 2). PCR was conducted on genomic DNA from young transgenic leaves using *AccuPower* Multiplex PCR PreMix (Bioneer Corp., Daejeon, Korea). The primers of primer set I were mixed within a single tube for DNA amplification and produced three different bands for high-throughput screening (data not shown).

Quantitative RT-PCR (qRT-PCR) was used to measure the coexpression of the three genes. At 9-10 days after flowering, siliques were taken from nine transgenic *Arabidopsis* plants differing in LC-PUFA output, and total RNA was extracted according to the method of Oñate-Sánchez and Vicente-Carbajosa [32]. Total RNA (1 μg) was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). The expression of the three genes was investigated with qRT-PCR using the specific primers shown in Table 2. The PCR product size was 150 bp for all three genes. The samples were amplified using SYBR *Premix Ex Taq* II (Takara) under the following conditions: 30 s of initial denaturation at 95°C and 40 cycles of 5 s at 95°C and 34 s at 60°C. All reactions were performed in triplicate. The relative amounts of mRNA were calculated using the comparative C_T method (User Bulletin number 2; Applied Biosystems, Foster City, CA, USA). The mRNA levels were normalized to that of *ACTIN1*, a housekeeping gene. Thermal cycling and fluorescence detection were performed with a 7300 Real-Time PCR System (Applied Biosystems).

2.6. Fatty Acid Analysis. One hundred milligrams of seeds were homogenized using a mortar and pestle with 5 mL of MeOH/$CHCl_3$ (2 : 1, v/v) and 1 mg PDA (pentadecanoic acid in MeOH) as an internal standard. Fatty acid methyl esters (FAMEs) were prepared with a lipid extraction method and analyzed by gas chromatography (GC) according to our

previous study [6]. The FAMEs were identified by reference to peaks of well-characterized commercial standards (Supelco; Sigma-Aldrich, Pennsylvania, USA), based on the GC retention time, and quantified using computer software. All analyses were performed in triplicate and replicated three times. Methanolic base (Sigma-Aldrich Canada Ltd., Ontario, Canada) and FAME standards (PUFA number II, 47015-U; PUFA number III, 47085-U; Supelco) were used as the reference standards.

3. Results and Discussion

3.1. Establishment of the Recombinant Plasmid pCAM::D6ELD5 in Arabidopsis. To optimize the production of VLC-PUFAs in oilseed plants, we conducted a stepwise engineering approach to generate a range of transgenic *Arabidopsis* lines carrying three genes, with each gene under the control of a seed-specific promoter. The expression construct (designated pCAM::*D6ELD5*) contained the minimal set of genes required for the synthesis of n-3 and n-6 C_{22} VLC-PUFAs (e.g., DPA and DTA) from endogenous C_{18} substrates (represented schematically in Figures 1 and 2(b)). The construct was verified by restriction analysis and sequencing of the resultant clones and introduced into *Arabidopsis* plants via floral dip transformation. From the T1 plants, 160 independent transgenic lines were selected with Basta. PCR analysis of genomic DNA revealed the presence of all three genes (*McD6DES*, *AsELOVL5*, and *PtD5DES*) in 104 of the selected plants (data not shown). Mature seeds from Basta-selected T2 plants were analyzed by GC for total fatty acid composition. No attempt was made to isolate homozygous lines from subsequent Basta-selected progeny.

3.2. Functional Analysis of PCAM::D6ELD5 in Arabidopsis. We examined the effect of this construct on functionality by examining the fatty acid composition in transgenic seeds. To reconstitute the biosynthetic pathway of C_{22} VLC-PUFAs from C_{18} precursors, the *McD6DES*, *AsELOVL5*, and *PtD5DES* open reading frames (ORFs) were coexpressed in *Arabidopsis* (Figure 2(b)). The transgenic seeds coexpressing the three ORFs produced DPA and DTA, in addition to STA, ETA, and EPA of n-3 PUFAs and GLA, DGLA, and ARA of and n-6 PUFAs (Figure 1 and Table 3). These nonnative PUFAs were not detected in wild-type *Arabidopsis* seeds. These results demonstrate that the coexpression of *M. cinereus* Δ^6-desaturase (McD6DES), an ELOVL5-like enzyme involved in VLC-PUFA elongation (AsELOVL5), and Δ^5-desaturase (PtD5DES) successfully reconstituted the n-3 and n-6 pathways in a heterologous system. Unexpectedly, however, our product of LC-PUFA was detected with only low levels in seeds (Table 3). As seen in Table 4, these experiments demonstrated the viability of using transgenic manners to modify seed oil PUFA content. Abbadi et al. [23] showed the analysis of transgenic seeds for tobacco and linseed. They described that different substrate requirements, namely, phospholipid-linked substrates for desaturases and acyl-CoA for elongases, resulted in a rate-limiting flux through the alternating desaturation and elongation steps. In addition, Kinney et al. [24] demonstrated that use of the endogenous acyltransferases which could accept nonnative substrates produced high level EPA in transgenic soybean (*Glycine max*). Cheng et al. [25] investigated the effects of host species on EPA biosynthesis using *Brassica carinata*. Moreover, Robert et al. [26] used the way to target both desaturase and elongase activities in one pool for bypassing the acyl exchange bottleneck. Hoffmann et al. [27] also tried to avoid the acyl exchange bottleneck by using acyl-CoA-dependent desaturases. However, disappointingly the seed levels of target VLC-PUFAs were low like our seeds. Our *M. cinereus* Δ^6-desaturase displayed preference for the n-3 substrate ALA, acting on this substrate with an efficiency of 21.3% compared to the n-6 substrate LA, of which only 7.0% was converted (Table 4). The *A. schlegelii* VLC-PUFA elongase demonstrated that this gene product has highly efficient activities (C18-elo and C20-elo). Yet although 42.6% of STA was elongated, only 6.5% of ETA was converted to EPA. It could be a likely explanation that repeat identical cassettes in the same orientation tend to be unstable, especially for the third cassette onward (D5-des in this case; Figure 2(b)). In addition, another approach is to use a strong acyl-CoA-dependent Δ^5-desaturase. We will be interested in seeing the capacity to identify another optimal combination of FA biosynthetic activities for the production of VLC-PUFAs in plants using methods of Petrie et al. [28].

3.3. Selection of Transgenic Arabidopsis. The 160 independent lines from the first Basta selection were grouped together in 65 groups of two or three lines. Analysis of total FAMEs from the seeds of T2 lines in 28 of the 65 groups indicated that plants expressing the pCAM::*D6ELD5* construct accumulated nonnative VLC-PUFAs (n-3 PUFAs: STA, ETA, EPA, and DPA; n-6 PUFAs: GLA, DGLA, ARA, and DTA) (Table 3). In lines of groups 12, 18, 33, 63, and 64 (named EPA group lines), EPA represented about 0.2% of total fatty acids; this level was higher than that observed in other *Arabidopsis* T2 lines. EPA accounted for 0.1% of total fatty acids in transgenic lines of groups 11, 14, 15, 20, 21, 23, 27, 41, 44, and 65. No EPA or only trace levels of EPA were detected in transgenic lines of groups 1, 2, 5, 6, 24, 26, 28, 39, 43, 45, 57, 61, and 62. The levels of ARA (n-6) were similar to those of EPA (n-3). Other nonnative PUFAs of STA, ETA, and DPA were shown by 4%, 3%, and 0.4% of total fatty acids in EPA group lines, respectively. Otherwise GLA, DGLA, and DTA were about 2%, 3%, and 0.1% of total fatty acids, respectively. These fatty acids are active in substrate of EPA or ARA products in metabolism of VLC-PUFAs (but not DPA and DTA) (Figure 1). The EPA group lines preferentially produced omega-3 (n-3) PUFAs rather than omega-6 (n-6). These results were to investigate the effect of introduction of the *McD6DES* gene which could be a useful tool for omega-3 synthesis. Our previous study reported that the *McD6DES* gene was a sustainable system for the production of dietary omega-3 fatty acids [7]. For subsequent experiments, we selected transgenic lines of groups 33, 44, 63, and 64, which produced some DPA and DTA in addition to EPA and ARA.

3.4. Quantification of Fatty Acids. Accumulation of nonnative fatty acids was monitored in the T3 generation derived

Table 3: Fatty acids composition (% w/w) produced by transgenic *Arabidopsis* T2 lines expressing pCAM::*D6ELD5* construct.

T2 group lines	Sample weight	16:0	18:0	18:1	LA	GLA	ALA	STA	20:1	DGLA	ARA	ETA	EPA	DTA	DPA
WT	0.1	8.5 ± 0.3	3.6 ± 0.2	15.1 ± 0.3	31.0 ± 0.3	0.0 ± 0.0	22.3 ± 0.1	0.0 ± 0.0	19.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	0.1	8.6 ± 0.2	4.4 ± 0.1	17.1 ± 0.1	30.8 ± 0.1	0.6 ± 0.1	18.0 ± 0.1	1.3 ± 0.3	17.4 ± 0.3	0.7 ± 0.3	0.1 ± 0.0	1.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.0
2	0.1	8.4 ± 0.3	4.3 ± 0.2	16.6 ± 0.3	30.2 ± 0.4	0.7 ± 0.2	19.1 ± 0.4	1.4 ± 0.1	17.2 ± 0.1	0.9 ± 0.1	0.1 ± 0.1	1.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1
5	0.1	8.8 ± 0.1	4.4 ± 0.3	15.6 ± 0.2	31.2 ± 0.1	0.7 ± 0.3	18.2 ± 0.4	1.5 ± 0.1	17.2 ± 0.1	1.0 ± 0.4	0.1 ± 0.1	1.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.0
6	0.1	8.6 ± 0.4	4.0 ± 0.3	15.9 ± 0.5	31.4 ± 0.2	0.2 ± 0.1	19.4 ± 0.2	0.4 ± 0.4	19.8 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
11	0.1	9.5 ± 0.3	5.5 ± 0.1	14.1 ± 0.3	29.2 ± 0.1	1.6 ± 0.1	16.5 ± 0.3	3.6 ± 0.1	14.6 ± 0.4	2.3 ± 0.1	0.2 ± 0.1	2.5 ± 0.4	0.1 ± 0.0	0.0 ± 0.1	0.3 ± 0.0
12	0.1	9.8 ± 0.5	6.1 ± 0.2	14.6 ± 0.3	28.6 ± 0.2	1.9 ± 0.3	14.8 ± 0.3	4.1 ± 0.4	13.5 ± 0.3	2.8 ± 0.2	0.2 ± 0.0	3.1 ± 0.4	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.0
14	0.1	9.2 ± 0.3	4.3 ± 0.4	14.7 ± 0.1	30.8 ± 0.1	1.0 ± 0.1	19.2 ± 0.1	2.4 ± 0.3	15.0 ± 0.1	1.4 ± 0.3	0.1 ± 0.1	1.9 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
15	0.1	8.8 ± 0.2	4.8 ± 0.3	15.5 ± 0.1	30.4 ± 0.1	1.1 ± 0.4	17.2 ± 0.2	2.9 ± 0.2	15.3 ± 0.1	1.6 ± 0.2	0.2 ± 0.1	2.0 ± 0.5	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
18	0.1	10.1 ± 0.3	5.0 ± 0.2	12.3 ± 0.1	30.3 ± 0.2	1.8 ± 0.2	17.5 ± 0.4	3.6 ± 0.4	14.1 ± 0.1	2.2 ± 0.1	0.2 ± 0.2	2.4 ± 0.4	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
20	0.1	9.4 ± 0.4	4.7 ± 0.4	13.0 ± 0.3	30.9 ± 0.4	1.2 ± 0.1	18.3 ± 0.2	2.5 ± 0.1	16.6 ± 0.3	1.4 ± 0.3	0.1 ± 0.1	1.6 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
21	0.1	8.8 ± 0.3	4.4 ± 0.1	16.6 ± 0.2	30.7 ± 0.4	1.1 ± 0.2	17.7 ± 0.4	2.2 ± 0.4	15.9 ± 0.4	1.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
23	0.1	8.7 ± 0.1	4.3 ± 0.5	15.0 ± 0.5	30.9 ± 0.1	0.5 ± 0.4	19.3 ± 0.1	1.2 ± 0.1	18.4 ± 0.3	0.7 ± 0.2	0.1 ± 0.0	0.8 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
24	0.1	8.8 ± 0.2	3.7 ± 0.4	14.2 ± 0.1	32.6 ± 0.3	0.1 ± 0.1	20.5 ± 0.1	0.2 ± 0.2	19.7 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
26	0.1	8.7 ± 0.3	4.4 ± 0.3	15.4 ± 0.2	31.2 ± 0.2	0.6 ± 0.3	18.3 ± 0.4	0.9 ± 0.1	19.0 ± 0.2	0.8 ± 0.1	0.0 ± 0.0	0.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
27	0.1	8.6 ± 0.4	4.2 ± 0.3	14.2 ± 0.3	31.7 ± 0.1	0.8 ± 0.1	18.4 ± 0.4	1.5 ± 0.3	17.8 ± 0.4	1.2 ± 0.3	0.1 ± 0.0	1.4 ± 0.4	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
28	0.1	8.7 ± 0.2	3.9 ± 0.2	14.5 ± 0.1	32.1 ± 0.1	0.2 ± 0.1	20.1 ± 0.2	0.4 ± 0.4	19.3 ± 0.4	0.3 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
33	0.1	9.0 ± 0.3	5.0 ± 0.5	15.1 ± 0.2	30.2 ± 0.2	2.1 ± 0.1	13.7 ± 0.1	3.4 ± 0.1	14.7 ± 0.2	3.2 ± 0.4	0.2 ± 0.0	3.0 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
39	0.1	8.5 ± 0.2	4.0 ± 0.3	15.9 ± 0.1	33.9 ± 0.1	0.1 ± 0.1	18.1 ± 0.2	0.2 ± 0.2	19.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
41	0.1	9.1 ± 0.1	4.8 ± 0.2	13.4 ± 0.1	31.1 ± 0.3	1.5 ± 0.4	16.9 ± 0.3	2.8 ± 0.3	16.5 ± 0.4	1.7 ± 0.1	0.2 ± 0.1	1.8 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
43	0.1	8.9 ± 0.4	5.4 ± 0.1	15.1 ± 0.1	29.2 ± 0.1	1.9 ± 0.2	16.7 ± 0.2	3.7 ± 0.3	15.5 ± 0.1	1.8 ± 0.3	0.0 ± 0.0	2.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
44	0.1	9.1 ± 0.3	5.2 ± 0.3	13.2 ± 0.3	30.5 ± 0.2	1.5 ± 0.1	16.6 ± 0.3	2.6 ± 0.2	16.7 ± 0.1	2.1 ± 0.1	0.2 ± 0.0	2.0 ± 0.3	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
45	0.1	9.0 ± 0.2	4.4 ± 0.2	15.4 ± 0.2	31.1 ± 0.1	0.9 ± 0.3	17.7 ± 0.3	1.8 ± 0.1	17.4 ± 0.2	1.2 ± 0.3	0.0 ± 0.0	1.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
57	0.1	9.2 ± 0.1	5.0 ± 0.3	13.6 ± 0.1	29.7 ± 0.1	1.1 ± 0.4	18.7 ± 0.4	2.6 ± 0.1	16.1 ± 0.4	1.7 ± 0.4	0.0 ± 0.0	2.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
61	0.1	9.1 ± 0.3	5.0 ± 0.4	14.2 ± 0.3	31.7 ± 0.5	1.2 ± 0.3	16.7 ± 0.3	2.7 ± 0.1	15.6 ± 0.1	1.9 ± 0.3	0.0 ± 0.0	2.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
62	0.1	11.2 ± 0.3	5.6 ± 0.3	0.0 ± 0.0	36.2 ± 0.1	1.9 ± 0.1	18.9 ± 0.3	4.3 ± 0.3	16.4 ± 0.3	2.6 ± 0.3	0.0 ± 0.0	2.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
63	0.1	9.5 ± 0.2	4.8 ± 0.2	14.0 ± 0.3	30.6 ± 0.2	2.3 ± 0.3	14.4 ± 0.3	3.9 ± 0.1	13.6 ± 0.4	3.1 ± 0.2	0.3 ± 0.0	2.9 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
64	0.1	9.3 ± 0.3	4.5 ± 0.3	14.3 ± 0.2	31.9 ± 0.1	2.1 ± 0.1	14.8 ± 0.1	3.0 ± 0.1	14.2 ± 0.4	2.8 ± 0.1	0.2 ± 0.1	2.4 ± 0.4	0.2 ± 0.0	0.1 ± 0.1	0.4 ± 0.0
65	0.1	8.7 ± 0.4	4.3 ± 0.4	16.8 ± 0.2	31.9 ± 0.2	1.3 ± 0.3	15.9 ± 0.2	2.6 ± 0.1	15.2 ± 0.3	1.8 ± 0.1	0.1 ± 0.0	1.1 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0

Each value is the mean ± SD from three independent experiments. Total lipid is mg lipid g^{-1} dry cells.

Table 4: Comparison of published transgenic lines producing VLC-PUFAs and biosynthetic intermediates.

Reference	Plant species	Tissue	GLA	STA	DGLA	ARA	ETA	EPA	DPA
This work [63 transgenic T2 line in Table 3] (Conversion efficiencies)	*A. thaliana*	Seed	2.3 **(7.0%, D6-des)**	3.9 **(21.3%, D6-des)**	3.1 **(59.2%, C18-elo)**	0.3 **(8.8%, D5-des)**	2.9 **(42.6%, C18-elo)**	0.2 **(6.5%, D5-des)**	0.3 **(60%, C20-elo)**
Abbadi et al. [23]	*N. tabacum*	Seed	29.3	—	1.8	1.5	—	—	—
	L. usitatissimum	Seed	16.8	11.4	1.2	1.0	0.9	0.8	—
Kinney et al. [24]	*G. max*	Seed	11.7	1.1	10.1	2.2	2.4	19.6	0.8
Cheng et al. [25]	*B. carinata*	Seed	26.9	5.4	2.2	5.7	2.5	20.4	4.0
Robert et al. [26]	*A. thaliana*	Seed	0.6	1.8	1.9	1.6	0.4	3.2	0.1
Hoffmann et al. [27]	*A. thaliana*	Seed	>0.5	>0.1	0.8	0.1	0.9	0.05	—

Where shown, conversion efficiencies are calculated as [product]/[product + substrate] × 100. This value represents the overall percent of conversion of total substrate into total product formed.

from the selected T2 plants (Table 5). Seeds of the transgenic T3 line 63-1 had the highest level of EPA, at 0.4% of total seed fatty acids; this represented a twofold increase in EPA level compared with seeds of the transgenic T2 line (Tables 3 and 5). The level of ARA in seeds of line 63-1 was 0.3% of total fatty acids, which was similar to the level found in T2 seeds. Levels of EPA and ARA in transgenic lines 33-1, 33-2, 33-4, 63-5, and 64-3 were half of those in line 63-1, in the range of 0.1–0.3% of total fatty acids (Table 5). Transgenic lines 44-1, 44-4, and 44-5 had only minor levels of EPA and ARA, in addition to DPA and DTA. In addition to having a higher level of EPA, seeds of line 63-1 also displayed increased levels of other LC-PUFAs, suggesting variation in the endogenous channeling of fatty acids into either pathway.

STA and GLA are intermediates in the early stages of EPA and ARA synthesis [33, 34], respectively, in the metabolism of VLC-PUFAs (Figure 1). STA in seeds of line 63-1, at 5.6% of total seed fatty acids, represented one and a half times increase compared with seeds of the transgenic T2 line (Tables 3 and 5). The level of STA was a threefold increase to GLA of line 63-1 which was similar to the level found in T2 seeds. ETA and DGLA were detected with similar level of total lipids in T3 plant of line 63-1, but ETA was increased by one times more compared with T2 plant. These contents are important for the production of EPA by the action of Δ^5-desaturase [35–37]. Use of *P. tricornutum* Δ^5-desaturase reached with 9.5% and 8.6% to EPA and ARA from substrates conversion of ETA and DGLA, respectively (Table 5).

DPA and DTA were 0.3 and 0.1% of the total fatty acids, respectively, in the transgenic T3 line 63-1. This suggests that DPA will be an important component of future metabolic engineering strategies for producing DHA with a combination of genes encoding Δ^4-desaturase in plants [38]. These results showed that transgenic line 63-1 of T3 plants was more stable than T2 plants for expressing appropriate heterologous enzymes and also a sustainable system for the production of dietary omega-3 fatty acids. To study variation in the endogenous capacity to synthesize VLC-PUFAs, we analyzed the coordinated tissue-specific expression of multiple genes in seeds of transgenic T3 plants.

3.5. Heterologous Expression of pCAM::D6ELD5 in Arabidopsis. After three rounds of Basta selection, nine independent transgenic T3 lines were obtained. The expression levels of the three transgenes in these lines were analyzed by qRT-PCR (Figure 3). The analysis indicated that the expression levels of the three genes encoded by *D6ELD5* were related to variation in the output of LC-PUFAs in transgenic seeds (Table 5 and Figure 3; see also the schematic representation in Figure 1). Overall levels of expression in line 63-1 were much higher than those in other transgenic lines, and the expression of *AsELOVL5* was highest in this line. The expression levels were quite different among the genes, with *AsELOVL5* being expressed at much higher levels than *McD6DES* or *PtD5DES*. The accumulation of ETA (or DGLA) and DPA (or DTA) was due to the activity of the elongase encoded by *AsELOVL5*. The sequential reactions catalyzed by the elongase, STA → ETA

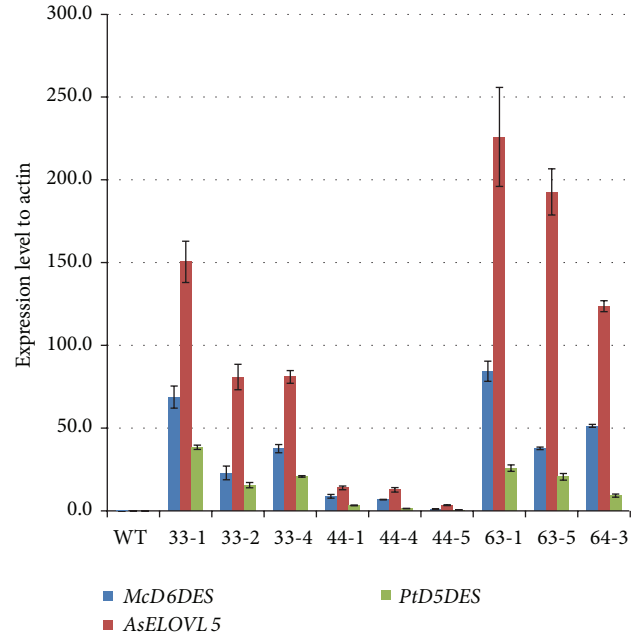

Figure 3: Analysis of pCAM::*D6ELD5* transcript accumulation in seeds of *Arabidopsis*. The genes *McD6DES*, *AsELOVL5*, and *PtD5DES* were assayed for transcript abundance. Data were normalized to *ACTIN1* mRNA levels and expressed as $2^{-\Delta\Delta CT}$. Mean values obtained from three independent experiments are shown by the line.

Table 5: Fatty acids composition (% w/w) produced by transgenic *Arabidopsis* T3 lines expressing pCAM::*D6ELD5* construct.

Usual FA	WT	33-1	33-2	33-4	44-1	44-4	44-5	63-1	63-5	64-3
					Fatty acid					
16:00	8.5 ± 0.3	8.8 ± 0.4	8.9 ± 0.1	8.7 ± 0.3	8.5 ± 0.2	8.6 ± 0.3	8.5 ± 0.1	9.4 ± 0.1	9.3 ± 0.3	9.4 ± 0.1
18:00	3.8 ± 0.1	4.2 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.2 ± 0.2	4.2 ± 0.3	3.9 ± 0.4	5.3 ± 0.3	5.1 ± 0.2	5.1 ± 0.2
18:01	13.5 ± 0.1	12.6 ± 0.1	13.6 ± 0.1	13.3 ± 0.3	13.6 ± 0.1	13.4 ± 0.4	12.6 ± 0.3	13.5 ± 0.1	13.4 ± 0.3	13.4 ± 0.1
LA	29.6 ± 0.3	28.4 ± 0.1	27.5 ± 0.2	27.6 ± 0.4	30.5 ± 0.3	29.5 ± 0.2	29.8 ± 0.1	27.4 ± 0.3	28.0 ± 0.2	28.2 ± 0.4
ALA	22.9 ± 0.1	19.7 ± 0.4	19.6 ± 0.3	19.2 ± 0.1	20.9 ± 0.2	21.7 ± 0.3	22.9 ± 0.2	15.3 ± 0.1	17.5 ± 0.4	17.2 ± 0.3
20:01	21.7 ± 0.4	17.0 ± 0.1	16.8 ± 0.1	17.4 ± 0.1	20.9 ± 0.2	21.1 ± 0.2	21.6 ± 0.2	13.1 ± 0.3	17.0 ± 0.4	16.2 ± 0.1
Total	100	90.7	91.1	91	98.6	98.5	99.3	84	90.3	89.5
					New *n*-3 PUFA					
STA	0.0 ± 0.0	3.3 ± 0.5	3.2 ± 0.4	3.1 ± 0.5	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	5.6 ± 0.1 (26.8%, D6-des)	3.2 ± 0.1	3.5 ± 0.4
ETA	0.0 ± 0.0	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	3.8 ± 0.1 (40.4%, C18-elo)	2.3 ± 0.1	2.5 ± 0.3
EPA	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0 (9.5%, D5-des)	0.2 ± 0.0	0.2 ± 0.0
DPA	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0 (42.9%, C20-elo)	0.2 ± 0.0	0.2 ± 0.0
Total	0	5.8	5.7	5.6	0.9	1	0.4	10.1	5.9	6.4
					New *n*-6 PUFA					
GLA	0.0 ± 0.0	1.3 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	2.3 ± 0.1 (7.7%, D6-des)	1.4 ± 0.3	1.7 ± 0.4
DGLA	0.0 ± 0.0	1.8 ± 0.4	1.7 ± 0.1	1.8 ± 0.2	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.2	3.2 ± 0.5 (58.2%, C18-elo)	2.1 ± 0.4	2.2 ± 0.2
ARA	0.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0 (8.6%, D5-dea)	0.2 ± 0.0	0.1 ± 0.0
DTA	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0 (25.0%, C20-elo)	0.1 ± 0.0	0.1 ± 0.0
Total	0	3.5	3.2	3.4	0.5	0.5	0.3	5.9	3.8	4.1
Total new FA	0	9.3	8.9	9	1.4	1.5	0.7	16	9.7	10.5
Total FA	100	100	100	100	100	100	100	100	100	100

Each value is the mean ± SD from five independent experiments. Total lipid is mg lipid g^{-1} dry cells. Where shown, conversion efficiencies are calculated as [product]/[product + substrate] × 100. This value represents the overall percent of conversion of total substrate into total product formed.

and EPA → DPA in the n-3 pathway and GLA → DGLA and ARA → DTA in the n-6 pathway, provide an abundance of precursor fatty acids for EPA and ARA synthesis (in the first reaction) and a progenitor of DHA (in the second reaction). Because the ELOVL5-like elongase yields both product and substrate, the high level of expression of *AsELOVL5* could provide some insight into the reasons for variation in the output of LC-PUFAs. Use of the efficient *A. schlegelii* VLC-PUFA elongase resulted in substrates conversion from STA and EPA to ETA and DPA reaching 40.4% and 42.9% in the n-3 pathway and from GLA and ARA to DGLA and DTA reaching 58.2% and 25.0% in the n-6 pathway, respectively (Table 5).

The step catalyzed by Δ^6-desaturase is considered the rate-limiting step in the conversion of dietary ALA or LA to VLC-PUFAs [39, 40]. STA and GLA are produced with the desaturation of ALA and LA, respectively, by Δ^6-desaturase (McD6DES). *McD6DES* was most highly expressed in line 63-1, and STA and GLA accumulated in this line. The STA accumulation was, as stated above, higher than GLA. These results were also shown in the conversion efficiency. Use of the efficient *M. cinereus* Δ^6-desaturase of line 63-1 resulted in substrates conversion from ALA and LA to STA and GLA reached 26.8% and 7.7%, respectively (Table 5). However, the accumulation of EPA and ARA, which are products of ETA and DGLA desaturation, respectively, could not be directly related to the level of *PtD5DES* expression. Although increased levels of EPA and ARA were observed in line 63-1, the expression level of *PtD5DES* in line 63-1 was below that in line 33-1. It may be that a number of different factors regulate the accumulation of Δ^5-desaturated fatty acids in the seeds. Therefore, the abundance of STA enabled abundant accumulation of EPA, and high expression of *AsELOVL5* resulted in the accumulation of EPA and DPA.

4. Conclusion

The modification of the lipid profile of oilseeds is an area of interest because the end-products have significant commercial value including foods, pharmaceuticals, or industrial raw material. However, the manipulation of plant seed oil composition is still a challenge. Because higher plants have no endogenous capacity for synthesis of VLC-PUFAs, we constructed a single recombinant plasmid from three gene expression cassettes to coexpress three genes using a single transformation step. The three primary enzymes needed for biosynthesis of VLC-PUFAs were expressed in transgenic *Arabidopsis* seeds as discrete transcription and translation products. The metabolism of endogenous cellular products, ALA and LA, to VLA-PUFAs was achieved in transgenic seeds by a series of desaturation and elongation reactions. Particularly, the introduction of the *McD6DES* gene could be a useful tool for omega-3 (n-3) STA synthesis is thought to be a rate-limiting step. Therefore, this study demonstrates that the success of this required expression of multiple genes, as three sequential nonnative enzymatic reactions are involved in the conversion of native plant FAs to VLC-PUFAs.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Docosahexaenoic Acid Induces Cell Death in Human Non-Small Cell Lung Cancer Cells by Repressing mTOR via AMPK Activation and PI3K/Akt Inhibition

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The anticancer properties and mechanism of action of omega-3 polyunsaturated fatty acids (ω3-PUFAs) have been demonstrated in several cancers; however, the mechanism in lung cancer remains unclear. Here, we show that docosahexaenoic acid (DHA), a ω3-PUFA, induced apoptosis and autophagy in non-small cell lung cancer (NSCLC) cells. DHA-induced cell death was accompanied by AMP-activated protein kinase (AMPK) activation and inactivated phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling. Knocking down AMPK and overexpressing Akt increased mTOR activity and attenuated DHA-induced cell death, suggesting that DHA induces cell death via AMPK- and Akt-regulated mTOR inactivation. This was confirmed in Fat-1 transgenic mice, which produce ω3-PUFAs. Lewis lung cancer (LLC) tumor cells implanted into Fat-1 mice showed slower growth, lower phospho-Akt levels, and higher levels of apoptosis and autophagy than cells implanted into wild-type mice. Taken together, these data suggest that DHA-induced apoptosis and autophagy in NSCLC cells are associated with AMPK activation and PI3K/Akt inhibition, which in turn lead to suppression of mTOR; thus ω3-PUFAs may be utilized as potential therapeutic agents for NSCLC treatment.

1. Introduction

Lung cancer is the main cause of cancer-related death worldwide. According to the latest statistics from the United States National Cancer Institute, it is estimated that 224,210 Americans will be diagnosed with lung cancer in 2014 [1]. There are two types of lung cancer, namely, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), with more than 80% of lung cancer cases being NSCLC [2]. Because NSCLC is much less sensitive to chemotherapy than SCLC [3], a new approach for treating NSCLC is required.

Autophagy is a lysosome-associated degradation process that is characterized by the formation of double-membraned autophagosomes, which encapsulate cytoplasmic constituents [4–6]. The degraded components can then be used for energy production and other cellular processes [7]. Autophagy-related (Atg) genes, such as Atg12 and Atg5, are key molecules in regulation of autophagy. Atg12 constitutively associates with Atg5 to form Atg12-Atg5 conjugate, which is essential for the formation of lapidated microtubule-associated light chain 3 (LC3-II, a mammalian homolog of Atg8-II) and autophagosomes [8, 9]. The most potent inhibitor of autophagy is mammalian target of rapamycin (mTOR), which acts upstream of Atg proteins to regulate cell growth/proliferation, survival, protein and lipid synthesis, lysosome biogenesis, and cytoskeletal organization [10–12]. mTOR is

activated by Akt, a downstream product of phosphatidylinositol 3-kinase (PI3K), whereas the major negative regulator of mTOR is 5′ AMP-activated protein kinase (AMPK), which regulates intracellular energy status by sensing the AMP/ATP ratio [13–15]. mTOR utilizes p70 ribosomal S6 kinases 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) as substrates [16]. Inhibiting mTOR not only blocks the phosphorylation of S6K1 and 4E-BP1, but also induces autophagy [16–18].

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (ω3-PUFA), induces apoptosis in tumor cells by regulating several key signaling mediators, including Wnt/β-catenin [19], p53-regulated activator protein 1 [20], and mitogen-activated protein kinase [21, 22]. In addition, we have previously reported that DHA simultaneously induces apoptosis and autophagy in human cervical cancer as well as prostate cancer cells, and this process involves mTOR repression [23, 24]. Despite several studies describing that the anti-lung cancer activity of DHA may be dependent on its proapoptosis effect [21, 25–27], it is still unclear whether it also induces autophagy *in vitro* and *in vivo*.

Here, we examined the mechanism(s) underlying DHA-induced cell death in human NSCLC cells. The results showed that DHA reduced cell viability and induced both apoptosis and autophagy. Moreover, DHA-induced cell death was associated with AKT-mTOR signaling inhibition and AMPK activation. Similarly, lung cancer cells implanted into Fat-1 transgenic mice exhibited higher levels of apoptosis, a higher autophagy index, and lower levels of phospho-AKT than cells implanted into wild-type mice. Taken together, these data show that DHA induces apoptosis and autophagy through AKT-mTOR signaling inhibition and AMPK activation, suggesting that ω3-PUFAs may be a potential treatment for human NSCLC.

2. Materials and Methods

2.1. Chemicals and Antibodies. DHA (Cayman Chemical, Ann Arbor, MI, USA; dissolved in absolute ethanol), chloroquine (CQ, Sigma, ST Louis, MO, USA), Bafilomycin A1 (Tocris, Bristol, UK; dissolved in phosphate buffered saline (Sigma)), and rapamycin (Tocris; dissolved in dimethyl sulfoxide (DMSO; Sigma)) were stored at −20°C before use.

The following antibodies were used in this study: PI3K (p85), Akt, phospho-Akt (Ser473), phosphophosphatase and tensin homolog deleted on chromosome 10 (PTEN), AMPK, phospho-AMPK (Thr172), phospho-mTOR (Ser2448), phospho-S6K1 (Thr389), Atg5, Atg7, 4E-BP1, and LC3B (all Cell Signaling Technology, Beverly, MA, USA) and poly(ADP-ribose) polymerase- (PARP-) 1/2 (H-250), phospho-Akt (Thr308), p27 (C-19), and actin (I-19)-R (all Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Calbiochem (Billerica, MA, USA).

2.2. Cells Lines and Cultures. Human NSCLC A549 cells and H1299 cells were purchased from American Type Cell Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO), penicillin, and streptomycin (GIBCO) in a humidified 5% CO_2 atmosphere at 37°C.

2.3. Cell Viability Assay. Cell viability was determined using thiazolyl blue tetrazolium bromide (MTT; Sigma). Cells were seeded into 96-well plates (7×10^3 per well) and incubated for 18 h at 37°C to allow adherence. The cells were then incubated with serum-free medium for 24 h and then treated with DHA for another 24 h. The cells were then incubated with MTT for 2 h and the formazan products dissolved in DMSO. Absorbance was assayed at 570 nm in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Western Blot Analysis. Western blot analysis was performed as described previously [28]. Briefly, cell lysates (30 μg) were resolved by 6–15% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked in 5% (w/v) skimmed-milk for 1 h, followed by incubation with appropriate primary antibodies (diluted 1 : 1000–1 : 5000) overnight at 4°C. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies and the blots developed using enhanced chemiluminescence (Millipore).

2.5. Apoptosis Analysis. Apoptosis was measured using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, flow cytometry, and an Annexin V assay as described previously [23, 29].

2.6. Immunofluorescence Analysis. Cells were grown to 70% confluence in growth medium for 18 h before infection with recombinant adenoviruses expressing GFP-tagged LC3 (GFP-LC3, a gift from Professor Chang Deok Kim, Chungnam National University, Korea). DHA was then added to the infected cell cultures for 24 h. The cells were observed by an Olympus iX70 fluorescence microscope.

2.7. Small Interfering RNAs (siRNAs) and Transfection. siRNAs targeting Atg5 and Atg7 were purchased from Invitrogen (Camarillo, CA, USA). A nontargeting control siRNA was purchased from Bioneer (Daejeon, Korea). Cells (4.5×10^5 cells in a 100 mm dish) were transfected with 50 nM of siRNA for 36–48 h using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were then incubated for 24 h with serum-free medium and then treated with DHA. The siRNA sequences were as follows: nontargeting control siRNA; *5′-ACG UGA CAC GUU CGG AGA AUU-3′*; Atg5, *5′-AUC CCA UCC AGA GUU GCU UGU GAU C-3′*; Atg7, *5′-CCA AGG AUG GUG AAC CUC AGU GAA U-3*; and AMPK *5′-GGU UGG CAA ACA UGA AUU GdTdT-3′*.

For the transfection of expression vectors, cells were grown to 70–80% confluence and then switched to serum-free medium for 2–4 h before being transiently transfected

with HA-tagged myr-Akt1 (Akt-wt) and HA-tagged kinase-dead (K179M) dominant negative Akt1 (Akt-dd) (kindly provided by Dr. Incheol Shin; Hanyang University, Korea) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 12–18 h, the cells were subjected to different treatments. Control transfections were carried out using an empty pcDNA3 vector.

2.8. Tumor Xenograft Study. Transgenic Fat-1 C57BL/6 mice were provided by Dr. J. X. Kang (Harvard University, USA). Control C57BL/6 mice were purchased from Central Lab Animal Inc. (Seoul, Korea). The control and Fat-1 C57BL/6 mice were kept under specific pathogen-free conditions and received care according to the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University. The Fat-1 C57BL/6 mice ($n = 5$) used in this study were heterozygous, male, and 6 weeks old at the time of the experiments. Each mouse was subcutaneously injected with Lewis lung cancer (LLC) cells (3×10^6 in 100 μL serum-free DMEM) on day 0. The tumor size was measured every other day using calipers for 10 days. Tumor size was calculated as length × wide and volume was calculated as 0.5 × length × $(\text{wide})^2$.

2.9. Immunohistochemistry. After deparaffinization and antigen retrieval, the implanted tumor cell tissues were blocked with Dako protein block (Dako, Glostrup, Denmark); stained with rabbit anti-phospho-Akt (Ser473; 1 : 250), rabbit anti-phospho-AMPK (1 : 250), and rabbit anti-LC3B (1 : 250) primary antibodies followed by TRITC-conjugated anti-rabbit IgG (1 : 500); and then counterstained with DAPI. Stained tissues were examined under an Olympus iX70 fluorescence microscope using the DP Controller software. Images from two separate channels were merged.

2.10. Statistical Analysis. Statistical analyses were done as recommended by an independent analyst. These included the unpaired Student's t-test. All values are expressed as mean ± SD, and statistical significance was accepted for P values of <0.05. *, **, and *** mean $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results and Discussion

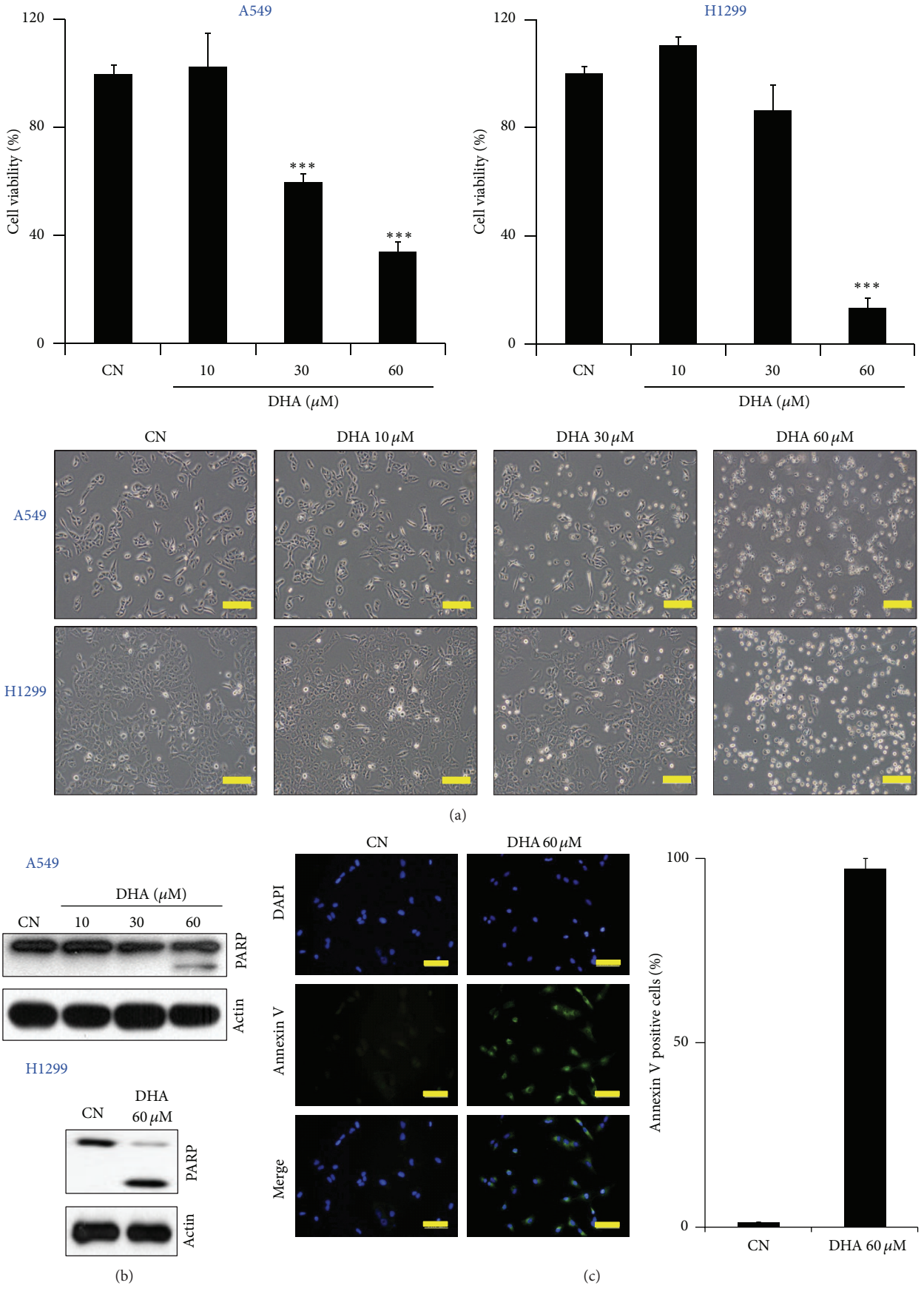
3.1. DHA Reduces Cell Viability and Induces Apoptosis in Human NSCLC Cells. It has been shown previously that DHA induces apoptotic cell death in several types of cancer cells [27, 30–32]. To examine whether DHA induces apoptosis in human NSCLC cells, we tested the effect of DHA on the viability of A549 and H1299 cells *in vitro*. DHA reduced the viability of both cell lines (Figure 1(a), upper). Moreover, when observed under a light microscope, DHA-treated cells appeared shrunken, rounded, and detached from the culture dishes (Figure 1(a), lower), characteristics suggestive of apoptosis.

To investigate whether this observed DHA-induced reduction in cell viability was actually due to apoptosis, we first examined the cleavage of PARP, an apoptotic marker. DHA treatment led to an increase in the levels of cleaved PARP in both A549 and H1299 cells (Figure 1(b)). Furthermore, DHA treatment caused a marked increase in the number of Annexin V-positive cells (Figure 1(c)), which is another early apoptotic indicator [33]. We next performed a TUNEL assay and cell-cycle analysis to look for nuclear DNA-strand breaks and hypodiploid DNA formation because both are increased during apoptosis [34]. DHA treatment increased the number of TUNEL-positive A549 cells (Figure 1(d)) and the percentage of A549 cells in the sub-G1 phase (Figure 1(e)). Taken together, these data indicate that DHA induces apoptosis in NSCLC cells.

3.2. DHA Triggers Autophagy as a Prerequisite for Apoptotic Cell Death. Apoptosis and autophagy are highly interconnected [35], and our previous studies showed that DHA activates both of these cascades simultaneously in cervical and prostate cancer cells [23, 24]. To determine whether autophagy is also involved in DHA-induced NSCLC cell death, we initially measured the expression LC3-II (the membrane-bound lipidated form of LC3), a biomarker of autophagic initiation [36]. Western blot analysis revealed that DHA caused a remarkable increase in LC3-II expression (Figure 2(a)). Similarly, DHA led to a dose-dependent increase in the number of GFP-LC3 puncta per virally infected A549 cell (Figure 2(b)). It is known that during autophagy, autophagosomes fuse with lysosomes in which both LC3-II and the cargo are degraded [23]. To examine whether DHA interferes with autophagic flux, we therefore next examined the colocalization of lipidated LC3-II with lysosomes using Lysotracker dye. DHA treatment led to a marked increase in the colocalization of lipidated LC3-II with Lysotracker compared with that in control cells (Figure 2(c)), suggesting that DHA does not block autophagosome-lysosome fusion. To further confirm this, A549 cells were exposed to DHA in the absence or presence of CQ and Bafilomycin A1 (inhibitors of lysosomal acidification) and LC3-II levels were analyzed by immunoblotting (Figure 2(d)). We found that the DHA-induced increase in LC3-II expression was further increased by CQ and Bafilomycin A1. Taken together, these results indicate that autophagy is activated and may play a role in DHA-induced cell death.

To unveil the relationship between DHA-autophagy and DHA-apoptotic cell death, we used siRNAs to knock down two essential autophagy gene products, Atg5 and Atg7. Although silencing of Atg5 and Atg7 had no effect on the viability of A549 cells, it strongly suppressed DHA-induced autophagy and apoptosis as evidenced by a reduction in LC3-II and viability (Figures 3(a) and 3(b)). These results imply that autophagy, at least partially, contributes to DHA-induced apoptotic cell death.

Autophagy has been shown to be essential for cell survival under certain stressful conditions. For example, hypoxia and the anticancer drug, Nelfinavir, induce autophagy by inactivating the growth factor receptors and by suppressing Akt signaling [37, 38], both of which play a positive role in cell survival; however, our data indicate that DHA induces autophagy, which enhances cell death. This observation is



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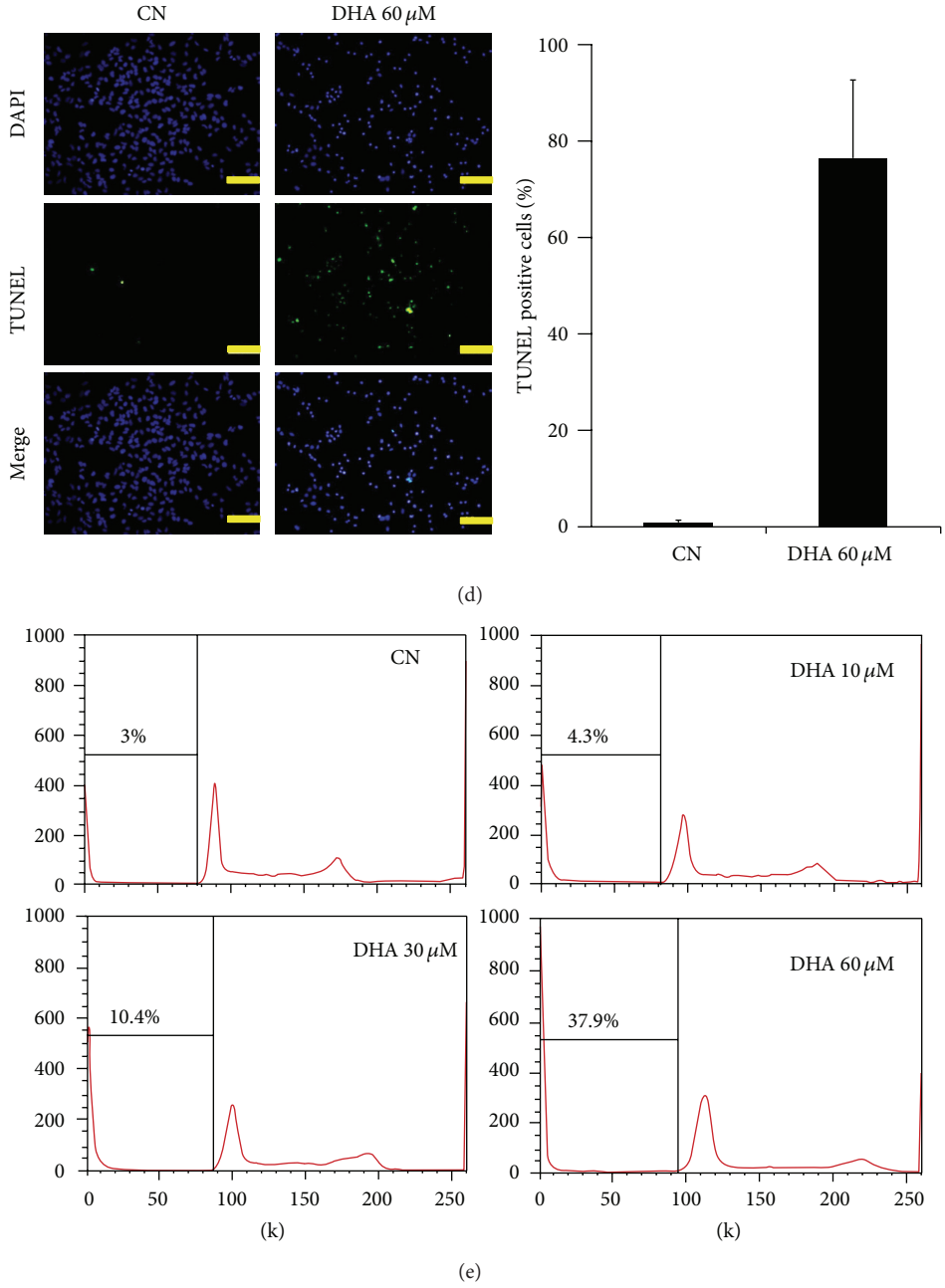


Figure 1: DHA inhibits cell viability and induces apoptosis in human cancer cells. (a) Upper panel: DHA reduces the viability of A549 and H1299 cells in a dose-dependent manner. Cells were exposed to the indicated doses of DHA for 24 h and the cell viability was measured in an MTT assay. Each bar represents the mean of three determinations. Each experiment was repeated three times. $^{***}P < 0.001$. Lower panel: representative images of A549 and H1299 cells treated with DHA for 24 h (scale bar: 200 μm). (b) DHA induces apoptosis. A549 (upper panel) and H1299 (lower panel) cells were incubated with the indicated doses of DHA for 24 h. The cells were then harvested and western blot analysis was performed with anti-PARP and anti-actin antibodies. (c) Left panel: evaluation of apoptosis by Annexin V staining. Green staining represents Annexin V-positive (apoptotic) cells (scale bar: 50 μm). Right panel: unfixed A549 cells were treated with FITC-Annexin V and then subjected to flow cytometry to examine changes in the plasma membrane. (d) DHA increases the number of TUNEL-positive cells. A549 cells were plated in a 12-well plate containing glass coverslips and then treated with 60 μM DHA for 6 h. Following treatment, apoptosis was detected using the DeadEnd Fluorometric TUNEL System. Left panel: representative fluorescence microscopy images (scale bar: 200 μm). Right panel: the percentage of TUNEL-positive cells in the presence or absence of DHA was expressed relative to the total number of DAPI-stained nuclei. TUNEL-positive cells were counted in three different fields and the numbers averaged. (e) DHA increases the number of NSCLC cells in the sub-G_1 phase. A549 cells were seeded and treated with the indicated doses of DHA for 24 h. The cell-cycle distribution of DHA-treated cells was analyzed by flow cytometry as described in Section 2. Data were analyzed using FlowJo software.

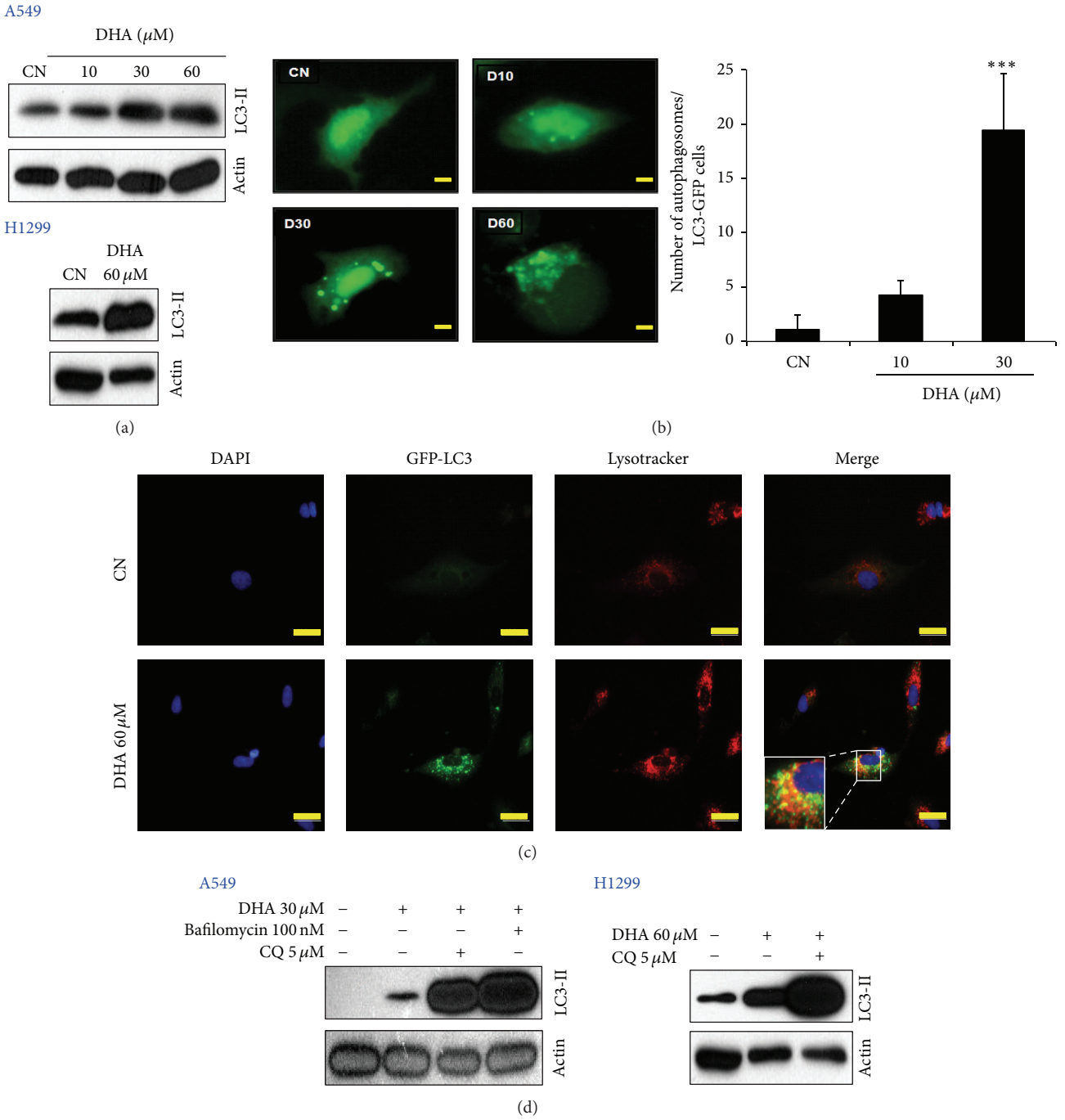


Figure 2: DHA induces autophagy. (a) DHA increased the expression of LC3-II in a dose-dependent manner. A549 (upper panel) and H1299 (lower panel) cells were exposed to the indicated doses of DHA for 24 h. Protein lysates were then prepared, separated in SDS polyacrylamide gels, and immunoblotted with antibodies against LC3-II and actin. (b) Formation of GFP-LC3 puncta in DHA-treated NSCLC cells. A549 cells were transfected with a GFP-LC3 plasmid and then exposed to the indicated doses of DHA for another 24 h before counterstaining with DAPI. Left panel: representative fluorescence microscopy images are shown (scale bar: 2 μm). Right: the number of autophagosomes was quantified as the number of GFP-LC3 puncta per transfected cell. Data are expressed as the mean ± SD of ten determinations (each in two separate experiments). $^{***}P < 0.001$. (c) DHA activates autophagic flux in NSCLC cells. Cells were transfected with the GFP-LC3 expression vector using Lipofectamine 2000 reagent for 17 h and then treated with 60 μM DHA for another 4 h. DHA-treated cells were then stained with Lysotracker dye. Representative fluorescence microscopy images are shown. Data are expressed as the mean ± SD of five determinations (each in three separate experiments) (scale bar: 10 μm). (d) DHA-induced autophagy increases NSCLC cell death. A549 (left) and H1299 (right) cells were incubated for 1 h in the presence or absence of the indicated doses of Bafilomycin and CQ before incubation with indicated doses of DHA for 24 h. Cell lysates were prepared and examined by western blotting.

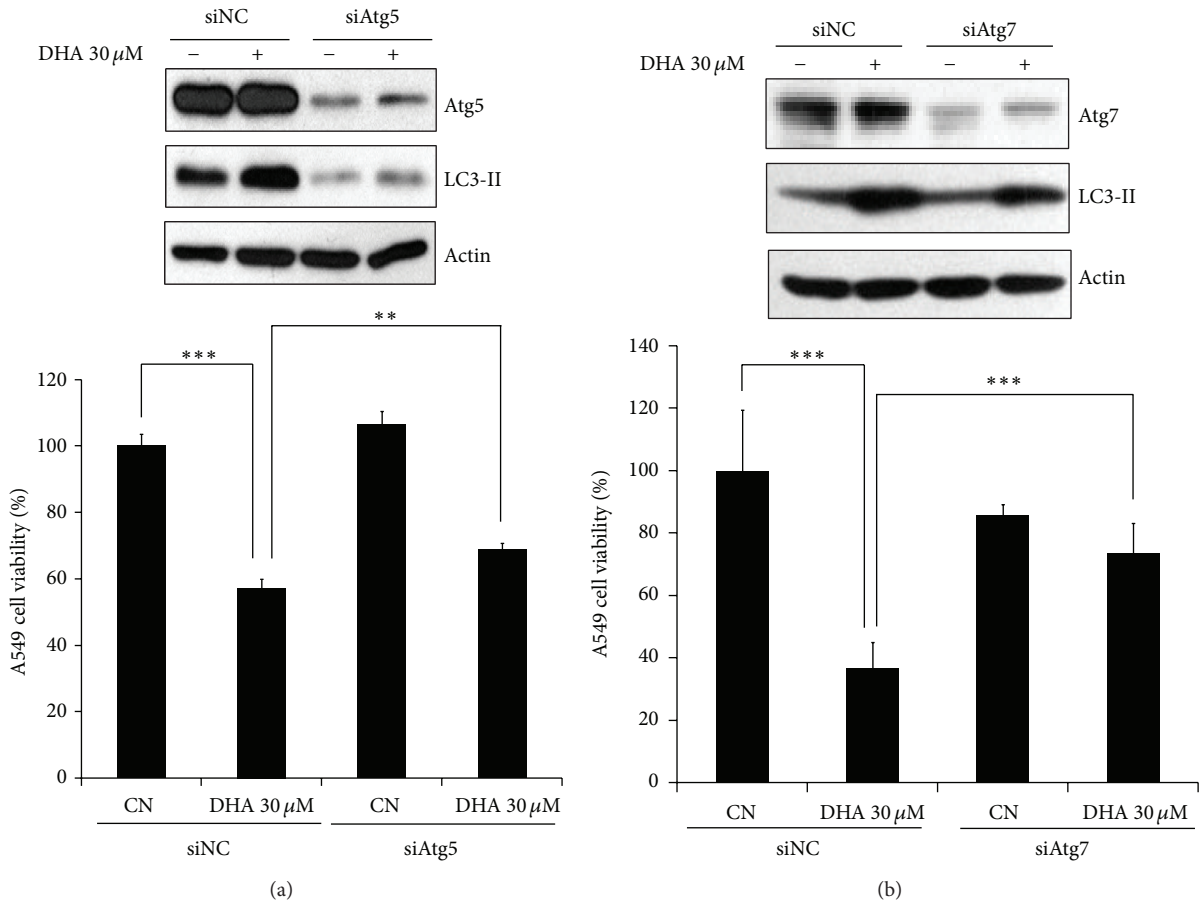
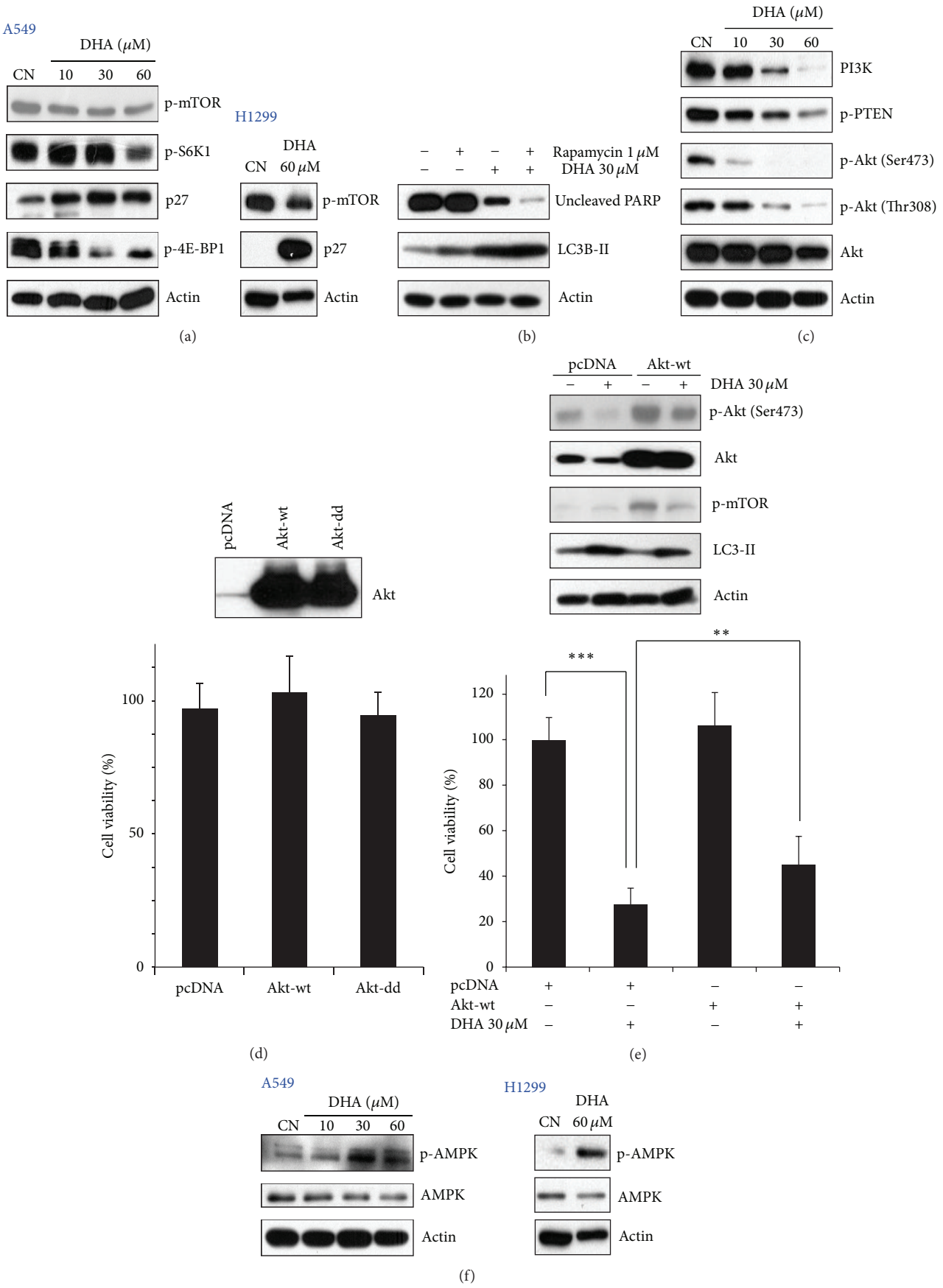


Figure 3: DHA-induced autophagy is required for apoptotic cell death. The indicated cancer cell lines were treated with nontargeting control small interfering RNA (siNC) or siRNAs specific for autophagy-related Atg5 (a) and Atg7 (b). At 18 h after transfection, cells were incubated with the indicated doses of DHA for 24 h. Next, cells were harvested and subjected to western blot analysis with the following antibodies: Atg5, Atg7, LC3-II, and actin (upper panel). Cell viability was measured in an MTT assay (lower panel). $^{**}P < 0.01$ and $^{***}P < 0.001$. Data are representative of three independent experiments, all with similar results.

in line with other reports showing that Clioquinol and Rhabdastrellic acid-A promote cell death in hepatocellular carcinoma, lung adenocarcinoma, myeloma, and leukemia cells lines by inducing autophagy [36, 39]. Although the exact mechanism by which autophagy contributes to DHA-induced apoptosis in NSCLC cells is still unclear, it is known that autophagy can promote cell death by selectively eliminating vital components, such as mitochondria and peroxisomes [40]. We have reported that DHA-induced apoptosis is associated with mitochondrial damage [24, 31]; therefore, it is possible that DHA-induced autophagy is triggered by eliminating components that are essential for survival, such as mitochondria.

3.3. DHA-Mediated Downregulation of mTOR Signaling Is Associated with Autophagy Induction. mTOR is the key negative regulator of autophagy [4]. To examine whether mTOR inhibition is involved in DHA-induced autophagy, we investigated the expression of mTOR signaling-related molecules in NSCLC cells after DHA treatment. DHA reduced the levels of phospho-mTOR in both A549 (Figure 4(a), left panel) and H1299 (Figure 4(a), right panel) cells, indicating that the activity of mTOR is repressed by DHA. Consistent with this, the levels of mTOR's two readout molecules, phospho-S6K1 and phospho-4E-BP1, were also found to be decreased in A549 cells. Meanwhile, increases in p27 (whose activation is indicative of mTOR inhibition) [23] were observed in DHA-treated A549 and H1299 cells (Figure 4(a)), suggesting that DHA indeed suppresses the mTOR signaling pathway. Next, to confirm the role of mTOR in DHA-induced cell death, we pretreated A549 cells with rapamycin followed by DHA. We found that DHA-induced decreases in uncleaved PARP and increases in LC3-II expression in A549 cells were enhanced by pretreatment with rapamycin (Figure 4(b)). These results imply that DHA-induced autophagy and apoptosis are associated with mTOR inhibition.

mTOR is directly linked to PI3K/Akt signaling [41], and the PI3K/Akt/mTOR signaling pathway plays an important role in cell proliferation and survival [42]. We then asked whether PI3K/Akt is associated with DHA-induced mTOR



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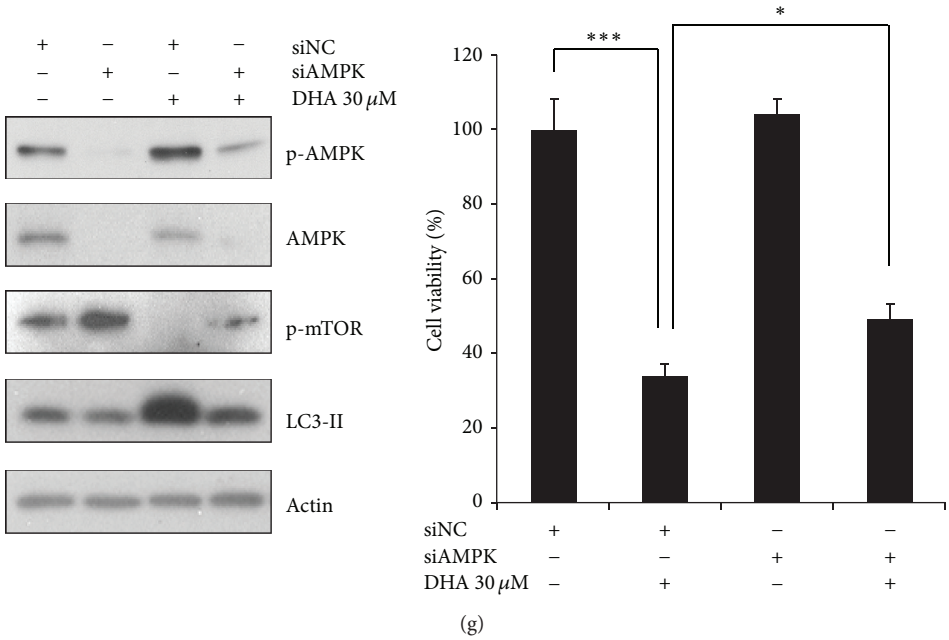
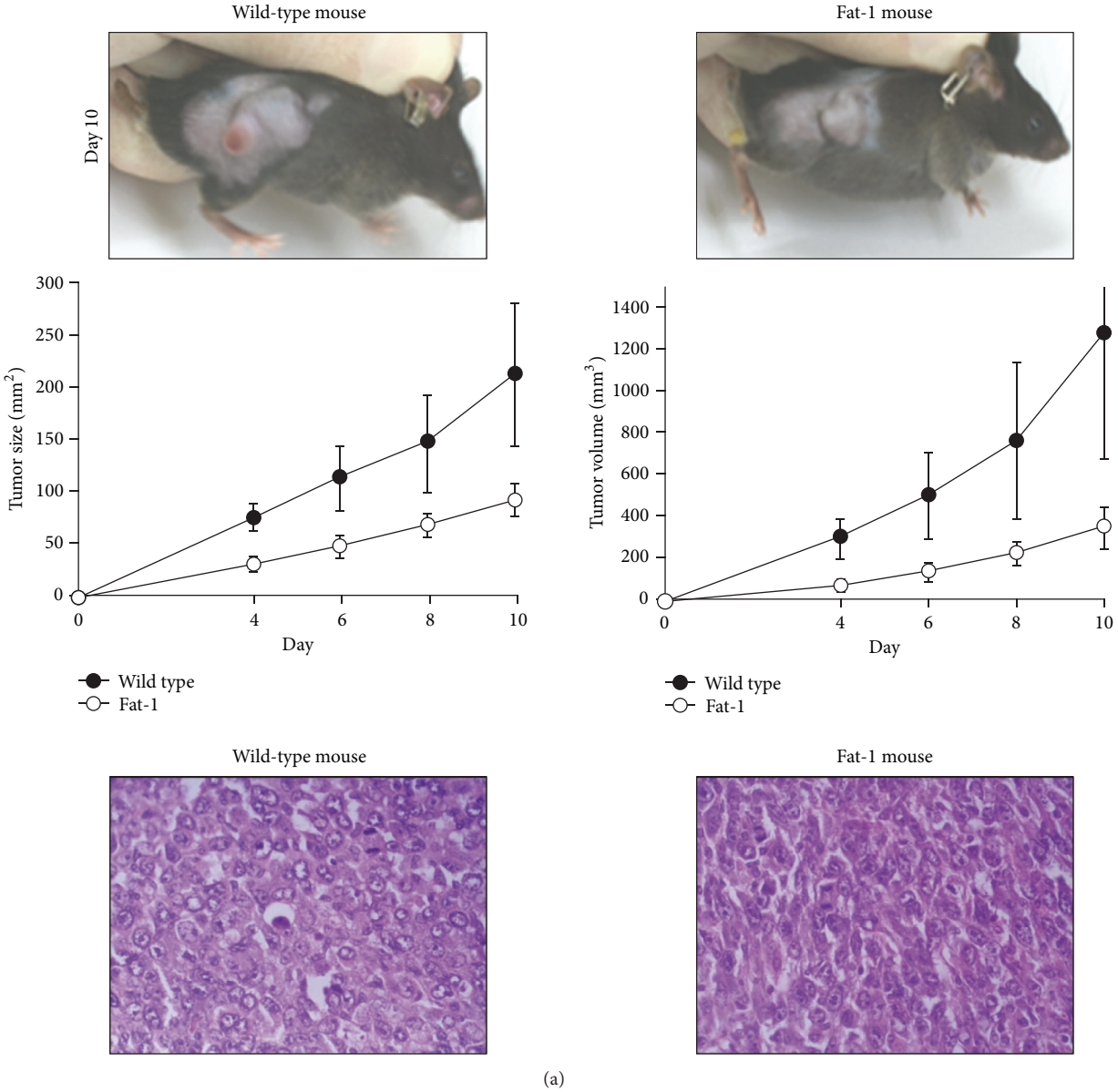


Figure 4: DHA-mediated downregulation of mTOR signaling is related to the induction of autophagy. (a) DHA downregulated mTOR signaling in a dose-dependent manner. A549 (left panel) and H1299 (right panel) cells were incubated with the indicated doses of DHA for 24 h and then subjected to western blot analysis with antibodies against phospho-mTOR, phospho-S6K1, p27, 4E-BP1, and actin. (b) Rapamycin accelerated autophagy and cell death by inhibiting mTOR. A549 cells were incubated for 1 h with or without 1 μM rapamycin before incubation for 24 h with 30 μM DHA. Cell lysates were prepared and examined by western blotting. (c) DHA reduces PI3K/Akt signaling pathway. Western blotting with antibodies against phosphatidylinositol 3-kinase (PI3K)/Akt signaling molecules showed that DHA downregulates PI3K/Akt signaling in a dose-dependent manner. (d)-(e) Expression of Akt-wt partially rescued DHA-induced NSCLC cell death. pcDNA and a Akt-wt vector were transfected into cells using Lipofectamine 2000 reagent for 12 h. The cells were then exposed to 30 μM for another 24 h. Cell viability was examined in an MTT assay ((d) and (e), lower panel) and the cell lysates were analyzed by western blotting with antibodies against phospho-Akt, Akt, phospho-mTOR, and actin ((e), upper panel). $^{***}P < 0.001$. (f) DHA treatment led to a dose-dependent increase in phospho-AMPK levels. A549 (left panel) and H1299 (right panel) cells were treated with indicated doses of DHA for 24 h and cell lysates were examined by western blotting. (g) siAMPK reduced DHA-induced autophagy and inhibited cell death in NSCLC cells by upregulating mTOR signaling. A549 cells were transfected with a siNC or siAMPK and then exposed to 30 μM DHA for 24 h. Left panel: western blot analysis of phospho-AMPK, AMPK, phospho-mTOR, LC3-II, and actin expression. Right panel: cell viability was measured in an MTT assay. $^{*}P < 0.05$ and $^{***}P < 0.001$.

inactivation. To test this, we examined the expression of PI3K/Akt signaling molecules and found that DHA induced a marked reduction in PI3K, Akt, and phospho-PTEN (a negative regulator of the PI3K) (Figure 4(c)). Next, to obtain evidence for the interconnection between decreased PI3K/Akt signaling and DHA-induced cell death, we overexpressed Akt in A549 cells before DHA treatment (overexpression in itself had no significant effect on the cell viability of A549 cells) (Figure 4(d)). DHA treatment led to a reduction in cell viability and increased the levels of phospho-mTOR and LC3-II; however, these phenomena were partially reversed by Akt overexpression (Figure 4(e)). These data suggest that DHA-induced cell death is also associated with Akt inhibition in NSCLC cells. Evidence suggests that DHA can disrupt the association between lipid rafts and epidermal growth factor receptor (EGFR), leading to inactivation of EGFR and its downstream PI3K/Akt signaling in lung cancer cells [43]. Accordingly, it is reasonable to speculate that DNA might inhibit the PI3K/Akt signaling pathway by disrupting EGFR phosphorylation and its association with lipid rafts; however, further studies are needed to investigate whether this is the mechanism underlying DHA-mediated Akt inactivation.

In addition to Akt, another key mediator of mTOR is AMPK, which has been demonstrated to negatively regulate mTOR activity [15]. We sought subsequently to determine whether DHA-induced mTOR inactivation involves AMPK in NSCLC cells. To this end, we first measured the levels of AMPK in DHA-treatment A549 and H1299 cells. DHA treatment led to increased AMPK activity in both A549 and H1299 cells, as evidenced by the promoted expression levels of phospho-AMPK (Figure 4(f)). Importantly, knockdown of AMPK not only rescued DHA-induced mTOR inactivation, but also inhibited DHA-induced autophagy and cell death in A549 cells (Figure 4(g)). These results demonstrated that DHA-induced autophagy mediated by mTOR inhibition, at least partly, attributes to AMPK activation. Previously, endoplasmic reticulum (ER) stress has been shown to elevate AMPK levels, leading to the upregulation of Ca^{2+}-CaMKKβ signaling and autophagy-related genes such as Atg5 and Atg12 [44]. We found that the levels of phosphoeukaryotic translation initiation factor-2α, which reflects ER stress,



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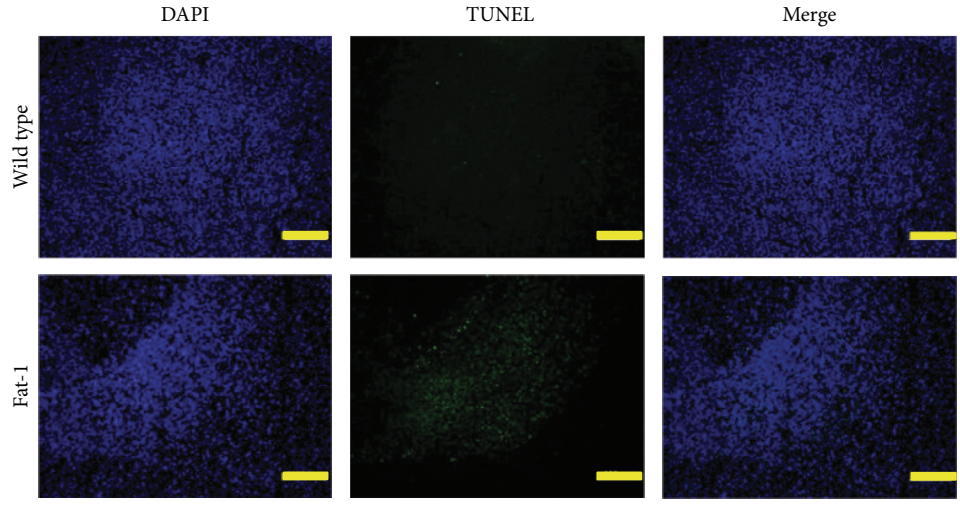

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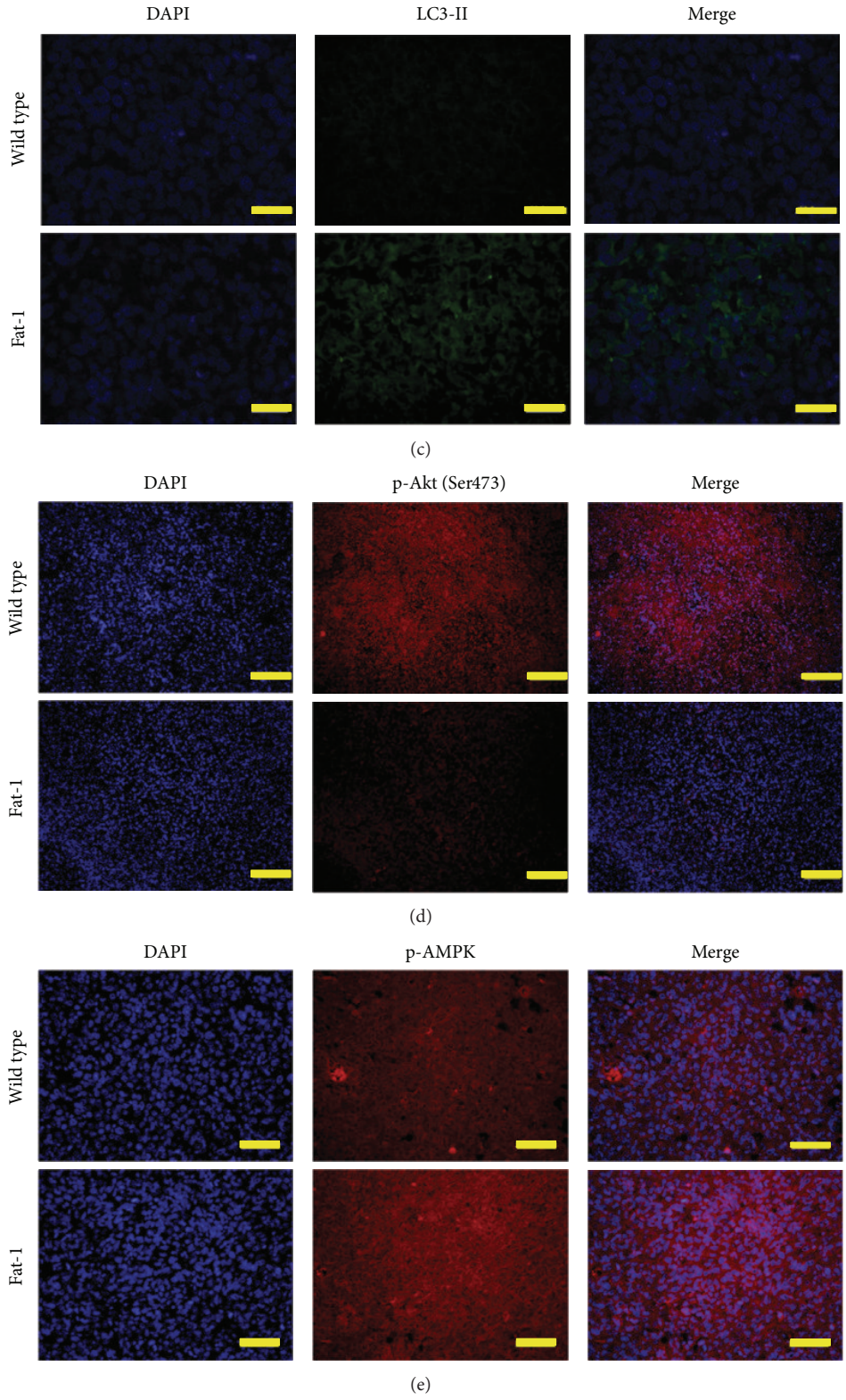

Figure 5: ω3-PUFAs suppress tumor growth *in vivo* by inhibiting phospho-Akt and phospho-AMPK, thereby inducing apoptosis and autophagy. (a) Effect of ω3-PUFA on tumorigenicity. Upper panel: LLC cells (3×10^6 cells) were injected subcutaneously into the flanks of wild-type and Fat-1 transgenic mice. Tumor size and volume were monitored every other day for 10 days. Tumor size and volume (middle) were calculated as described in Section 2. Lower panel: hematoxylin and eosin (H&E) staining. ((b)–(e)) Representative fluorescence images showing the TUNEL assay results (b), LC3-II (c), phospho-Akt (Ser473) (d), and phospho-AMPK (e). Fluorescently stained tissues were observed under a fluorescence microscope using DP Controller software (Olympus) for image acquisition. Scale bars: 200 μm.

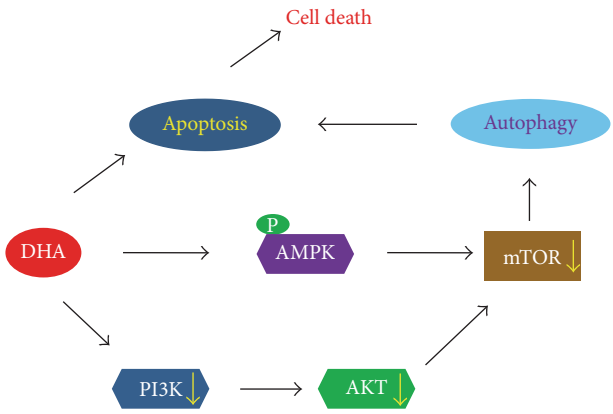

Figure 6: Schematic model of DHA-induced apoptosis and autophagy in NSCLC cells. DHA-induced autophagy and apoptosis in lung cancer cells are triggered by inhibition of mTOR activation via AMPK activation and PI3K/Akt inhibition.

increased in DHA-treated A549 cells in a time-dependent manner (data not shown). Thus, although the exact mechanism underlying DHA-mediated AMPK activation remains unknown, it is possible that AMPK activation in DHA-treated cells is induced by the activation of Ca^{2+}-CaMKKβ and autophagy-related genes via ER stress.

3.4. ω3-PUFAs Suppress Tumor Growth In Vivo by Inhibiting the Expression of Phospho-Akt and Phospho-AMPK, Thereby Inducing Apoptosis and Autophagy. The *in vitro* results presented above show that DHA reduced the viability of human NSCLC cells. We next examined the effect of DHA on tumor formation and growth in Fat-1 transgenic mice. Fat-1 transgenic mice express ω3-desaturase and thus produce higher levels of ω3-PUFAs than wild-type (WT) mice [45]. The mouse origined NSCLC, LLC cells were subcutaneously injected into WT mice and Fat-1 transgenic mice, and tumor size and volume were measured. We found that both the size and volume of the tumors in Fat-1 transgenic mice were markedly lower than those in WT mice (Figure 5(a)), suggesting that ω3-PUFAs suppress the growth of NSCLC cells *in vivo*. To verify whether apoptosis and autophagy played a role in this process, we performed TUNEL assays and indirect immunofluorescence assays on tumor tissue sections to examine apoptosis and autophagy levels, respectively. The number of TUNEL-positive cells and the number of LC3-II puncta were higher in Fat-1 tumors than in WT tumors (Figures 5(b) and 5(c)), indicating that both apoptosis and autophagy played a role in inhibiting tumor growth in Fat-1 transgenic mice. Next, to determine whether PI3K/Akt and AMPK signaling were responsible for the increased levels of apoptosis and autophagy, we examined the levels of Akt and AMPK in tumor tissues by immunohistochemistry. As shown in Figures 5(d) and 5(e), the levels of phospho-Akt (Ser473) decreased and the levels of phospho-AMPK increased in Fat-1 tumor tissues. Together, these data demonstrate that ω3-PUFAs induce apoptosis and autophagy *in vivo* by regulating AMPK and PI3K/Akt signaling.

4. Conclusions

In conclusion, we describe for the first time that DHA triggers autophagy and apoptosis in NSCLC cells, which simultaneously promotes cell death. Our results indicate that the DHA-induced autophagy and apoptosis are controlled by repressing mTOR through AMPK activation and PI3K/Akt inhibition (Figure 6). These data suggest that DHA may represent a potentially useful reagent for treating human NSCLC in clinical settings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Nayeong Kim and Soyeon Jeong contributed equally to this work.

Acknowledgments

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Research Article

The Pharmacokinetic Profile of a New Gastroresistant Capsule Preparation of Eicosapentaenoic Acid as the Free Fatty Acid

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Supplementation with n-3 polyunsaturated fatty acids (n-3 PUFAs) may be beneficial for patients with inflammatory bowel diseases (IBD). In this study we analyzed the pharmacokinetic profile of eicosapentaenoic acid (EPA), as the free fatty acid (FFA), in an enteric-coated preparation, in 10 ulcerative colitis (UC) and 10 Crohn's disease (CD) patients and 15 healthy volunteers (HV). Subjects received 2 g daily of EPA-FFA for 8 weeks. Plasma phospholipid and red blood cell (RBC) membrane fatty acid content were measured by gas chromatography-mass spectrometry. There was a rapid incorporation of EPA into plasma phospholipids by 2 weeks and a slower, but highly consistent, incorporation into RBC membranes (4% total fatty acid content; coefficient of variation 10–16%). There was a concomitant reduction in relative n-6 PUFA content. Elongation and desaturation of EPA into docosahexaenoic acid (DHA) via docosapentaenoic acid (DPA) were apparent and DHA content also increased in membranes. EPA-FFA is well tolerated and no difference in the pharmacokinetic profile of n-3 PUFA incorporation was detected between IBD patients and HV. Our data support the concept that EPA can be considered the "universal donor" with respect to key n-3 PUFAs and that this enteric-coated formulation allows long term treatment with a high level of compliance.

1. Introduction

The major natural dietary source of long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs) is cold-water, oily fish, which can be consumed safely in large quantities. However, despite the large scale and safe consumption reported in Eskimos [1], this dietary habit only occurs in a small proportion of individuals in the industrialised and developing world. Fish oil can be administered in pharmaceutical form for many different therapeutic purposes such as chronic inflammatory diseases [2–4], treatment of hyperlipidaemia [5], and after myocardial infarction [6].

Oral administration of fish oil containing the two main bioactive components C20:5n3 eicosapentaenoic acid (EPA) and C22:6n3 docosahexaenoic acid (DHA) can replace C18:2n6 linoleic acid (LA) and C20:4n6 arachidonic acid (AA) in a time- and dose-dependent manner in plasma and cellular phospholipid membranes [2].

Plasma n-3 PUFA level is the easiest marker of EPA and DHA intake for measuring compliance in taking supplements as various fish oil preparations. However, it is established that the plasma phospholipid fatty acid profile may change within a period of hours, depending on the type and timing of food intake [7]. Analysis of red blood cell (RBC) membrane n-3 PUFA content is understood to be a more reliable measure [8]. The relatively long half-life of the RBC (120 days) provides a more stable measure of the incorporation of fatty acids into cellular phospholipid membranes [9]. The Omega-3 index (the combined percentage EPA, docosapentaenoic acid-DPA, and DHA content in RBC phospholipid membranes) can reach ≥8% with achievable n-3 PUFA intake [10].

It is clear that although fish oil has no serious toxicity, minor adverse events (AEs) such as dysgeusia, flatulence, pyrosis, halitosis, belching, and abdominal discomfort are common and may limit compliance [11, 12]. Enteric coating of the capsules containing the fish oil may help to minimise upper gastrointestinal effects.

There are conflicting data on the comparative bioavailability and adverse event profiles of n-3 PUFAs conjugated to a glycerol chain, as an ethyl ester conjugate and as the free fatty acid [13], but direct comparison of the three forms suggests that bioavailability is highest with the free fatty acid and lowest with the ethyl ester [14].

We decided to test compliance with and the pharmacokinetic profile of a new preparation containing EPA in the free fatty acid (FFA) form presented in gastroresistant capsules.

Since supplementation with n-3 polyunsaturated fatty acids (n-3 PUFAs) has been claimed to be beneficial in patients with chronic inflammatory conditions, even if results were controversial [3, 4, 15–17], we enrolled a group of patients with inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD). The patients were in stable clinical remission (≥3 months), our main purpose being to supplement patients with presumed good absorption characteristics, whilst avoiding inclusion of those with diarrhoea. A control group of healthy volunteers (HV) was recruited from medical students.

2. Materials and Methods

2.1. Study Design. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee and the Clinical Board of Sant'Orsola-Malpighi Hospital, Bologna. Written, informed consent was obtained from 20 IBD patients (10 CD and 10 UC), who were in stable clinical remission according to routine clinical scores (Crohn's Disease Activity Index—CDAI < 150 and Simple Clinical Colitis Activity Index—SCCAI = 0) [18, 19] for at least 3 months, attending the outpatient clinic, and 15 HV recruited among medical students.

Exclusion criteria were previous allergy/intolerance to n-3 PUFAs, existing use of n-3 PUFA-containing supplements, previous bowel resection more than 1 metre, pregnancy, or desire to become pregnant. During the study, subjects were asked not to change their dietary habits.

All subjects received 2 g EPA-FFA daily as two 500 mg capsules (ALFA) twice a day with food for 8 weeks.

Venous blood was taken in an EDTA tube after an overnight fast at baseline (time zero) and at 2, 4, and 8 weeks to obtain plasma and RBCs. Routine screening blood tests, including blood count, erythrocyte sedimentation rate, serum creatinine, and liver function tests, were performed at baseline and the end of the study.

Adherence to the dosing regimen was evaluated at each clinic visit by interview and by capsule count. Subjects were considered adherent if they used at least 80% of the capsules between each visit, with no interruption of supplementation for more than 14 consecutive days. At each visit, diary cards were checked for adverse events (AEs).

2.2. Fatty Acid Analysis. The detailed description of the phospholipid fatty acid assay has been previously published [20].

In brief, total lipids were extracted from 2 mL of plasma and from 2 mL of packed RBCs. Phospholipids were separated from the total lipid fraction by one-dimensional thin-layer chromatography [21].

Red blood cell membranes were then processed following the procedure of Popp-Snijders et al. [22]. Red cell membrane lipids were extracted according to Dodge and Phillips [23], using a 2 : 1 (v : v) mixture of chloroform and methanol containing 0.01% butylated hydroxytoluene (2,6 di-*tert*-bury-p-cresol, Sigma) as antioxidant. Samples were then stored under nitrogen at −20°C for a maximum of 2 weeks prior to fatty acid analysis.

Fatty acids were transmethylated using 1 N potassium hydroxide in methanol and boron trifluoride in 14% methanol for 10 minutes at 80°C [24]. Fatty acid methyl-esters were then extracted in hexane, resuspended in 100 μL of benzene, and analysed by gas chromatography-mass spectrometry. Individual fatty acid methyl-esters were identified by comparison with authentic standards (Sigma). Heptadecanoic acid (17 : 0) was used as an internal calibrating standard (1 mg/mL in benzene) and the results are expressed as the percentage of total phospholipid fatty acids [25].

The following PUFAs were analysed: LA, AA, EPA, C22:5n3 docosapentaenoic acid (DPA), and DHA.

2.3. Statistical Methods. The geometric mean and standard deviation (SD) of the mean are provided for each fatty acid. The plasma and RBC phospholipid fatty acid content of each fatty acid in each group over time were compared by the Kruskal-Wallis test or the Wilcoxon rank test. Absolute intraindividual changes in PUFA content were analysed using the one-sample t-test and data are quoted as the mean and 95% confidence interval. Variability of the change in PUFA content over time between individuals was described using the percentage (%) coefficient of variation.

Differences between groups were compared using the Mann-Whitney (MW) U test.

In all cases, statistical significance was assumed if $P \leq 0.05$.

3. Results

3.1. Subjects. Characteristics of the study subjects are summarized in Table 1. There were no statistically significant differences between subjects at baseline.

3.2. Baseline PUFA Profile in Plasma Phospholipids and RBC Membranes. Baseline plasma and RBC PUFA profiles in IBD patients and HV are noted in Tables 2 and 3. As expected, in individuals consuming a "western" diet, the n-6 PUFA content was in greater than 10-fold excess of the relative n-3 PUFA content.

The plasma n-3 PUFA content in IBD patients was significantly higher than in HVs (MW: $P < 0.01$; Table 2).

Table 1: Subject characteristics.

Characteristic°	Crohn's disease ($N = 10$)	Ulcerative colitis ($N = 10$)	Healthy volunteers ($N = 15$)
Age (years) mean ± SD	39 ± 11	34 ± 10	28 ± 8
Male *n* (%)	6 (60)	4 (40)	5 (33.3)
Current smoker *n* (%)	3 (30)	1 (10)	4 (26.6)
Duration of disease (months)			
Mean ± SD	68 ± 32	65 ± 28	—
Previous intestinal resection *n* (%)	2 (20)	0 (0)	0 (0)
CDAI*			
Median (range)	82 (38–102)	/	/
SCCAI*			
Median (range)	/	0 (0)	/
Site of involvement *n* (%)			
Ileum	4 (40)	/	/
Ileum and colon	3 (30)	/	/
Colon	3 (30)	/	/
Pancolitis	/	3 (30)	/
Left-sided colitis	/	7 (70)	/
Ongoing drug therapy *n* (%)			
Immunosuppressive agents	4 (40)	2 (20)	/
Mesalamine	6 (60)	8 (80)	

*CDAI: Crohn's disease activity index; SCCAI: simple clinical colitis activity index; °P = ns.

Table 2: Fatty acid content of plasma phospholipids in healthy volunteers (HV) and inflammatory bowel disease (IBD) patients.

	Fatty acid (% of total)		Baseline	2 weeks	4 weeks	8 weeks	Change in fatty acid content from baseline to week 8*	%CV
HV	n-6 PUFAs	C18:2 LA	19.1 ± 2.2**	17.3 ± 1.3	16.4 ± 1.3	15.9 ± 1.1	−3.2 (−4.6 to −1.9)***	75.2
		C20:4 AA	13.6 ± 0.9	11.6 ± 1.7	10.5 ± 0.9	10.3 ± 0.8	−3.2 (−3.7 to −2.8)	25.0
	n-3 PUFAs	C20:5 EPA	0.3 ± 0.2	4 ± 0.8	4.5 ± 1	4.6 ± 0.6	4.3 (4.0–4.7)	14.5
		C22:5 DPA	0.3 ± 0.3	2.6 ± 0.7	2.7 ± 0.5	2.8 ± 0.5	2.5 (2.2 to 2.8)	19.8
		C22:6 DHA	2.7 ± 0.8	4.6 ± 0.7	4.0 ± 0.5	4.2 ± 0.7	1.5 (0.9–2.1)	70.9
IBD	n-6 PUFAs	C18:2 LA	18.5 ± 1.6	16 ± 1.1	15.7 ± 1	15.2 ± 1	−3.4 (−4.1 to −2.6)	45.2
		C20:4 AA	14.2 ± 1	10.7 ± 0.9	11 ± 0.8	10.6 ± 0.9	−3.6 (−4.1 to −3.1)	32.0
	n-3 PUFAs	C20:5 EPA	0.6 ± 0.2°	4.2 ± 0.7	4.8 ± 0.6	4.7 ± 0.5	4.1 (3.8 to 4.3)	12.3
		C22:5 DPA	0.5 ± 0.2°	2.6 ± 0.5	2.9 ± 0.4	3.1 ± 0.6	2.6 (2.3 to 2.9)	24.0
		C22:6 DHA	3.3 ± 0.6°	4.5 ± 0.5	4.4 ± 0.5	4.9 ± 0.4	1.6 (1.4 to 1.8)	32.1

**Values are mean ± SD.
°$P < 0.01$ for the difference in individual n-3 PUFA content between IBD patients and HV.
*Absolute change in % fatty acid content.
***Data are the mean and 95% confidence interval. All $P < 0.01$.
PUFAs: polyunsaturated fatty acids.
LA: linoleic acid.
AA: arachidonic acid.
EPA: eicosapentaenoic acid.
DPA: docosapentaenoic acid.
DHA: docosahexaenoic acid.
CV: coefficient of variation.

However, the RBC n-6 PUFA content in IBD patients was significantly higher than HV ($P < 0.01$; Table 3). Both of these observations are consistent with previous reports [9, 26].

There was no significant difference in baseline PUFA profiles between CD and UC patients in clinical remission. CD and UC site involvement did not predict the baseline plasma or RBC PUFA profile (data not shown).

The mean baseline Omega-3 indices in HV and IBD patients were 4.1% and 3.7%, respectively.

3.3. Changes in Plasma and RBC PUFA Content during EPA-FFA Treatment. Tables 2 and 3 detail the changes in the plasma and RBC membrane content of n-3 and n-6 PUFAs during 8-week treatment with EPA-FFA.

Table 3: Fatty acid content of red blood cell membranes in healthy volunteers (HV) and inflammatory bowel disease (IBD) patients.

	Fatty acid		Baseline	2 weeks	4 weeks	8 weeks	Change in fatty acid content from baseline to week 8*	%CV
HV	n-6 PUFAs	C18:2 LA	10.5 ± 1.4**	9.1 ± 1	7.7 ± 1	6.5 ± 1	−4.0 (−4.5 to −3.5)***	24.0
		C20:4 AA	14.5 ± 0.7	12.2 ± 1.2	10.1 ± 0.7	9.7 ± 0.6	−4.8 (−5.2 to −4.3)	17.9
	n-3 PUFAs	C20:5 EPA	0.3 ± 0.1	1.7 ± 0.4	4 ± 0.5	4.5 ± 0.4	4.3 (4.0 to 4.5)	9.8
		C22:5 DPA	0.8 ± 0.2	1.4 ± 0.4	2.2 ± 0.4	3.8 ± 0.7	2.9 (2.5 to 3.4)	27.3
		C22:6 DHA	3 ± 0.5	3.3 ± 0.5	3.6 ± 0.7	4.2 ± 0.8	1.2 (0.7 to 1.7)	69.4
IBD	n-6 PUFAs	C18:2 LA	12.2 ± 1°	11.1 ± 0.8	9.7 ± 0.8	7.8 ± 0.8	−4.4 (−4.8 to −3.9)	20.4
		C20:4 AA	18 ± 2.1°	15.7 ± 1.9	14.1 ± 1.7	11.6 ± 1.5	−6.5 (−7.4 to −5.5)	31.0
	n-3 PUFAs	C20:5 EPA	0.2 ± 0.1	1.1 ± 0.3	2.1 ± 0.4	4.3 ± 0.7	4.1 (3.7 to 4.4)	16.3
		C22:5 DPA	0.4 ± 0.1	1.1 ± 0.6	2.2 ± 0.5	3.3 ± 0.4	2.8 (2.6 to 3.0)	15.5
		C22:6 DHA	3.1 ± 0.5	3.7 ± 0.4	4.5 ± 0.4	5 ± 0.4	1.9 (1.6 to 2.2)	33.5

**Values are mean ± SD.
°$P < 0.01$ for the difference in individual n-3 PUFA content between IBD patients and HV.
*Absolute change in % fatty acid content.
***Data are the mean and 95% confidence interval. All $P \leq 0.01$.
PUFAs: polyunsaturated fatty acids.
LA: linoleic acid.
AA: arachidonic acid.
EPA: eicosapentaenoic acid.
DPA: docosapentaenoic acid.
DHA: docosahexaenoic acid.
CV: coefficient of variation.

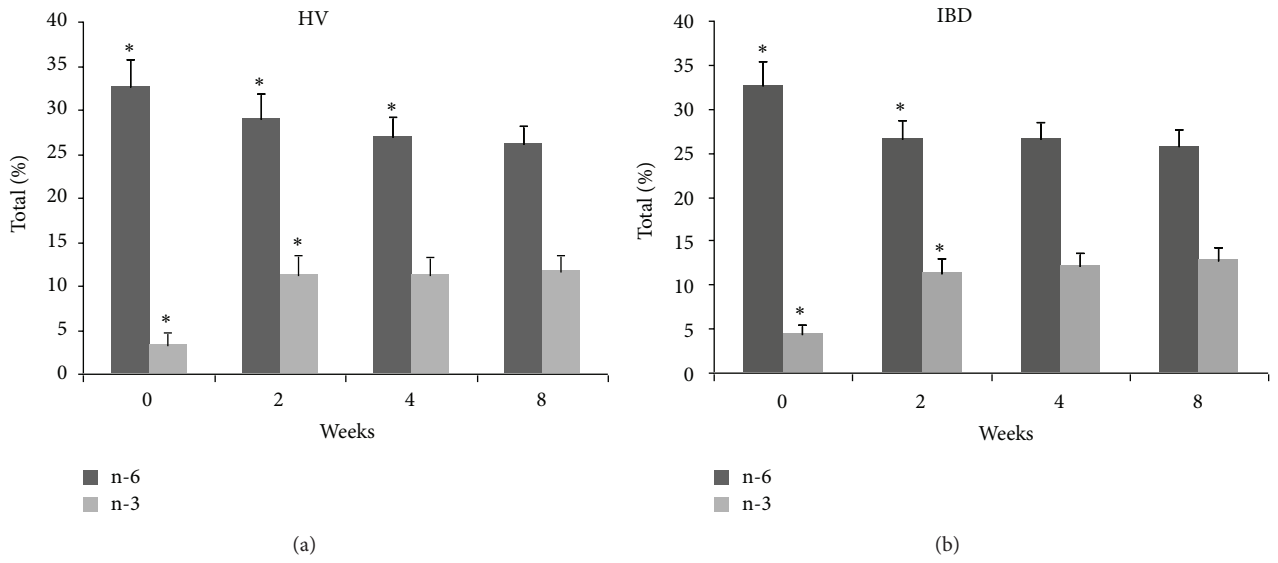

Figure 1: Plasma polyunsaturated fatty acids (PUFAs) profile during eicosapentaenoic acid-free fatty acids (EPA-FFA) treatment in healthy volunteers (HV) and inflammatory bowel disease (IBD) patients. Data are the mean (column) and standard deviation (bar) of the total n-6 PUFAs (linoleic acid + arachidonic acid; dark shade) and n-3 PUFAs (eicosapentaenoic acid + docosapentaenoic acid + docosahexaenoic acid; light shade); % of total PUFA content at each time point. *$P < 0.001$.

The change in plasma PUFA profile occurred rapidly, being evident after only 2 weeks of supplementation with % content reaching steady-state between the 4th and the 8th week (Table 2 and Figure 1). By contrast, changes in the RBC membrane PUFA profile occurred more slowly and were progressive up to 8 weeks (Table 3 and Figure 2). In both phospholipid compartments, there was a statistically significant increase in incorporation of all n-3 PUFAs and a concomitant decrease in n-6 PUFA content (in all cases $P < 0.001$; Kruskal-Wallis test). Individual changes in fatty acid content in plasma and RBC membrane were similar in both HV and IBD groups with little interindividual variability in EPA and DPA incorporation but larger variability in the reduction of n-6 PUFAs and DHA (Tables 2 and 3, Figure 3).

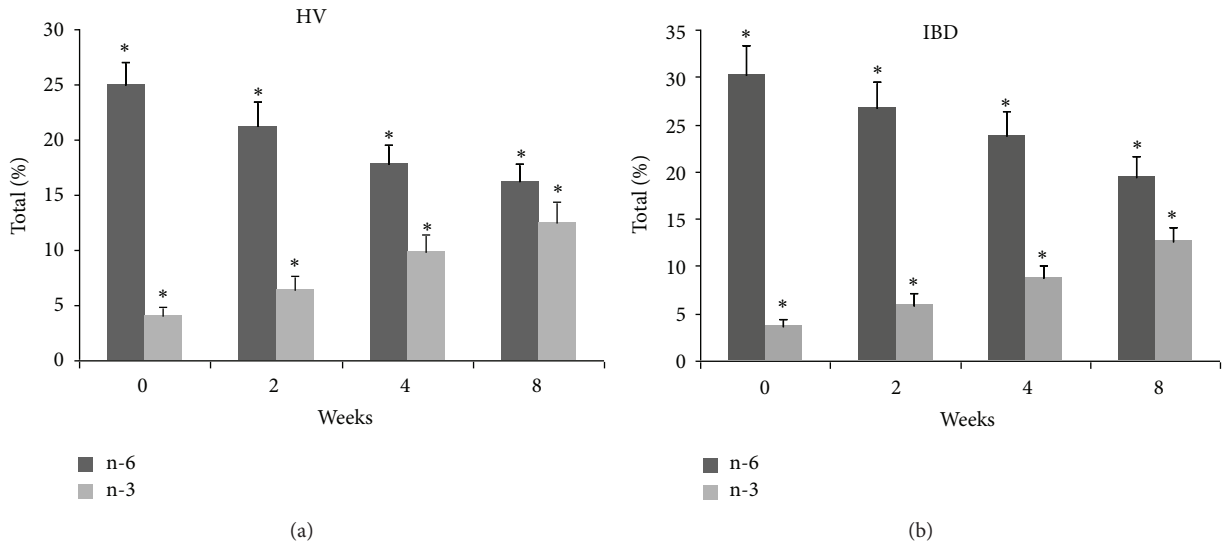

Figure 2: Red blood cell membrane polyunsaturated fatty acids (PUFAs) profile during eicosapentaenoic acid-free fatty acids (EPA-FFA) treatment in healthy volunteers (HV) and inflammatory bowel disease (IBD) patients. Data are the mean (column) and standard deviation (bar) of the total n-6 PUFAs (linoleic acid + arachidonic acid; dark shade) and n-3 PUFAs (eicosapentaenoic acid + docosapentaenoic acid + docosahexaenoic acid; light shade); % of total PUFA content at each time point. $^{*}P < 0.001$.

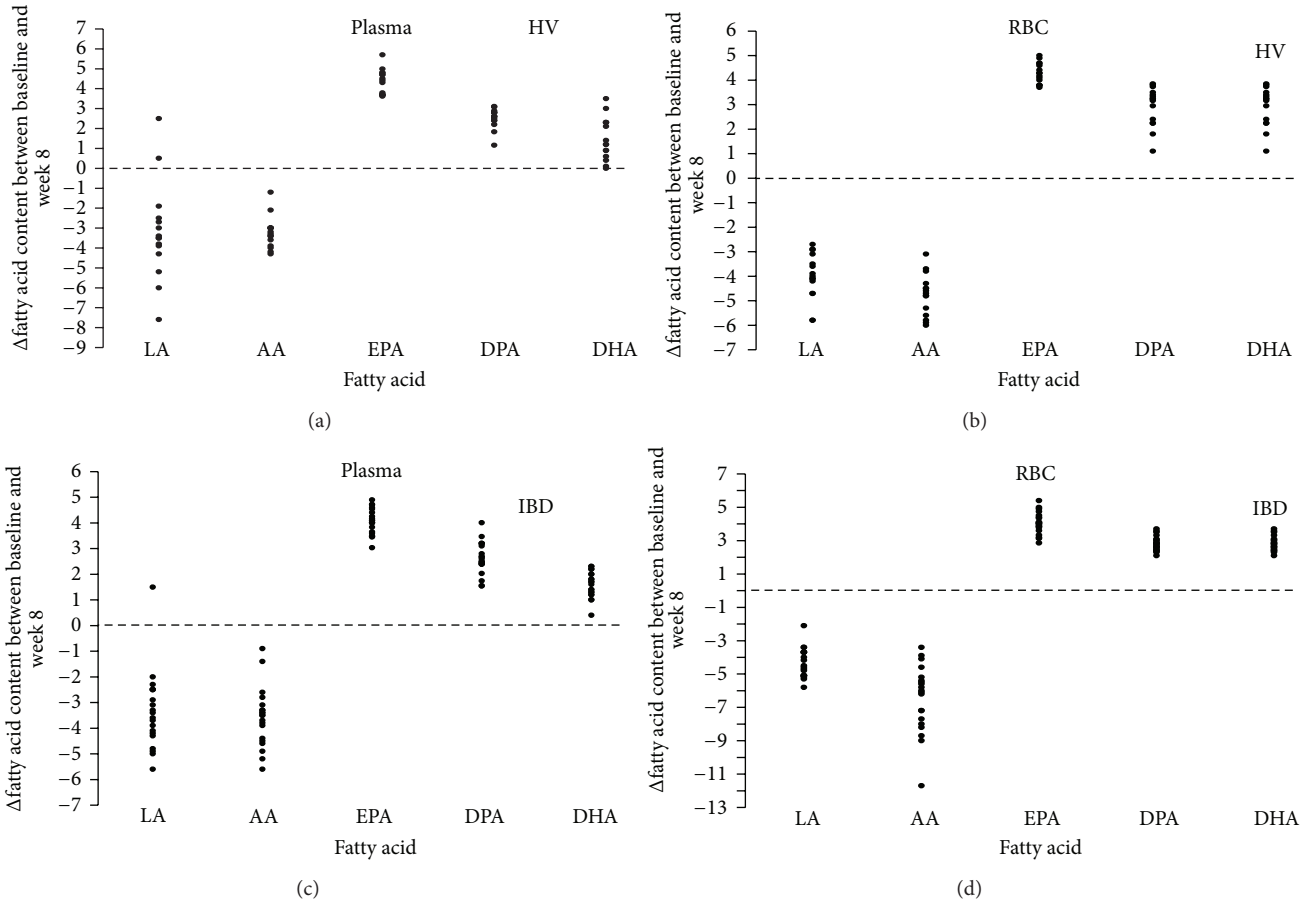

Figure 3: Interindividual variability in the absolute change in polyunsaturated fatty acids (PUFAs) content of plasma and red blood cell (RBC) membrane from baseline to week 8 in healthy volunteers (HV) and inflammatory bowel disease (IBD) patients. LA: linoleic acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.

There was no significant correlation between the change in plasma PUFA content in the first two weeks and the change in RBC PUFA content at 8 weeks (data not shown) suggesting that early changes in plasma FA content do not predict longer-term tissue PUFA incorporation during EPA-FFA treatment.

The mean Omega-3 indices in HV and IBD patients were 12.5% and 12.6%, respectively, which is consistent with previous studies [10].

There were no significant differences in PUFA content between HV and IBD patients, suggesting that there is comparable incorporation (and, by inference, absorption) in both groups. There was also no significant difference in the PUFA profile change between CD and UC patients overall or on the basis of the site of IBD involvement (data not shown).

At the end of the treatment, the n-6 PUFA/n-3 PUFA ratio was much lower in both HV and IBD patients being 3 : 1 and 2 : 1 in plasma and RBC membranes.

3.4. Compliance and Side Effects. EPA-FFA 2 g daily was well tolerated and there was no participant drop out. All subjects satisfied the criteria for compliance (i.e., less than 20% of capsules returned and no reported interruption for more than 14 days). Some minor/mild side effects were reported and these are summarised in Table 4. The most commonly reported side effects were mild gastrointestinal disturbance, which did not result in treatment cessation. Importantly, IBD patients did not report high incidence of diarrhoea. No serious adverse events were reported.

Table 4: Adverse event profile of eicosapentaenoic acid-free fatty acid (EPA-FFA).

	n (%)	Frequency
HV		
Diarrhoea	1 (6.7)	4
Nausea	2 (13.3)	4
Abdominal pain/distension	2 (13.3)	4
Epigastric discomfort	2 (13.3)	3
Headache	1 (6.7)	3
IBD patients		
Diarrhoea	3 (15)	5
Nausea	2 (10)	4
Abdominal pain/distension	3 (15)	5
Epigastric discomfort	3 (15)	3
Headache	2 (10)	4

Frequency is the absolute number of times given adverse events occurred.
HV: healthy volunteers.
IBD: inflammatory bowel disease.

4. Discussion

To our knowledge, this is the first pharmacokinetic study to evaluate the incorporation of n-3 PUFAs into RBCs with an EPA-only preparation over a period of more than 4 weeks in humans.

Treatment with EPA-FFA has been evaluated in several randomised trials and has proved to be safe and well tolerated [27, 28]. However, the kinetics of EPA incorporation and changes in the content of other PUFAs during EPA-FFA ingestion have not been studied systematically. Herein, we demonstrate efficient and consistent EPA incorporation into plasma phospholipids and RBC membranes. Consistent with the concept that EPA is the "universal donor" [29], EPA-FFA treatment led to increased relative content of both EPA and DHA. Importantly, early changes in plasma phospholipid PUFA content did not predict the eventual PUFA profile in RBCs at 8 weeks. Therefore, we can confirm that the plasma PUFA level is not a reliable biomarker of longer-term tissue incorporation.

We and others have demonstrated that the FFA form of n-3 PUFAs provides the most favourable PK profile in comparison with ethyl ester and triglyceride preparations. Improved absorption of the FFA is believed to be due to its ability to cross the intestinal wall directly, without the requirement for lipase activity [5, 14, 30].

We have demonstrated that EPA-FFA is rapidly and consistently incorporated into plasma phospholipids and RBC membranes in healthy volunteers as well as IBD patients in remission. Our data show that the absorption and the metabolism of n-3 PUFAs are similar in patients with IBD and HVs, with a comparable level of omega-3 absorption and incorporation. This is a fundamental requirement when considering long term treatment with omega-3 PUFAs. The benefit of n-3 PUFAs in patients with IBD is still a matter of debate. Recent reviews and meta-analyses still leave open the question as to the efficacy of n-3 PUFA supplementation in IBD patients, and the results of the trials over the years are not in favour of chronic omega-3 supplementation [4, 17, 31, 32]. Nevertheless a recent paper by Costea et al. [33] reopens the debate, showing that, in a subgroup of children with a specific gene pattern who developed CD, there was a profound imbalance between n-6/n-3 PUFAs, suggesting that supplementation with n-3 PUFAs would be beneficial.

Our previous experience in this field is in favour of supplementation with n-3 PUFAs and we would wish to have available the most efficacious and well tolerated omega-3 preparation for our patients.

We tested ALFA EPA-free fatty acid capsules in a group of IBD patients in stable clinical remission and, encouragingly, we found that data on incorporation and compliance were comparable to those of healthy volunteers.

After just 2 months' treatment, the total relative n-3 PUFA content (the Omega-3 index) was greater than 12% in both HV and IBD patients, which compares favourably with that attained after mixed EPA/DHA supplementation [34] and which exceeds the recognized threshold Omega-3 index after n-3 PUFA treatment [10].

DHA content also increased in membranes, implying elongation and desaturation of EPA into DHA via DPA. The increase in RBC DHA content that we observed is similar to that seen after daily dosing for 8 weeks with six capsules containing a total of 1,296 mg EPA and 864 mg DHA [34]. It is recognized that DHA content after supplementation with n-3 PUFA (EPA+DHA) exceeds that associated with EPA alone, which is consistent with our data [34, 35]. Supplementation

with DHA alone would be important to compare the effects of the two different n-3 PUFAs in humans.

In keeping with experience with EPA-FFA in randomized trials [27, 28], only a small number of mild side effects were registered which may be explained by the formulation of EPA-FFA in gastroresistant capsules.

5. Conclusions

Our data confirm that administration of EPA-FFA results in rapid tissue EPA incorporation. EPA can be quickly converted into DHA via DPA so that EPA can be considered the "universal donor" of n-3 PUFAs. As the EPA-FFA gastroresistant capsules employed in the study enable n-3 PUFA supplementation in high doses with a small number of capsules, this formulation overcomes the poor compliance observed with mixed PUFA fish oil derivatives that require dosing with large numbers of capsules.

Abbreviations

PUFAs: Polyunsaturated fatty acids
LA: Linoleic acid
AA: Arachidonic acid
EPA: Eicosapentaenoic acid
DPA: Docosapentaenoic acid
DHA: Docosahexaenoic acid
FFA: Free fatty acid
IBD: Inflammatory bowel diseases
UC: Ulcerative colitis
CD: Crohn's disease.

Conflict of Interests

Andrea Belluzzi and Mark A. Hull have received a travel grant from SLA Pharma AG. Eleonora Scaioli, Carla Cardamone, Elisa Liverani, and Alessandra Munarini have no conflict of interests.

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Review Article

Effect of Dietary ω-3 Polyunsaturated Fatty Acid DHA on Glycolytic Enzymes and Warburg Phenotypes in Cancer

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The omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are a class of lipids that has been shown to have beneficial effects on some chronic degenerative diseases such as cardiovascular diseases, rheumatoid arthritis, inflammatory disorders, diabetes, and cancer. Among ω-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) has received particular attention for its antiproliferative, proapoptotic, antiangiogenetic, anti-invasion, and antimetastatic properties, even though the involved molecular mechanisms are not well understood. Recently, some *in vitro* studies showed that DHA promotes the inhibition of glycolytic enzymes and the Warburg phenotype. For example, it was shown that in breast cancer cell lines the modulation of bioenergetic functions is due to the capacity of DHA to activate the AMPK signalling and negatively regulate the HIF-1α functions. Taking into account these considerations, this review is focused on current knowledge concerning the role of DHA in interfering with cancer cell metabolism; this could be considered a further mechanism by which DHA inhibits cancer cell survival and progression.

1. Introduction

ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are essential fatty acids (FAs) that have beneficial effects on some chronic degenerative diseases such as cardiovascular diseases [1, 2], rheumatoid arthritis [3], diabetes [4], several autoimmune diseases [5, 6], and cancer [7, 8]. The EPA is a long-chain ω-3 PUFA that has 20 carbon atoms and 5 double bonds (20 : 5); DHA has a longer chain, 22 carbon atoms, and 6 double bounds (22 : 6). DHA and EPA, as well as the other FAs, once ingested, give a substantial contribution to the physical properties of biological membranes, including membrane organization, ion permeability, elasticity, and eicosanoid formation [9–11]. Taking into account these considerations, dietary DHA and EPA were established as significant nutrients involved in metabolic regulation. Moreover, some studies have established the capability of EPA, as well as in particular of DHA, to influence cancer proliferation [12], apoptosis [12, 13], and differentiation [12], as well as to inhibit angiogenesis [14], tumour cell invasion [15], and metastasis [16]. These data suggest that DHA can exert antitumour activity [17]. Despite the knowledge gained about the mechanisms associated with the anticancer effects of ω-3 PUFAs, still today, studies report new discoveries to clarify the complex system of the involved pathways.

Metabolic dysfunction is one of the emerging hallmarks of cancer: cancer cells show a shift in energy production that is abnormally dependent on aerobic glycolysis, and thus some of the key effectors of glycolysis (enzymes and transporters) can be considered as promising targets for the therapeutic intervention against cancer [18].

Recently, some studies have reported that DHA could act as metabolic modulator of several metabolic pathways in cancer cells [19–21].

This review focuses on the investigations on the potential use of DHA as modulator of some targets of aerobic glycolysis and Warburg effect.

2. Warburg Effect

Glycolysis is a catabolic pathway that converts a glucose molecule into two pyruvate molecules, and finally it yields

2 ATPs. In normal cells, pyruvate is oxidized to CO_2 and H_2O generating 36 ATPs in the mitochondrial oxidative phosphorylation pathway. When adequate oxygen supply is not available, normal cells use anaerobic glycolysis, because mitochondrial functions are suppressed in absence of oxygen. Under anaerobic condition, the conversion of pyruvate to lactic acid is favoured because this is the only mechanism available to regenerate NAD^+, the coenzyme for glyceraldehyde-3-phosphate dehydrogenase. The conversion of glucose into lactate generates only a fraction of energy from glucose (2 moles of ATP/mole of glucose). Therefore, normal cells use this less efficient pathway, in terms of energy production, only under anaerobic conditions.

In contrast cancer cells, even under highly aerobic conditions, primarily derive energy from glucose via glycolysis to lactic acid, a property first observed by Otto Warburg [22]. Since then, this "aerobic glycolysis" is known as the "Warburg effect." Because glycolysis is far less efficient for ATP production compared to mitochondrial oxidative phosphorylation, it is usually associated with marked increases in glucose uptake and consumption [23], a phenomenon clinically exploited to visualize cancer using the glucose similar 18-fluorodeoxyglucose by positron electron tomography [24]. The preference of cancers for aerobic glycolysis, over the more energy-efficient oxidative phosphorylation pathway, has many advantages for cancer. Warburg initially proposed that there was a defect within the mitochondria of tumour cells and they were unable to use oxygen to produce ATP. This hypothesis has been largely disproven, because the majority of cancers are able to revert back to oxidative phosphorylation when lactic acid generation is inhibited [25]. Further studies suggested that aerobic glycolysis has arisen as an adaptation to hypoxic conditions. Tumours commonly are located in an environment with fluctuating oxygen levels, alternating between normoxic and hypoxic conditions. The use of oxygen-independent glycolysis would confer a proliferative advantage to cancer cells, making them less susceptible to hypoxic stress during episodes of spontaneous hypoxia [26, 27]. However, this theory does not explain why these cells still undergo aerobic glycolysis when adequate oxygen amount is available. A more likely theory is that cancerous cells could favour aerobic glycolysis because of the large number of produced carbon-based intermediates, which may be useful in proliferative processes. Advantage of aerobic glycolysis lies in the incomplete utilization of glucose, allowing upstream intermediates to be redirected for biosynthesis, thereby providing cancer cells with an abundance of building blocks for synthesis of essential cellular components such as macromolecules. For example, glucose-6-phosphate, a metabolic intermediate of glycolysis, is used for nucleic acid synthesis through pentose phosphate pathway to support cell proliferation, as well as the large amount of pyruvate that is shunted from tricarboxylic acid cycle (TCA cycle) in mitochondria to lactate production through the upregulation of pyruvate kinase M2 isoform (PK-M2) and lactate dehydrogenase A (LDH-A) [18, 28, 29]. Another advantage of Warburg effect is the acidification of the microenvironment by lactic acid: aerobic glycolysis leads to an accelerated lactate secretion, which can acidify the surrounding extracellular matrix and facilitate angiogenesis and tumour metastasis [30]. In addition to the dependency on glycolysis, cancer cells exhibit other metabolic characteristics, such as increased fatty acid synthesis and glutamine metabolism. A pyruvate amount is utilized by a truncated trycarboxylic acid for lipid synthesis required for cell membrane formation during division, by exporting acetyl-CoA from the mitochondrial matrix to the cytoplasm. Enhanced fatty acid synthesis allows a quick tumour cell proliferation, conferring both a growth and survival advantage [31]. Glutamine is the most abundant amino acid in plasma and it constitutes an important additional energy source in tumour cells, especially when glycolytic energy production is low. The degradation products of glutamine (glutamate and aspartate) are necessary for rapidly proliferating cells by acting as aminoacid precursors [32]. Although the mechanisms underlying the Warburg's effect have not been completely understood, complex interactions between the major oncogenic pathways have been known to promote the glycolytic phenotype in cancer cells [33]. Since oncogenic activation is often thought as an early event in cancer development and progression, aerobic glycolysis could be a consequence of the oncogenic alteration.

2.1. Oncogenic Signalling and the Glycolytic Phenotype of Cancer Cells. The altered metabolic phenotype of cancer usually does not result from mutations in specific metabolic genes, except for rare mutations in two enzymes of the TCA, succinate dehydrogenase (SDH) and fumarate hydratase (FH), but rather is the result of mutations in metabolic regulators. A number of oncogenes, such as c-Myc, some tumour suppressors like p53, and hypoxia inducible factors-1 (HIF-1) have been linked to the dysregulation of glucose transport, TCA cycle, glutaminolysis, glycolysis, and hypoxic protection [34, 35]. Several genes are involved in hypoxic protection and their alteration results in an upregulation of glycolysis. Among them, the hypoxia-inducible transcription factors alpha (HIF-1α) is one of the most important factors involved in this mechanism. Indeed, under hypoxic conditions, HIF-1α becomes stabilized and forms a heterodimeric transcription complex with HIF-1β, which activates over 100 downstream genes important in hypoxic survival. Its targets include glycolytic enzymes (hexokinase, aldolase, and lactate dehydrogenase A), glucose transporters (GLUT family transporters), angiogenic factors (VEGF), haematopoietic factors (erythropoietin), and antiapoptotic factors (Bcl-2, IAP-2). In the presence of oxygen, HIF-1α activity is negatively regulated at post-translational level, by a family of oxygen-dependent prolyl asparagine hydroxylases (PHD), which begin the enzymatic sequence that leads to ubiquitination and proteolytic degradation, mediated by the Von Hippel-Lindau (VHL) protein [36]. In different types of tumours, even in conditions of normoxia, the HIF-1α protein levels are elevated, resulting from loss-of-function mutations targeting its negative regulator VHL tumour suppressor. Moreover, HIF-1α was found to be evoked in response to other stimuli, including radiation and reactive oxygen species (ROS), besides oncogenic signalling by ras, v-src, MEK-ERK, EGFR, and PI3K-AKT-mTOR pathways [37]. In

particular, the PI3K-AKT-mTOR pathway plays a central role in growth factor signalling and glucose homeostasis. Indeed, mTORC1 (mammalian target of rapamycin complex 1), besides increasing HIF-1α protein by inducing its mRNA translation, promotes cell growth by regulating multiple biosynthetic processes, including ribosome biogenesis and protein and lipid synthesis. Two classes of direct downstream targets of mTORC1 are the ribosomal protein S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E-(eIF4E-) binding proteins (4E-BP1 and 4E-BP2), both of which control specific steps in the initiation of cap-dependent translation [38, 39]. Aberrantly elevated mTORC1 activity detected in the majority of human cancers is mainly due to dysregulation of upstream signalling pathway. The serine/threonine kinase Akt was recognized as a major upstream activator of mTORC1; Akt inactivates the tuberous sclerosis complex (TSC) proteins, which is a negative regulator of mTORC1. Akt is regulated in turn by phosphatidylinositol 3-kinase (PI3K), which is a transducer of growth factor effects on cell survival [40, 41]. The Akt-activating ability of PI3K is opposed to the tumour suppressor PTEN (for phosphatase and tensin homolog deleted on chromosome 10), a phospholipid phosphatase that directly antagonized the PI3K activity [42]. Another way by which Akt activates mTORC1 is through the indirect inhibition of the AMP-kinase (AMPK), the central regulator of cell metabolism. Cells with hyperactive Akt accumulate high levels of ATP, which inactivate AMPK; instead elevated cytosolic levels of AMP activate AMPK, which phosphorylates and activates TSC. The activation of TSC by AMPK leads to inhibition of mTORC1 signalling [43]. AMP causes allosteric change that promotes the phosphorylation of Thr-172 in the activation loop of AMPK. This phosphorylation is necessary for the full activation of AMPK, which may be performed by the serine-threonine kinase LKB1 [44]. The AMPK phosphorylation is also mediated by the tumour suppressor p53. Besides AMPK, p53 has most well characterized targets which are involved in cell cycle regulation and apoptosis, but some studies have identified also several metabolic enzymes p53-regulated, like glucose transporter proteins 1–4 (GLUTs 1–4), hexokinase (HK), phosphofructokinase (PFK), and pyruvate dehydrogenase kinase (PDK) [45]. Therefore, the suppressive effects of AMPK signalling on glycolytic phenotype require the presence and the activation of tumour suppressive mechanisms mediated by p53, PTEN, TSC, and LKB1, but since they are frequently inactivated in cancers, the expression of the Warburg phenotype is promoted [37].

3. DHA as Modulator of the Metabolic Functions of Cancer Cells

Several biological mechanisms and pathways have been proposed to explain the health benefits of ω-3 PUFAs, including the ability to interact with energetic metabolism [46]. The metabolic changes induced in several tissues by ω-3 PUFAs have been long known. In particular EPA and DHA have been shown to act as hypolipidemic agents exerting prophylactic effects on cardiovascular diseases and improving insulin sensitivity [47, 48]. In liver ω-3 PUFAs inhibit the expression of genes encoding glycolytic and lipogenic enzymes both *in vivo* and *in vitro* [49, 50], and in white adipose tissues EPA and DHA regulate mitochondrial function, especially oxidative phosphorylation [51]. Since DHA and EPA are able to interfere with metabolic functions, it is tempting to affirm that these effects on cancer cell metabolism could be a further possible mechanism for inhibiting cancer survival and progression. This assumption is sustained by recent works, in which it has been demonstrated that ω-3 PUFAs are able to counteract the Warburg effect. In a proteomic, metabolomic, and interactomic integrated study, realized on human pancreatic PACA-44 cell line, it was found that DHA-induced apoptosis is preceded by a metabolic switch from glycolysis towards Kreb's cycle [19]. Indeed, in this paper proteomic and interactomic analysis identified several proteins related to overactivated oxidative metabolism in DHA treated cells. This result suggested that DHA causes an increase of energy production through mitochondrial pathway, resulting in the activation of aerobic metabolism. This was confirmed by metabolomic analysis in which metabolites related to glycolysis, like lactate and phosphoenol piruvate, decreased in DHA-treated cells, while metabolites involved in Kreb's cycle and pentose phosphate pathway, like α-ketoglutarate and NADPH, are significantly accumulated upon DHA-treatment. Moreover, from metabolomic analysis it was shown that glutathione/oxidized-glutathione (GSH/GSSG) ratio remained unaltered in DHA-supplemented cells in comparison to controls, despite the increase of oxidative stress in DHA-treated cells. The absence of the GSSG intracellular accumulation is explained by considering that the switch from glycolytic pathway towards pentose phosphate pathway leads to the accumulation of NADPH, essential coenzyme in the reduction processes of several antioxidant enzymes, and biomolecules, like GSH. For the first time, this proteomic and metabolomic study highlights the DHA ability to modulate glycolytic metabolism that might represent a further mechanism of growth inhibition and apoptosis activation established by ω-3 PUFAs treatment.

In a recent work, it was shown that DHA decreases the bioenergetic functions and metabolic reprogramming of breast cancer cell lines [20]. In this study, two metabolically distinct breast cancer cell lines were utilized, BT-474 and MDA-MB-231, representing mitochondrial and glycolytic phenotypes, respectively, and nontumorigenic breast epithelial cell line, MCF-10A, to identify the efficacy of DHA in multiple metabolic pathway. The extracellular acidification rate (ECAR), representative of glycolysis, and the oxygen consumption rate (OCR), representative of oxidative phosphorylation, were analysed in response to DHA treatment. Both parameters significantly decreased in the two cancer cell lines in a dose-dependent manner in response to DHA supplementation, compared with untreated cells but not in nontumorigenic control. These findings suggest that, independently of metabolic phenotype of cancer cells, DHA is able to change the bioenergetic profile. Moreover, DHA selectively targets malignant cell lines, since no effect was observed in the MCF-10A nontransformed cell line. The authors argue that

the ability of DHA to interfere, not only with the glycolytic activity, but also with the mitochondrial respiration, is due to its capacity to alter the mitochondrial structure and function. Indeed, from the literature it is known that DHA may modify the mitochondrial phospholipid composition and alter the activity of essential inner membrane proteins and channels; this could lead to a reduction of mitochondrial bioenergetic function [52]. The reduction of oxidative phosphorylation is an effect that counteracts with the results obtained in the above discussed work of D'Alessandro et al., where the DHA-treated pancreatic cancer cell line showed a shift from glycolysis to Kreb's cycle [19]. It is possible that the DHA effects on mitochondrial functions are different among cell types. This may depend on the functional state of mitochondria themselves, as demonstrated in work of Suchorolski et al. [53]. In this work, it was compared with ECAR and OCR in four cell lines derived from Barrett's oesophagus (BE), a premalignant condition associated with an increased risk of oesophageal adenocarcinoma (EA), in response to metabolic inhibitors. The treatment with 2-deoxyglucose (2-DG), a competitive inhibitor of glycolytic pathway, increases the OCR value, only in the cell line CP-D. From the analysis of nuclear and mitochondria genome it was found that the CP-D line had the fewest number of mitochondrial genome mutations, among all cell lines. Since this cell line has functional mitochondria, it is able to revert the glycolytic metabolism towards oxidative phosphorylation [53]. Moreover, it is possible that the increased activity of Kreb's cycle, as a result of glycolysis inhibition, may be associated with the ability of some cells to oxidize alternative substrates like glutamine or fatty acids, which provide TCA cycle metabolites [54]. In the work of Mouradian et al., it was shown that the decrease of bioenergetic functions is associated with the reduction of HIF-1α expression and activity in DHA-treated breast cancer cell lines [20] (Figure 1). Further investigation found a reduction of downstream transcriptional targets of HIF-1α, glucose transporter 1 (GLUT1), and lactate dehydrogenase (LDH). The authors hypothesize that the DHA-induced decrease of HIF-1α can occur by two modalities: the first hypothesis expected that DHA induces degradation of HIF-1α protein through activation of PPARα. This consideration comes primarily from extensive scientific evidences that showed the ability of DHA and its metabolites to activate peroxisome proliferator-activated receptors (PPARs) [55, 56]. Moreover, in a recent work it has been demonstrated that the activation of PPARα by clofibrate suppressed HIF-1α signalling by increasing degradation of HIF-1α. The activated PPARα would seem to increase the interaction of HIF-1α with VHL, which enhances the ubiquitin-proteasome degradation pathway [57]. The other hypothesized mechanisms provide that the decrease of HIF-1α is due to a dysfunction of the HSP90 complex, which is required for a correct folding of this transcription factor [58]. Decreases of intracellular ATP levels attenuate the function of the HSP90 molecular chaperone; DHA treatment determines a reduction of ATP and so the disruption of the HSP90 function (Figure 1). The metabolic stress induced by DHA is demonstrated also by an increase in phospho-Thr172-AMPK in treated cells. This result is important evidence that DHA is able to modulate the AMPK pathway, which is implicated in reducing cell proliferation and in regulation of cell metabolism.

In another work, a further mechanism has been proposed by which DHA is able to regulate the AMPK signalling [21]. Indeed, in this paper it was shown that DHA enhanced the tumour suppressor function of LKB1 in breast cancer cell lines. AMPK is a direct target of LKB1, and, by activating LKB1 signalling, DHA treatment leads to phosphorylation and activation of AMPK (Figure 1). The results have shown that pAMPK, in turn, suppresses the mTORC1 signalling and the relative downstream targets, S6K and eIF4E. The suppression of mTORC1 decreased the capacity of cells to execute glycolysis; in fact, the expression of glycolytic enzymes, like hexokinase 2 and lactate dehydrogenase, was decreased in presence of DHA. Consequently, with the decrease of these enzymes a reduction of lactate production has occurred and then of extracellular acidification. These events lead to a decrease in the migration potential of the cells, as demonstrated in this study by migration assay on breast cancer cell lines treated with DHA. The DHA-induced reduction of the lactate concentration may be considered another potential mechanism by which DHA exerts the anti-invasive capacity [17].

4. Conclusions

Based on the results obtained from these studies, it can be stated that DHA interferes with the glycolytic phenotype of cancer cells. However, the bioenergetic dysfunction and the metabolic reprogramming induced by DHA differ among cell lines. Indeed, in pancreatic PACA-44 cell line, the DHA-induced glycolysis depression is followed by Kreb's cycle activation; instead, in some breast cancer cell lines DHA treatment leads to the inhibition of both glycolytic and mitochondrial activity. The decrease of the bioenergetic functions in breast cancer seems to be due to the ability of DHA to activate the AMPK protein, decreasing the ATP levels and activating the LKB1 protein. Probably these mechanisms are connected and contribute to the negative regulation of HIF1-α, because both reduction of ATP and inhibition of mTOR signalling lead to suppression of this transcription factor. Further studies are needed to demonstrate whether DHA is able to interfere with cell metabolism in other types of cancer and if other mechanisms are involved. Nevertheless, the studies reported here show that alteration of cell metabolism may be considered as a further mechanism by which DHA can contribute to impair cancer cell growth and survival and so this provides a new innovative strategy for cancer therapy through targeting cancer cell metabolism.

Conflict of Interests

The authors declare that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

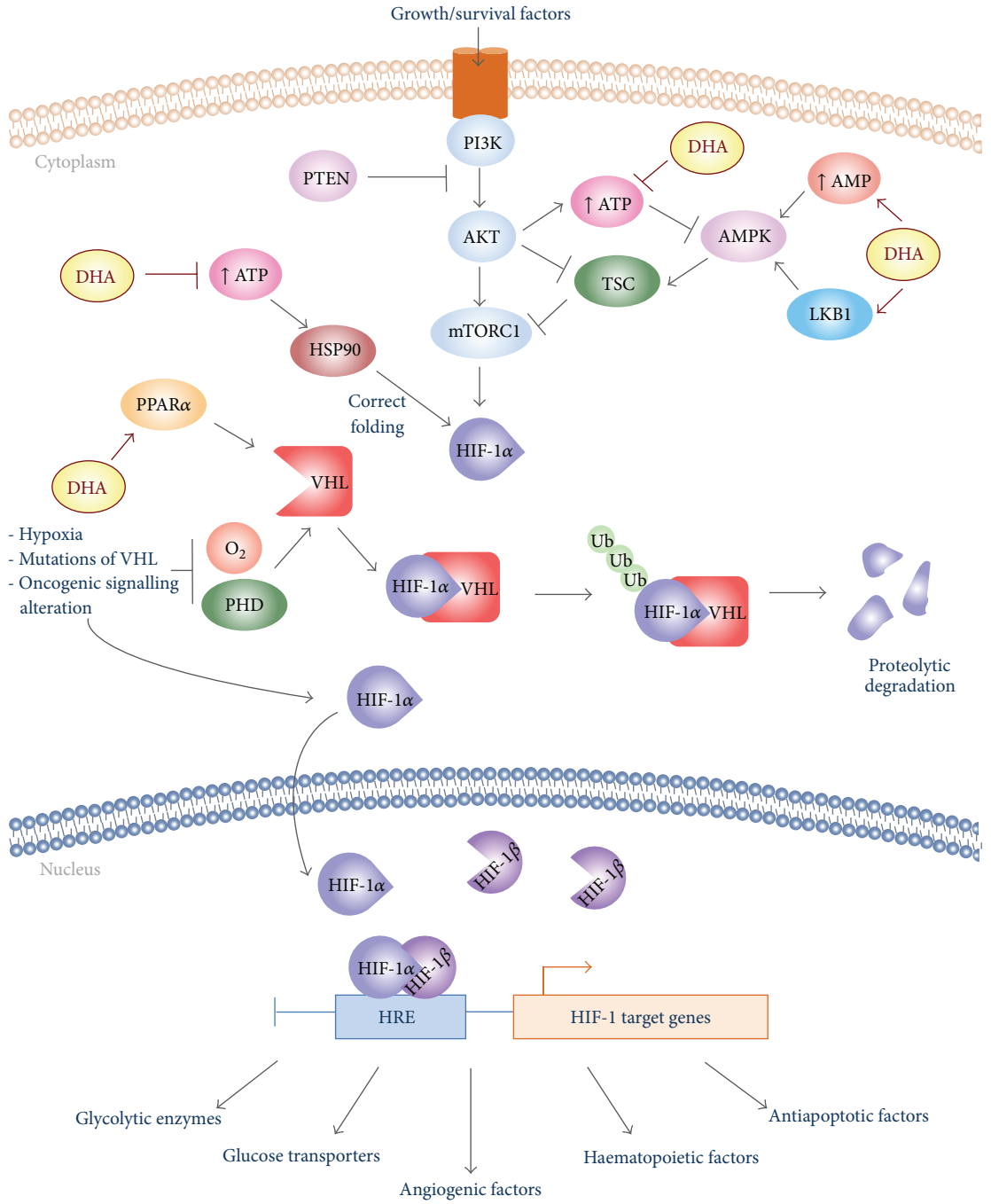

Figure 1: Schematic illustration of the mechanism by which DHA may interfere with the molecular signalling by activating glycolytic phenotype. The PI3K-Akt-mTORC1 pathway promotes the glycolytic phenotype, principally activating the transcription factor HIF-1α. HIF-1α is activated also by hypoxia, as well as by mutations of its regulator VHL. The accumulation of HIF-1α in the cytosol determines its heterodimerization with the subunit HIF-1β, forming the active HIF-1 complex. HIF-1 upregulates a wide network of genes by binding to hypoxia response elements (HRE). DHA interferes at various sites of this pathway, and then it is able to attenuate bioenergetic function and Warburg metabolism. DHA treatment increases the LKB1 protein expression and AMP cytosolic levels, necessary events to activate the AMPK pathway. Active AMPK inhibits mTORC1 signalling, via phosphorylation of TSC protein. Moreover, DHA alters cancer cell metabolism by interfering with the processes implicated in the stabilization of HIF-1α. Indeed, the reduction of cytosolic ATP levels induced by DHA prevents the proper functioning of HSP90, molecular chaperon necessary for folding of HIF-1α. Moreover, DHA destabilizes HIF-1α promoting its proteolytic degradation via PPARα activation.

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Review Article

Biological Roles of Resolvins and Related Substances in the Resolution of Pain

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Endogenous pain-inhibitory substances have rarely been found. A group of powerful pain suppressor molecules that are endogenously generated are now emerging: resolvins and related compounds including neuroprotectins and maresins. These molecules began to be unveiled in a series of inflammation studies more than a decade ago, rapidly shifting the paradigm that explains the mechanism for the inflammatory phase switch. The resolution phase was considered a passive process as proinflammatory mediators disappeared; it is now understood to be actively drawn by the actions of resolvins. Surprisingly, these substances potently affect the pain state. Although this research area is not fully matured, consistently beneficial outcomes have been observed in a various *in vivo* and *in vitro* pain models. Furthermore, multiple hypotheses on the neuronal and molecular mechanisms for alleviating pain are being tested, deriving inspiration from existing inflammation and pain studies. This paper serves as a brief summary of the proresolving roles of resolvins and related lipid mediators in inflammation and also as a review for accumulated information of their painkilling actions. This also includes potential receptor-mediated mechanisms and discusses future scientific perspectives. Further diverse approaches will help to construct a hidden axis of natural protection principles and establish proofs of concept for pain relief.

1. Introduction

Inflammation plays a key role in the pathogenesis of numerous health conditions and is characterized by a series of inflammatory responses. Interestingly, local inflammation promptly occurs after injuries or infections and subsequently involves mechanisms underlying the resolution of inflammation and the wound healing process. Once impaired, these mechanisms may cause chronically harmful conditions. The resolution of inflammation is not simply associated with the reduction of proinflammatory mediators followed by their decreased influences. In fact, different mediators become active for resolving purposes at a certain time point during the inflammatory process, which may define what is currently called the resolution phase. These mediators dynamically stimulate the cellular and biochemical resolution processes that restore the normalcy rather than merely antagonizing the functions of proinflammatory mediators.

Once released due to tissue injuries, proinflammatory mediators induce peripheral pain sensitization; these include bradykinin, prostaglandins, nerve growth factors (NGF), proinflammatory cytokines (e.g., tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, and IL-6) and proinflammatory chemokines (e.g., chemokine (CC-motif) ligand 2 (CCL2)) [1–3]. It is noteworthy that all of these proinflammatory mediators bind to and stimulate specific receptors expressed in the peripheral terminals of nociceptors (pain-mediating sensory nerve fibers) [4]. Once activated, the receptors engage hyperactivation of key transduction molecules, such as transient receptor potential vanilloid subtype 1 (TRPV1) and ankyrin subtype 1 (TRPA1) ion channels, and conduction molecules such as tetrodotoxin-insensitive voltage-gated sodium channels Nav1.7, 1.8, and 1.9. The increases in nociceptor excitability are often more amplified by activation of protein kinases (which is also caused by different downstreams of the same proinflammatory mediator

signaling), such as protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) [3–10].

This excessive nociceptor activation initiated in the peripheral terminals is followed by the increased release of neurotransmitters (e.g., glutamate) and neuromodulators (e.g., substance P and brain-derived neurotrophic factor (BDNF)) from the central terminals in the spinal cord [11, 12]. Accordingly, postsynaptic receptors such as N-methyl-D-aspartic acid (NMDA) type glutamate receptors and metabotropic neurokinin-1 receptors are overstimulated, hyperactivating postsynaptic neurons: the central sensitization in the spinal cord dorsal horn [13–15]. Central sensitization plays a key role in the development of chronic pain and even in its persistent spread outside injured areas [16]. The extracellular signal-regulated kinase (ERK), a member of the MAPK family, is specifically phosphorylated in the nociceptive postsynaptic dorsal horn neurons in a hyperactive state. Therefore, phosphorylated ERK (pERK) serves as a marker for central sensitization [17]. In turn, pERK persistently contributes to increasing the activity of NMDA receptors, inhibiting the activity of Kv4.2 potassium channel, inducing AMPA receptor trafficking, and transcribing other pronociceptive genes [14, 17, 18]. Recent studies on a variety of inflammatory responses in the central nervous system (CNS) and postoperative pain have shown that, not only neurons, but also glial cells, such as microglia and astrocytes, play an important role in developing central sensitization of pain [12, 19, 20]. When peripheral tissue is injured, TNF-α and IL-1β are released from microglia and astrocytes in the spinal cord, where the terminals of the primary nociceptors that cover the injured area form their central synapses [21–23]. This nonneuronal glial activation causes a persistent increase in the pre- and postsynaptic neuronal excitations (e.g., by elevating glutamate and neuropeptide release and by upregulating glutamate receptors) and decreases in the inhibitory mechanisms (e.g., by downregulating inhibitory Cl^- signals) in the dorsal horn, thus contributing to central sensitization [21].

In addition, sustained excitation of the cerebral cortex and brain stem on the pain pathway retrogradely (e.g., releasing nitric oxide) facilitates efferent activities of afferent nociceptor neurons in the spinal cord dorsal horn, which also contributes to persistent pain after the onset of tissue injury [16, 24]. Primary nociceptor neurons in a chronic sensitization state also perform efferent activities on injured tissues: the peripheral terminals of those neurons may release substance P and calcitonin gene-related peptide (CGRP) towards the injured site. This may maintain or exacerbate inflammation and widen the inflamed area [25]. Independent of such changes in inflamed areas, the influence of increased synaptic strength and glial activation may extend to neighboring synapses, which can cause a chronic hyperalgesic or allodynic state in a neuronal pathway that covers uninjured areas [26].

TRPV1 and TRPA1 are two critical types of TRP ion channels that participate in pain generation [27]. Besides the influences of proinflammatory cytokines and chemokines which indirectly stimulate TRP channels in a receptor-mediated signal transduction-dependent manner, these two TRPs can also be directly hyperactivated by protons, oxidative stress, tissue stretches, excessively hot or cold temperatures, and proinflammatory lipids such as leukotrienes and isoprostanes, all of which may often occur during inflammation. The consequences from their direct hyperactivations are the same, somatic inflammatory pain *via* central and peripheral sensitization, in which increased glutamate release strongly facilitates the synaptic transmission in the spinal cord [4, 12, 28]. Not surprisingly, sustained TRP activation has also been shown to contribute to chronic pain state. Currently, considerable efforts are being made to develop small-molecule pharmacological inhibitors of these two TRP channels to improve chronic pain [12].

Using unbiased liquid chromatography-mass spectrometry- (LC-MS/MS-) based lipidomics, Dr. Serhan's group at Harvard Medical School uncovered two families of endogenous lipid mediators, including resolvins (e.g., RvD1, RvD2, and RvE1) and protectins (e.g., PD1 or NPD1) in inflammatory exudates [29, 30]. They are biosynthesized from ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Unlike most known lipid mediators (prostaglandins, leukotrienes, and oxygenated eicosanoids) which are strongly proinflammatory, resolvins and protectins show remarkable resolving potency when tested in animal models of inflammatory diseases [31]. Maresins (e.g., maresin 1), a newly found group of macrophage-derived mediators, are also metabolized from DHA and constitute another strong resolver family during inflammation [32]. Surprisingly, evidence is emerging that resolvins, protectins, and maresins are all able to inhibit the nociceptor activities associated with inflammatory pain [31, 33]. Recent studies have demonstrated that RvE1, RvD1, RvD2, NPD1, and maresin 1 potently suppress somatic inflammatory pain, to some extent by modulating the activities of TRPV1 and TRPA1 and by regulating TRPV1 and TRPA1 activation-elicited synaptic plasticity [28, 33, 34].

In this review paper, we discuss the latest updates on proresolving lipid mediators and attempt to define their roles in the body's natural protective principles, obtain a better understanding of their molecular and cellular mechanisms, and speculate on unanswered questions and therapeutic implications regarding tissue injury-induced inflammatory pain.

2. Resolution of Inflammation and Pain

The process by which proinflammatory mediators trigger inflammatory pain is well established as mentioned above. In contrast, little is known about the mechanisms underlying the resolution of inflammatory pain. The hypothesis has often been overlooked that novel anti-inflammatory or proresolving local mediators as well as proinflammatory mediators can naturally occur inside injured tissues. The discovery of such putative resolving molecules would contribute to our understanding of mechanisms for spontaneous or self-limited recovery of acute inflammation and inflammatory pain. Indeed, the resolution of acute inflammation was presumed

to be a passive process in the past, but it is now considered to involve homeostatic recruitment of active biochemical programs that return inflamed tissues to their preinflammatory states [12, 42]. Accumulating evidence indicates that anti-inflammation and proresolution are distinct mechanisms that are important for controlling the inflammation [43]. Moreover, proresolving mediators show different actions than known, widely used anti-inflammatory therapeutics such as cyclooxygenase-2 (COX-2) inhibitors [42, 44]. In fact, COX-2 inhibitors are able to disrupt endogenous resolution mechanisms. COX-2 inhibitors reduce not only the production of proinflammatory lipid mediators, but also those of key proresolving lipid mediators, since COX-2 also produces these lipid resolvents. Thus, despite their anti-inflammatory effect, COX-2 inhibitors impair the inflammatory resolution process [30, 42, 44–46]. This is an example. According to an early animal study of pleuritis using rats, the degree of the inflammatory response was decreased at 2 h but was increased even at 48 h after the treatment of NS-398, a COX-2 inhibitor. It was then proposed that COX-2 may have a proinflammatory effect during the early phase of acute inflammation, predominantly seen in polymorphonuclear leukocytes (PMNLs). However, it may also be involved in the resolution of inflammation in the later phase, predominantly seen in mononuclear leukocytes (MNLs) [44]. COX-2 is now known to be required for the biosynthesis of proresolving mediators, such as lipoxins and resolvins, in the resolution phase [42]. This leads to a speculation that effects of COX-2 inhibitors on pain relief should be determined based on the balance between its anti-inflammatory action (or antinociceptive action in pain) and its antiresolving (or pronociceptive) action. It has been suggested that COX-2 inhibitors may transiently exhibit antihyperalgesic effects in some experimental models of chronic inflammation and pain. However, they may also prolong posttreatment pain by disrupting endogenous resolution circuits [47].

The reduction in leukocyte recruitment (influx) to the site of inflammation is indicative of anti-inflammatory responses. Likewise, the increased leukocyte exit (efflux) might be associated with proresolution action [42]. If left unresolved, acute inflammation shifts into chronic inflammation; this transition paradigm has been ignored in the field of pain research. This may be because a lower degree of the inflammation, a feature of local inflammation, seems to represent a chronic state and also it frequently lacks inflammatory signs in the blood indices that are indicative of clear acute inflammation. However, unresolved inflammation can often lead to neuroinflammation that can occur in the CNS and peripheral nervous system (PNS), characterized by the activation of glial cells (e.g., microglia, astrocytes, and satellite glia), which are key contributors to pain exacerbation. Furthermore, persistent chronic inflammation would play an essential role in the maintenance of synaptic plasticity in the neural pain pathway and eventually in chronic pain.

Of note, chronic pain is not an extension of acute pain but originates from plastic changes in pain processing, also known as neuroplasticity [11]. Targeting the transition phase from acute to chronic pain would be critical for prevention of chronic pain that appears to result from chronic inflammation. It should also be noted, however, that it would not be sufficient to treat chronic pain by simply lowering the degree of the inflammation in a chronic phase in the injured tissue because independent chronic mechanisms are already initiated inside the relevant neural circuit in parallel. It is therefore imperative that novel therapies based on "neural" plasticity along the circuit in both the CNS and PNS are developed to control the chronic pain.

3. Resolvins

Resolvins are a new family of local mediators that are present in resolving inflammatory exudates. These are lipids that are enzymatically biosynthesized from PUFAs by multiple types of lipoxygenases, or also by COX-2 as mentioned. Resolvins were initially identified using a systems approach with LC-MS/MS-based lipidomics and informatics. Further research has elucidated the chemical structure and the identities of related intermediate metabolites of resolvins. Resolvins (that were sometimes called resolution-phase interaction products because of their unique nature in biosynthesis: transcellular biosynthesis) are referred to as endogenous compounds synthesized from major ω-3 fatty acids, such as EPA and DHA, and are thus denoted as E-series (RvE) and D-series (RvD) resolvins, respectively (Figures 1 and 2) [29]. Like lipoxins, some resolvin members are also produced by the COX-2 pathway in the presence of aspirin, yielding "aspirin-triggered" (AT) forms. Evidence is accumulating that, besides their proresolving mechanisms explained below, resolvins also display potent anti-inflammatory and immunoregulatory effects by inhibiting the syntheses of proinflammatory mediators and modulating the leukocyte trafficking to inflammatory sites as well as clearance of neutrophils from mucosal surfaces.

3.1. E-Series Resolvins. Experimental models of inflammation have shown that specialized proresolving lipid mediators (SPMs: a collective term, recently coined for all proresolving lipids) including resolvins have both proresolving and anti-inflammatory actions. Anti-inflammatory actions of resolvins are largely based on downregulation of the recruitment and function of PMNLs and the decreased synthesis and secretion of proinflammatory mediators. For example, they have inhibitory effects against the transendothelial migration of the PMNLs *in vitro* and infiltration *in vivo* [29]. On the other hand, proresolving effects seem to be accomplished by promoting the recruitment and nonphlogistic phagocytosis of monocytes/macrophages and the secretion of IL-10.

The effects of resolvin E1 (RvE1) appear to arise from its specific and direct actions on G-protein coupled receptors (GPRs). RvE1 antagonizes type 1 leukotriene B4 receptor (BLT1) in neutrophils and dendritic cells, and it activates chemokine receptor-like 1 receptors (CMKLR1, also called chemR23) in monocytes. BLT1 antagonism finally suppresses leukocyte infiltration, intracellular signaling of nuclear factor Kappa B (NFκB), and mitogen-activated kinase (MAP kinase), thereby reducing the biosyntheses and secretions

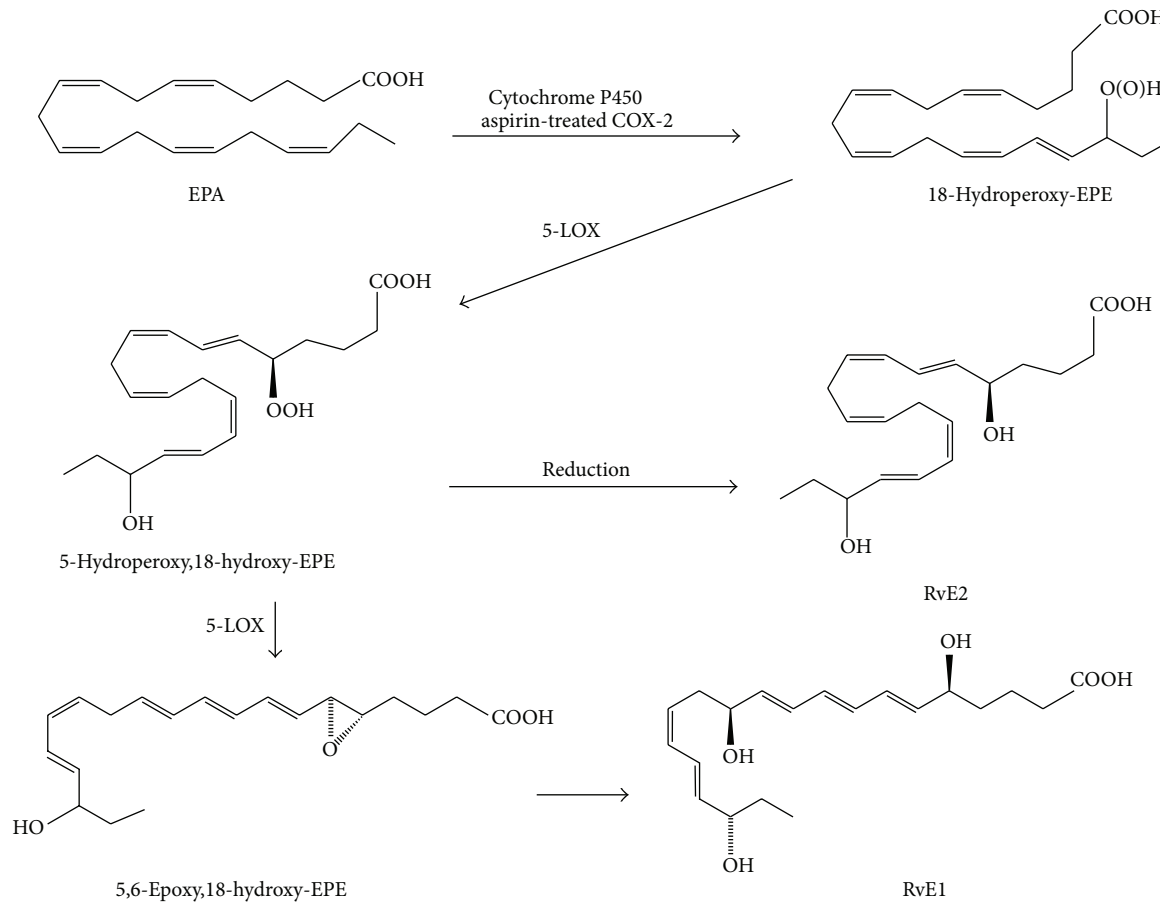

Figure 1: Endogenous biosynthetic pathways of the E-series resolvins (RvEs). Eicosapentaenoic acid (EPA) is converted into 18-hydroperoxy-EPE (18-HpEPE) by aspirin-treated cyclooxygenase-2 (COX-2) or cytochrome P450 (CYP450) and subsequently transformed by 5-lipoxygenase (5-LOX) into RvE1 and RvE2.

of proinflammatory mediators. Strong ChemR23 expression is mostly detected in monocytic cell types, including macrophages, microglia, and dendritic cells. Its activation promotes phagocytic clearance of apoptotic neutrophils. Chemerin is a chemoattractant protein and is a known activator of chemR23. This protein ligand has been shown to exhibit immunological effects similar to those of RvE1 in both *in vivo* and *in vitro* experimental models [48–52]. RvE2 plays a similar role to RvE1, based on receptor-mediated mechanisms for resolving the inflammation [53].

A multitude of experimental inflammation models have been examined to define the resolving outcomes of RvE1 treatment. An experimental model of peritonitis demonstrated RvE1-induced resolution where changes in neutrophil recruitment, dendritic cell migration, and levels of cytokines and chemokines were obvious [54, 55]. Also in the gastrointestinal tract, RvE1 suppressed leukocyte infiltration in animals with colitis. In addition, diverse parameters were explored in the respiratory tract inflammation, and those were again improved upon RvE1 treatment; in an experimental animal model of allergic airway inflammation, the migratory and cytotoxic functions of natural killer (NK) cells were enhanced whereas T helper-17 cells were downregulated. One of the causes seemed to be decreases in levels of proinflammatory cytokines such as IL-23 and IL-6 [56, 57]. Furthermore, RvE1 mitigated antiapoptotic signals and therefore resulted in phagocytosis-induced apoptosis of neutrophils in an experimental model of acute lung inflammation [58]. In a murine model of aspiration pneumonia, neutrophils were less accumulated and the clearance of *Escherichia coli* improved with RvE1 treatment [59].

Topical inflammation was also challenged; in an atopic dermatitis model, proinflammatory signs and indices including inflammatory cytokine levels (IFN-γ and IL-4), degrees of CD4(+), CD8(+) T cell infiltration, mast cells and eosinophils, and skin swelling were improved [60]. In a rabbit model of periodontitis, RvE1 resolved local inflammation and attenuated osteoclast-mediated bone loss [61]. Also in a study using human components, RvE1 improved the phagocytic function of macrophages in patients with localized aggressive periodontitis [62]. In a mouse ocular stromal keratitis model infected with herpes simplex virus, there was a significant decrease in the degree of angiogenesis and the frequency and severity of lesions following treatment of RvE1 [63]. RvE1,

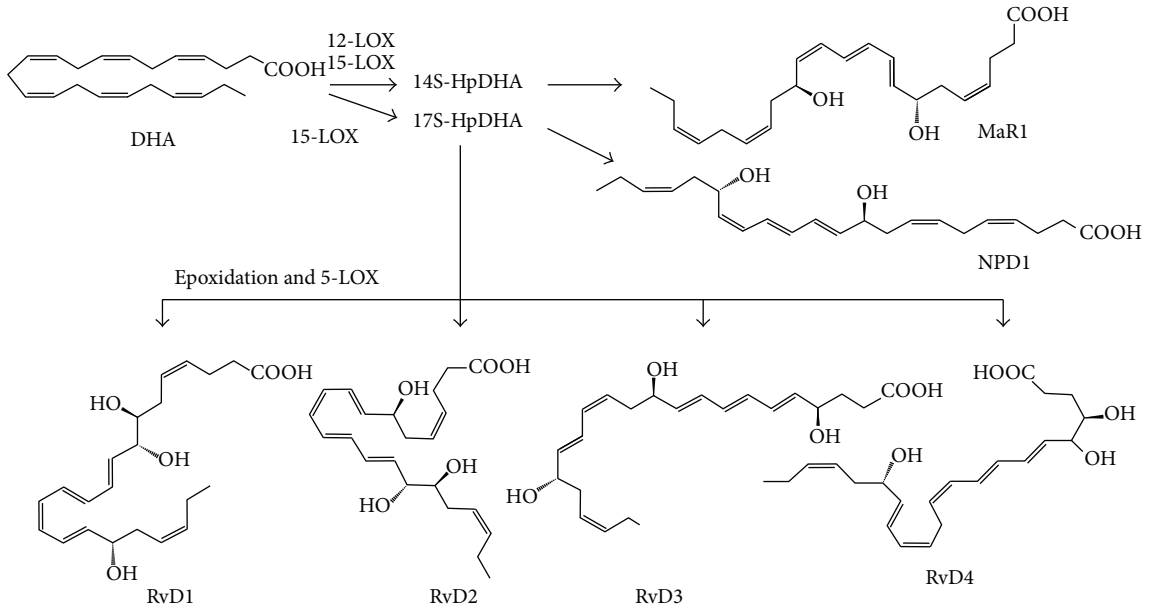

Figure 2: Endogenous biosynthetic pathways of the D-series resolvins (RvDs), maresin 1, and neuroprotectin D1. Docosahexaenoic acid (DHA) is converted into 17S-hydroperoxy-DHA (17S-HpDHA) by 15-LOX. 17S-HpDHA is further transformed into RvD1, RvD2, RvD3, RvD4, or neuroprotectin D1. DHA can be transformed by 12-LOX into 14S-hydroperoxy-DHA (14S-HpDHA), from which subsequent epoxide hydrolysis produces maresin 1.

RvD1, and NPD1 were once shown to display strong effects in protecting neovascularization in a murine model of oxygen-induced retinopathy [49].

3.2. D-Series Resolvins. A study with a murine inflammation model using a dorsal-skin air pouch first demonstrated the effects of RvD1 [29]; it interfered with the neutrophil recruitment. Interestingly, despite their similarity in proresolving outcomes, separate GPR types appear to be engaged in the action mechanisms for RvDs and RvEs. That is, RvD1 restricts neutrophil recruitment *via* the activation of lipoxin A4/annexin-A1 receptor/formyl-peptide receptor 2 (ALX/FPR2), which indicates that RvD1 and another SPM lipoxin A4 at least partly share the signaling mechanism for their resolvent actions [64]. RvD1 also seems to utilize a different GPR, GPR32. Recent studies have shown that 17R-RvD1, also known as aspirin-triggered RvD1 and AT-RvD1, activates the same GPRs. However, it remains to be elucidated if the actions of other RvDs involve the same GPR mode [65].

From the molecular actions of RvDs described above one may extrapolate its resolving effects in cellular, tissue, and animal levels. A series of studies on practical inflammation models using experimental animals confirmed it. Like RvEs, RvDs have been shown to be effective in ameliorating inflammatory pathologies of the respiratory and gastrointestinal tracts. RvD1 and AT-RvD1 reversed allergic responses in airway inflammation [66]. RvD1 also dampened inflammation in lipopolysaccharide-induced acute lung injury [67]. In experimental colitis models using dextran sulfate sodium or 2,4,6-trinitrobenzene sulfonic acid, disease parameters were markedly improved upon treatment of D-series resolvins, PMNL infiltration, colonic damage, and body weight loss [68].

Particularly in recent studies using D-series resolvins, the effects in bacterial infection models have been carefully examined. RvD1, RvD5, and NPD1 greatly enhanced bacterial clearance [69]. Furthermore, those resolvin species also elevated the antibiotic effectiveness of bacterial clearance, which leads to a consideration that D-series resolvins may possibly become candidates for novel therapeutics for bacterial infections. For the molecular and cellular mechanisms, RvD5 appears to be the predominant form among endogenous resolvins that work against infection and also utilizes the known RvD1 receptor, GPR32. Downregulations of NF-κB and TNF-α expressions upon their treatments were detected. The effect of RvD2 was explored in a sepsis model [70]. RvD2 treatment prevented the induction of sepsis in a murine model of microbial colitis. The mechanism seems to be that RvD2 potently regulates leukocyte function; RvD2 attenuated leukocyte/endothelial interactions *in vivo*, by increasing the production of endothelium-dependent nitric oxide and directly decreasing the expression of leukocyte adhesion receptor. In microbial sepsis initiated by caecal ligation and puncture, RvD2 sharply reduced local and systemic bacterial burden, excessive cytokine production, and neutrophil recruitment. RvD2 also elevated the number of peritoneal mononuclear cells and augmented macrophage phagocytosis. Such multilayered proresolving actions of RvD2 eventually resulted in prolonged survival from sepsis. Taken together, D series resolvins appear to be powerful regulators of both typical and explosive inflammatory responses under infection, by controlling multiple cellular mechanisms mostly aiming to preserve immune vigilance.

Mounting evidence from other disease states and inflamed tissues supports the role of RvDs as potent proresolvents. For example, RvD1 treatment led to less accumulation of apoptotic thymocytes and enhanced the resolution of peritonitis and to wound closing in diabetic mice [71]. Also, RvD1 diminished the production of TNF-α and IL-6 in macrophages of the postmortem spinal cord from patients with amyotrophic lateral sclerosis, showing over a thousand times more potency than its precursor lipid, DHA [72]. In the IFN-γ/LPS-induced T helper-1 cells of inflamed obese adipose tissues, RvD1 led to reduction of cytokine secretions such as TNF-α, IL-6, and monocyte chemotactic protein-1 (MCP-1). Moreover, RvD1 promoted nonphlogistic phagocytosis by macrophages in the stromal vascular fraction. Again, similar effects were obtained from RvD1 and RvD2, both of which reversed the impaired expression and secretion of adiponectin and decreased those of proinflammatory adipokines in inflamed obese adipose tissues, such as IL-6, TNF-α, IL-1β, and MCP-1 [71, 73]. Those two D-series resolvents also attenuated leukotriene B4-stimulated monocyte adhesion to adipocytes and their transadipose migration.

Further detailed molecular mechanisms for RvD1 actions are now being explored. For example, microRNA- (miRNA-) mediated mechanisms were proposed. In resolving exudates from an experimental model of self-limited murine peritonitis, miR-21, miR-142, miR-146b, miR-203, miR-208a, miR-219, and miR-302d were selectively regulated [74]. Among them, nanomolar RvD1 could upregulate miR-21, miR-146b, and miR-219 and downregulate miR-208a. miR-146b targets NF-κB signaling, and miR-219 targets 5-lipoxygenase, which may cause reduced leukotriene production. Overexpressed miRNA upregulated IL-10 production in macrophages [65]. In ALX/FPR2 knockout mice, RvD1 failed to affect the levels of miR-208 and IL-10 and to decrease leukocyte infiltration, which highlights the GPR specific manner of this mechanism. Toll-like receptor (TLR) involvement was also suggested. RvD1 was detected in mouse kidney under bilateral ischemic insults [75]. RvD1 reduced interstitial fibrosis and leukocyte infiltration and inhibited TLR-mediated macrophage activation, thus contributing to the improvement of kidney morphology and function.

RvD1 also ameliorated oxidative stress-induced inflammation [76]. Oxidative stress finally generates endogenous lipid peroxidation products (LPOs) including 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE). In the pain research field, LPOs are known to cause acute pain via direct activation of TRPA1, a major peripheral sensor, but in the inflammation research field, LPOs have been more extensively studied as inflammation inducers that covalently modify protective proteins, for example, glutathione conjugation. RvD1 limited the infiltration of Gr-1(+) leukocytes into the experimentally inflamed region caused by glutathione-conjugated HNE injection.

4. Effects of Resolvins on Inflammatory Pain

D and E series resolvins are now known to powerfully lead tissue situation to the resolving phase in a variety of forms of inflammation. By this notion, a hypothesis can be born that pain, an important aspect of inflammation (so-called "dolor" in *Latin*), may be strongly affected by the actions of resolvins. At a different point of view, resolvins may also directly regulate the function of the sensory neural circuit, independent of their control mechanisms for the inflammatory state; in fact unsaturated nature and lipoxygenase-involved biosynthetic pathways closely resemble a group of lipidergic mediators that directly act on the nerve [77, 78]. No matter what mechanism explains the action of resolvins on pain, a large number of inflammatory pain models have been tested to determine whether resolvins affect pain states, and as a result surprising analgesic effects have consistently been reported [30, 31, 79] (Table 1). A pioneering result from the study by the Ji lab stands at the forefront of the resolvin-pain research [35]. This study focused on inflammatory pain and commonly on multiple types of experimental animal models such as inflamed animals using complete Freund's adjuvant (CFA), carrageenan, and TNF-α and on an acute pain model using capsaicin, a pain receptor TRPV1-specific agonist; RvE1 administration drastically decreased pain symptoms even at nanogram levels.

The underlying mechanisms that realize such powerful analgesic outcomes might be complex and still require further elucidation, but clear evidence for some downstream signalings was provided. First, a typical mechanism as shown above in the inflammation research field also works in the inflamed tissues of pain models. When intraplantar injections were given into inflamed paws, RvE1 disturbed neutrophil infiltration and paw edema formation and reduced expression of proinflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α, MCP-1, and macrophage inflammatory protein-1α (MIP-1α) [35]. Another mechanism is via direct actions on the neuronal circuit (Figure 3). RvE1 has been shown to exert direct effects on the primary nociceptor neurons. TRPV1 is a major pain receptor present in the tissue-innervating terminals of nociceptor neurons, detecting and electrically transducing damage signals including heat, anisotonicity, acid, and capsaicin, in normal and inflammatory conditions. Thus, TRPV1 has been a key peripheral analgesic target for nearly two decades. RvE1 blocked capsaicin-evoked acute pain and TRPV1-mediated heat pain. Its detailed molecular mechanisms presumably merge into the GPR signaling mentioned above, Gαi signaling- (which is GPR's intracellular downstream signaling-) mediated modification of TRP functions [28, 35]. ChemR23 GPR is expressed in the dorsal root ganglia where nociceptor neuronal cell bodies are clustered. Its colocalization with TRPV1 in DRG neurons has already been demonstrated [35].

This GPR-mediated molecular mechanism may work not only in the peripheral part of the TRPV1-positive nerve circuit, but also in the central synapses. This is because the same signaling machinery is preserved in both pre- and postsynapses in the dorsal horn of the spinal cord and even in the adjacent microglia that may support synaptic strength. It can therefore be inferred that similar pain-inhibitory mechanisms might be involved when RvE1 is intrathecally injected or endogenously generated. However, the final postsynaptic effectors that lead to pain alleviation should be different

Table 1: Summary of important antinociceptive actions of resolvins in various pain models.

Resolvins		Genus	Pain models	Pain symptoms that were improved by resolvin treatment [references]
D-series	RvD1	Mouse	CFA	Mechanical allodynia, heat hyperalgesia [35, 36], mechanical hyperalgesia [36]
			Carrageenan	Chemical pain [36]
			Formalin	Phase 1 and 2 inflammatory pain [35, 36]
			Prostaglandin E2	Acute mechanical pain [36]
			TRP agonists	Acute chemical pain [36]
		Rat	Incision	Mechanical allodynia [35]
			Incision	Mechanical allodynia and hyperalgesia [37]
			Trinitrobenzene sulfonic acid-induced chronic pancreatitis	Mechanical allodynia [38]
	RvD2	Mouse	CFA Carrageenan	Mechanical allodynia and heat hyperalgesia [34]
			Formalin	Phase 2 inflammatory pain [34]
	AT-RvD1	Mouse	CFA	Heat hyperalgesia [39]
			Carrageenan	Chemical pain [39]
			TRP agonists	Heat hypersensitivity [39]
		Rat	Carrageenan	Mechanical allodynia [40]
E-series	RvE1	Mouse	CFA Carrageenan Intrathecal TNF-α	Mechanical allodynia and heat hyperalgesia [35]
			Formalin	Phase 1 and 2 inflammatory pain [35]
			TRP agonists	Acute capsaicin-evoked pain [35]
			Spinal nerve ligation	Heat hyperalgesia [35]
			Chronic constriction injury	Mechanical allodynia and heat hyperalgesia [41]
Others	NPD1	Mouse	CFA	Heat hyperalgesia [28]
			Formalin	Phase 2 inflammatory pain [28]
			TNF-α	Mechanical allodynia and heat hyperalgesia [28]
			TRP agonists	Acute capsaicin-evoked pain [28]
	Maresin 1	Mouse	TRP agonists	Acute capsaicin-evoked pain [32]
			Vincristine chemotherapy	Mechanical allodynia [32]

from those present in the peripheral terminals or central presynaptic terminals of the DRG nociceptor population. The peripheral or presynaptic effectors of the nociceptors are TRP channels as mentioned, while the central postsynaptic effectors expressed in dorsal horn neurons appear to be NMDA receptors. On the other hand, both mechanisms seem to share extracellular signal-regulated kinase (ERK) inhibition as an intermediate downstream process [80, 81].

The microglia located in the spinal cord dorsal horn play a critical role in maintaining pathologic plasticity in the nociceptive synapses between DRG and dorsal horn neurons. The monocytic origin of spinal microglia leads to a strong possibility that the microglia may transcellularly synthesize and secrete resolvins and also express the resolvin-specific GPRs, as already shown in the monocytes and macrophages [41]. Further research may investigate the quantity and type of resolvins that are secreted from microglia upon injury or inflammation and how much the GPRs contribute to the analgesic effects when extraneous resolvins are administered in pain synapses.

It is also noteworthy that intrathecal RvE1 had a greater efficacy in dampening the second pain phase in formalin-induced models, as compared with NS-398, a COX-2 inhibitor, and even with morphine, the famous opioid. Furthermore, the analgesia seems to follow an indiscriminate manner, for example, when administered intrathecally, RvE1 or RvD1 alleviates both heat and mechanical hypersensitivity. In the same experimental models of pain, chemerin, a ChemR23-specific GPR ligand, also exhibited similar effects. Such GPR signalings seem to be a predominant mechanism underlying the analgesic effects of resolvins on the neuronal circuit in multiple types of pain, which is based on the series

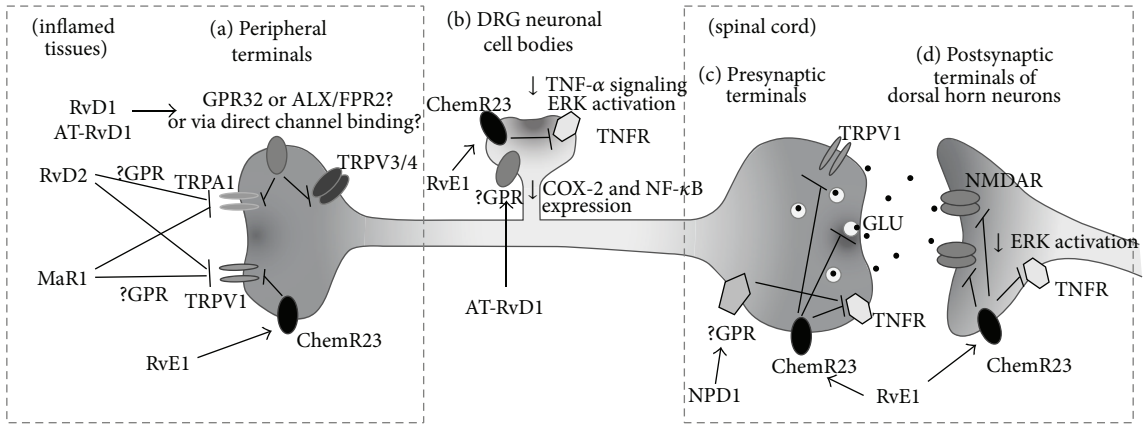

Figure 3: Signaling mechanisms of resolvin actions on the pain-mediating DRG neurons and their synapses. (a) RvE1 suppresses TRPV1 activity, and RvD1 suppresses TRPA1, TRPV3, and TRPV4 activities in DRG nerve terminals. (b) RvE1 attenuates TNF-α signaling and ERK activation in DRG neuronal cell bodies. In the same regions, AT-RvD1 downregulates COX-2 and NF-κB expressions. (c) RvE1 reduces excitatory neurotransmitter releases and TNF-α signaling at the central presynaptic terminals. (d) RvE1 suppresses overactivated ERK and NMDA receptor at postsynaptic dorsal horn neurons in the spinal cord. DRG, dorsal root ganglion; GLU, glutamate; NMDAR, NMDA receptor; TNFR, TNF-α receptor; TRPA1, transient receptor potential ankyrin subtype 1; TRPV1, transient receptor potential vanilloid subtype 1; TRPV3, transient receptor potential vanilloid subtype 3; TRPV4, transient receptor potential vanilloid subtype 4.

of Ji group's experiments [35]. Particularly in the central mechanism underlying the modification of synaptic strength, this GPR-dependent aspect appears to be obvious. In the second pain phase of the formalin-induced pain model, the analgesic effects of RvE1 were blocked by the intrathecally injected Gαi-coupled GPR inhibitor (pertussis toxin) and were also inhibited when spinal ChemR23 was genetically knocked down. In addition, as mentioned above, chemerin successfully replicated the intrathecal effects of resolvins. However, little is known about the relationships between the inhibitory effects of D series resolvins and GPR32 or ALX/FPR2 for painkilling mechanisms in the spinal synapses. It has once been reported that ALX/FPR2 is expressed in spinal astrocytes [82].

For resolvins' analgesic actions, not only the mechanism in the spinal synapses, but also the peripheral mechanisms seem to depend on the GPR signalings. In DRG neurons, RvD2 inhibited both of activities of TRPV1 and TRPA1 (Table 2) [34]. The effect of RvD2 was prevented by the pretreatment of pertussis toxin or GDPβS which is able to block Gαi-coupled signaling. This suggests that the TRP channel blockade is mediated by GPR action. However, identities of RvD2-specific GPRs remain obscure. Interestingly, these unknown GPR to Gαi-cascades are likely coupled only to specific subtypes of TRP channels. Accordingly, pain-inhibitory effects of resolvins might be limited to the sensory modalities (e.g., heat, cold, pressure, and poking) that the target TRP channel conveys. That also seems to be true for RvE1, since RvE1 applications resulted in clear inhibition of TRPV1 activity, but not of TRPA1 [34, 35].

TRP channel specificity of resolvins' actions was further explored by the Hwang lab. Unlike the sharp specificity of RvE1 to TRPV1 activity, RvD1 displayed broader inhibitory actions on the activities of TRPA1, TRPV3, and TRPV4 [36, 39]. Activities of other nociceptive sensory TRP channels including TRPV1, TRPV2, and TRPM8 were inert to

Table 2: IC_{50} (nM) values for the inhibition of TRPV1 or TRPA1 activity by resolvins, maresin 1, NPD1, and their fatty acid precursors as obtained from studies using DRG neurons. Capsaicin (100 nM) and allyl isothiocyanate (300 mM) were used as basal agonists for activation of TRPV1- and TRPA1-mediated currents, respectively.

Inhibitors	TRPV1 IC_{50} (nM)	TRPA1 IC_{50} (nM)
RvE1	1 ± 0.14	—
RvD1	—	8.5 ± 0.13
RvD2	0.09 ± 0.02	2.1 ± 0.53
DHA	1200 ± 20	—
EPA	224 ± 10	—
MaR1	0.16 ± 0.01	—
NPD1	0.36 ± 0.05	—

RvD1 applications. Different TRP channels are known to be in charge of different pain phenotypes according to their sensory modalities. That is, TRPA1 and TRPV4 are mechanosensitive channels and thus mediate mechanical pain, and TRPV3 and TRPV4 are heat-gated channels and therefore contribute to heat pain. TRPV4, which is also activated by hypotonicity, is responsible for hypotonicity-induced pain. Each individual TRP also mediates acute pain induced by its specific chemical agonists such as cinnamaldehyde or formalin for TRPA1 and camphor for TRPV3. Therefore, *in vivo* specificities of RvD1 on such particular pain phenotypes could be presumed from the *in vitro* TRP channel results. Predictably, the peripheral treatment of RvD1 prevented agonist-specific acute pain. TRPA1 and TRPV4 mediate mechanical hyperalgesia and allodynia under complete Freund's adjuvant- (CFA-) induced inflammation, which is explained by their sensory modalities as mentioned above. Inflammatory pain was also prevented

by RvD1 injections. Hypotonicity-induced pain (which is presumably mediated via TRPV4 activation) of animal paws primed with an inflammatory mediator prostaglandin E2 was also suppressed by local RvD1 treatment. Heat hyperalgesia (which TRPV3 and TRPV4 activations contribute to) in CFA-induced inflammation was also reversed. Formalin is a standard substance that causes pain in examining the peripheral (phase 1) and central (phase 2) mechanisms of an analgesic compound [83]. The phase 1 mechanism is based on acute nociception *via* TRPA1-direct activation by formalin, and RvD1 significantly blocked this phase. Collectively, broad specificity of RvD1 to pain receptor TRP channels appears to explain such an extended analgesic spectrum for variable pain phenotypes. More importantly, pain alleviation by extraneous resolvin injections at least in the acute phase can be independent of a typical inflammatory mechanism where leukocytes should be key players.

AT-RvD1 has shown different specificity; it only has an inhibitory effect on TRPV3. This was consistent with behavioral phenotypes seen following the use of TRPV3 agonists. In addition, AT-RvD1 was also effective at inflammatory heat responses (which is TRPV3's physical modality) triggered by CFA or carrageenan injections [39]. However, no changes in mechanical phenotypes were detected at least immediately after the peripheral application of AT-RvD1. This time course is, again, in an earlier phase than leukocyte-mediated resolving mechanism begins. Different from the GPR-mediated interaction between RvE1 and TRPV1, a distinctive mode may work for interactions between AT-RvD1 and TRPV3 [39]. For example, no inhibitory effect was replicated with the treatment of different agonists for the same RvD receptor (ALX/FPR2), such as cathelicidin LL-37 and Trp-Lys-Tyr-Met-Val-Met [36]. In addition, the inhibitory effects of AT-RvD1 against TRPV3 were maintained following treatment of gallein (a G$\beta\gamma$ inhibitor that incapacitates GPR signalings). These results suggest that it is not likely that inhibitory effects of RvDs depend on GPR signaling. Furthermore, the potency of RvD actions on TRP channels is different from the known potency of RvD actions on leukocytes that likely involve GPR signaling, indicating that different receptors with different affinities are engaged [84].

Many PUFAs and their metabolites have been demonstrated to directly bind to and modulate TRP channels. Resolvins seem to share the biosynthetic pathways and structural features including carbon chain length and number of double bonds with known TRP channel binding lipids, which stimulates the curiosity whether resolvin inhibits channel gating by direct TRP binding. Further studies, such as binding assays, mutation studies on the putative binding sequences, or even deorphanizing hidden target GPRs, need to precede the conclusion. The Ji lab and the Hwang lab have utilized different resolvins for their molecular mechanism studies. The GPR-mediated mechanism and the TRP-specific mechanism might be differentially important for each tested resolvin. Further, either of the molecularly different mechanisms may be differentially available, or their contribution indices may vary depending on cell types or pathologic conditions.

No matter what the predominant intracellular and molecular mechanism is, the above independent results from neurons and neural circuits clearly verified that neuronal cells and microglia are important targets for resolvin actions. Moreover, outcomes from all these actions are surprisingly consistent and those aim to fight pathologic pain and few adverse effects are predicted. In addition, both Ji and Hwang labs have shown that resolvins did not alter thresholds for normal pain despite their high efficacy and potency [34–36, 39]. To verify this, Ji et al. further measured the strength of the central synapse and no change in the basal spontaneous excitability of postsynapses was detected after RvE1 treatment. This indicates that it might be more appropriate explanation that resolvins can "normalize" neuronal function rather than simply "downregulating" it when synaptic strength is exaggerated due to pathologic inflammation [34, 35].

5. Neuroprotectin D1

In 15-lipoxygenase metabolism, DHA generates D series resolvins and NPD1 [85, 86]. The term "protectin" is based on its anti-inflammatory and protective actions in the nervous system. To describe their origin more specifically, the prefix "neuro-" is sometimes added, as in "neuro-" protectin D1 (NPD1) but NPD1 is detected in other nonneuronal regions including the lungs and blood [87]. Like resolvins, NPD1 exerts inhibitory effects on PMNL infiltration [88]. In the nervous system, NPD1 seems to be produced mostly by glial cells and also acts on them in an autocrine or paracrine manner, reducing cytokine expression. Multiple murine experimental models have shown that NPD1 ameliorated damaging pathology in corneal injuries, oxidative-stress-induced retinal epithelial pigmentation, and brain cells exposed to β-amyloid. It also reduced stroke occurrence and promoted wound healing in the cornea [6, 89, 90]. Regardless of its origin, when extraneously administered, NPD1 is also able to display protective effects. In a peritonitis model using zymosan A, the effects of nanogram NPD1 injections were examined in terms of leukocyte infiltration. Collected peritoneal lavages at 4 h showed a greater than 90% reduction in PMNL infiltrations, which indicates that their migration and infiltration were powerfully suppressed *in vivo* [91]. An interesting result was obtained from a murine peritonitis study using NPD1 and RvE1. Separate injections of nanomolar NPD1 and RvE1 led to significant inhibitions of PMNL infiltrations and the degree of NPD1 effect was lower than that with RvE1. When administered concomitantly, greater inhibitory effects were detected on the infiltration of PMNLs, indicating that an *in vivo* additive outcome can be acquired using coapplication of NPD1 and RvE1 [87, 92]. Observations of NPD1 occurrence and function in nonneuronal tissues become active. In the immune system, eosinophils were demonstrated to be able to produce NPD1 during the resolution of inflammation [93]. NPD1 is also generated in murine joints during the resolution of Lyme disease [94]. NPD1 exhibited renoprotective effects in ischemic renal injury models [95]. The production of adipokines could be regulated by NPD1 in an obesity model [96]. Stem cell differentiation is also affected; the incubation in nanomolar NPD1-containing

media greatly promoted neuronal differentiation whereas leukotrienes lacked this effect [97].

Eventually, Park et al. demonstrated that NPD1 is also a highly potent endogenous inhibitor of TPRV1, a negative regulator for spinal cord synaptic transmission, and consequently a suppressor of inflammatory pain [28]; NPD1 blocked TRPV1- and TNF-α-mediated synaptic transmission and long-term potentiation (LTP) in the spinal cord. NPD1 blocked capsaicin-induced TRPV1 currents in DRG sensory neurons at very low concentrations. The IC_{50} of NPD1 (0.36 nM) for TRPV1 inhibition was ~500 times lower than that of AMG9810, a widely used synthetic TRPV1 antagonist. NPD1's action on TRPV1 is likely mediated by activation of specific pertussis toxin-sensitive/Gαi-coupled GPCRs and subsequent inhibition of adenylyl cyclase, PKA, and ERK signaling pathways. Low dose spinal injection of NPD1 (0.1–10 ng) prevented spinal long-term potentiation of the synaptic transmission and reduced TRPV1-dependent inflammatory pain, again, without affecting baseline pain. NPD1 also reduced TRPV1-independent but TNF-α-dependent pain hypersensitivity.

6. Maresins: Macrophage-Derived Resolvents for Inflammation and Pain

Macrophages play a central role in maintaining homeostasis against injuries, infections, and diseases by orchestrating resolution and tissue repair [98]. Correspondingly, macrophages utilize (secrete and respond to) resolving lipid mediators as mentioned above for accomplishing their purposes and also participate in transcellular biosynthesis of those lipids. Besides known lipid resolvents found so far, the presence of other mediators is still possible regarding the extended influences of this cell type on our body protection and the detection of undesignated lipid intermediates the roles and origins of which still remain obscure. Consequently, attempts to identify a novel lipid mediator generated from macrophages and challenges to define its actions and biosynthesis led to the discovery of a novel group of DHA-derived resolvents, maresins [99].

In a lipidomics study using self-resolving inflammatory exudates from a murine model of peritonitis, accumulations of 17S-hydroxyDHA (17S-HDHA), 14S-HDHA were detected [29, 99]. 17S-HDHA is known to be a marker for the biosynthesis of D-series resolvins and NPD1 since it is an upstream intermediate. On the other hand, 14S-HDHA was not known for its further metabolism. It could be hypothesized that 14S-HDHA might be further metabolized to serve as a marker on a novel biosynthetic pathway for a certain bioactive final product. Incubating macrophages with either DHA or 14S-hydroperoxyDHA (14S-HpDHA, the immediate precursor metabolite for 14S-HDHA) resulted in an occurrence of a converted form with highly potent anti-inflammatory and proresolving actions. A series of experiments such as isotope incorporation, intermediate trapping, and characterizations of physical and biological properties showed that the macrophage-produced mediator is 7R,14S-dihydroxy-4Z, 8E,10E,12Z,16Z,19Z-DHA, which is synthesized *via* a novel 12-lipoxygenase action. This substance was termed as maresin 1 (macrophage mediators in resolving inflammation 1 (MaR1) [99]; recently, MaR2 (13R,14S-diHDHA) was found [100]). As briefly mentioned above, maresins exert potent anti-inflammatory and proresolving actions. Cellular mechanisms appear to be similar to other resolvents. MaR1 inhibits the infiltration of PMNLs and promotes the macrophage phagocytosis of apoptotic cells.

MaR1 also has recently been shown to exert potent analgesic actions. Intraplantarly administrations of MaR1 alleviated mechanical allodynia in a neuropathic pain model in mice mimicking chemotherapy-induced pain, by injecting vincristine intraperitoneally. MaR1 also attenuated capsaicin- or allyl isothiocyanate- (a TRPA1 agonist-) induced acute pain, which suggests that MaR1 may modulate TRPV1 and TRPA1 directly or indirectly. Indeed, MaR1 suppressed TRPV1 activity in cultured neurons of DRG [32]. MaR1 blocked capsaicin- (100 nM) induced inward currents (IC_{50}: 0.49 ± 0.02 nM) in a dose-dependent manner, which was reversed by pertussis toxin treatment, indicating a GPR-mediated TRPV1-inhibitory mechanism. However, *in vitro* TRPA1 activity was resistant to MaR1 application, suggesting an unknown indirect mechanism for the effects of MaR1 on allyl isothiocyanate-evoked pain and mechanical allodynia.

Collectively, resolvin series substances have consistently been confirmed to possess significant pain resolving effects and the related substances, NPD1 and maresins, are emerging in the research field in the same context.

7. Conclusions and Perspectives

This review summarizes information on resolvins and related substances, which are important both as endogenous proresolvers and as therapeutic candidates preventing deterioration of inflammation and pathologic pain. Indeed, clinical applications related to inflammation are seriously being considered; a clinical phase II trial of a resolvin-derived synthetic analog RX-10045 for eye dryness was recently completed. A phase I study of oral administration of RvE1 was also completed, and its possible clinical applications will likely include rheumatoid arthritis, asthma, and colitis. Studies on the total synthesis processes of resolvins will help to establish a platform to generate more chemically and metabolically stable analogs which guarantees sufficient proresolving duration when clinically applied [101]. Resolvin studies, as mentioned above, are also evolving into ones investigating practical utilities for various pain diseases. In the initial stage, a relatively stable analog already began to be examined regarding painkilling efficacy [35].

Although the powerful potencies and negligible adverse effects of resolvins appear to promote their transitions to clinical settings, many aspects of the biological mechanisms remain to be clarified. Early termination of inflammation by resolvin treatment seems to be beneficial in that it prevents conversion into chronic inflammation and chronic inflammatory pain. On the other hand, if it is a premature termination, clearance of initial damaging insults or microorganisms might be incomplete. Surprisingly beneficial indices

were obtained in this regards; RvDs and RvE1 contribute to bacterial clearance [59, 69]. In addition, resolvins appear to promote injury repair by elevating phosphoinositide 3 kinase-dependent migration and reducing apoptotic cell accumulation [102, 103].

Although resolvins are found in inflammatory exudates, which is the reason we call these endogenous substances, most studies about the effects and mode of actions have been conducted using exogenous administration. Quantitative information on the tissue-level oscillations needs to be more accumulated. In the same context, it remains to be clarified which type of cells (or blood albumin) is the major source for their metabolic precursors, DHA and EPA [104]. During transcellular synthesis, rate-limiting cell types for resolvin production have yet to be identified. In pain-mediating neural circuit research, such observations on the endogenous regulation might be more complex because, particularly near the central synapses, changes in the leukocyte content are limited. Changes in functional parameters (resolvin production, receptor expression, receptor sensitivities, etc.) of neural components, but not cell numbers, might become dynamic and critical for relaying resolving signals unlike peripheral tissues. Possibility of the presence of other unknown target receptors or ion channels should be also speculated. In addition, the potencies of other lipid mediators that are not classified as resolvin species but share biosynthetic pathways need to be carefully compared for resolution and anti-inflammation [105].

Taken together, recent efforts initiated the definition of the powerful proresolving and analgesic actions of resolvins and related lipidergic molecules. Studies are now examining their cellular and molecular mechanisms. Further studies will explore and examine the utilities for more specific types of pain diseases including mostly chronic ones and will also expand the information to explain an axis of the body's natural protective principles.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Omega-3 Polyunsaturated Fatty Acids in the Treatment of Patients with Acute Respiratory Distress Syndrome: A Clinical Review

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Acute respiratory distress syndrome (ARDS) is defined as the acute onset of noncardiogenic edema and subsequent gas-exchange impairment due to a severe inflammatory process. Recent report on the prognostic value of eicosanoids in patients with ARDS suggests that modulating the inflammatory response through the use of polyunsaturated fatty acids may be a useful strategy for ARDS treatment. The use of enteral diets enriched with eicosapentaenoic acid (EPA) and gamma-linolenic acid (GLA) has reported promising results, showing an improvement in respiratory variables and haemodynamics. However, the interpretation of the studies is limited by their heterogeneity and methodology and the effect of ω-3 fatty acid-enriched lipid emulsion or enteral diets on patients with ARDS remains unclear. Therefore, the routine use of ω-3 fatty acid-enriched nutrition cannot be recommended and further large, homogeneous, and high-quality clinical trials need to be conducted to clarify the effectiveness of ω-3 polyunsaturated fatty acids.

1. Introduction

Acute respiratory distress syndrome (ARDS), defined as the acute onset of noncardiogenic edema and subsequent gas-exchange impairment due to a severe inflammatory process, affects 78.9 cases per 100,000 person-years in the US and is one of the main reasons for ICU admission [1]. Despite recent advances in overall support, it is still associated with high rates of mortality [1, 2], reduced quality of life [3, 4], and increased healthcare costs [5].

Adjunctive nutritional support is a strategy that is currently receiving growing attention in the management of critically ill patients. In particular, it has been suggested that supplementation with ω-3 fatty acids may modulate the inflammatory response in ARDS, although the evidence compiled so far is limited. The aim of this review is, therefore, to discuss recent evidence regarding the role of ω-3 fatty acids-enriched diets in patients with ARDS.

2. Acute Respiratory Distress Syndrome

According to the latest review by a panel of experts from the European Society of Intensive Care Medicine, the Society of Critical Care Medicine, and the American Thoracic Society [6] (Table 1), ARDS is defined as the onset of acute respiratory failure ($P_aO_2/F_IO_2 < 300$ mmHg, with a minimum level of positive end expiratory pressure) with bilateral opacities within a week of a known clinical insult, not fully explained by cardiac failure or fluid overload (using objective assessment tools such as echocardiography to exclude hydrostatic oedema). The clinical insult may be either intra or extrapulmonary. The most frequent causes of ARDS are pneumonia and extrapulmonary severe sepsis [7–9].

Regardless of the primary insult, the lung response has classically been considered as a stereotypical process, characterized by the activation of inflammatory, coagulation, and fibrinolytic systems that lead to lung inflammation

Table 1: Berlin definition of ARDS.

Timing	Within one week of a known clinical insult or new or worsening respiratory symptoms.
Chest imaging	Bilateral opacities not fully explained by effusions, lobar/lung collapse, or nodules.
Origin of oedema	Respiratory failure not fully explained by cardiac failure or fluid overload. Need for objective assessment (e.g., echocardiography) to exclude hydrostatic oedema if no risk factors are present.
Oxygenation	
Mild	200 mmHg < P_aO_2/F_IO_2 ≤ 300 mmHg with PEEP or CPAP ≥ 5 cmH_2O
Moderate	100 mmHg < P_aO_2/F_IO_2 ≤ 200 mmHg with PEEP ≥ 5 cmH_2O
Severe	P_aO_2/F_IO_2 ≤ 100 mmHg with PEEP ≥ 5 cmH_2O

P_aO_2: partial pressure of arterial oxygen; F_IO_2: fraction of inspired oxygen; PEEP: positive end expiratory pressure; CPAP: continuous positive airway pressure.

and the subsequent epithelial-endothelial barrier injury [10]. Inflammatory mediators may also cause loss of the vascular tone leading to vasoconstriction and creating parched areas of lung destruction [11].

The inflammatory cascade involves large numbers of inflammatory cells and mediators, many of which are directly produced in the lung. It is accompanied by a change in bronchoalveolar lavage (BAL) cellularity, due to neutrophils migration [12]. Neutrophil migration and activation may, then, be the main trigger of lung injury. They produce toxic molecules, chemokines (e.g., IL-1, IL-8, NTFa, L-selectin, and CXCL-CXCR complex), adhesion molecules (e.g., ICAM, PECAM) [13, 14], and procoagulant substances [15]. It has been proved that during lung injury there are proinflammatory as well as anti-inflammatory mediators in BAL fluid and serum, suggesting that it may be the balance between them that regulates lung damage and repair [13].

ARDS is, therefore, a heterogeneous syndrome with no specific treatment. In addition to addressing the primary insult and providing support measures, many therapeutic options have been tested in order to improve the clinical outcome in these patients. Until now, protective mechanical ventilation [16] and prone-positioning in the more severe patients [17] are the only strategies that have clearly demonstrated their usefulness for ARDS management. Other treatments such as neuromuscular blockage, vasodilators, anti-inflammatory drugs, extracorporeal support, or high frequency oscillating ventilation have obtained inconclusive results [18–21]. The effect of protective ventilation strategies on mortality seems to be related to the decrease in cytokine response induced by mechanical ventilation, minimizing the degree of ventilation-induced lung injury and subsequent biotrauma [22]. The possibilities of nutritional support have also been investigated in depth: recent studies suggest that lipid emulsions and enteral ω-3 fatty acid supplementation may have an effect on the inflammatory process, not only in ARDS but in other critical conditions as well.

3. ω-3 Polyunsaturated Fatty Acids

Fatty acids are crucial to human life: they are a main source of energy, they have structural functions as part of the cell membrane, and they participate in cell signalling and response [23]. Essential polyunsaturated fatty acids (PUFA) are linoleic acid (LA, PUFA ω-6 series) and linolenic acid (LNA, PUFA ω-3 series), which must be obtained through the diet. Linoleic acid is the endogenous precursor of arachidonic acid (AA), which joins the phospholipids in the cell membrane, while LNA is related to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, direct ingestion of these acids (from fish, shellfish, and algae) seems to be a more efficient source. Oxygenation and lipoxygenase enzymes transform AA into eicosanoids such as prostaglandins, thromboxanes, and leukotrienes, which have a high biological activity and play a major role in the inflammatory response (Figure 1). In contrast, LNA derivatives are much less active. As they both use the same metabolic routes, ω-3 fatty acids compete with AA for the conversion to lipid mediators, balancing the negative effects of ω-6 fatty acids [24] (Figure 2).

The prognostic value of eicosanoids in patients with ARDS was studied by Masclans et al. [25] in a prospective study including 21 patients. Plasma levels of thromboxane B2 (TXB2), prostaglandin F1-alpha (PGF1-alpha), and leukotriene B4 (LTB4) were measured both in peripheral and pulmonary arterial samples and in venous samples within 48 h of ARDS onset. ARDS patients showed significantly higher levels of eicosanoids than a reference group of healthy subjects. However, only LTB4 correlated with lung injury score (in peripheral and pulmonary blood). Nonsurvivors presented a lower systemic-pulmonary arterial gradient of eicosanoid levels than survivors. A more recent study assessed the value of LTB4-levels as clinical markers for predicting pulmonary complications such as ARDS, respiratory failure, pulmonary embolism, or pneumonia in multiply traumatized patients [26]. The results showed that LTB4 levels were significantly higher in patients who presented pulmonary complications, suggesting that this mediator may play a role in their pathogenesis.

The possible prognostic value of leukotriene B(4) in ARDS was again reported by Masclans et al. in a prospective study of 16 ARDS patients admitted to the ICU [27]. These authors found higher plasma concentrations of thromboxane B(2), 6-keto-prostaglandin F(1alpha), and leukotriene B(4) compared to the general population, but only leukotriene B(4) was higher in arterial plasma than in mixed venous plasma. Baseline P_aO_2/F_IO_2 correlated with levels of arterial thromboxane B(2) and arterial leukotriene B(4) and the transpulmonary gradient of leukotriene B(4). A correlation between the transpulmonary gradient of leukotriene B(4)

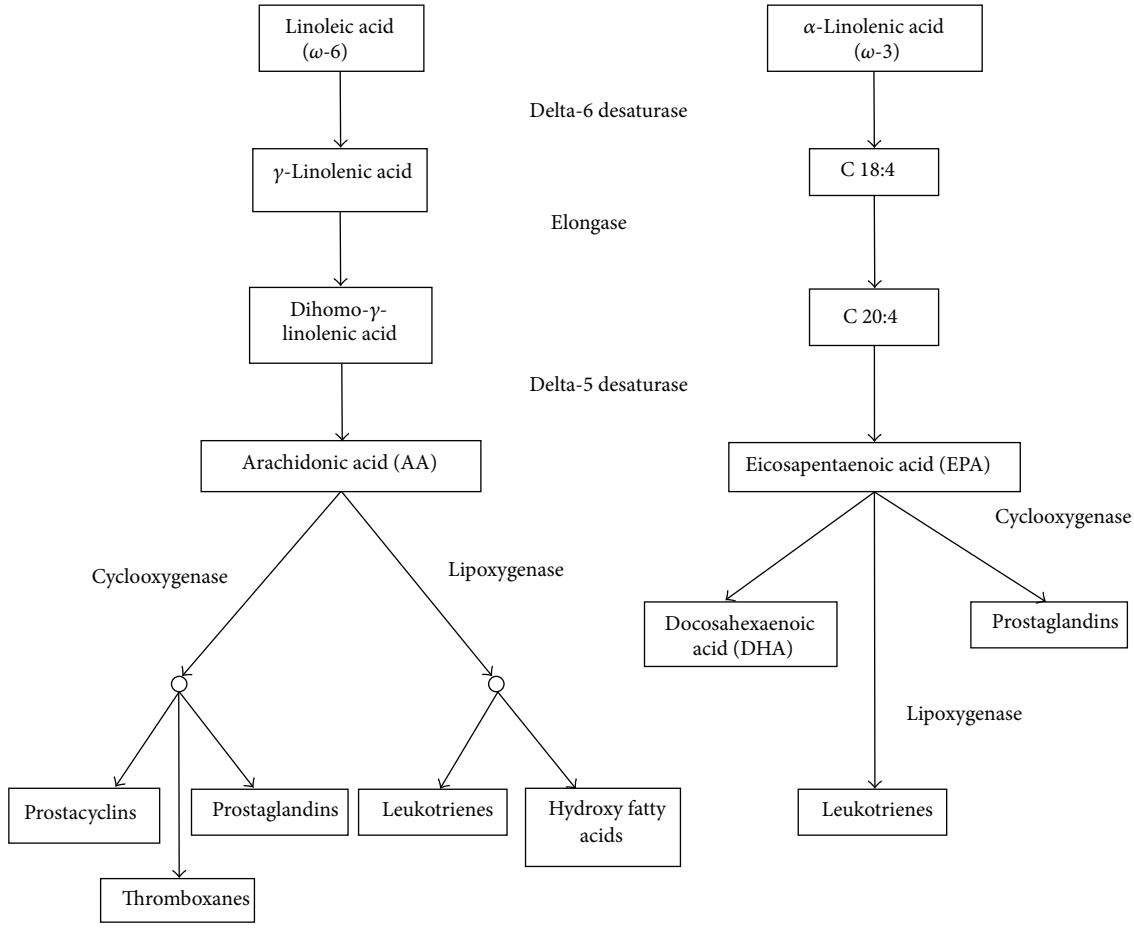

Figure 1: Eicosanoid metabolic pathways.

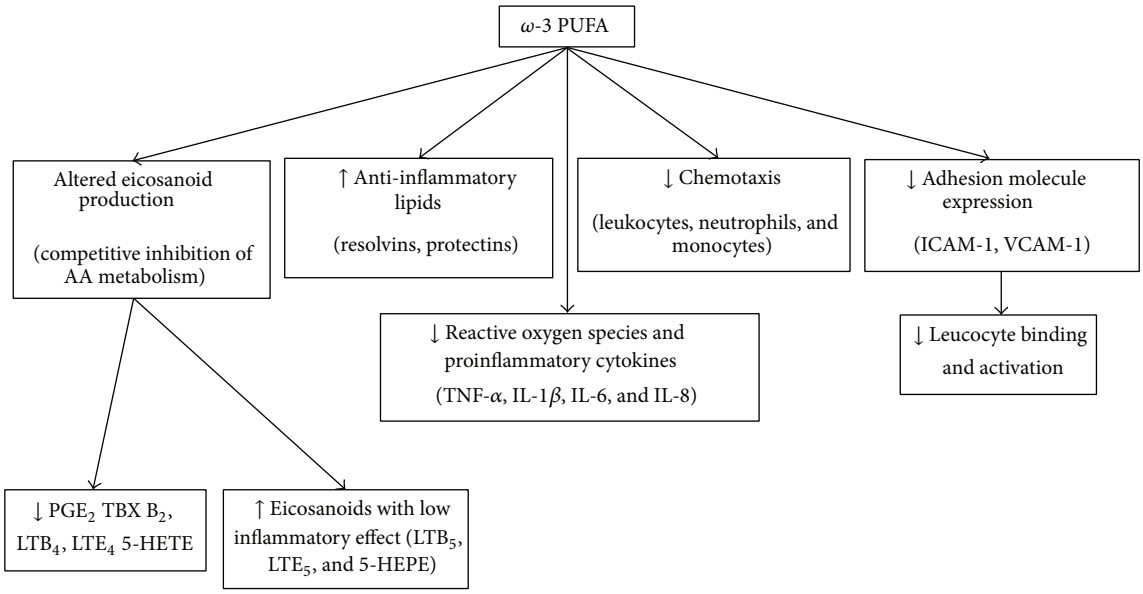

Figure 2: Effects of ω-3 polyunsaturated fatty acids in ARDS.

and the lung injury score was also found. Moreover, among nonsurvivors a substantial positive gradient of leukotriene B(4) was detected. Thus, leukotriene B(4) may correlate with lung injury severity and outcome in patients with ARDS.

These results suggest that modulating the inflammatory response through the use of PUFA may be a useful strategy for ARDS treatment.

Classically, the nutrition of critically ill patients was particularly rich in long-chain triglycerides such as linoleic acid. LA may have a harmful effect on the immune and pulmonary function due to its proinflammatory properties, as it is the precursor of AA and eicosanoids. For this reason, in recent years several strategies have been developed in order to minimize these effects: for example, emulsions with a mixture of long-chain and medium-chain fatty acids, enriched with olive oil or with ω-3 fatty acids [28]. To date, however, the results regarding the administration of mixed emulsions are inconclusive and the use of olive oil in patients with ARDS has not been studied in depth.

As for the most recent ω-3 fatty acids enriched formulas, they may have a beneficial effect on ARDS as they compete with ω-6 PUFA and minimize the synthesis of proinflammatory eicosanoids. They contribute to the modulation of nuclear receptor activation (i.e., NF-kappaB suppression), the suppression of arachidonic acid-cyclooxygenase-derived eicosanoids (primarily prostaglandin E(2)) and the alteration of the plasma membrane microorganization related to the function of Toll-like receptors (TLRs), and immune cell recruitment [29].

4. Recent Findings in the Use of ω-3 PUFA in Patients with ARDS

4.1. Experimental Studies. Several experimental studies have reported the effect of ω-3 PUFA in animal models. In a murine model, Mancuso et al. [30] compared the effects of fish oil, fish and borage oil, and corn oil. Fish oil and fish and borage oil seemed to improve endotoxin-induced acute lung injury by suppressing the levels of proinflammatory eicosanoids in bronchoalveolar lavage fluid and reducing neutrophil accumulation in lungs. Palombo et al. [31] studied the effect of short-term enteral feeding with eicosapentaenoic acid-enriched or eicosapentaenoic with gamma-linolenic acid-enriched diets in rats. These diets seemed to modulate the fatty acid composition of alveolar macrophage phospholipids, minimizing the formation of less inflammatory eicosanoids.

4.2. Human Studies. Research on patients with ARDS dates back to some 15 years (Table 2). Initially, the focus was mainly on the use of a mixture of long-chain and medium-chain fatty acids.

Masclans et al. [32] conducted a randomized trial to evaluate the effect on gas exchange and pulmonary haemodynamics of two different intravenous fat emulsions in 21 patients with ARDS. Patients were randomized to receive a long-chain triglyceride-enriched emulsion (20% LCT), a medium-chain triglyceride/long-chain triglyceride emulsion (20% MCT/LCT: 50/50), or placebo, at a slow rate (2 mg/Kg/min). Increases in cardiac output, oxygen consumption, and oxygen delivery were found during LCT infusion. However, no differences in pulmonary haemodynamics and arterial oxygen tension were detected, suggesting that, at a slow rate, the beneficial effect on cardiac output of LCT infusion offsets the detrimental effect of increased oxygen consumption. No changes were observed in the MCT/LCT group. In this case, the effect of ω-3 was not analysed.

Suchner et al. [33] studied the effect of fat emulsions on pulmonary haemodynamics and gas exchange in patients with ARDS or sepsis. In a prospective crossover study, eight patients with ARDS and 10 patients with sepsis were randomized to receive intravenous fat emulsions (LCT/MCT) over 6 h (rapid fat infusion) or 24 h (slow fat infusion) along with a routine parenteral nutrition regimen. In the ARDS group, patients who received rapid fat infusion presented increased prostaglandin I2/thromboxane A2 (P/T) ratios, higher pulmonary shunt, and a decrease in oxygenation. As for the haemodynamics, patients presented decreased pulmonary and systemic vascular resistances while their cardiac indices increased. Increasing plasma concentrations of TxA2 were associated with improved respiratory performance. All values returned to baseline after 12 h. It was therefore suggested that fat emulsion-derived vasodilatory PGI2 may increase pulmonary shunt and affect gas exchange by increasing pulmonary blood flow and decreasing pulmonary vascular tone. The conclusion is that the speed of infusion also plays an important role.

In contrast, patients with severe sepsis showed reduced pulmonary shunt and increased oxygenation index, while the P/T ratio and haemodynamics remained unchanged.

Faucher et al. [34] conducted a crossover study including 18 patients with ARDS who were randomized to receive either a 6 h infusion of a fat emulsion containing LCT or an infusion of 50% LCT/50% MCT. LCT emulsion showed no effect on oxygenation, whereas MCT/LCT emulsion significantly improved P_aO_2/F_IO_2 by 16% 1 h after initiating the infusion and cardiac output as well. However, the changes were transitory and oxygenation and haemodynamic parameters were similar to baseline at the end of the infusion.

The results regarding the administration of mixed emulsions (LCT/MCT) were not conclusive, probably because the administration of LCT decreases but the ratio ω-6/ω-3 remains unchanged. Subsequent research focused on the use of ω-3 enriched nutrition and obtained promising results. A meta-analysis of three trials including 296 patients published by Pontes-Arruda et al. [35] suggested that enteral supplementation with ω-3 fatty acids and gamma-linolenic acid (GLA) could significantly reduce the risk of mortality, the duration of mechanical ventilation, and ICU stay in patients with ARDS.

In 1999, Gadek et al. [36] studied the effect of enteral feeding with eicosapentaenoic acid, gamma-linolenic acid, and antioxidants in patients with ARDS. One hundred and forty-six patients with ARDS were randomized to receive either eicosapentaenoic acid (EPA) + gamma-linolenic acid (GLA) or isonitrogenous, isocaloric standard diet for 4–7 days. Significant improvements in oxygenation were found in patients fed with EPA + GLA compared with controls. These

Table 2: Omega-3 polyunsaturated fatty acids in ARDS.

Author/year	Design	*N*	Intervention	Main outcomes
Masclans et al., 1998 [32]	RCT, single-centre	21	LCT/MCT (versus LCT versus placebo), 12 hr	↑CO, O_2 consumption and delivery = Pulmonary haemodynamics and arterial O_2 tension
Suchner et al., 2001 [33]	RCT (crossover)	18	Rapid (6 hr) versus slow (24 hr) fat emulsion, LCT/MCT	Rapid: ↑P/T, pulmonary shunt and CO; ↓PVR, SVR, and P_aO_2/F_IO_2
Faucher et al., 2003 [34]	RCT (crossover)	18	LCT/MCT (versus LCT), 6 hr	Transitory ↑P_aO_2/F_IO_2
Gadek et al., 1999 [36]	RCT, multicentre	146	EPA + GLA, 4–7 days	↑P_aO_2/F_IO_2 ↓Days of MV and UCI LOS
Sabater et al., 2008 [37]	RCT, single-centre	16	LCT/MCT/ω-3 (versus LCT), 12 hr	= Oxygenation, haemodynamics
Sabater et al., 2011 [38]	RCT, single-centre	16	LCT/MCT/ω-3 (versus LCT), 12 hr	↓LTB4, TXB2, 6-keto-PG during infusion
Singer et al., 2006 [39]	RCT, single-centre	100	EPA + GLA (versus standard), 14 days	Transitory ↑P_aO_2/F_IO_2 = Mortality
Pontes-Arruda et al., 2011 [40]	RCT, multicentre	115	EPA + GLA (versus standard), 7 days	↓Severe sepsis and SS ↓Cardiac and respiratory failure ↓Days of MV and UCI LOS = 28-day mortality
Rice et al., 2011 [41]	RCT, multicentre	272	EPA + GLA supplementation (versus standard) twice daily	↓MV-free days, ICU-free days, nonpulmonary organ failure-free days Stopped for futility
Stapleton et al., 2011 [42]	RCT, multicentre	90	EPA + DHA (versus placebo), 14 days	↑Serum EPA = IL-8 in BALF, organ failure score, MV-free days, ICU-free days, and 60-day mortality
Grau-Carmona et al., 2011 [43]	RCT, multicentre	132	EPA + GLA (versus standard)	= Oxygenation and organ failures
Elamin et al., 2012 [44]	RCT, multicentre	17	EPA + GLA (versus standard), 7 days	↓LIS, ICU LOS, 28-day multiorgan dysfunction score = Mortality

RCT: randomized controlled trial; LCT: long-chain triglycerides; MCT: medium-chain triglycerides; CO: cardiac output; EPA: eicosapentaenoic acid; GLA: gamma-linolenic acid; P_aO_2/F_IO_2: partial pressure of arterial oxygen/fraction of inspired oxygen; MV: mechanical ventilation; ICU LOS: intensive care unit length of stay; P/T: prostaglandin I2/thromboxane A2 ratio; PVR: pulmonary vascular resistance; SVR: systemic vascular resistance; LTB4: leukotriene B4; TXB2: thromboxane B2; 6-keto-PG: 6-keto-prostaglandin; IL-8: interleukin 8; BALF: bronchoalveolar lavage fluid; LIS: lung injury score.

patients also required significantly fewer days of mechanical ventilation and a shorter length of stay in the intensive care unit.

Sabater et al. studied the effect of ω-3 fatty acid-enriched lipid emulsion on haemodynamics and gas exchange in patients with ARDS. In a randomized, parallel group study [37], 16 patients were included and randomized to receive the control emulsion (100% LCT) or the study emulsion (50% MCT, 40% LCT, 10% ω-3) for 12 h. No differences in gas exchange or haemodynamics were found, except for pulmonary capillary pressure, which was lower in the group with the study emulsion; these results suggested that both emulsions were clinically safe.

In those patients, significant short-term changes in eicosanoid synthesis have been identified [38]. Levels of LTB4, TXB2, and 6-keto-prostaglandin F1-alpha in arterial and mixed venous blood samples increased during infusion in the LCT group and returned to baseline after discontinuation. In the study group, mediator levels decreased during infusion and then behaved erratically. It has been suggested that leukotrienes, particularly LTB4, play a crucial role in lung injury, and their activity may help to modulate the immune response.

Singer et al. [39] reported a beneficial effect of an enteral diet enriched with eicosapentaenoic acid (EPA) and gamma-linolenic acid (GLA) in oxygenation and lung dynamics in 95 patients with ARDS. Those authors found significant differences in P_aO_2/F_IO_2, but this improvement was lost by day 14. Improvements in static compliance and length of mechanical ventilation were also observed, although these differences were not clinically relevant. No changes in mortality were reported.

However, the characteristics of the control diet must also be taken into consideration. In some of these studies (i.e.,

Gadek et al., Singer et al.) the control group received a diet with a high content of fatty acids, particularly linoleic acid, which may have had a deleterious effect. What is more, in addition to ω-3 FA, formulas also included vitamins, antioxidants, and other elements that could have an effect on the inflammatory reaction, making it difficult to establish the real benefit of each single component on its own. These studies also have some other limitations: for instance, no information was provided on other therapeutic strategies such as mechanical ventilation or fluid administration, which we now know that they have a major role in the management of ARDS. The design of later studies attempted to overcome these limitations.

Studying the effect of enteral nutrition with EPA/GLA and antioxidants in patients in early stages of sepsis without associated organ dysfunction, Pontes-Arruda et al. [40] showed a more frequent progression to severe sepsis and septic shock in the control group, particularly due to development of cardiovascular and respiratory failure. Enteral nutrition with EPA/GLA may then play a beneficial role by slowing the progression of severe forms of sepsis. The study group showed shorter length of stay, lower incidence, and shorter duration of mechanical ventilation, but no differences were found in 28-day all-cause mortality.

The OMEGA study was a randomized multicentre trial which lasted one year and included 272 patients with ARDS [41]. Patients received a twice daily supplementation of ω-3 fatty acids, GLA, and antioxidants or an isocaloric control (enteral nutrition was delivered separately). Surprisingly, the study had to be stopped for futility. The enteral diet was reported to be less well tolerated than in previous studies, with a higher incidence of diarrhoea. Patients in the ω-3 group had fewer ventilator-free days, ICU-free days, and nonpulmonary organ failure-free days. The authors therefore concluded that enteral supplementation with ω-3 fatty acids, GLA, and antioxidants did not improve outcomes and might be harmful. In spite of its early termination this study presented certain methodological advantages over previous trials: mechanical ventilation and fluid administration were controlled in both groups, and the control group received a less proinflammatory diet, as well as a high intake of proteins, which may have had a beneficial effect.

In a phase II multicentre, randomized placebo-controlled trial, Stapleton et al. [42] reported that mechanically ventilated patients with acute lung injury receiving enteral fish oil (EPA and DHA) had increased levels of EPA in BALF with no differences in other biomarkers (e.g., IL-8), organ failure score, ventilator-free days, ICU-free days, or 60-day mortality. Those authors suggested that the positive results of previous studies may have been related to the deleterious effect of control diets. Therefore, the beneficial effect could not be attributed to ω-3 PUFA but possibly to other components of the diet or to their combined action.

Grau-Carmona et al. [43] conducted a randomized, open-label study in 11 Spanish intensive care units, including 132 patients with sepsis and established ARDS [31]. Patients were randomized to receive an enteral diet enriched with eicosapentaenoic acid (EPA), gamma-linolenic acid (GLA), and antioxidants or a control diet. No differences in gas exchange or novel organ failures were observed between the two groups.

In contrast, another multicentre randomized trial conducted by Elamin et al. [44] showed an association between an EPA- and GLA-supplemented diet and improvement in gas exchange. Patients in the study group presented reductions in lung injury score, ICU length of stay, and 28-day multiorgan dysfunction score. No differences in survival were detected. However, the sample comprised only 17 patients and so the results should be considered with caution.

In view of the contradictory nature of these results, a systematic review and meta-analysis were recently performed to investigate the beneficial or harmful effect of enteral supplementation of ω-3 fatty acids in patients with ARDS [45]. The meta-analysis of seven trials including 955 patients showed no significant decrease in all-cause 28-day mortality or ICU-free days with the use of enteral ω-3 fatty acids. Obviously, the analysis had its limitations: most of the trials were performed in small samples and used different control formulas. Nevertheless, it was significant that the ω-3 fatty acid-enriched enteral diet was generally well tolerated, with no reports of adverse events.

Nearly all studies considering the effect of ω-3 have been conducted using the enteral route. It should be borne in mind that patients with ARDS may present digestive intolerance, due to deep sedation, neuromuscular blockage, or mechanical ventilation, which may limit the use of enteral nutrition. Little is known about the parenteral route in this particular group of patients. This route is worth exploring, as it offers several potential advantages such as the rapid incorporation of the FA into the cell membrane and the possibility of administering higher doses of these lipids.

5. Conclusion

The effect of ω-3 fatty acid-enriched lipid emulsion or enteral diets on respiratory and cardiovascular variables in patients with ARDS remains unclear. These diets appear to interfere in eicosanoid synthesis, modulating the inflammation response in patients with lung injury. Although enteral ω-3 PUFA-enriched diets have a robust physiopathological basis and some promising results were initially reported in experimental and human studies, recent research has cast doubt on their real impact on patients with ARDS. In fact, scientific societies such as the Canadian Society for Clinical Nutrition are currently lowering the level of evidence of these strategies.

The interpretation of the studies is limited by several factors, principally their heterogeneity and methodology. For example, most of the trials are single-centre studies with small sample sizes and include heterogeneous groups of patients with different aetiologies and degrees of severity. Many of them lack information concerning clinical management, such as mechanical ventilation strategies, and different formulas, route of administration, rate of infusion, and treatment duration have been used. The ideal doses, route, and time of administration are still to be established. In general, the effects on oxygenation are transitory and no clear differences in important clinical outcomes such as mortality have been

reported. However, the use of lipid formulas seems to be safe and well tolerated.

Based on this evidence, the routine use of ω-3 fatty acid-enriched nutrition cannot be recommended. Further large, homogeneous, and high-quality clinical trials need to be conducted to conclusively determine its effectiveness.

Conflict of Interests

There is no conflict of interests to disclose.

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Review Article

Endogenous Generation and Signaling Actions of Omega-3 Fatty Acid Electrophilic Derivatives

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Dietary omega-3 polyunsaturated fatty acids (PUFAs) are beneficial for a number of conditions ranging from cardiovascular disease to chronic airways disorders, neurodegeneration, and cancer. Growing evidence has shown that bioactive oxygenated derivatives are responsible for transducing these salutary effects. Electrophilic oxo-derivatives of omega-3 PUFAs represent a class of oxidized derivatives that can be generated via enzymatic and nonenzymatic pathways. Inflammation and oxidative stress favor the formation of these signaling species to promote the resolution of inflammation within a fine autoregulatory loop. Endogenous generation of electrophilic oxo-derivatives of omega-3 PUFAs has been observed in *in vitro* and *ex vivo* human models and dietary supplementation of omega-3 PUFAs has been reported to increase their formation. Due to the presence of an α,β-unsaturated ketone moiety, these compounds covalently and reversibly react with nucleophilic residues on target proteins triggering the activation of cytoprotective pathways, including the Nrf2 antioxidant response, the heat shock response, and the peroxisome proliferator activated receptor γ (PPARγ) and suppressing the NF-κB proinflammatory pathway. The endogenous nature of electrophilic oxo-derivatives of omega-3 PUFAs combined with their ability to simultaneously activate multiple cytoprotective pathways has made these compounds attractive for the development of new therapies for the treatment of chronic disorders and acute events characterized by inflammation and oxidative stress.

1. Introduction

Dietary intake of omega-3 polyunsaturated fatty acids (PUFAs) has been associated with beneficial effects for human health, leading to a reduced cardiovascular risk both in primary and in secondary prevention, contrasting systemic inflammation and neurodegeneration as well as the development of chronic disorders including cancer and inflammatory airways diseases [1–4]. Upon dietary intake, omega-3 PUFAs are readily incorporated into lipid membranes and modify cellular signaling through multiple mechanisms. Omega-3 fatty acid membrane enrichment occurs mainly at expense of arachidonic acid (AA), resulting in reduced production of AA-derived proinflammatory prostaglandins and leukotrienes [5]. A second mechanism of action is related to the high degree of unsaturation of omega-3 PUFAs which results in altered membrane fluidity and leads to the disruption of lipid raft-related proinflammatory signaling [6, 7]. In addition to these established mechanisms, in the last decade a third mechanism of action has emerged related to the conversion of omega-3 PUFAs into oxygenated bioactive derivatives to promote the resolution of inflammation. Once incorporated into cell membranes, omega-3 PUFAs become available for conversion into bioactive oxidized derivatives. Consistently, dietary intake of omega-3 PUFAs significantly enhances the production of omega-3 PUFA derived oxidized species [8–11]. Formation of bioactive oxygenated derivatives of omega-3 PUFAs occurs via enzymatic and nonenzymatic pathways and uses both free and esterified fatty acids as substrates. In particular, electrophilic oxo-derivatives are generated during oxidative reactions and represent a recently discovered class of bioactive omega-3 PUFAs. These species are released by the cell during oxidative stress and inflammation to exert autocrine and paracrine signaling. Omega-3 PUFAs electrophilic derivatives appear to be the active mediators that transduce the beneficial actions observed

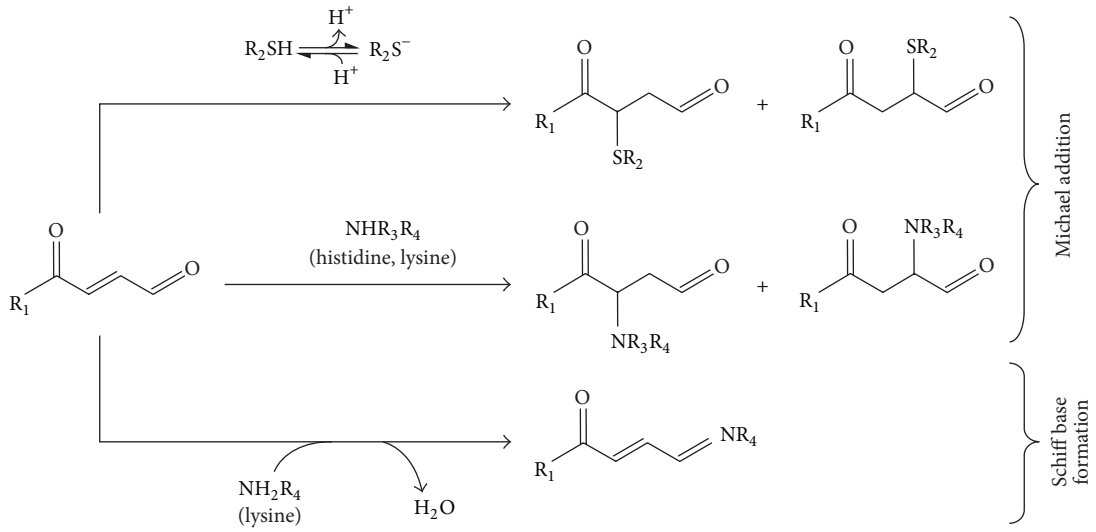

Figure 1: Reaction scheme of electrophilic lipid derivatives. Electrophilic α,β-unsaturated ketone moieties react with nucleophilic residues on target proteins (thiolates of cysteines and amino groups of histidine and lysine) via Michael reaction. In the case of bifunctional electrophiles, the aldehyde group reacts with primary amines of lysine generating Schiff base adducts.

upon dietary administration of omega-3 PUFAs and therefore there has been a growing interest in characterizing their formation and signaling actions in health and disease. While originally viewed as toxic mediators, these compounds have recently been appreciated for their anti-inflammatory role and oxidative stress suppression through the expression of phase II genes. These actions are triggered by the covalent reaction of electrophilic PUFAs with nucleophilic residues on target proteins leading to the activation of several cytoprotective pathways. Due to their endogenous nature and ability to simultaneously activate multiple signaling pathways, these electrophilic compounds have attracted great attention for the development of new drugs for the treatment of diseases characterized by inflammation and oxidative stress [12–18].

2. Chemistry of Electrophilic Lipids

Enzymatic and nonenzymatic oxidation of omega-3 PUFAs generates a broad range of oxygenated species containing electrophilic α,β-unsaturated ketone moieties. The presence of a double bond conjugated to a ketogroup renders the β-carbon electron poor and is therefore susceptible to nucleophilic attack. Reaction of α,β-unsaturated ketones with nucleophiles occurs via Michael addition during which the electron-poor β-carbon accepts the pair of electrons of the electron-rich nucleophile forming a covalent bond (Figure 1). The chemistry governing the reaction between electrophiles and nucleophiles is described by the hard/soft acid-base theory [19] that provides a framework for understanding the reactivity of these species in which soft (polarizable) electrophiles preferentially react with soft nucleophiles while hard (nonpolarizable) electrophiles favorably react with hard nucleophiles. The "electrophilicity index" was later introduced by Parr et al. to better describe the chemical properties of electrophilic species [20]. The electrophilicity index combines softness and chemical potential and can be used to predict the reactivity of an electrophile and to anticipate its biological activity and potential toxicity [21, 22]. For example, several mutagenic compounds present a high electrophilicity index and are hard electrophiles thus reacting more favorably with hard nucleophilic groups found in purine and pyrimidine bases leading to irreversible modification of DNA [23]. In contrast, α,β-unsaturated ketones are soft electrophiles that preferentially react with soft nucleophiles, including cysteine thiols and to a lesser extent primary and secondary amines of lysine and histidine residues, respectively. More specifically, the thiolate anion form of cysteine is the preferred target for α,β-unsaturated ketones [24, 25]. In this regard, the pKa of a cysteine is defined as the pH at which 50% is in an ionized state (deprotonated) and is between 8 and 9 for most biologically relevant thiols, close to the physiological pH range. This means that small changes of cysteine pKa that can be caused by conformational modifications, changes of intracellular distribution, or protein-lipid interaction will significantly affect thiolate levels. This modulation of cysteine reactivity provides a framework for fine-tuning of posttranslational modifications within physiological pH ranges [25]. In addition to cysteine pKa, the reactivity of a given electrophile towards a nucleophilic residue will depend on structural factors including the accessibility of the nucleophilic site and the presence of a microenvironment that stabilizes protein-lipid interaction thus favoring Michael addition. Polar and hydrophobic interactions between the electrophilic fatty acid and exposed amino acids are crucial for the right positioning of the reactive carbon in order for the Michael addition to occur. In this respect, extensive structural investigations on the covalent binding between electrophilic lipids (oxo-fatty acids and nitroalkenes) and Cys-285 within the ligand binding pocket of the peroxisome proliferator-activated receptor γ (PPARγ) provided important mechanistic information

[26–28]. In this particular case, the fatty acid is bound to the receptor so that the carboxylate and the electron-withdrawing groups (either nitro- or keto-) interact with polar residues in the binding pocket while the aliphatic chain is stabilized through hydrophobic interactions [26–28]. Moreover, it has been proposed that polar side chains close to the electrophilic carbon may enhance the electron-withdrawing effect of the ketogroup thus promoting Michael addition reactions [26].

By covalently reacting with multiple target proteins, electrophilic derivatives of long chain PUFAs activate a complex cascade of signaling events. In addition to the rate of Michael reaction, the biological activity of a given electrophile in the cellular environment will depend on multiple factors, including the reversibility of the covalent binding through beta elimination, intracellular concentration, and levels of glutathione (GSH), glutathione S-transferases, and multidrug resistance proteins [24, 29]. Covalent binding of soft electrophiles to cysteine thiols does not provide a static modification as it reverses via beta elimination and participates to inter- and intramolecular electrophile exchange between thiols. Beta elimination reactions occur via nonenzymatic mechanisms and are favored at high pH values, with the ratio of product to substrate being determined by the equilibrium constant. Enzymatic catalysis of beta elimination reactions has been reported for GS-electrophile conjugates and involves the enzyme glutathione-S-transferase (GST) [30, 31]. Alternatively, the binding of an electrophile with a nucleophilic residue can be reversed through exchange reactions that are favored in the presence of high concentrations of low molecular weight acceptor nucleophiles, such as GSH [32–34]. More recently, it has been proposed that the addition of electrophiles to protein thiols can also be reversed via an enzyme thioredoxin 1 (Trx1) catalysed transalkylation reaction in the presence of GSH [35].

While reversible binding of an electrophilic lipid may represent an important signaling mechanism, irreversible binding and high concentration are generally associated with cytotoxicity due to glutathione depletion, protein misfolding, and irreversible modification of enzyme activities [36]. For example, HNE has been historically viewed as a toxic mediator contributing to oxidative damage related to its elevated concentration found under pathological conditions and the formation of stable, irreversible adducts mainly via Schiff base formation with lysine residues (Figure 1) [37].

3. Nonenzymatic Formation of Omega-3 PUFA Electrophilic Derivatives

Nonenzymatic generation of electrophilic derivatives of long chain PUFAs occurs through free-radical-catalyzed lipid peroxidation of both free and esterified fatty acids. Due to their high unsaturation degree, long chain omega-3 PUFAs are highly prone to free-radical-mediated autoxidation generating a wide range of oxidized metabolites including small reactive α,β-unsaturated aldehydes and electrophilic cyclopentenone isoprostanes (IsoPs) and neuroprostanes (NPs). Lipid autoxidation reactions are triggered when bisallylic hydrogen is abstracted by an initiating free radical species, such as hydroxyl or superoxide radicals whose production is enhanced under conditions of oxidative stress. This reaction generates a lipid radical that rapidly reacts with molecular oxygen to form a peroxyl radical that in turn abstracts hydrogen from an adjacent PUFA. This results in the formation of a lipid hydroperoxide and a new radical species that starts the chain reaction. Since molecular oxygen (O_2) is required for this propagation phase, lipid peroxidation proceeds at a higher rate in the hydrophobic environment of cell membranes where oxygen concentrates. The hydroperoxide formed during the propagation phase is highly unstable and can be reduced to an alkoxy radical followed by cleavage of the carbon-carbon bond via β-scission or can undergo a Hock rearrangement leading to lipid cleavage [38]. Chain breakdown results in the release of short-chain α,β-unsaturated aldehydes, including 4-hydroxynonenal and 4-hydroxyhexenal (released from omega-6 and omega-3 PUFAs, resp.) [38, 39]. These short-chain, highly reactive compounds are bifunctional molecules that can undergo both Michael addition and Schiff base formation (Figure 1) and are historically viewed as toxic mediators of oxidative stress.

In addition to generating the highly unstable lipid hydroperoxide, lipid peroxyl radicals can undergo endocyclization followed by further addition of molecular oxygen leading to the formation of prostaglandin-like bicyclic endoperoxide intermediates that are further metabolized to IsoPs. Arachidonic acid-derived E_2 and D_2-IsoPs readily dehydrate in aqueous solution to cyclopentenone-containing electrophilic A_2/J_2-IsoPs [40, 41]. Similarly, autoxidation of EPA and DHA generates electrophilic A_3/J_3-IsoPs and A_4/J_4-NPs, respectively, which covalently react with target proteins promoting anti-inflammatory and cytoprotective actions [13, 17, 42, 43].

4. Enzymatic Generation of α,β-Unsaturated Ketoderivatives of Omega-3 PUFAs

Several enzymatic mechanisms lead to the formation of oxygenated electrophilic derivatives of omega-3 fatty acids. Three enzyme families are mainly responsible for the oxygenation of omega-3 PUFAs, namely, cyclooxygenases (Cox), lipoxygenases (LOs), and cytochromes P450 [10, 11, 44, 45]. By acting alone or in concerted transcellular biosynthetic mechanisms, these enzymes generate epoxy- as well as mono-, di-, and three-hydroxyl species that can be further oxidized to electrophilic α,β-unsaturated keto-derivatives by cellular dehydrogenases. The pattern of oxidized lipids released by a given cell type will depend on substrate availability, enzyme expression and activation state, and overall oxidative status. For example, the expression of Cox-2 is controlled at the transcriptional level and is induced during inflammation [46]. In contrast, 5-LO is constitutively expressed and its activity depends on the translocation to the nuclear membrane, association with 5-LO activating protein (FLAP), and access to substrate [47]. In addition to these mechanisms, the activity of these enzymes is modulated by the lipid peroxide tone which in turn depends on the oxidative status

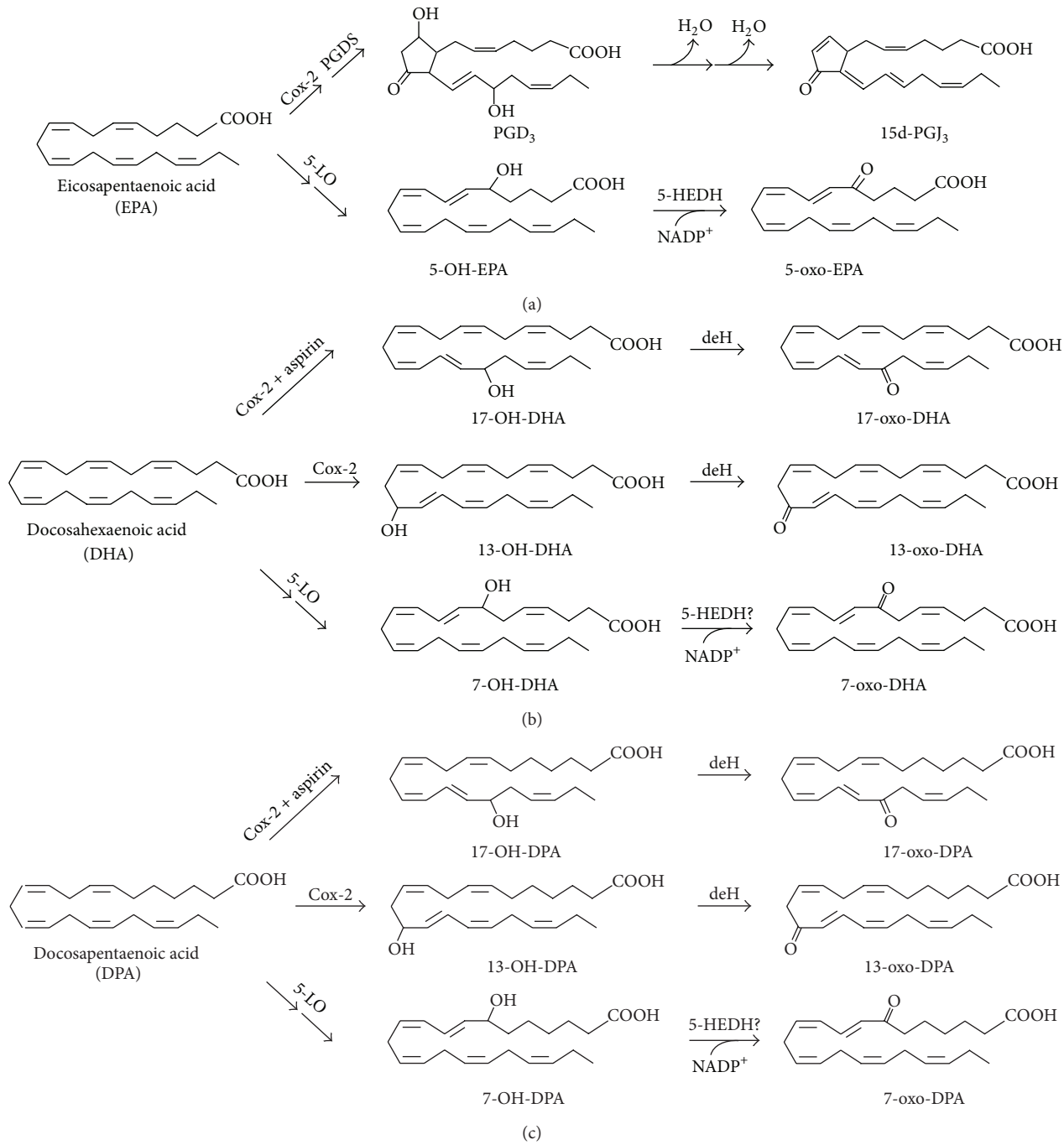

Figure 2: Enzymatic generation of electrophilic ketoderivatives of EPA (a), DHA (b), and DPA (c). Cox-2, cyclooxygenase-2; PGDS, prostaglandin D synthase; 5-LO, 5-lipoxygenase; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; deH, cellular dehydrogenases.

of the cell [48]. Similarly, the activity of dehydrogenase enzymes, including 5-hydroxyeicosanoid dehydrogenase (5-HEDH) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH), depends on the availability of the cofactor NAD(P)+ which increases after exposure of cells to oxidative stress or, in phagocytic cells, during the activation of respiratory burst [49–51].

Endogenous generation of electrophilic α,β-unsaturated derivatives of omega-3 PUFAs has been reported in several cell types. In activated macrophages, 13-oxo-derivatives of DHA and DPA are formed in two enzymatic steps involving Cox-2 and a cellular dehydrogenase. In the presence of aspirin, Cox-2 converts DHA and DPA into 17-OH-derivatives which are then oxidized to 17-oxo-DHA and 17-oxo-DPA (Figure 2) [14]. Primary alveolar epithelial cells (AEC) supplemented with DHA generate the electrophilic 14-oxo-DHA via a 15-PGDH dependent mechanism [52]. These electrophilic compounds display anti-inflammatory

and cytoprotective properties [12, 14]. When using EPA as substrate, Cox-2 catalyses the conversion of this omega-3 PUFA into PGH_3 which is further metabolized to 3-series prostaglandins. In aqueous environment, PGD_3 undergoes two nonenzymatic dehydration steps to give the electrophilic cyclopentenone-containing 15d-PGJ_3 (Figure 2) [15, 16]. In human neutrophils, 5-LO-dependent generation of electrophilic 5-oxo-EPA, 7-oxo-DHA, and 7-oxo-DPA has been reported to be increased upon dietary supplementation with the precursors DHA and EPA (Figure 2) providing evidence that endogenous generation of electrophilic derivatives of omega-3 PUFAs can be effectively modulated through dietary interventions [9, 53].

5. Electrophile-Sensitive Signaling Pathways

Electrophilic derivatives of long chain PUFAs promote cytoprotective and anti-inflammatory actions by covalently and reversibly adducting to target proteins inducing a complex cascade of cytoprotective signaling events. Growing evidence supports that the beneficial actions of dietary omega-3 PUFAs are partly mediated by their electrophilic oxygenated derivatives. The Nrf2-dependent antioxidant response, the heat shock response, the NF-κB inflammatory pathway, and the PPARγ are among the most studied pathways regulated by electrophiles and participate in transducing the beneficial actions of electrophilic omega-3 PUFAs. Recently, growing evidence supports that electrophilic lipids also contribute to epigenetic control of gene expression through direct binding to histones or histone-modulating enzymes and through regulating microRNA expression.

5.1. Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) and Its Inhibitor Kelch-Like ECH-Associated Protein 1 (Keap1). Cells are equipped with highly efficient protective mechanisms to overcome chemical and oxidative insults. These include a large number of detoxification proteins such as phase II enzymes, like NAD(P)H:quinone oxidoreductase 1 (NQO1), the enzymes required for the synthesis and metabolism of glutathione, and the heme oxygenase 1 (HO-1). The expression of these proteins is controlled at their transcriptional level and depends on the presence of a cis-acting promoter element called the antioxidant or electrophile responsive element (ARE/EpRE) which is specifically recognized by the transcriptional factor Nrf2, the master regulator of the inducible antioxidant response [54]. Under basal conditions, Nrf2 binds to its negative regulator, Keap1, an adaptor for the ubiquitin ligase Cul3, which targets Nrf2 to ubiquitination and proteasomal degradation. In response to electrophilic inducers, Keap1-mediated ubiquitination of Nrf2 is inhibited and de novo synthesized Nrf2 protein accumulates in the nucleus [55, 56]. Upon nuclear translocation, Nrf2 forms heterodimers with small Maf proteins and recruits other transcriptional factors required for the activation of ARE elements thus starting its transcriptional program (Figure 3(a)). In human Keap1, Cys-273 and Cys-288 located in the intervening region (IVR) are crucial for basal turnover of Nrf2 [56, 57]. The highly reactive cysteine at position Cys-151, which is 100% conserved between species, appears to be critical for a subset of Nrf2 activators, including the electrophilic sulforaphane and 4-HNE [57, 58]. Cysteine 151 is located in the N-terminal BTB domain of Keap1 which is required for proper interaction with Cul3 [57, 59]. Covalent adduction of electrophiles to Cys-151 inhibits Keap1-mediated ubiquitination of Nrf2 leading to stabilization and nuclear accumulation of newly synthesized protein [56]. The electrophilic Nrf2-inducers 15d-PGJ_2 and nitrofatty acids primarily form adducts with Cys-273 and Cys-288 on Keap1, displaying a much lower reactivity towards Cys-151, suggesting that different patterns of cysteine modification can lead to Keap1 inhibition and Nrf2 activation [60, 61]. Nuclear accumulation of Nrf2 and induction of its target genes have been reported in different experimental models in response to several omega-3 PUFAs derived electrophiles, including the DHA-derivatives 4-HHE and 17-oxo-DHA, the DPA-derivative 17-oxo-DPA, and the EPA-derivatives A_3/J_3-IsoPs [12–14, 62].

Multiple alterations of the Nrf2 pathways have been associated with the development and progression of chronic disorders. For example, a mutation of the gene DJ-1, encoding a positive regulator of Nrf2, leads to development of a monogenic form of Parkinson's disease (PD) [63, 64]. Decline of Nrf2 expression has been reported in the lung of chronic obstructive pulmonary disease (COPD) patients [65] and dysfunction of Nrf2 has been correlated with severe asthma in children [66]. In mice exposed to cigarette smoke, disruption of Nrf2 enhanced the susceptibility to emphysema, increased neutrophils influx to the lung, and decreased the expression and activity of HDAC2 thus enhancing oxidative stress-induced inflammation and contributing to steroid resistance [67, 68]. In murine models of asthma, Nrf2 deficiency has been associated with increased eosinophils infiltration into the lungs and enhanced severity of the asthmatic response due to the reduced expression of the antioxidant genes [69].

Based on the growing evidences supporting the central role of Nrf2 in controlling the oxidative status of the cell and the inflammatory response, there has been a growing interest towards the development of new small molecules activators of Nrf2 as drugs for chronic degenerative disorders [54].

5.2. Heat Shock Response. Heat, oxidative stress, and other cellular insults induce the heat shock response (HSR) that protect the cell from misfolded and aggregated protein damage through the induction of a large family of genes encoding factors involved in protein synthesis, folding, trafficking, and degradation [70]. This response is mainly controlled at the transcriptional level and depends on the activity of a family of heat shock factors among which Hsf1 is essential for the regulation of heat shock proteins (HSPs) expression. Under homeostatic conditions, Hsf1 is an inactive monomer located in the cytoplasm and bound to the chaperones Hsp70 and Hsp90. Under conditions of heat shock and oxidative stress or in presence of electrophilic inducers, Hsp90 and Hsp70 dissociate from Hsf1. Once released, Hsf1 undergoes multistep processing involving phosphorylation, nuclear translocation, trimerization, and binding to the heat shock elements (HSE)

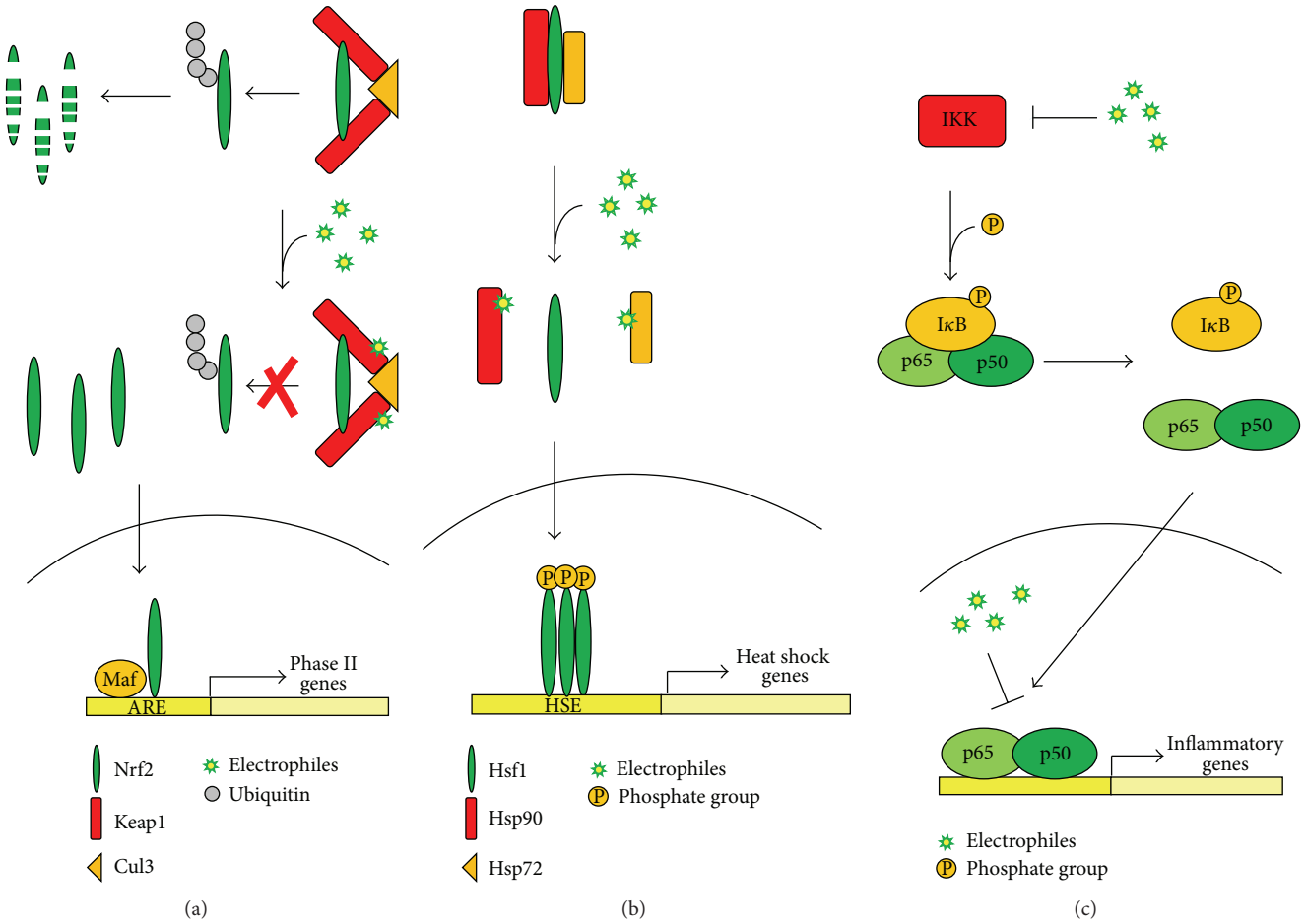

FIGURE 3: Signaling pathways modulated by electrophilic lipids. (a) Nrf2-dependent antioxidant response. Under basal conditions, Keap1 binds to Nrf2, sending it to proteasomal degradation via cullin-3-dependent ubiquitination. Electrophilic lipids react with target cysteines on Keap1 inhibiting ubiquitination of Nrf2 and promoting nuclear accumulation of Nrf2 which leads to the activation of ARE-dependent genes; (b) Heat shock response. Under basal conditions, inert Hsf1 is found in the cytoplasm bound with Hsp72 and Hsp90. Electrophiles react with Hsp72 and Hsp90 and promote Hsf1 release, phosphorylation, trimerization, and translocation to the nucleus where Hsf1 activates the transcription of heat shock response genes. (c) NF-κB pathway. Under basal conditions, IKK phosphorylates IκB, causing the release of the heterodimer p50/p65 which translocates to the nucleus and activates a variety of proinflammatory mediators. Electrophiles react with IKK leading to kinase inhibition and blocking NF-κB activation. In addition, covalent reaction of electrophiles with target cysteines in the DNA-binding domain of p65 and p50 inhibits their binding to DNA.

ultimately leading to transcriptional activation of the heat shock genes [70] (Figure 3(b)).

It has been shown that 4-HNE and the mild electrophile sulphoxythiocarbamate alkyne (STCA) form stable adducts with Hsp90 and Hsp72 (the inducible form of Hsp70) [71, 72]. More specifically, STCA covalently reacts with Cys-412, Cys-564, and Cys-589 (or Cys-590) in human recombinant Hsp90, most likely impairing its chaperone activity, leading to Hsf1 release and HSR activation [72]. In a rat model of ethanol-induced oxidative stress, covalent reaction of 4-HNE with the Cys-267 in the ATPase domain of Hsp72 and with Hsp90 Cys-572 has been reported leading to a reduced chaperone activity [73, 74]. Although the complete pattern of adduction by electrophilic lipids has not been clearly identified and the molecular mechanisms of action still remain to be defined, induction of the Hsf1-dependent HSR has been reported for several electrophilic derivatives of long chain PUFAs, including nitro-fatty acids (nitro-FAs) and 15d-PGJ_2 [75–77].

Growing evidence indicates that electrophilic inducers of the Nrf2 pathway are also activators of the heat shock response through covalent binding with Hsp90 and Hsp72 and activation of Hsf1 [72, 78–80]. This supports a model in which the two most prominent cellular cytoprotective pathways, namely, the Nrf2-dependent antioxidant response and the HSR, can be induced through similar pharmacological mechanisms within a common regulatory network.

Since the induction of the HSR plays a central role in protecting the cell from external insults and protein damage, the sensitivity of this pathway to activation by electrophilic species further supports that some of the beneficial actions that have been associated with this class of compounds rely on the activation of this specific pathway.

5.3. Nuclear Factor-Kappa B (NF-κB) Proinflammatory Pathway. Inhibition of the NF-κB proinflammatory pathway is one of the best-defined mechanisms through which PUFAs electrophilic derivatives promote anti-inflammatory and cytoprotective actions. The NF-κB signaling pathway controls the onset of innate and adaptive immune response by activating the expression of cytokines, adhesion molecules, proinflammatory enzymes, and transmembrane receptors in response to several stimuli. The activity of this transcriptional factor is controlled at multiple levels and electrophilic PUFAs have been reported to interfere with most of them.

In unstimulated conditions, NF-κB resides in the cytoplasm, mostly as a heterodimer composed of p65 and p50 and bound to the inhibitor IκB. In response to proinflammatory stimuli, the complex IκB kinase (IKK) becomes activated by phosphorylation and in turn phosphorylates IκB, sending it to ubiquitination and proteasomal degradation. This results in the release and nuclear translocation of NF-κB, leading to transcriptional activation of its target genes [81] (Figure 3(c)).

A well-described inhibition mechanism of NF-κB by electrophiles occurs through covalent binding to the highly conserved cysteines at position Cys-38 of p65 and Cys-62 of p50, within their DNA-binding domain. More specifically, 15d-PGJ_2 and nitro-FAs covalently react with these residues leading to the loss of DNA binding activity [82–85]. The electrophilic DHA and DPA derivatives, 17-oxo-DHA and 17-oxo-DPA, also suppress p65 DNA binding activity, although the precise mechanism of action has not been established yet [14]. Inhibition of the IKK kinase by alkylation of Cys-179, located in the activation loop of IKKβ, represents an alternative mechanism through which electrophiles suppress the NF-κB pathway. This modification, which has been demonstrated for the DHA-derived cyclopentenone-NPs and for 15d-PGJ_2, results in the suppression of kinase activity, IκB stabilization, and consequent NF-κB inhibition [17, 84, 86]. More recently, an additional mechanism has been reported through which electrophilic nitro-FAs can suppress the activation of this proinflammatory pathway, that is, by interfering with the recruitment into lipid rafts of the signaling mediators required for triggering the NF-κB pathway [87]. Since alterations of lipid-raft-related proinflammatory signaling have been identified as a mechanism through which omega-3 PUFAs exert anti-inflammatory actions, bioactive electrophilic derivatives provide an alternative mechanism to the reported changes in membrane composition and fluidity [6, 7].

Persistent activation of the NF-κB pathway represents a common feature of virtually all chronic diseases, including neurodegenerative disorders, asthma, and COPD. Increased nuclear accumulation of NF-κB has been measured in the Parkinsonian brain as well as in neurons and peripheral blood mononuclear cells (PBMCs) of Alzheimer's patients, and the neurotoxic amyloid-beta (Aβ) peptide is a strong inducer of the NF-κB [88–90]. Dysregulation of this pathway has been reported in asthmatic patients and in lungs of COPD subjects, where cigarette smoke contributes to persistent activation of the NF-κB [91–93]. In these cases, and for most of the chronic inflammatory disorders, targeting the NF-κB through pharmacological approaches appears to be a promising therapeutic strategy. In this regard, electrophilic lipids represent a class of compounds with a great pharmacological potential.

5.4. Peroxisome Proliferator-Activated Receptor γ (PPARγ). The PPARγ is a member of the nuclear hormone receptor superfamily of transcription factors that is highly expressed in adipose tissue, macrophages, and dendritic cells (DCs) [94]. Upon ligand binding, the PPARγ forms a heterodimer with the retinoid X receptors (RXRs), binds to PPARγ response element (PPRE), and recruits transcriptional coregulators that control the expression of genes involved in adipogenesis, glucose metabolism, and macrophage and DCs function [95]. Its association with coregulatory proteins occurs through interactions with the surface of the ligand binding domain (LBD) and is controlled by the conformational changes induced by ligands ultimately modulating gene expression [95]. The PPARγ LBD is a hydrophobic pocket that can accommodate a wide range of lipophilic ligands, including long chain PUFAs and oxidized fatty acids. The presence of a reactive cysteine within the LBD (Cys-285) confers a special sensitivity for electrophilic lipids, which are best described as partial agonists and are able to covalently bind to PPARγ [26]. This provides evidence for the particular activation by electrophiles, resulting in activation at lower concentrations, and for prolonged periods of time when compared to non-electrophilic PUFAs [26]. Covalent addition to Cys-285 and activation of the PPARγ have been reported for nitro-FAs, oxo-ETEs, 15d-PGJ_2 and for the electrophilic omega-3 PUFA derivatives 4-oxo-DHA, 17-oxo-DHA, 17-oxo-DPA, and 15d-PGJ_3 [14, 16, 26, 28, 96, 97]. The activation of the PPARγ produces a cascade of events that differ based on the cell type and condition ranging from antidiabetic to neuroprotective, anti-inflammatory, and cardioprotective actions [96, 98–103]. For example, in murine model of diabetes, activation of PPARγ by nitro-FAs restored insulin sensitivity and blood glucose levels [96] and in experimental models of COPD, treatment with PPARγ agonists contrasted cigarette smoke-induced inflammation and downregulation of HDAC2 [93, 102, 103]. Overall, there is increasing evidence that activating the PPARγ promotes beneficial effects in several pathological conditions. As potent activators of this transcriptional factor, electrophilic PUFAs enhance PPARγ-dependent signaling which becomes part of the complex salutary cascade of events triggered by these lipid derivatives. However, because of the complex network of signaling pathways that are activated in response to electrophilic PUFAs, it is still a challenge to define to what extent the PPARγ is responsible for the observed effects.

5.5. Epigenetic Modulation by Electrophiles. Epigenetic control of gene expression involves DNA, RNA, and protein modification as it occurs during DNA methylation, covalent modification of histones, and posttranscriptional regulation of gene expression by noncoding microRNAs (miRNAs) [104, 105]. Growing evidence supports that electrophilic lipids participate in epigenetic mechanisms at multiple levels, that is, by directly adducting histones, by regulating the activity

of histone-modifying and DNA methylating enzymes, and by controlling miRNA expression.

Histones are lysine- and histidine-rich proteins that are required for the control of chromatin structure. A recent study has shown that, under physiological conditions, the electrophilic 4-ONE covalently adds to histones H2A (His-123), H2B (His-82, His-109, Lys-116), H3 (Lys-23, Lys-27), and H4 (Lys-79) [106]. Interestingly, modifications of H3 Lys-23 and Lys-27 (known sites of acetylation and methylation) interfered with the process of nucleosome assembly. These findings support that electrophilic adduction to histones is a mechanism through which these reactive species control gene expression under physiological and pathological conditions.

While limited reports are available on histone adduction by electrophiles, more data exist on electrophilic modification of histone-modifying enzymes, including histone deacetylases (HDACs) and acetyl transferases (HATs) [107–111]. In this regard, electrophilic lipids containing an α,β-unsaturated carbonyl moiety covalently bind to two highly conserved cysteines that are present in class I histone deacetylases HDAC1, HDAC2 and HDAC3, namely, Cys-261 and Cys-273 in HDAC1 [108, 109]. These modifications disrupt the interaction of histones with their substrate and reduce their enzymatic activity. Similarly, 15d-PGJ_2 was shown to inhibit the activity of the histone deacetylase Sirt1 due to its electrophilic carbon [107]. The p300 HAT is also a target for electrophilic addition by 15d-PGJ_2. More specifically, it has been shown that 15d-PGJ_2 undergoes Michael addition with the catalytic cysteine at position 1438, within the substrate binding site of p300, leading to inhibition of its enzymatic activity [111]. The enzyme DNA methyltransferase 1 (DNMT1) possesses a reactive catalytic cysteine at position 1226 that covalently reacts with soft electrophiles with Michael addition. This reaction was characterized for curcumin, a dietary electrophile with protective anti-inflammatory actions, and leads to inhibition of enzyme activity resulting in DNA hypomethylation [112]. Finally, it has been reported that electrophiles can modulate the expression of several miRNAs thus providing an additional mechanism through which these reactive species participate in controlling gene expression via epigenetic mechanisms [113, 114].

Several data support that omega-3 PUFAs contribute to epigenetic control of gene expression. Dietary supplementation of omega-3 PUFAs has been correlated with reduced histone acetylation levels, changes in histone methylation/phosphorylation status, and modification of global DNA methylation [115–118]. Also, it has been reported that the oxygenated derivative of DHA, Resolvin D1, is able to modulate the expression of specific miRNAs [119]. It remains to be established whether these effects are mediated by electrophilic derivatives of omega-3 PUFAs.

6. Therapeutic Potential of Electrophilic Derivatives of Omega-3 PUFAs

In recent years, a growing number of electrophilic drugs have entered clinical development. The interest towards this class of compounds for drug development relies on their ability to simultaneously activate multiple antioxidant and cytoprotective pathways that are involved in the pathophysiology of several diseases where inflammation and oxidative stress play a central role. The therapeutic potential of the naturally occurring electrophile sulforaphane has been investigated in several clinical trials for diseases ranging from cancer to diabetes and COPD [120–123]. Very recently, the Food and Drug Administration (FDA) has approved the use of the electrophilic dimethyl fumarate for the treatment of relapsing forms of multiple sclerosis [124]. Until now, the activity of electrophilic derivatives of long chain PUFAs such as nitro-FAs and cyclopentenone prostaglandins has been assessed in preclinical models, including *in vitro* and *ex vivo* studies and animal models of disease. In murine models, nitro-FAs displayed antidiabetic actions, reduced vascular inflammation, attenuated hypoxia-induced pulmonary hypertension, and reduced the severity of allergic airways disease being more effective than fluticasone propionate in contrasting neutrophilic inflammation [87, 96, 125, 126].

Increasing evidence shows that oxygenated derivatives of omega-3 PUFAs transduce the beneficial effects that have been associated with DHA and EPA dietary intake. Several reports have demonstrated the protective effects of mono-, di-, and trihydroxyl derivatives of DHA and EPA in murine models of disease [127, 128]. However, preclinical data on the therapeutic actions of omega-3 PUFAs electrophilic oxo-derivatives remain very limited. In murine models of leukemia, the EPA metabolite 15d-PGJ_3 selectively targeted leukemia stem cells (LSCs) for apoptosis in the spleen and bone marrow, displaying superior performance compared to available chemotherapeutic approaches [15]. Furthermore, the anti-inflammatory and cytoprotective actions of 13- and 17-oxo-DHA, A_4/J_4-NPs and 15d-PGJ_3 have been demonstrated in several *in vitro* models of disorders ranging from neurodegeneration to airways inflammatory diseases [12, 14, 16, 17].

The use of endogenous omega-3 PUFAs electrophilic derivatives as drugs would offer several advantages including the possibility to increase their concentration via two routes, that is, by oral supplementation of the fatty acid precursor and by direct administration of the electrophilic compound [8–11]. In the specific case of airways inflammatory disorders, a further advantage is correlated to the possibility of using these compounds for inhalation therapy as recently reported for Resolvin D1 [127]. In this regard, recently published data suggest that, in presence of oxidative stress, as it occurs in the lung of COPD patients, hydroxyl derivatives of DHA, including Resolvin D1, may be further oxidized to electrophilic ketoderivatives which could be the final mediators of the observed beneficial actions [12]. Regarding the possibility of enhancing the formation of electrophilic derivatives of omega-3 PUFAs through dietary administration of their fatty acid precursors, therapeutic doses should be carefully evaluated. Recent reports suggest that high intake of omega-3 PUFAs may be not without risk. In fact, high doses of omega-3 PUFAs may dampen the immune system altering pathogen clearance or interfere with tumor surveillance mechanisms thus leading to adverse outcomes [129, 130].

Finally, when considering direct administration of electrophilic drugs, important factors should be taken into consideration, which are related to the typical reactivity of these compounds. In fact, electrophiles covalently react with target cysteines and their signaling can accumulate over time. To determine bioavailability and pharmacokinetics of these compounds, classical pharmacological methods are not applicable calling for the development of new approaches. Furthermore, high doses of electrophilic inducers of Nrf2 may promote cancer cell proliferation and chemoresistance in the long run [131]. To better evaluate this and other potential toxic effects, well-designed long-term clinical trials should be conducted.

Overall, growing evidence supports that electrophilic oxo-derivatives of omega-3 PUFAs promote the beneficial effects that are observed upon dietary supplementation of these fatty acids. However, research aiming at translating these findings into new therapeutic applications is still at the beginning and preclinical and clinical studies should be conducted to assess the potential of these compounds as drugs for the treatment of inflammatory disorders.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

N-3 Polyunsaturated Fatty Acids and Inflammation in Obesity: Local Effect and Systemic Benefit

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Overwhelming consensus emerges among countless evidences that obesity is characterized by a chronic low-grade inflammation in the adipose tissue (AT), which subsequently develops into a systemic inflammatory state contributing to obesity-associated diseases. N-3 Polyunsaturated fatty acids (n-3 PUFA), known as important modulators participating in inflammatory process, turn out to be an effective mitigating strategy dealing with local and systemic inflammation observed in obesity. Some of the effects of n-3 PUFA are brought about by regulation of gene expression through interacting with nuclear receptors and transcription factors; other effects are elicited by modulation of the amount and type of mediator derived from PUFAs. The metabolic effects of n-3 PUFA mainly result from their interactions with several organ systems, not limited to AT. Notably, the attenuation of inflammation in hard-hit AT, in turn, contributes to reducing circulating concentrations of proinflammatory cytokines and detrimental metabolic derivatives, which is beneficial for the function of other involved organs. The present review highlights a bridging mechanism between n-3 PUFA-mediated inflammation relief in AT and systemic benefits.

1. Introduction

There are mounting evidences demonstrating that obesity and associated disorders pose a daunting threat on global public health problem, given their morbidity and mortality. Obesity is associated with a chronic low-grade inflammatory response characterized by abnormal cytokine production, increased acute phase reactants, and activation of inflammatory signaling pathways [1, 2]. It is specifically the excessive accumulation of body fat in AT that initiates local inflammation and later systemic response [3]. AT is more like an important endocrine and immune organ, secreting a considerable variety of adipokines, including inflammatory mediators [3, 4]. Increased circulating concentration of these cytokines is triggered by obesity and proven associated with pathogenesis of metabolic syndrome [1, 5]. That partly explains why obese individual is vulnerable to serious complications, ranging from diabetes to cardiovascular disease. AT inflammation is never considered as a mere local reaction. Instead, locally inflamed AT exerts profound influences on other organs and systems.

The anti-inflammatory properties of n-3 PUFA are not novel concepts, and the benefits of dietary supplementation with n-3 PUFA are well documented in several inflammatory and autoimmune diseases, including obesity [6, 7]. Under the condition of obesity, catabasis of inflammation manifestation mediated by n-3 PUFA is never restricted to AT. In parallel, it indeed benefits the improvement of other metabolic sites. According to our observation, even during normal physiological processes, n-3 PUFA suppresses the expression of proinflammatory cytokines and decreases the circulatory TNF-α concentration [8]. It is conceivable that the anti-inflammatory potential of n-3 PUFA might be stretched under the inflammatory status. However, it seems reasonable to suppose that n-3 PUFA may mediate some of its beneficial effects on whole-body homeostasis by resetting the function of AT. Since the peroxisome proliferator-activated receptor-gamma (PPARγ) functions as an effector of adipocyte-derived T helper 2 (Th2) cytokines [9] whose activation is required for the circulating monocytes differentiation into M2 macrophages and anti-inflammatory gene transcription, as discussed later in this review, typically, PPARγ might

function as communicator integrating inflammation relief in AT into systemic benefits. The emphasis of present review is upon benefits from n-3 PUFA supplementation in models of chronic inflammatory conditions companied with obesity. On a global level, the potential adipocentric beneficial effects are expended into systemic effects. Given its desirable anti-inflammatory effect, dietary manipulation with n-3 PUFA may therefore offer a logical strategy for preventing or treating obesity and obesity-induced complications.

2. Adipose Tissue Inflammation in Obesity

Interestingly, inflammation emerging in the context of obesity seems to be triggered and to reside predominantly, in the AT [10], although other organs actively involved in metabolism also inevitably suffer from a chronic low-grade inflammation during the development of the diseases. More than a notable characteristic of obesity, AT inflammation is considered as causative factors connecting obesity with its metabolic complications [3, 11–13].

In addition to matrix of extracellular proteins, adipocytes are surrounded by a wide variety of cells, including endothelium, fibroblasts, preadipocytes, and immune cells [14]. In contrast to previous understanding, adipocytes are increasingly recognized as an integrator of various physiological pathways rather than a passive energy storage depot with a droplet which is completely out of the proportion [15]. This recognition begins with the observation that adipocyte is a significant source of endogenous tumor necrosis factorα (TNF-α) at which secretion is substantially stimulated by obesity [10, 16]. Adipocytes are equipped with the ability to secrete a large number of adipokines (interleukin-6 (IL-6), interleukin-1 (IL-1), chemokine monocyte chemotactic protein, leptin, adiponectin, resistin, etc.) [15]. Following adipocytes, resident immune cells constitute the second largest AT cellular component [3]. Obesity-induced infiltration of immune cells into AT leads to increased synthesis and subsequent secretion of proinflammatory factors into circulation [17].

Obesity is featured by progressive infiltration of immune cells into AT. Adipocytes are studied with increasing intensity, while some pursuit concentrates on the possibility that the cellular source of these inflammatory changes derives not only from adipocytes. Actually, since the majority of cytokines produced in obese AT are adipose tissue macrophage (ATM) derived, it is speculated that recruitment and proinflammatory activation of ATM are required for the development of obesity-associated complication [18, 19]. Histologically, there is evidence of significant infiltration of macrophages into white adipose tissue (WAT) of obese mice, which well supports earlier microarray analysis demonstrating that gene product expressed in macrophage is markedly increased in obese (*ob/ob*) AT [20–22] and unequivocally explains previous discoveries that macrophage accumulation is positively correlated with body mass index (BMI) and adipocyte size [14, 21]. With the deterioration of obesity, macrophages recruit in the AT, sometimes contributing as much as half of the cellularity [21]. The state of chronic low-grade inflammation in WAT is powerfully augmented through the infiltration of macrophages [23]. Instead, fat reduction leads to a dramatic decrease in number and modified distribution of macrophages together with decreased expression of inflammatory markers [22]. Not all ATM are programmed for proinflammation, since even, for lean mice, moderate quantity of ATM is present in the context of low or undetectable inflammatory signals [21]. Diet-induced obesity leads to a shift in the activation state of ATM from an M2-polarized state in lean animals to a proinflammatory M1 polarization state [24]. The mechanism responsible for this phenotypic switch of macrophage polarization remains unclear. It is speculated that dietary n-3 PUFA servers as the monitor switch, which will be discussed in following context. It is worthwhile to mention that macrophage, overwhelmingly localized to necrotic adipocytes, is the predominant or significant source of proinflammatory adipokines in WAT [25]. Adipocyte death is one of the putative mechanisms explaining the initiation of macrophage infiltration into adipose [26].

In addition to macrophages infiltration, adipocyte hypertrophy and hyperplasia are followed by enhanced angiogenesis and extracellular matrix (ECM) overproduction. The protein composition and dynamics of ECM are critical for physiologic role of the AT [27]. Additionally, an emerging view is that adipocytes hypertrophy sets obstacle for sufficient oxygen supply to the cells, which creates a state of hypoxia followed by apoptosis of some cellular components [28, 29], once physical limit to adipocyte growth hardly copes with increased volume. Virtually, if disproportionate accumulation of ECM does not allow an adequate expansion of adipocytes, adipocytes are more susceptible to necrosis [30]. Presumably, as a process pathologically accelerated in obesity, unhealthy AT remodeling, not AT expansion, may be the root of the attraction of macrophages and final inflammation, at least necrosis adipocytes mainly caused by nutrition and oxygen deprivation are unignorable stimulus that drives immune cells infiltration as discussed before. More important, in spite of an enlarged fat mass, well controlled healthy AT expansions do not exhibit pathological changes and metabolic disorders [26].

Another marked feature of obesity is the dramatic upregulation of Inflammation and macrophage-specific genes in AT [1]. Earlier microarray performed in AT from wild-type and *ob/ob* mice [20] and subsequent research concerning the treatment with PPARγ ligand in Zucker diabetic fatty (ZDF) rats [31] both disclose the notable regulation of inflammatory genes in AT during obesity development. In addition, extensive transcriptional profiling studies using multiple tissues taken from mice with genetic or diet-induced obesity indicate the majority of gene regulated in obesity comprises macrophage and inflammatory genes [1]. Similar conclusion is observed in the WAT of mice suffering from varying degrees of obesity [21].

Although macrophage invasion into visceral adipose tissue (VAT) is regarded as a dominant driving force leading to AT inflammation, there has also been a resurgence of interest into a unique population of VAT-resident regulatory T (T_{reg}) cells characterized by the expression of forkhead box

P3 (Foxp3). T_{reg} cells-mediated suppression turns out to be an effective defense against aberrant or excessive immune responses [32]. T_{reg} cells are abundant in the VAT of normal but not obese individuals, suggesting that they are engaged in control of the inflammatory state of AT [33]. What is more, they have a different T cell receptor repertoire compared with T_{reg} cells residing in other tissues, which also indicates their novelty. Interestingly, VAT T_{reg} cells reduce strikingly and specifically in insulin-resistant models of obesity. Conversely, their expansion improves insulin sensitivity [33]. Loss-of-function and gain-of-function experiments further confirm their influence on the surrounding adipocytes, inflammatory state of AT, and insulin sensitivity, coupled with differentially synthesized cytokines [33]. It is likely that the shortage of VAT-resident T_{reg} cells offers the answer why obesity-associated inflammation can escape the powerful armamentarium of cells and molecular ready for curbing a runaway immune response.

Exposed to obesity-susceptive environment, complex events take place in AT in a defined order with a number of critical cell types participating in this process, as shown in Figure 1.

3. Systemic Consequences in Response to Local Inflammation

Elevated TNF-α expression in AT from rodent models of obesity was first proposed in 1993 [16]. Subsequently, the novel function of TNF-α is verified in different rodent obesity models as well as in obese humans [10, 34–36]. The absence of TNF-α results in significantly improved insulin sensitivity in both diet-induced and *ob/ob* model of obesity [37]. In sharp contrast to elevated TNF-α expression in AT, circulating TNF-α concentrations in obesity are found unchanged or disproportionately increased [16, 38, 39]. Demonstration of elevated expression of transmembrane forms of TNF-α in obesity well confirms the spatial restriction of TNF-α, suggesting the action of this cytokine is restricted to AT because of TNF-α retention on the cell surface [36]. Attempts to reverse insulin resistance with an injection of anti-TNF binding proteins finally fail [40, 41], indirectly supporting the speculation that TNF-α functions locally at AT via a paracrine or autocrine fashion. However, it would be premature to conclude that AT inflammation is an isolated system.

TNF-α is able to amplify inflammatory response via activating other cytokine networks and proinflammatory pathways [42]. Apart from TNF-α, there is a significant graded increase of proinflammatory cytokines, such as IL-6 as well as acute phase markers, which work in a coordinated manner to impact whole-body hemostasis. More direct and detailed discussions concerning the linkage between these cytokines and inflammation associated diseases are reported before [42]. Studies concerning the origins of the accumulated triacylglycerol (TAG) in the liver during the development of nonalcohol fatty liver disease clarify that the primary contributor to hepatic TAG is serum nonesterified fatty acid (NEFA) pool, most of which derived from AT fatty acid flux [43]. Enhanced release of fatty acid from hypertrophic fat cells results in lipotoxicity caused by accumulation of lipid in nonadipose tissues, contributing to systemic insulin resistance [44]. However, a recent discovery, contrary to previous demonstration, not only challenges former opinion by confirming downregulated rates of NEFA delivery from AT, but also demonstrates that the implicit reduction in AT fatty acid uptake goes beyond the downregulation of systemic NEFA release from AT in obesity [45]. No matter what mechanism dominates the excess fat deposition in non-adipose tissue, accretive fatty acid release originated from AT or impaired AT storage of ingested fat, the dysfunction, and destruction necessarily associated with ectopic fat depots cannot be too strongly emphasized. Ectopic fat depots with predominantly systemic effects include VAT, intrahepatic fat, and intramuscular fat, whereas pericardial (or the related epicardial or pericoronary fat), renal sinus fat, myocardial steatosis, and perivascular fat are postulated to have potential local effects [46].

By analogy to proinflammatory mediators and free fatty acid (FFA), several adipokines, especially leptin and adiponectin protecting peripheral tissues against the lipotoxic damage by promoting oxidation of fatty acids and sensitizing insulin actions, are also an integral part of AT-derived factors which bridge the physiology of AT and function of non-AT tissues [44]. Unfortunately, obesity is always accompanied with increased leptin and decreased adiponectin in serum [47], negatively associated with desirable metabolic parameters.

A direct relationship between ATM with other metabolic or nonmetabolic disarrangements suggests that ATM plays integrated role that goes beyond local AT inflammation [48], with emphasizing on the effects of polarization state of ATM on systemic inflammation and insulin action [49, 50]. The mechanism underlying this process remains deeper investigation. According to achieved evidences, it is clear that vastly increased production of proinflammatory mediators derived from M1 macrophages results in their entry into the circulation to cause dysfunction of actively metabolized tissue.

In conclusion, the label of endocrine organ reflects the systemic effects of AT. Excessive accumulation of surplus body fat lies at the core of all these problems and initiates the release of a number of proinflammatory cytokines from adipocytes as well as tissue-resident macrophages, followed by a rapid recruitment of monocytes from circulation to the AT. Infiltrating cells deteriorate the state of inflammation by representing an additional source of proinflammatory cytokines, leading to disruption of normal homeostatic control of metabolism locally and systemically via endocrine or paracrine effects. In addition to dysregulation of secretory functions, impairment of storage function results in excessive release of free fatty acids and ectopic deposition in non-adipocyte cells, which dramatically worsens the situation [51, 52].

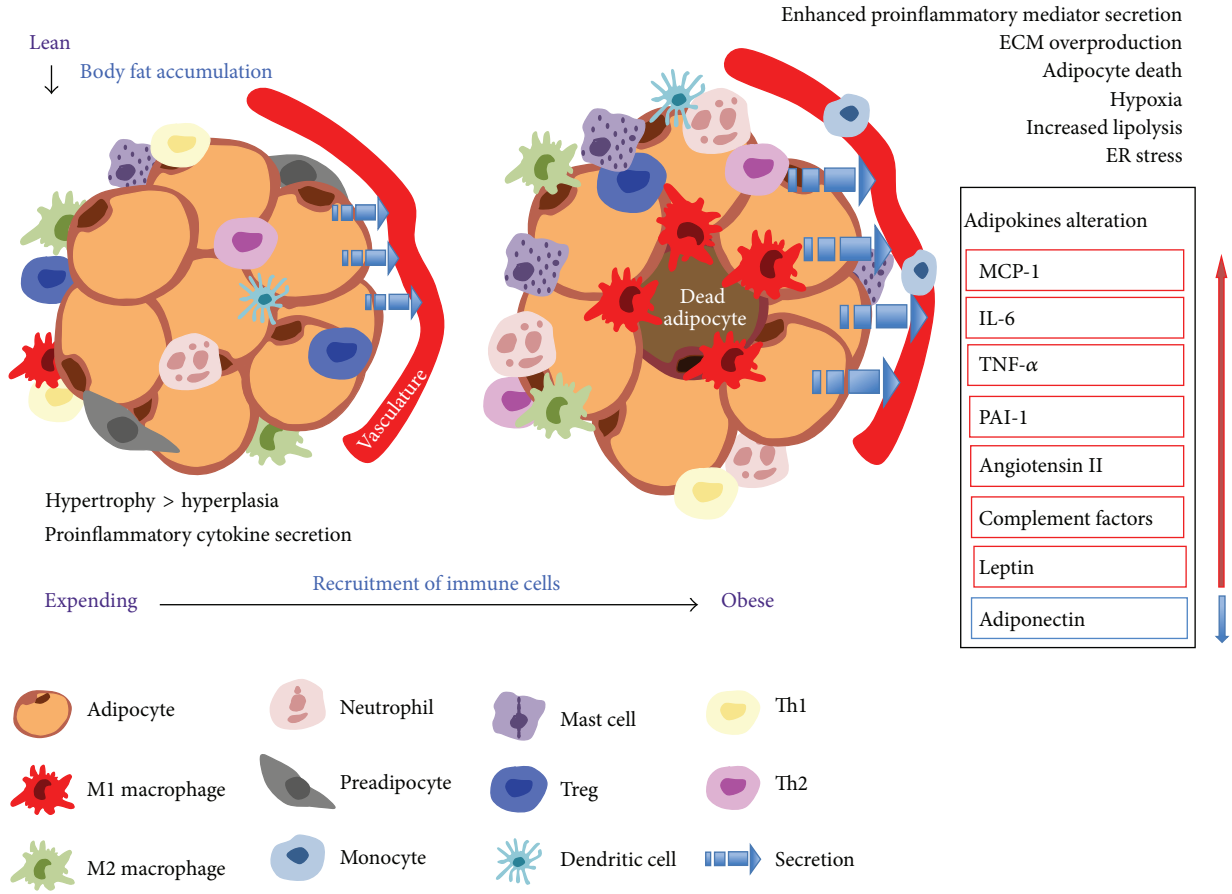

Figure 1: Alteration occurring in adipose tissue when it suffers from excessive TAG accumulation. In the face of chronic overnutrition, excessive adipose tissue expansion is initiated and dominated by adipocyte hypertrophy, whereas adipocyte number becomes fixed with obese individuals achieving a higher plateau. Activated adipocytes trigger production of proinflammatory mediators, together with resident immune cells, exerting functions in an endocrine and paracrine fashion. Infiltration of immune cells is secondary consequence followed by adipocyte activation. Mature adipocytes promote diapedesis of monocytes through microvascular endothelial cells, facilitating monocyte-derived macrophages accumulation. Progressive recruitment of immune cells underpins inflammation in AT by enhancing proinflammatory mediator secretion. Additionally, obese state pathologically accelerates AT remodeling featured by ECM overproduction, necrotic adipocyte, and hypoxia, along with dysregulation in fatty acid fluxes. Macrophages are predominantly localized around dead adipocytes. Sensibly, lean individuals exhibit higher ratios of M2:M1 macrophages, Th1:Th2 T cells. With the development of obesity, major shift in the above cell ratios favors a modest inflammation status (originality is inspired by previous review written by Evan D. Rosen and Bruce M. Spiegelman. Based on former illustration focusing on recruitment of immune cells, more detail and latest information are injected into the figure).

4. N-3 PUFA-Mediated Prevention and Reversal of Metabolic Syndrome

The structural feature, metabolic interconversion, and food source of n-3 PUFA are well demonstrated in previous literatures [53]. Numerous researches, in vivo or in vitro, confirm the positive effects of n-3 PUFA supplement on lipid and glucose metabolism, such as lower triacylglycerol concentration and higher high density lipoprotein (HDL) cholesterol levels in plasma, improved insulin sensitivity as well as reduced blood pressure [54, 55]. Animal dietary intervention trials demonstrate that n-3 PUFA limits development of obesity and reduces cellularity of AT in the context of the diet rich in fat, with improvement of lipid and glucose metabolism [56–58]. Importantly, n-3 PUFA administration alleviates high-fat diet (HFD)/obesity-induced insulin resistance, which is equal to or greater than the effects of clinically used insulin sensitizing drug [59]. Clinical studies in humans also report that n-3 PUFA contributes to a significant decrease of body fat and improves glucose metabolism and plasma lipid profile simultaneously [60–62]. The division of scientific opinions focuses on the issue that n-3 PUFA consumption significantly reduces food intake [63], since some clearly observe less weight again in animals fed fish oil-based diets, an effect cannot be explained by less lower caloric intake [64]. The discrepancy likely comes from the level of energy intake and experimental design, since the weight gain of control animals varies distinctly between different studies, and the conclusion is based on the comparison with control treatments different in fatty acid content. Notably, the metabolic and molecular effects of different high-fat diets with varying fatty acid

compositions are systemically compared. Unlike other high-fat diets tested, n-3 PUFA dietary regimen appears to be the only treatment successful in fighting against high-fat induced metabolic deterioration associated with obesity [64].

As said before, inflammation is an inevitable feature in the development of obesity and contributes to obesity-related metabolic derangements. Conversely, ameliorating the low-grade inflammation companied with obesity by n-3 PUFA benefits obesity and its sequelae via modulating adipokines secretion and improving insulin sensitivity [44].

Actually, the ratio of dietary n-6 to n-3 PUFA, rather than the absolute amount of n-3 PUFA, is important in determining the development of inflammatory response [65, 66]. Linoleic acid and α-linolenic acid (ALA) are the precursors responsible for the synthesis of n-6 and n-3 series, respectively. Despite different metabolites, the enzymes involved in their metabolism turn out to be the same, which means excessive intake of linoleic acid leads to reduced synthesis of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) because of competition for relevant enzymes. Increased ingestion of n-6 PUFA also inhibits EPA incorporation into neutrophil membranes and reduces the inhibition of production of inflammatory mediators [67, 68]. But in vivo, n-3 PUFA prevents the arachidonic acid- (AA-) induced increase in proinflammatory eicosanoids in fat cells [69]. Given the inefficiency of transformation from ALA to n-3 long-chain polyunsaturated fatty acid (LC-PUFA) [70, 71], increasing dietary consumption of EPA and DHA helps to reach the maximum beneficial effects. Besides that, precise difference exists between DHA and EPA and their precursor ALA, because longer carbohydrate chains seem more potent to exert stronger effects [7]. In most cases, it is active metabolites derived from n-3 PUFA, rather than themselves, that exerts anti-inflammatory effects.

5. Anti-Inflammatory Effects of n-3 PUFA

Clinical investigations of dietary supplementation with n-3 PUFA indicate their beneficial impact on some certain diseases, especially those companied with inflammation [6]. Anti-inflammatory effects of n-3 PUFA are primarily demonstrated by two standpoints: inhibitory secretion of proinflammatory mediators [72, 73] and greatly reduced macrophage migration into AT [74]. On the other hand, as outlined in the later paragraphs, n-3 PUFA assists with body fat reduction by multiple mechanisms including prevention of adipocytes proliferation, increased fatty acid oxidation, and inhibited hepatic lipogenesis, which can be considered as an indirect way to exert its anti-inflammatory effects given the immune role played by AT. Last, incorporation of the n-3 PUFA into adipocyte membrane is also regarded as a metabolism for its favorable effect in AT. Alterations occurring in AT finally result in systemic benefits.

5.1. Production of Proinflammatory and Anti-Inflammatory Cytokines. Among these identified factors contributing to uncontrolled inflammation in obesity, special attention is given to bioactive lipid mediators derived from the cyclooxygenase and 5-lipoxygenase pathways, which convert the membrane-derived AA into potent proinflammatory eicosanoids (such as prostaglandin and leukotriene). Notably, active metabolites derived from PUFA indeed play indispensable roles in the development of inflammation [44]. In contrast to proinflammatory potential of eicosanoids derive from n-6 PUFA, eicosanoids derived from n-3 series are generally gifted in anti-inflammation [75]. A relatively small increase in n-3 LC-PUFA consumption significantly inhibits AA conversion to proinflammatory eicosanoids generated by the 5-lipoxygenase pathway of neutrophils, monocytes, and macrophages [76, 77]. Thus, increase in dietary n-3 PUFA can shift the balance of the produced eicosanoids to a less inflammatory mixture, resulting from competitive inhibition of proinflammatory cytokines conversion.

In parallel, an arsenal of new lipid mediators is isolated and identified, suggesting that these novel families of anti-inflammatory mediators contrast with the earlier n-3 PUFA-derived oxygenated products previously known as eicosanoids [78]. As we know, in most cases, inflammation is normally well regulated and followed by complete resolution that enables inflamed tissues to return to homeostasis due to the activation of negative feedback mechanisms, which means influx of specialized leukocytes induces mononuclear leukocytes recruitment to phagocytose apoptotic leukocytes and cells debris from inflamed site until the injurious stimulus is cleared and infiltrated leukocyte is removed [79]. Contrarily, chronic inflammation is featured by continual activation of the adaptive immune system that exacerbates the inflammatory response. Of special interests in this process are some previously ignored factors that signal the termination of inflammation. As endogenous local mediators, these bioactive substances derived from EPA and EHA during the resolution phase are termed protectins and resolvins, because specific members of these families are widely appreciated for their ability to stimulate and accelerate resolution [80, 81]. In addition to controlling the magnitude and duration of inflammation, resolvins and protectins also function as inflammation terminators facilitating removal of chemokines from the resolving milieu [78]. Emerging evidence demonstrates that resolvins and protectins exert a strict control towards the resolution process of AT inflammation and pave the way for monocyte migration and their differentiation into macrophages and even elicit macrophage polarization toward an M2-like phenotype [79, 82].

Besides the mediators derived from PUFA, it is confirmed that, for healthy volunteers, supplementation of n-3 PUFA suppresses the production of secreted proinflammatory mediators [83–85]. Indeed, monocytes pretreated with n-3 PUFA significantly decrease proinflammatory production after lipopolysaccharide (LPS) stimulation [86–88]. Aligning with the emerging phenotype, a conceivable reduction in the expression of a number of inflammatory markers is achieved, in partial analogy to the situation with anti-inflammatory drugs treatment [63].

5.2. Alteration Occurring in Adipose Tissue. In addition to dietary PUFA-originated metabolites and n-3 PUFA-mediated

cytokines secretion, recent studies implicate n-3 PUFA influence on the polarization and recruitment of macrophages [52]. ATM in lean individuals is of an anti-inflammatory phenotype but capable of excessive proinflammatory mediator production [89]. Incorporation of n-3 PUFA in diet completely prevents macrophage infiltration induced by high-fat diet [90], which is consistent with another research revealing dramatic reduction in ATM content, along with transformation from M1 to M2 polarization state [59]. Similar macrophage phenotypic switch considered as a benefit of n-3 PUFA is observed in Kupffer cells [91].

Undeniably, adipocyte is the other target through which n-3 PUFA prevents AT inflammation. One possible mechanism lies in the prevention of AT expanding and proliferation of adipocytes by n-3 PUFA. Antiadipogenic effect of n-3 PUFA significantly prevents fat accumulation by reducing cellularity of AT, with a preferential inhibition in the epididymal fat [58]. Compatible with animal experiments, n-3 PUFA inhibits adipocyte differentiation, induces apoptosis in postconfluent preadipocytes, and promotes lipolysis [92]. Additionally, oxidation may be also involved in the profound effects of n-3 PUFA on prevention of enlarged adipocytes. The effect of n-3 PUFA in abdominal fat is associated with increased expression of genes engaged in mitochondrial biogenesis and oxidative metabolism, contributing to the shrinkage of adipocytes [93]. It is worthwhile to mention that dietary n-3 PUFA counteracts accretion of body fat without inducing mitochondrial uncoupling protein 1 (UCP1) in AT, suggesting that the antiobesity effect is independent of adaptive thermogenesis [94]. However, conflicting results give rise to the question whether the reductive effect on body fat accumulation exerted by n-3 PUFA is attributed to both limited hypertrophy and hyperplasia of adipocytes. Treatment with n-3 PUFA results in a significant decrease in the size of mature adipocytes and accumulation of smaller adipocytes [95], possibly along with increased total number of adipocytes. It cannot be excluded that the formation of small adipocytes results not only from shrinkage of existing mature adipocytes, but also from the proliferation of preadipocyte due to the activation of PPARγ [96]. Taken together, it is reasonable to speculate that n-3 PUFA ameliorates adipocyte stress and normalizes adipocyte functions, especially secretory function.

It was initially assumed that inclusion of n-3 PUFA is associated with remarkable changes in the plasma levels of two key adipokines, adiponectin and leptin. Further studies demonstrate that the induction of adiponectin by n-3 PUFA is adiposity-independent, since reduction of fat content due to caloric restriction hardly regulates adiponectin levels [90, 97, 98]. Based on emerging data highlighting the negative regulation of n-3 PUFA on leptin [99], conflicting results demonstrate that, in contrast to adiponectin, plasma leptin levels decrease with caloric restriction, an effect depending on body fat mass [100]. We cannot exclude the possibility that alteration in leptin is secondary to inhibition of enlarged AT caused by n-3 PUFA, rather than a direct consequence of n-3 PUFA manipulation. Other adipokines visfatin and apelin are also regulated corresponding to n-3 PUFA treatment without alteration in circulating levels, suggesting that both of them are incompetent to mediate systemic influence [101]. With regard to proinflammatory mediators derived from AT, dietary interventions with n-3 PUFA have been found to reduce TNF-α, IL-6 levels in AT [102, 103]. Given depot-specificity, n-3 PUFA reduces the expression of inflammatory genes of both gonadal and subcutaneous fat depots, suggesting the prevention against AT inflammatory may be apply to different fat depots [90]. The mechanism concerning adipocyte biological functions unveils that n-3 PUFA efficiently incorporates into AT, making their way into the membrane phospholipids and TAG lipid droplets, which is regarded as a mechanism for its preferential effects in AT [104–107]. Overall, these observations indicate that n-3 PUFA brings about a shift from a proinflammatory microenvironment to one of reduced inflammations (Figure 2).

5.3. Systemic Benefits. As mentioned, AT is responsible for the major form of crosstalk between insulin-responsive tissues. Thus, any treatment aimed at normalizing AT storage and secretory functions will alleviate the inflammatory state and lead to a global improvement in insulin sensitivity. Admittedly, the anti-inflammatory benefits of dietary n-3 PUFA involve modification in different tissues (liver, skeletal muscle, and AT) [108], which is not the interest of present review. Instead, this part is focused on systemic benefits of n-3 PUFA mediated by AT.

A large body of evidences demonstrates that n-3 PUFA reduces lipolysis in AT, resulting in reduced NEFA release into circulation [52, 108]. The modulation of lipoprotein lipase, hormone-sensitive lipase and fatty acid synthase is proposed as a possible mechanism in which n-3 PUFA inhibits lipolysis in AT [109].

In parallel, beneficial effect of n-3 PUFA on systemic glucose and lipid homeostasis heavily relies on circulating levels of adiponectin that is recognized as a critical mediator in improving insulin sensitivity. Given its insulin-sensitizing effect, it makes sense that improved insulin action observed with n-3 PUFA treatment in adiposity, liver, and skeletal muscle is mediated by adiponectin. Of note, this adipocyte-derived hormone directly increases glucose utilization and fatty acid oxidation in liver and skeletal muscle via stimulating the activation of 5′-AMP-activated protein kinase (AMPK) [110] and PPARγ [111], which is considered as the answer to this insulin-sensitizing effect of adiponectin. Actually, recent molecular studies illuminate the signal transduction of adiponectin in muscle cells [112], warranting that stimulation of adiponectin secretion induces mitochondrial biogenesis.

Recent results have increased interest in the role of hypothalamus inflammation playing in the pathogenesis of obesity. As an early and determining factor in the installation and progression of obesity, hypothalamic inflammation is a cardinal mechanism leading to the anomalous control of energy intake and expenditure [113, 114]. The substitution of saturated fat by n-3 PUFA in the diet reduces diet-induced hypothalamic inflammation and corrects the response to nutrient sensing signals [63].

N-3 PUFA minimizes the insulin resistance in high fat-fed mice in an adiponectin-dependent manner [100].

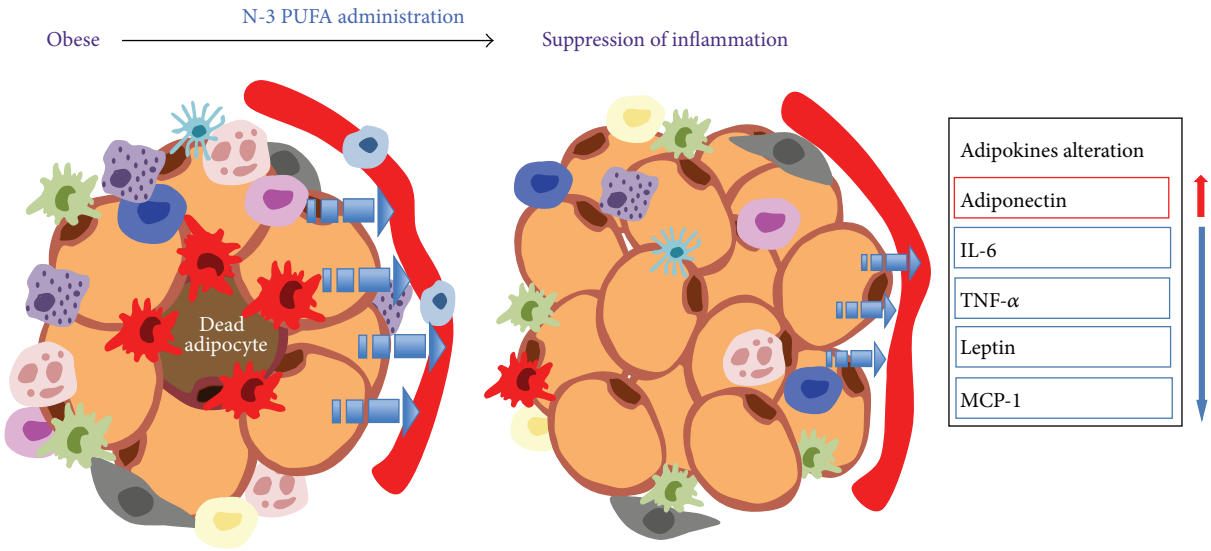

Figure 2: Mitigation of AT local inflammation by n-3 PUFA as a result from normalization of AT storage and secretory function. In the presence of n-3 PUFA, it is clear that the population of small adipocytes increases, possibly with unchanged total fat mass due to reduced large adipocyte. Alteration in cellularity also includes reduced ATM content, along with transformation from M1 to M2 polarization state. The last confirmed observation is dramatically modified adipokine secretion.

Additionally, insulin and nonesterified fatty acid are also affected predominantly by n-3 PUFA administration [100]. Taken together, the beneficial effects of n-3 PUFA on metabolic derangements are mediated in part by alleviation of AT inflammation, especially resulting from decreased secretion of proinflammatory adipokines, increased secretion of adiponectin, and induced synthesis of proresolving lipid mediators.

6. Intracellular Regulatory Mechanism Affected by n-3 PUFA

The previous section summarizes the broad range of anti-inflammatory actions exerted by n-3 PUFA. Various mechanisms are directly or indirectly involved in explaining the influence of n-3 PUFA on metabolic events, including, but not limited to, the changes in membrane composition and intracellular metabolite levels concerning signaling pathways (Figure 3). Here, four approaches which are identified as the molecular targets of the anti-inflammatory effect of n-3 PUFA are described in detail, since the transcription of inflammatory genes determines the production of inflammatory cytokines by certain cells, which plays a direct as well as central role in inflammatory cascade. Activity of transcription factors mastering the inflammatory signaling will potentially modulate inflammation. Moreover, the integration of important cell-signaling pathways engaged in n-3 PUFA-mediated anti-inflammatory functions has only begun to be investigated and has not yet been clearly defined. A former review summarizes that the major role for PPARs is in the trans-suppression of inflammatory gene activation by negatively interfering with the nuclear factor κB (NF-κB) [115]. In C2C12 myotubes, we have discovered that the suppressive effect of EPA on NF-κB activation is mediated via PPARγ activation [116]. Similarly, the expression of PPARγ in LPS-stimulated macrophages augments the inhibitory effect of inhibitor on NF-κB activity [117]. As a complement to above observation, Zúñiga and colleagues [118] figure out an antagonistic effect of PPARα on NF-κB-controlled transcription of pro-inflammatory mediators.

6.1. Membrane Phospholipid Fatty Acid Profile. PUFA is a key structural and functional component of the phospholipids in cell membranes and the most common PUFA in the membrane phospholipids of macrophages, neutrophils, and lymphocytes is the n-6 PUFA AA [53]. In contrast to high proportion of AA, much less EPA and DHA are found in the cell membrane phospholipids. However, n-3 PUFA supplement in the diet of animals or healthy human demonstrates a significant incorporation of EPA and DHA into total AT lipids, liver TAG and phospholipid fractions, and brain phospholipids [119]. Corresponding to n-3 PUFA supplementation, mice exhibit the accumulation of n-3 PUFA in membrane phospholipids of immune cells coupled with reduced AA content [77]. The increased proportion of n-3 PUFA in the membrane phospholipids likely contributes to its anti-inflammatory effect of inhibiting production of proinflammatory mediators secreted by mononuclear cells [85]. As mentioned before, similar incorporation is observed in adipocytes. Researches attempting to illuminate the influence of dietary n-3 PUFA on the lipid composition and metabolism of adipocytes disclose that rats fed the n-3 PUFA have significantly lower concentrations of serum triglycerides, cholesterol, and insulin, concomitant with higher unsaturated to saturated fatty acid ratio observed in adipocyte membrane phospholipids [105]. Conceivably, the improvement of insulin sensitivity is also positively correlated with

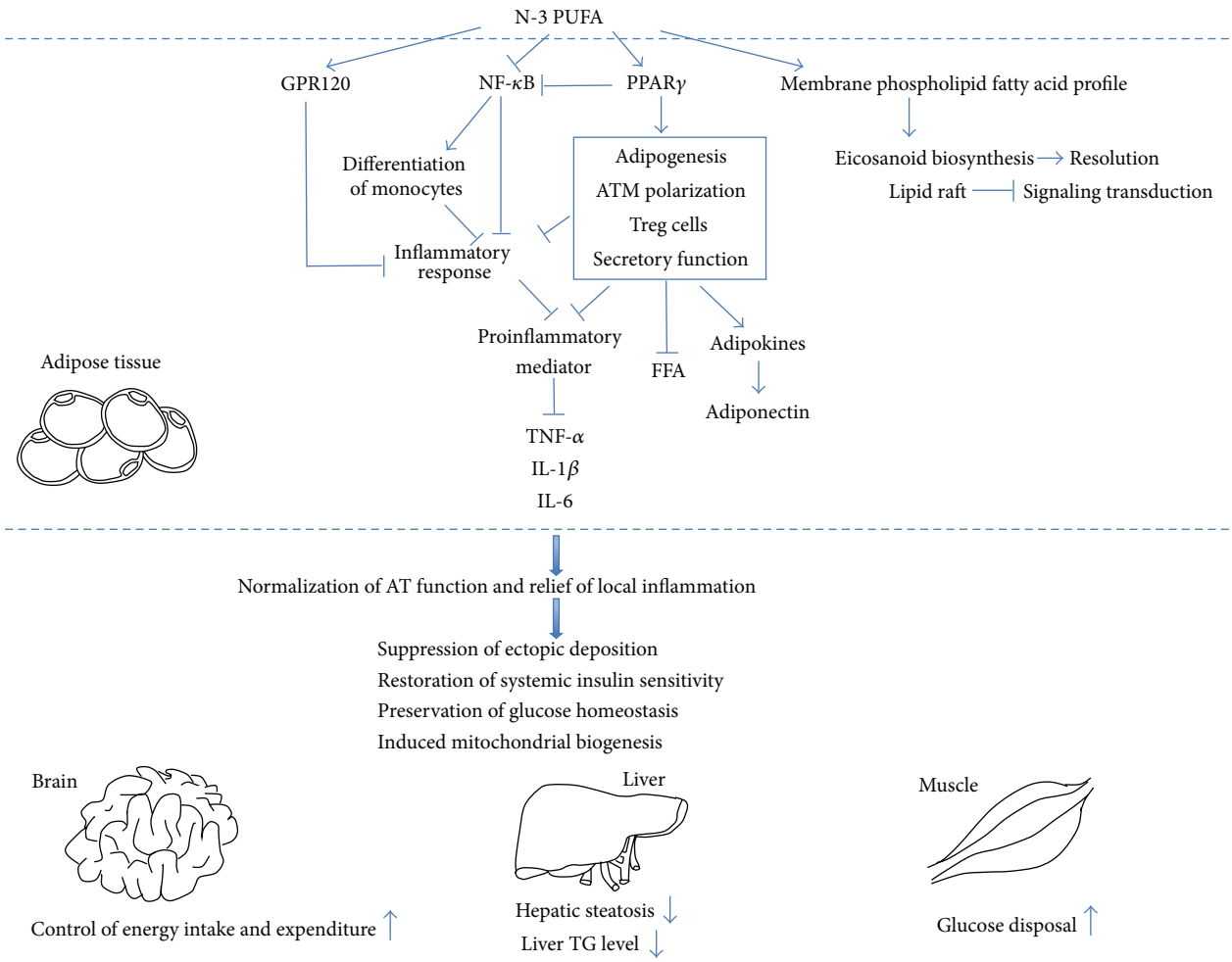

Figure 3: Putative mechanism by which adipose tissue mediates the systemic benefits derived from n-3 PUFA consumption. N-3 PUFA reduces adipose tissue inflammation via changing membrane phospholipid composition and activity of signaling pathways. The manipulation of membrane phospholipids composition and lipid raft microdomain with n-3 PUFA modify downstream processes mediated by membrane, such as signaling transduction, gene expression, and eicosanoid biosynthesis. Increased biosynthesis of resolvin and protectin contributes to resolution process. PPARγ activation mediated by n-3 PUFA leads to increased number of small adipocytes, M2 macrophages, and Treg cells, while normalizing secretory function featured by induced adiponectin synthesis, inhibited lipolysis, and reduced pro-inflammatory mediator secretion. As anticipated, NF-κB inhibition, which is partly dependent on PPARγ activation, downregulates the expression of proinflammatory gene directly and indirectly via determining the differentiation of monocytes. GPR120 is the functional receptor making response to n-3 PUFA and mediates the anti-inflammatory benefits by repressing several inflammatory signalings. The improved storage and secretory functions of adipose tissue lead to relief of AT-specific inflammation, followed by global improvement of metabolic profile. Adipose-specific blunting of inflammation primarily favors the functional improvement of several organ systems involving liver, skeletal muscle, and brain.

higher incorporation of n-3 PUFA in adipocyte membrane phospholipids [104].

It is thought that n-3 PUFA displaces AA from plasma membranes, decreasing its availability as a precursor of inflammation associated mediators. Inhibitory effect of n-3 PUFA on the release of substrate AA also is attributed to the suppressed phospholipase activity [76]. Irrespective of substrate accessibility, alteration of metabolites results from decreased AA metabolism due to the inhibition of cyclooxygenase (COX) activity [120]. This seems the possible explanation for the mechanism in which n-3 PUFA affects lipid-mediator profiles. The n-3 PUFA substitution of AA into adipocyte membrane phospholipids results in decreased level of prostaglandin E_2 (PGE_2) and subsequent downregulated fatty acid synthesis enzyme activity [69], which curbs adipocyte hypertrophic. On the other hand, decreased formation of prostaglandin D2 (PGD2) and its derivatives, known as PPARγ ligands, presumably explains the effects of n-3 PUFA on proliferation and maturation of adipocytes [44]. Interestingly, insulin action is positively correlated with the fatty acid unsaturation index in membrane [104], whereas related mechanism remains obscure.

A second aspect of the alternation in cell membrane phospholipid fatty acid involves lipid raft in which key signal transduction proteins are localized, such as the tyrosine kinase lck and the signaling molecule linker of activated T

cells [121]. Exposed to n-3 PUFA treatment, Jurkat T cell ends in marked enrichment of n-3 PUFA in lipids from isolated raft, with selective displacement of signaling proteins from raft in PUFA-treated T cells due to altered raft lipid composition [122]. Later data collectively demonstrate that modified raft lipid environment affects the membrane subdomain distribution of proteins involved in the interleukin-2 receptor (IL-2R) and toll-like receptor (TLR-4) signaling pathway [123, 124]. For now, disrupting rafts with n-3 PUFA and subsequent impact on immune cell function are centered on T cells, macrophages, and B cells, considered as the answer to immunosuppression property of n-3 PUFA.

The manipulation of membrane phospholipids composition and lipid raft microdomain with n-3 PUFA modify downstream processes mediated by membrane, such as signaling transduction, gene expression, and eicosanoid biosynthesis.

6.2. Peroxisome Proliferator-Activator Receptors (PPARs). As lipid-activated transcription factors, PPARs contribute to attenuating inflammatory response. Numerous agonists of the related receptor PPAR have anti-inflammatory activity [125, 126]. Thiazolidinedione- (TZD-) induced activation of PPARγ is reported to relieve hyperglycemia, hyperinsulinemia in vivo, reducing secretion of FFA, TNF-α, and leptin concomitantly [96]. PPARγ, originally described in differentiating adipocytes, severs as a master regulator of adipogenesis and the target for the insulin-sensitizing drugs [127, 128]. Admittedly, forced expression of PPARγ in the fibroblasts makes them differentiate into adipocytes [129] and activation of PPARγ indeed contributes to increasing population of small adipocytes, but the total mass or the total triglyceride content of WAT remains unchanged with the number of large adipocytes decreasing [96]. Compared with large mature adipocytes, small immature ones seem less competent in secreting proinflammatory cytokines [130]. This shift toward smaller adipocytes seems to be partially responsible for the favorable metabolic effects of PPARγ activators. With regard to the secretion of adipokines, PPARγ agonists have been reported to increase circulating levels of adiponectin [131]. Moreover, PPARγ null mice and in vitro experiments all demonstrate that the increased synthesis and secretion of adipokines by n-3 PUFA are dependent on PPARγ [132, 133].

The expression of PPARγ is also observed in cells of immune system and central nervous system. PPARγ activation stimulated by short-term glitazones treatment increases infiltration of M2 in AT [134]. Macrophage-specific disruption of PPARγ impairs development of alternatively activated M2 phenotype and predisposes individuals to diet-induced obesity [135]. Providing that PPARγ is indispensable in the development of M2 phenotype, PPARγ inactivation indirectly increases production of proinflammatory cytokine [136]. Similarly, PPARγ activation suppresses monocyte elaboration of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6 [137]. Unexpectedly, inactivation of PPARγ in macrophages has unimaginable broad effects on significant glucose intolerance and impaired whole-body insulin resistance in lean mice fed a normal diet [136]. VAT-resident Foxp3$^+$ Treg cells also specifically express PPARγ which interacts with Foxp3 [138]. Foxp3-dependent PPARγ conditional knockout mice demonstrate that Treg cell-specific deletion of PPARγ leads to reduced number of Treg cells specifically in VAT, resulting in an increase in VAT infiltration of macrophages and monocytes. Additionally, stimulation with TZD drugs specifically increases VAT-resident Treg cells in the obese mice fed high-fat diet, with improving insulin sensitivity synchronically. These results collectively indicate that the therapeutic effect of TZD drugs partly is attributed to accumulation of Foxp3$^+$ Treg cells in VAT. Naturally occurring Foxp3$^+$ Treg cells produced by thymus possibly sense activator, as a result, PPARγ expression is upregulated with migration of Foxp3$^+$ Treg cells to the VAT. Alternatively, in parallel with accumulation of VAT-resident Foxp3$^+$ Treg cells, augmentation of such suppressive activities of PPARγ-expressing Treg cells achieved by ligand activation may enable better control of inflammation in obesity.

In conclusion, PPARγ may act on differentiating adipocytes, macrophages, and Treg cells to alleviate AT inflammation and restore insulin sensitivity. The other mechanism involved in the effect of PPARγ on anti-inflammation remains further investigation. High concentration of n-3 PUFA has been reported to be active PPARγ, whereas their metabolites are stronger agonists [139]. Anti-inflammatory effects of n-3 PUFA are known to act at least in part through activation of PPARγ. Although this speculation seems arguable, since n-6 PUFA, the stronger activator of PPARγ, exhibits opposite effects. However, we are supposed to keep in mind that n-6 PUFA likely assumes their proinflammatory responsibility via other mechanism which is powerful enough to counteract the anti-inflammatory effect mediated by activation of PPARγ.

Another candidate mechanism responsible for the anti-inflammatory action of n-3 PUFA is the removal of detrimental eicosanoids via β-oxidation, which is likely stimulated by PPAR activation [140].

6.3. Nuclear Factor κB (NF-κB). Consistent with its central role in inflammatory signaling pathways, NF-κB is widely investigated in the linkage between inflammatory and metabolic responses. Once activated by extracellular stimuli, NF-κB promotes inflammation through initiating expression of genes encoding for inflammatory-related proteins in a very wide range of cell types, including macrophages, hepatocytes, and adipocytes. Additionally, NF-κB directs the differentiation of distinct immune cell types by regulating expression of inflammatory mediators. NF-κB-dependent differentiation of monocytes into either M1 or M2 macrophages in response to cytokines produced by immune cells also accounts for the involvement of NF-κB in inflammation associated metabolic disorders, taking distinctive functions of M1 and M2 macrophages into consideration. Furthermore, activated NF-κB promotes macrophage relocalization and activation, and more proinflammatory cytokines secretion augments macrophage activation and recruitment to the inflamed site [141]. Unexpectedly, different from a local and modest inflammatory response caused by IKK-β induced

NF-κB activation in hepatocytes [142], inactivation due to IKK-β deficiency in myeloid cells favors global insulin sensitivity [143]. Additionally, inactivation of NF-κB by IKK-β deletion significantly diminishes the expression of inflammatory mediators [144]. Not only in peripheral metabolic tissues, recent study even discloses that in hypothalamus, NF-κB which functions as the pro-inflammatory master switch is blamed for dysregulation of energy balance after sensing metabolic signals produced by overnutrition [113]. Brightly discriminated from the inflammatory reactions featured by chaotic release and powerful action of many inflammatory cytokines in nonneuronal cells, forced activation of hypothalamic IKK-β/NF-κB interrupts central insulin/leptin signaling and actions in a neuron-specific and noncytokine way, resulting in increased intake of high-fat food and weight gain. By contrast, suppression of IKK-β either broadly or locally significantly protects against obesity and glucose intolerance [113], suggesting that inflammatory response in the peripheral metabolic loci trigged by overnutrition no longer causes functional defect in tissue-specific manner. Instead, metabolic inflammation and related inflammatory mediator are connected to the dysfunctions in the central nervous system.

In the quiescent state, NF-κB remains inactive in the cytoplasm binding to the inhibitory protein IκB. Phosphorylation of this inhibitory subunit releases NF-κB, followed by NF-κB translocation into nucleus where it controls the transcription of its target genes. Consistent with other researches based on THP-1 cells [87], we have found that n-3 PUFA inhibits NF-κB activity in myotubes in vitro by preventing the degradation of IκBα [145]. The explanation for the regulation of cytokines production caused by n-3 PUFA is the changes in inflammatory gene expression, which is partly ascribed to NF-κB suppression with decreased IκB phosphorylation [86, 87]. Notably, NF-κB is enhanced or unaffected with saturated fatty acids and n-6 PUFA treatments [86, 146], validating that the inactivation of NF-κB is exclusive to n-3 PUFA instead of a general lipid effect. Based on accessible reports, inhibitory effects of n-3 PUFA on NF-κB activation lead to related gene expression, including IL-1, IL-1β, and TNF-α.

6.4. G Protein-Coupled Receptor 120. It has been reported previously that G protein-coupled receptor 120 (GPR120) functions as a signaling molecule for a wide array of cellular functions in response to unsaturated long-chain FAs [147]. Tissue expression pattern further indicates that GPR120 is the only lipid sensing GPR highly expressed in specialized proinflammatory tissue and cells [59], suggesting its critical role in development of obesity. Subsequent researches using human genetics approaches identify loss-of-function GPR120 gene variants that caused increased incidence of obesity and related sequelae, especially in the context of high-fat diet [148]. Interestingly, dysfunctional receptor underscores the inefficiency to transduce the signal of long-chain fatty acid [148], opening novel avenues of researches for antiobesity effects associated with n-3 PUFA. Actually, it has been already confirmed that GPR120 is the functional receptor making response to n-3 PUFA and mediates the anti-inflammatory benefits by repressing TRL2/3/4 and TNF-α inflammatory signaling in a β-arrestin2 dependent way, coupled with insulin sensitizing actions [59]. In more detail, following n-3 PUFA-stimulated internalization of the GPR120/β-arrestin2 complex, β-arrestin2 associates with TAK1 binding protein 1 (TAB1), blocking the association of TAB1 with activated kinase 1 (TAK1), which dampens downstream signaling to the IKKβ/NF-κB and JNK/AP1 system [59]. A similar receptor dependency for the anti-inflammatory effects of n-3 PUFA is observed in hypothalamus and Kupffer cells [63, 91].

7. Conclusion

Prolonged nutrient excess elicits infiltration of macrophages and other immune cells in AT, resulting in uncontrolled secretion of proinflammatory cytokines and a state of chronic low-grade inflammation. In turn, these released cytokines affect other organs, such as skeletal muscle, liver, and brain, ultimately ending up with metabolic abnormalities systemically. Undoubtedly, AT is the epicenter of the obesity, since inflamed AT is the primary source blamed for proinflammatory cytokines. The overall impacts of n-3 PUFA on AT biology and metabolism fall into three categories: storage, secretory function, and inflammation. Decreased storage and normalized secretion finally achieve adipose-specific blunting of inflammation. Aligning with the emerging view, AT lies at the crossroad of nutrient sensing, metabolism, inflammation, and endocrine. As anticipated, results from clinical and molecular studies have converged to highlight the broad spectrum of protective effects of n-3 PUFA in obesity and comorbidity. There is considerable crosstalk between AT and a wide array of organs, underscoring the difficulty in dissecting tissue-specific effects of n-3 PUFA and additional benefits mediated by AT. Recapitulating the phenotypical and metabolic date, we easily come to the conclusion that specific treatment results in a certain phenotype. However, elevated circulating adiponectin derived from AT due to dietary n-3 PUFA is an easily ignored contributor to restoration of insulin signaling in liver and muscle. Identification of the "missing link" between treatment and direct executor is an important next step towards our understanding of the actions of n-3 PUFA.

More recently, nutrients and environmental factors have been shown to induce epigenetic modifications [149], which is definitely a most rapidly expanding field in biology. Mapping of epigenetic marks, such as DNA methylation, histone modifications, and nucleosome positioning, is critical for understanding the regulatory mechanism for gene expression, chromatin remodeling factors, and noncoding RNA expression influenced by diet constitutes. Since the pathophysiology of obesity is concomitant with extensive gene expression changes, special emphasis is put on identification epigenetic changes induced by obesity and mechanism through which epigenetics contribute to obesity [150]. It would appear that future studies might be needed to establish and validate the relationship between dietary n-3 PUFA and antiobesity effect and how epigenetic mechanism may link them. Although the effect of dietary n-3 PUFA on the

increased expression of leptin seems unrelated to promoter CpG methylation, given that feeding mice with n-3 PUFA diet is unable to affect CpG methylation in the leptin gene promoters [151]. The reasonable explanation may be the irrelevance between n-3 PUFA and increased leptin, which has been suggested before. Otherwise, we cannot rule out the possibility that histone modification, rather than DNA methylation, is the determinant elucidating the regulatory influence of n-3 PUFA on leptin expression in obesity.

The fact that not all AT expansion is necessarily associated with pathological changes makes us rethink our understanding towards obesity. Despite having excessive fat accumulation, a unique subset of obese individuals seem metabolically healthy, bypassing all of the aforementioned pathological phenotype associated with obesity [152]. It is supposed to reconsider and revalue obesity professionally in terms of metabolic disturbances and metabolic parameters, rather than arbitrary judgment based on fat mass. Accordingly, n-3 PUFA is never a panacea for dramatic loss of AT mass compared with energy restriction, but it is proficient in protecting from obesity-induced metabolic abnormalities, regardless of weight loss. In this regard, n-3 PUFA is a qualified nutritional regimen fighting against metabolic disorders. The anti-inflammatory and immune-regulatory effects of n-3 PUFA would be the hot issue worthy of further investigation rather than having antiobesity effects as the endpoint.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Docosahexaenoic Acid on Apoptosis and Proliferation in the Placenta: Preliminary Report

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Introduction. Observational studies confirm a higher incidence of preeclampsia in patients with low erythrocyte concentrations of omega-3 fatty acids. Observations point to an association of disorders of pregnancy, such as intrauterine growth restriction (IUGR) and preeclampsia, with excessive apoptosis. One potential mechanism of action of docosahexaenoic acid (DHA) promoting a reduction in the risk of pathological pregnancy may be by influencing these processes in the placenta. *Materials and Methods.* We investigated 28 pregnant women supplemented with a fish oil product containing 300 mg DHA starting from pregnancy week 20 until delivery (DHA group). The control group consisted of 50 women who did not receive such supplementation (control group). We determined the expression of Ki-67 and p21 as markers of proliferation and caspase 3 activity as a marker of apoptosis and DHA levels in umbilical cord blood. *Results.* Caspase 3 activity was significantly lower in the DHA group in comparison to the control group. Umbilical cord blood DHA concentration was higher in the DHA group. The expression of the proteins p21 and Ki-67 did not differ significantly between the groups. *Conclusions.* We observed an association between DHA supplementation and inhibition of placental apoptosis. We did not find an association between DHA and proliferation process in the placenta.

1. Introduction

The use of omega-3 acids in pregnant women dates back to the 1980s, when Olsen conducted the first observational study of the effect of dietary fish oil on the pregnancy outcome [1]. Further research revealed that docosahexaenoic acid (DHA) was responsible for many of observed effects. DHA is a long-chain fatty acid belonging to the omega-3 group. These essential unsaturated fatty acids need to be supplied in the diet as they cannot be synthesised by the body. Fatty marine fish are the richest source of long-chain fatty acids. At present, many international scientific societies, including the Polish Gynaecological Society, recommend the use of DHA for the prevention of premature labour [2, 3]. They are based on meta-analyses and large RCT studies conducted by Makrides et al. and Carlson et al. [4, 5]. Currently in Australia a large clinical trial takes place among 5500 women. The aim of this study is to observe the effects of DHA from fish oil (800 mg) on the risk of preterm delivery and adverse pregnancy outcome [6]. The role of DHA in the development of the central nervous system and its influence on cognitive processes have also been appreciated by paediatricians, who recommend DHA consumption by pregnant women and breast-feeding mothers. The prenatal and early postnatal period are critical for the development of the infant brain [7]. An animal model has shown that DHA plays a role in the prevention of cerebral hypoxia. If this effect is confirmed in human studies, it will be a high clinical significance [8]. Studies investigating the link between DHA consumption and the development of preeclampsia or postpartum depression have not been consistent [5, 9]. Available data do not make it clear which doses should be used in the presence of specific disorders of pregnancy since various studies have used a wide range of daily dosages from 500 mg to 2.7 g [10]. In spite of so many unknowns, many experts agree that DHA should be regarded as an important component of a pregnant woman's diet and an element of nutritional programming [11]. New data from animal studies and a cell model indicate a protective effect of omega-3 acids, and particularly DHA, on central nervous system cells [12]. It has been

proved that this effect is caused by antiapoptotic activity. In women, apoptosis plays a significant role in placentation. Intense apoptosis in the placenta is believed to be a primary factor in the pathogenesis of such disorders of pregnancy as preeclampsia and IUGR [13]. Increased apoptosis in peripheral blood is also associated with the destruction of fetal DNA fragments [14]. Intense placental apoptosis leads to cell destruction, decreasing the area available for oxygen exchange and transfer of nutrients and metabolites and, later, leading to hypoxia and, consequently, the development of disorders of pregnancy [15]. Animal studies of the effect of DHA on apoptosis and observations of excessive apoptosis in IUGR and preeclampsia in conjunction with observational evidence of a correlation between the incidence of these pathologies and low DHA levels prompted us to study the effect of DHA on placental apoptosis in pregnant women.

2. Objective

The present paper aims to improve our understanding of the effect of DHA on cellular proliferation (Ki-67 and p21 expression) and to assess placental apoptosis (caspase 3 activity) in women with normal uncomplicated pregnancies without disorders such as IUGR, preeclampsia, or premature delivery. The correlation between DHA supplementation and cord blood DHA concentration is also investigated.

3. Material and Methods

The study involved a group of 28 parturient women who were supplemented with fish oil from pregnancy week 20 until delivery. The women from the supplementation group gave birth at the Department of Obstetrics and Perinatology, Independent Public Teaching Hospital number 4 in Lublin, between March and August 2012. There was a control group of 50 women who did not receive supplementation. Women were enrolled in the study on the basis of their history of supplementation in pregnancy. The women in the supplementation group took one capsule of a supplement containing 300 mg DHA (Prenatal DHA, Holbex). The supplementation and control groups were matched for age, fertility, parity, and pregnancy age. All women in the study had single pregnancies with alive fetuses, were negative for clinical and biochemical markers of inflammation, did not smoke tobacco, and had no chronic medical conditions that could lead to angiopathy. The study protocol was approved by the Ethical Committee at the Medical University in Lublin.

DHA concentration was determined in all patients in the serum of venous cord blood. The profile of fatty acids in the spectrum range for DHA was analysed by gas chromatography using for lipids separation Folch method [16].

The p21 protein is a potent inhibitor of cyclin-dependent kinases. P21 protein binds to cyclin complexes to inhibit their activity, thus acting as a cell cycle regulator in phase G1. P21 expression was determined in freshly frozen placental tissue sections by immunohistochemistry using standard sets of monoclonal antibodies and an avidin-biotin detection system from Dako [17]. Positive p21 reaction was defined as the fluorescence of more than 10% of the cells at a magnification of 100x.

The expression of the Ki-67 protein was detected with the PP-67 standard murine anti-human monoclonal antibody kit (Sigma-Aldrich) [18]. The Ki-67 antigen is found in the cell nucleus, especially in the nucleoli during the interphase. It is the protein marker of cellular proliferation. It was determined immunohistochemically in freshly frozen sections based on detection system for biotinylated samples (Dako) [19]. Ki-67 positivity was defined as the presence of a positive immunohistochemical reaction in more than 30% of cells per field at a magnification of 100x.

Caspase 3 activity was determined by colorimetry in homogenates of slices of full sections through placentae obtained after delivery. The commercially available caspase 3 colorimetric assay kit code CASP-3-C (Sigma-Aldrich, USA) was used. Caspase 3 activity was converted to μmols of p-nitroaniline release per minute (min) per millilitre (mL) of a placental cell lysate or a positive control [20].

Differences in the different assay results between the groups were analysed with the Mann-Whitney U test ($P(U)$). The correlation between cord blood DHA levels and the apoptosis index was analysed by estimating Spearman's rank correlation coefficients. Differences were considered statistically significant at $P < 0.05$.

4. Results

The statistical analysis of the data obtained in the supplementation versus control groups did not reveal statistically significant differences in the distribution of results with regard to age, duration of pregnancy, and body weight at birth between the groups. The supplementation and control groups were assumed to be comparable with regard to these indices. There were significant differences between the groups with regard to placental weight ($P(U) = 0.0071$), which was significantly higher in the control group. Apgar scores did not differ significantly between the groups ($P(U) = 1.0000$). The index of apoptosis, that is, caspase 3 activity, was significantly different between the groups ($P(U) = 0.0001$) (Figure 1): it was significantly lower in the DHA supplementation group as compared to the control group. The clinical details of the patients and their children and caspase 3 activity are presented in Table 1.

DHA concentration in the serum of venous cord blood differed significantly between the groups ($P(U) < 0.0001$). Cord blood DHA levels were higher in the DHA supplementation group (Figure 2). Overall, there was a highly significant negative rank correlation between cord blood DHA levels and the index of apoptosis ($P < 0.01$). Overall for all values we have Spearman's correlation −0,3082. For DHA group we received −0,0670 and for control group 0,0622, respectively. This finding means that as DHA levels increase in cord blood caspase 3 activity decreases (Figure 3). The correlation was calculated with Spearman's coefficient, which is robust for nonnormal distributions and outliers.

Table 1: Clinical data and the activity of caspase 3 and the concentration of DHA in the serum of venous cord blood in patients from both analyzed groups.

Variable	Median	Min–max	95% CI	$P(U)$ value
Maternal age (y)				
DHA group ($n = 28$)	25,5	19–39	25,2–26,7	**0,5399**
Control group ($n = 50$)	26	18–35	24,2–26,8	
Gestational age (w)				
DHA group ($n = 28$)	39,5	37,8–41,2	39,26–39,64	**0,2151**
Control group ($n = 50$)	39,20	37,6–41		
Birth weight (g)				
DHA group ($n = 28$)	3675	2990–4350	3589–3760	**0,8815**
Control group ($n = 50$)	3785	3170–4400	3725–3844	
Placenta weight (g)				
DHA group ($n = 28$)	510	440–650	497–522	**0,0071**
Control group ($n = 50$)	530	410–610	522–537	
Apgar score at 5 minutes (p)				
DHA group ($n = 28$)	10	8–10	9,87–10,13	**1,0000**
Control group ($n = 50$)	10	7–10	9,88–10,12	
Caspase 3 activity (μmol pNa/min/mL)				
DHA group ($n = 28$)	0,053	0,0084–0,048	0,0511–0,0549	**0,0001**
Control group ($n = 50$)	0,059	0,0047–0,049	0,0582–0,0598	
Cord DHA (ng/μL)				
DHA group ($n = 28$)	3,86	3,54–4,13	3,83–3,89	**0,0001**
Control group ($n = 50$)	3,61	3,49–3,79	3,60–3,62	

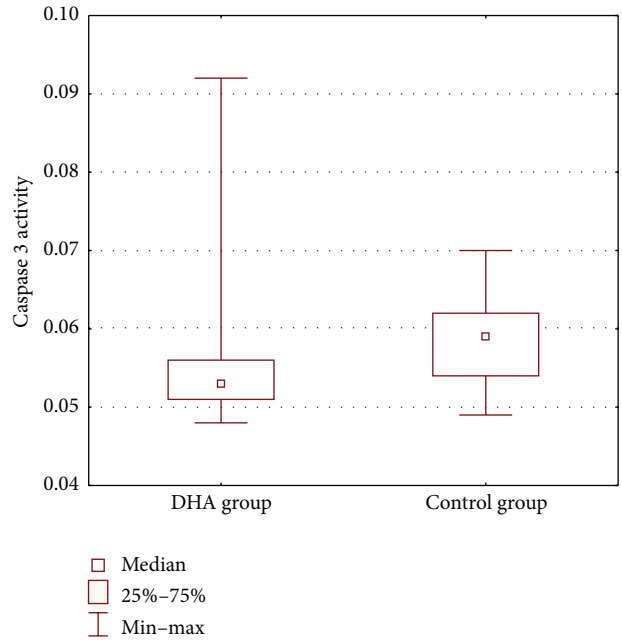

Figure 1: Average activity of caspase 3 (μmol pNA/min/mL) in both analyzed groups ($P(U) = 0.0001$).

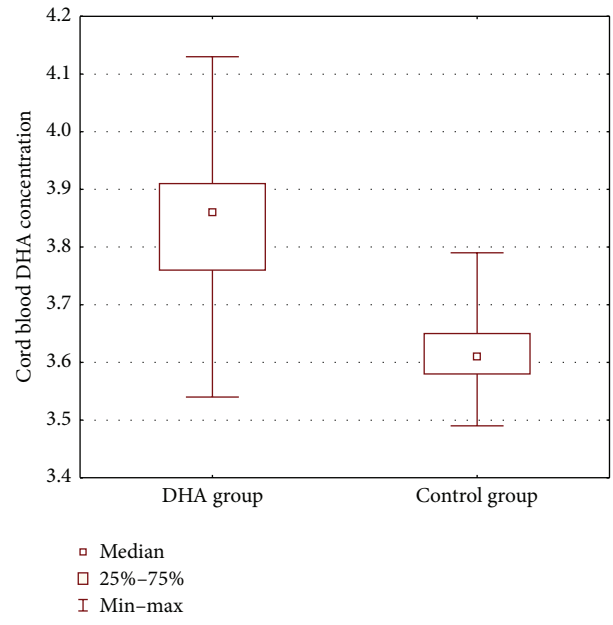

Figure 2: Average concentrations of DHA (ng/μL) in the serum of venous cord blood in both analyzed groups ($P(U) = 0.0001$).

Expression of the proteins p21 and Ki-67 was not significantly different between the groups ($P > 0.05$). For p21 protein we observed a positive reaction in 17% and 15% of cells for the DHA and control group, respectively. Positive reaction for Ki-67 was received for 21% and 20% cases for DHA and control group, respectively. It indicates that the DHA dose used in the study had no effect on placental proliferation in the patients.

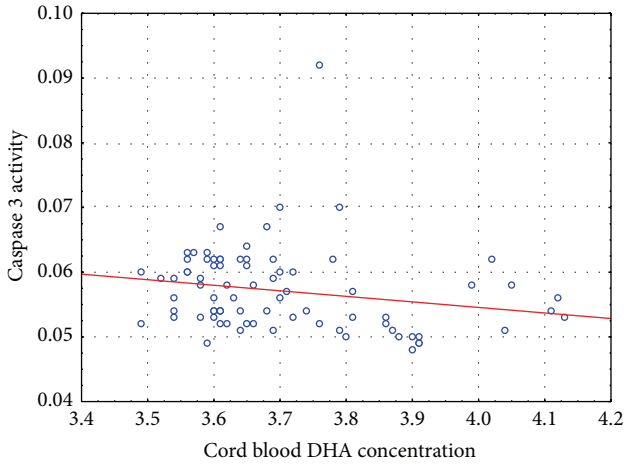

Figure 3: Distribution of caspase 3 activity in the placenta depending on the concentration of DHA in cord blood.

5. Discussion

The diet of citizens of the Western countries is characterised by a predominance of omega-6 acids over omega-3 acids, which may lead to intensified proinflammatory activity, narrowing of vessels, a prothrombotic effect, and, consequently, preeclampsia in pregnant women. Observational studies confirm greater incidence of this pathology in women on a fish-deficient diet or in those with low omega-3 acid levels in erythrocytes [21, 22]. In a 2011 meta-analysis, Duley pointed to the lack of sufficient data to assess the effectiveness of fish oil in the prevention and treatment of preeclampsia [9]. In contrast to this, the Canadian Hypertension Society has published recommendations on nonpharmacological management and prevention of hypertension, advising the use of fish oil in cases of hypertension in pregnant women. They particularly recommend fish oil supplementation in women from risk groups who experienced hypertensive episodes in early pregnancy [23]. The latest data indicate that women with preeclampsia have a reduced pool of long-chain fatty acids and reduced placental synthesis and fetal transport. That is why the supplementation of omega-3 fatty acids may improve their status both in the women and in children born to mothers with preeclampsia [24]. One potential mechanism of action of DHA promoting a reduction in the risk of pathological pregnancy may be by influencing cellular apoptosis and proliferation in the placenta.

Placental apoptosis is physiological in normal pregnancy and intensifies over time. Placental apoptosis has been particularly studied in two pathologies of pregnancy: preeclampsia and IUGR [13]. A finding shared by both of these conditions is intense placental apoptosis, which is probably a key mechanism leading to placental dysfunction. The attenuation of proliferation in pathological pregnancy is not an established fact, with published studies presenting contradictory results [25–27].

The effect of omega-3 acid supplementation on proliferation and apoptosis in various cells of the placenta has been described to date in only one paper by Klingler et al. [28]. The authors put forward the hypothesis that greater proliferation could be expected towards the end of pregnancy on the basis of earlier reports that high dosages of DHA can prolong pregnancy and increase infant birth weight [29]. Klingler et al. reported that supplementation did not influence the duration of pregnancy or the birth weight of the infants. There were also no changes in apoptosis between the groups. Only a group using fish oil with folate demonstrated an effect on proliferation. Our results do not confirm an effect of DHA on cell proliferation, which could be due to an insufficient dosage used (300 mg DHA compared to 500 mg in the study by Klingler et al.). At the same time, we did demonstrate an influence of DHA supplementation on apoptosis. The effect on proliferation observed in Klingler's study might have been due to the synergistic action of DHA and folate in the study preparation. Even though DHA had no effect on proliferation in our study, placental weight in the DHA group was significantly higher, which may be attributed to DHA-mediated reduction of oxidative stress as observed in an animal model by Jones et al. [30].

Importantly, DHA-mediated inhibition of neuronal apoptosis has been demonstrated in an animal model. In a Japanese study, pregnant rats, some of which had been fed a DHA-rich diet, were placed in hypoxia. The DHA group demonstrated fewer apoptotic neurons, which may be a sign of neuroprotection by inhibition of oxidative stress in nerve cells [8]. The latest reports also indicate an inhibitory effect on neuronal apoptosis following the addition of omega-3 acids to the diet in postnatal hyperoxia [12].

Our report also describes an effect of daily supplementation of 300 mg DHA on the levels of this compound in cord blood, which were significantly higher than in the control group. In previous studies, higher DHA cord blood levels were obtained at higher DHA dosages [31, 32]. According to earlier data, a dosage of 200 mg did not raise DHA levels in cord blood [33]. Cord blood DHA levels are a marker not only of dietary DHA (including DHA supplementation) but also of DHA synthesis from its precursors, which is increased in pregnancy. Accordingly, the determination of DHA in cord blood appears to be the most appropriate measure of the amount of DHA reaching the fetus. Recent data suggest that cord blood DHA levels may be influenced by other factors than maternal DHA consumption, which may account for the different conclusions from the assessment of the infants in studies where DHA supplementation was the responsibility of the pregnant women [34].

We currently know little about the effect of DHA on placental proliferation and apoptosis, with only one paper of relevance to this issue in the global literature. A confirmation of our results by a study with a larger sample may in the future lead to the development of recommendations and prophylactic use of DHA to prevent primary disorders of pregnancy associated with too shallow placentation, that is, preeclampsia and IUGR.

Our findings are the outcome of a pilot study of a group of pregnant women with no history of disorders related to placental dysfunction. The limitations of our study comprise the dosage of 300 mg DHA, which was consistent with clinical recommendations at the time of the study. Current

recommendations suggest the need to use higher doses of DHA at least 600 mg. New clinical trials at dose 800 mg are currently being designed. It seems that in our study too low dose was used, to assess the effect on apoptosis and proliferation rates. The second limitation was the small number of participants in this pilot study.

Accordingly, a continuation of this study should involve women at risk for these disorders and be conducted on a larger sample with higher DHA dosages. Since placentation is already influenced by DHA in the first trimester of pregnancy, it is advisable to conduct a study of DHA supplementation from the beginning of pregnancy [35]. Regarding the timing of supplementation we postulate that the effect of DHA, which improves the clinical outcome, is to prevent defective deep placentation. Therefore, the more early in pregnancy the DHA starts, the greater the effect on improving placentation is, making it possible to achieve a significant improvement in clinical outcomes. A limitation of our study was the lack of assessment of the effects of DHA supplementation on placentogenesis using placental bed biopsies, which so far has not been studied [35].

6. Conclusions

Cord blood DHA levels were significantly higher among the women who received DHA supplementation in comparison with the controls. We did not find an association between DHA and proliferation process in the placenta. The finding of reduced caspase 3 activity furnishes evidence for confirming the association of DHA supplementation with inhibition of placental apoptosis. The intensity of apoptosis was correlated with DHA levels in venous cord blood, which suggests an influence of this compound on the incidence of disorders of pregnancy associated with excessive apoptosis in cases of placental failure. Further research is necessary to more precisely determine the role of DHA in placenta-related disorders of pregnancy.

Disclosure

The study was conducted as part of statutory original research of the Faculty of Medicine, Medical University of Lublin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Krill Oil Ameliorates Mitochondrial Dysfunctions in Rats Treated with High-Fat Diet

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In recent years, several studies focused their attention on the role of dietary fats in the pathogenesis of hepatic steatosis. It has been demonstrated that a high-fat diet is able to induce hyperglycemia, hyperinsulinemia, obesity, and nonalcoholic fatty liver disease. On the other hand, krill oil, a novel dietary supplement of n-3 PUFAs, has the ability to improve lipid and glucose metabolism, exerting possible protective effects against hepatic steatosis. In this study we have investigated the effects of krill oil on mitochondrial energetic metabolism in animals fed a high-fat diet. To this end, male Sprague-Dawley rats were divided into three groups and fed for 4 weeks with a standard diet (control group), a diet with 35% fat (HF group), or a high-fat diet supplemented with 2.5% krill oil (HF+KO group). The obtained results suggest that krill oil promotes the burning of fat excess introduced by the high-fat diet. This effect is obtained by stimulating mitochondrial metabolic pathways such as fatty acid oxidation, Krebs cycle, and respiratory chain complexes activity. Modulation of the expression of carrier proteins involved in mitochondrial uncoupling was also observed. Overall, krill oil counteracts the negative effects of a high-fat diet on mitochondrial energetic metabolism.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition in which fat accumulates in the cells of liver. It occurs when the rate of hepatic fatty acid uptake from plasma and *de novo* lipogenesis is greater than the rate of fatty acid oxidation and export. Traditionally, NAFLD is a histological disease which progresses from pure fatty liver (simple steatosis) through nonalcoholic steatohepatitis (NASH) to liver fibrosis, cirrhosis, and eventually hepatocellular carcinoma.

Several evidences indicate that hepatic mitochondrial dysfunction is crucial to the pathogenesis of NAFLD [1–3]. However, the whole spectrum of mitochondrial adaptations during hepatic steatosis remains to be characterized. A recent study [4] proposed a model describing the development of NAFLD in which overflow of free fatty acids to hepatocytes leads to an increase in mitochondrial energetic metabolism. This seems necessary to supply the energy needed for triglycerides storage. Later, mitochondrial oxidative stress, morphological abnormalities, and finally hepatocytes apoptosis lead to the development of the second hit of steatosis.

As liver fat accumulation can originate from dietary intake, it is of critical importance to understand how different diets and their macronutrient composition can influence the development of NAFLD. Despite contradictory results regarding the role of different diets on NAFLD, it is reasonable to propose that overconsumption of either fat or carbohydrates may promote the development of NAFLD [5]. It seems that specific fatty acids or carbohydrates are more prone to induce or improve NAFLD. Indeed, diets rich in fatty acids, particularly saturated, or in refined carbohydrates, can actually exacerbate NAFLD [5].

Interestingly, it has been demonstrated that dietary fats not only influence the pathogenesis of NAFLD or NASH, but also may prevent or reverse its expression [6]. Therefore, a condition of fatty liver was induced in rodent animal models by the use of high-fat (HF) diets [2, 7] whereas supplementation of the diet with n-3 PUFAs helped in the prevention

and/or in the treatment of steatotic liver [2]. Moreover, there is much stronger evidence that polyunsaturated oils are responsible for the progression from simple steatosis to NASH and these effects may result from a high intake of total PUFAs or may result from a high ratio of n-6 to n-3 fatty PUFAs [8].

In this context, it has been reported that a dietary krill oil (KO) supplementation has the capacity to reduce fatty liver in mice and rats [2, 9, 10]. Recently, it has been also demonstrated that this oil stimulated the catabolization of fat excess introduced by hypercaloric diets, while inhibiting *de novo* fatty acid synthesis and therefore preventing the onset of fatty liver [2, 10].

KO is a novel dietary supplement extracted from Antarctic krill (*Euphausia superba*), a shrimp-like zooplankton, which has become increasingly popular during the last decade. This oil contains two n-3 PUFAs, eicosapentaenoic acid (EPA, 20 : 5) and docosahexanoic acid (DHA, 22 : 6), in amounts similar to those present in fish oil. However, in KO these long-chain n-3 fatty acids are present in the form of phospholipids rather than triglycerides as in the case of fish oil [11]. Furthermore, the ratio of EPA to DHA present in KO is higher than that in fish oil. It has been proposed that these peculiar characteristics of KO composition render it more efficient than fish oil in the modulation of activity and expression of many enzymes involved in lipid metabolism in animal models [10, 12].

In this study we have investigated the molecular mechanism by which KO exerts a possible protective effect on mitochondrial energetic metabolism in animals fed a diet enriched in fat. To this end, we have thoroughly analyzed mitochondrial respiration by monitoring oxygen consumption and the activity of the single respiratory chain complexes in mitochondria. The expression of proteins related to the respiratory chain function was also investigated. The obtained results led us to depict a possible framework for the protective effects of KO against hepatic steatosis.

2. Materials and Methods

2.1. Materials. Bio-Rad protein assay kit was purchased from Bio-Rad; sodium pyruvate, malic acid, succinic acid, ascorbic acid, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), palmitoyl-L-carnitine, rotenone, antimycin A, oligomycin, coenzyme A trilithium salt (CoA-SH), acetyl-CoA, oxaloacetic acid, thiamine pyrophosphate (TPP), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,6-dichlorophenolindophenol (DCPIP), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (CCCP), phosphoenolpyruvate (PEP), ADP, ATP, NAD^+, NADH, NADPH, KCN, pyruvate kinase, lactate dehydrogenase, cytochrome *c*, and decylubiquinone were from Sigma; KO was a generous gift of Aker BioMarine ASA (Oslo, Norway). Antibodies against AAC and UCP2 were from Santa Cruz Biotechnology (sc-11433 and sc-6526); antibodies against OXPHOS proteins were from Mitosciences (ab110413). Kits for the assay of triglycerides and total cholesterol were purchased from Futura System. Plasma insulin concentration was analyzed with a Mercodia Ultrasensitive Mouse Insulin kit. Luciferase ATP assay kit was from Sigma and Lipid Hydroperoxide (LPO) assay kit was from Merck. All other reagents were of analytical grade.

2.2. Ethics Statement. This study was carried out in strict accordance with the European Committee Council 106 Directive (86/609/EEC) and with the Italian animal welfare legislation (art 4 and 5 of D.L. 116/92). The Italian Ministry of Health specifically approved this study.

2.3. Animals and Diets. Male Sprague-Dawley rats (70–100 g) were obtained from Harlan (Carezzana, Italy) and housed individually in animal cages at a temperature of 22 ± 1°C with a 12 : 12 hour light-dark cycle and 30–40% humidity. After 1 week of acclimatization, 15 rats were divided into three groups of 5 animals each. The first group (control group) received a standard diet, also known as standard rodent chow, containing 6.2% fat and 44.2% carbohydrates (Global Diet 2018S from Harlan Teklad). The second group received a diet with a higher content (35%) of fat (and therefore referred to as HF group) and 36.1% of carbohydrates (Diet TD.03584 from Harlan Teklad). The third group of animals (HF+KO group) was fed with the above reported HF diet supplemented with 2.5% (w/w) KO. Diets composition is described in Table 1. The animals were treated for 4 weeks with *ad libitum* access to diets and water.

At the end of dietary treatment (after 4 weeks), body and liver weight were determined when rats were killed by decapitation, according to the guidelines for the care and use of laboratory animals. For the determination of plasma lipids, glucose, and insulin, control and treated rats were starved overnight before killing. Homeostasis model assessment (HOMA) index was calculated according to the following formula: HOMA = (fasting glucose (mM) × fasting insulin (μU/mL))/22.5 [13].

Liver lipids were extracted and quantified as described in [14].

2.4. Assay of Enzymes Involved in Glucose and Fatty Acid Oxidation. Rat liver mitochondria were prepared by standard procedures and protein concentration was determined by the Bradford method [15].

Mitochondria (0.25 mg of mitochondrial protein/mL) were incubated in a buffer containing 25 mM MOPS and 0.05% Triton X-100 at pH 7.4. Solubilization of mitochondria with 0.05% Triton X-100 inhibited complex I of the respiratory chain preventing consumption of NADH. Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically as the rate of NAD^+ reduction to NADH upon addition of 2.5 mM pyruvate, 0.1 mM CoA-SH, 0.2 mM TPP, 1 mM NAD^+, and 5 mM $MgCl_2$ (pH 7.4) [16].

Citrate synthase (CS) activity was determined in a medium containing 100 mM Tris-HCl (pH 8.1), 0.1 mM DTNB, and 0.25 mM acetyl-CoA. The reaction was started by adding 0.25 mM oxaloacetate, followed by monitoring changes at 412 nm for 3 min [17].

Total carnitine palmitoyl-CoA transferase (CPT) and CPT I activity was determined spectrophotometrically at

Table 1: Composition of diets (%).

	Control	HF	HF+KO
Proteins	18.6	20.4	19.9
Lipids	6.2	35.2	36.8
Fatty acids			
14:0	—	0.5	0.6
16:0	0.7	8.7	9.0
18:0	0.2	4.3	4.3
16:1	—	—	0.1
18:1 (n9-7-5)	1.2	15.8	16.0
18:2 (n6)	3.1	3.5	3.5
18:3 (n3)	0.3	—	—
20:5 (n3) EPA	—	—	0.3
22:6 (n3) DHA	—	—	0.2
$\sum$ SFA	0.9	13.5	13.9
$\sum$ MUFA	1.3	15.8	16.1
$\sum$ PUFA	3.4	3.5	4.1
$\sum$ PUFA n-3	0.3	—	0.6
Carbohydrates	44.2	36.1	35.2
kcal/100 g	310	540	549
Calories from protein (%)	24.0	15.0	14.0
Calories from fat (%)	18.0	58.0	60.0
Calories from carbohydrates (%)	58.0	27.0	26.0

The control group of animals received a standard diet (Global Diet 2018S from Harlan Teklad). The HF group received a diet with 35% fat (Diet TD.03584 from Harlan Teklad) and the KO group was fed with the above reported HF diet supplemented with 2.5% (w/w) KO. Fatty acids were extracted from the three diets and analyzed by gas-liquid chromatography. Energy content was calculated using 4 kcal/g for protein and carbohydrate and 9 kcal/g for lipid.

412 nm in freshly isolated rat liver mitochondria, essentially as described previously [18].

2.5. Mitochondrial Respiration Efficiency. Mitochondrial respiration (0.3 mg of mitochondrial protein/mL) was measured in a medium consisting of 220 mM sucrose, 20 mM KCl, 2.5 mM KH_2PO_4, 1 mM EDTA, 20 mM Hepes, 5 mM $MgCl_2$, and 0.1% BSA, pH 7.4, by a Clark oxygen electrode at 25°C [2]. The addition of different substrates permitted to evaluate mitochondrial respiration when respiratory complexes I (5 mM pyruvate, 2.5 mM malate), II (5 mM K-succinate, 5 μM rotenone), and IV (10 mM ascorbate, 0.2 mM TMPD, 5 μM rotenone, 5 μM antimycin A) were stimulated [19, 20]. Instead, the addition of 40 μM palmitoyl-L-carnitine and 2.5 mM malate permitted revealing oxygen consumption from β-oxidation pathway.

For each substrate, after 2 min, state 3 respiration was induced by the addition of 0.3 mM ADP. Respiratory control ratio (RCR) was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (state 3) to the rate observed when added ADP had been completely phosphorylated to ATP (state 4).

2.6. Assay of Mitochondrial Respiratory Complex Activity. Complex I activity was determined using decylubiquinone as electron acceptor and NADH as donor. Rat liver mitochondria (40 μg of protein) were pretreated first by freeze-thawing the samples two or three times in hypotonic medium consisting of 25 mM KH_2PO_4 (pH 7.2), 5 mM $MgCl_2$ followed by a hypotonic shock in H_2O to disrupt mitochondrial membranes. The reaction started after the addition of 50 mM Tris (pH 8.0) medium supplemented with 5 mg/mL BSA, 0.8 mM NADH as donor, 240 μM KCN, 4 μM antimycin A, and 50 μM of the acceptor decylubiquinone. The oxidation of NADH was measured following the decrease in absorbance at 340 nm for 3 min [20].

Complex II activity assay was performed following the decrease in absorbance at 600 nm resulting from the reduction of DCPIP as electron acceptor and using succinate as donor. Rat liver mitochondria (40 μg of protein) were pretreated first by freeze-thawing following by an incubation with succinate 10 min at 30°C in a medium containing 10 mM KH_2PO_4 (pH 7.8), 80 μM DCPIP as acceptor, 4 μM rotenone, and 0.2 mM ATP. The reaction started by addition of 80 μM decylubiquinone and the activity was measured for 5 min [20].

Complex III activity was determined by measuring the reduction of oxidized cytochrome *c* at 550 nm. Rat liver mitochondria (40 μg protein) were incubated for 1 min at 30°C in a reaction medium containing 50 mM KH_2PO_4 (pH 7.2), 0.01% Tween-20, 50 mM EDTA, 4 mM KCN, and 40 mM oxidized cytochrome *c*. The reaction was initiated by adding the ubiquinol analog, decylubiquinol (DBH_2), to a final concentration of 50 μM, and the rate of cytochrome *c* reduction was calculated from the absorbance increase at 550 nm [2].

Complex IV activity assay was performed following the decrease in absorbance at 550 nm resulting from the oxidation of reduced cytochrome *c*. Rat liver mitochondria (20 μg of protein) were incubated in 1 mL of isosmotic medium containing 250 mM sucrose, 10 mM KH_2PO_4 (pH 6.5), and 10 μM reduced cytochrome *c*. Cytochrome *c* solution was freshly reduced by adding some crystals of sodium dithionite. The addition of 2.5 mM lauryl maltoside permitted permeabilization of the outer mitochondrial membrane to cytochrome *c* and the decrease in absorbance at 550 nm was followed for 3 min [20].

ATP-synthase activity (ATP hydrolysis) was measured spectrophotometrically by a coupled assay using lactate dehydrogenase and pyruvate kinase as the coupling enzyme [20]. For each ATP molecule that is hydrolyzed, a molecule of NADH is oxidized. Rat liver mitochondria were incubated first in distilled water for 30 s at 37°C and then in a medium containing 200 μL of 50 mM Tris (pH 8.0), 5 mg/mL BSA, 20 mM $MgCl_2$, 50 mM KCl, 15 μM CCCP, 5 μM antimycin A, 10 mM PEP, 2.5 mM ATP, 4 units of lactate dehydrogenase and pyruvate kinase, and 1 mM NADH, previously incubated for 5 min at 37°C. Reaction was started by the addition of the medium to mitochondria and monitored for 3 min. Finally, the addition of 3 μM oligomycin permitted distinguishing the ATPase activity coupled to the respiratory chain [20].

2.7. Western Blot Analysis. The expression of oxidative phosphorylation system (OXPHOS) proteins and mitochondrial

Table 2: Body and liver weight, serum parameters, and liver fat content.

	Control	HF	HF+KO
Body weight (g)	245.8 ± 7.8	277.2 ± 15.8*	235.2 ± 25.2#
Liver weight (g/100 g body weight)	3.9 ± 0.6	3.5 ± 0.5	4.1 ± 0.3
Glycemia (mM)	2.9 ± 0.1	4.9 ± 0.7*	4.1 ± 0.4*#
Insulin (μU/mL)	50.1 ± 7.5	142.8 ± 10.0*	77.7 ± 5.0*#
HOMA	6.4 ± 0.1	31.2 ± 0.3*	14.2 ± 0.1*#
Serum triglycerides (mg/dL)	137.3 ± 12.9	147.1 ± 8.8	138.6 ± 9.2
Serum cholesterol (mg/dL)	92.6 ± 8.3	95.6 ± 4.2	94.1 ± 6.2
Liver triglycerides (mg/g liver)	8.6 ± 0.4	13.6 ± 1.0*	8.3 ± 0.5#
Liver cholesterol (mg/g liver)	2.7 ± 0.4	6.6 ± 0.4*	3.5 ± 0.5#

Each point represents the mean ± SD for 5 animals. * $P < 0.05$ versus rats fed a control diet; # $P < 0.05$ versus rats fed HF diet.

carrier proteins AAC (ADP/ATP carrier) and UCP2 (uncoupling protein 2) was determined by western blotting analysis. Polyacrylamide gel electrophoresis was performed in the presence of 0.1% SDS (SDS-PAGE) according to standard procedures. The mitochondrial proteins that have been separated by SDS-PAGE were transferred to a nitrocellulose membrane. For the OXPHOS proteins, a cocktail directed against NDUFB8 (Complex I), SDHB (Complex II), COR2 (Complex III), COX1 (Complex IV), and ATP5A (ATP-synthase) was used at a dilution $1:1\times10^{1}$ and the immunoreaction was carried out with monoclonal antibody by chemiluminescence. For AAC, UCP2, and porin (a protein used as a control) detection, antisera were used at a dilution of $1:1\times10^{3}$ and the immunoreaction was carried out with polyclonal antibody by chemiluminescence.

2.8. ATP and Lipid Hydroperoxide (LPO) Levels. ATP levels in liver homogenates were determined by using the luciferase ATP assay kit. The concentration of ATP in the samples was calculated from interpolation of a standard curve prepared with known amounts of ATP. Results were expressed as nmoles of ATP per mg of protein.

Lipid peroxidation levels in liver samples were determined using a lipid hydroperoxide assay kit which measures the redox reactions with ferrous ions. LPO content was expressed as LPO per mg of protein.

2.9. Statistical Analysis. Experimental data represent the means ± SD. The data were analyzed by one-way ANOVA and a Tukey-Kramer *post hoc* analysis was used to detect significant differences between the means at a level of $P > 0.05$.

3. Results

3.1. Body and Liver Weight, Serum Parameters, and Liver Fat Content. As shown in Table 2, a significant increase in body weight was recorded in HF animals after 4 weeks of dietary treatment, in agreement with the high caloric content of the diet. Interestingly, the supplementation of the HF diet with 2.5% KO significantly prevented this effect. Instead, no statistically significant variations were found in the liver weight of animals fed with the different experimental diets.

The blood glucose concentration increased in both HF and HF+KO animals after 4 weeks of dietary administration, in comparison to control rats. Such an increase was less evident in the case of the HF+KO fed animals. A massive increase in the levels of insulin was revealed in the plasma of HF animals, in comparison to control rats. Interestingly, the KO supplementation significantly mitigated this increase. HF rats showed a marked increase in HOMA index, demonstrating typical insulin resistance induced by the high-fat diet. In the HF+KO animals, HOMA decreased, showing reduced insulin resistance induced by the addition of KO.

No significant difference was detected in the serum lipid (triglyceride and cholesterol) levels between the three groups of animals. On the contrary, liver lipid content was dramatically enhanced in HF animals, thus reflecting the presence of hepatic steatosis (Figure 1). The supplementation with KO of the HF diet reversed this effect, thereby keeping the lipid concentrations similar to those found in control rats. Accordingly, the liver histologic examination revealed a beginning of microvesicular fat depositions only in HF rats.

3.2. Pyruvate Dehydrogenase (PDH) Complex, Carnitine Palmitoyltransferase (CPT), and Citrate Synthase (CS) Activity. Mitochondria play a central role in energy production. Sugars and fatty acids undergo glycolysis and mitochondrial β-oxidation, respectively, to produce acetyl-CoA (Figure 2(a)). PDH complex activity is the major determinant of glucose oxidation in animal cells. We found that PDH activity remained unchanged in the three groups of animals, suggesting that glucose catabolism was not affected by a HF diet or by KO supplementation (Figure 2(b)).

The limiting step for catabolic pathway of fatty acid oxidation is represented by the activity of CPT I, which is responsible for the entry of fatty acids into the mitochondrial matrix, where β-oxidation of fatty acids occurs. As shown in Figure 2(c), in the HF animals a clear decrease (about 40%) in the CPT I activity was detected. On the contrary, a strong increase in CPT I activity was found in the HF+KO rats. Thus, KO supplemented to the HF diet strongly stimulates fatty acid oxidation.

Acetyl-CoA, generated essentially from glucose or from fatty acids, is channeled into the Krebs cycle, where CS (the rate-limiting enzyme of the Krebs cycle) catalyzes its

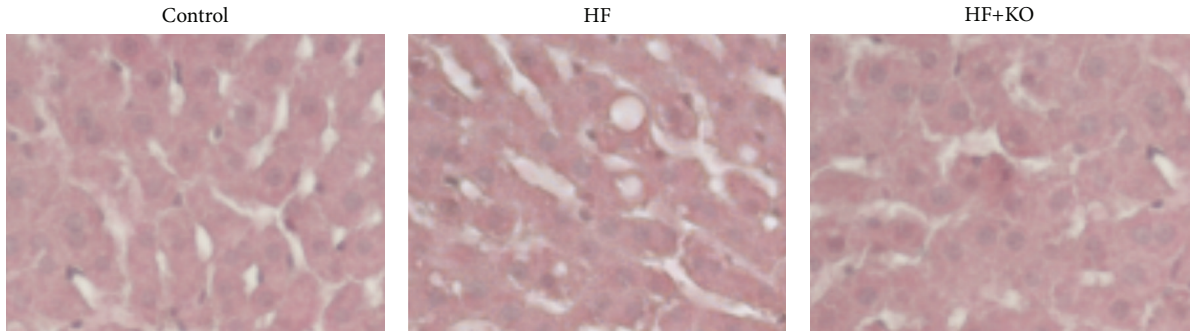

FIGURE 1: Liver samples were stored in neutral formaldehyde and embedded in paraffin wax. Sections (15 μm) were stained with hematoxylin and eosin.

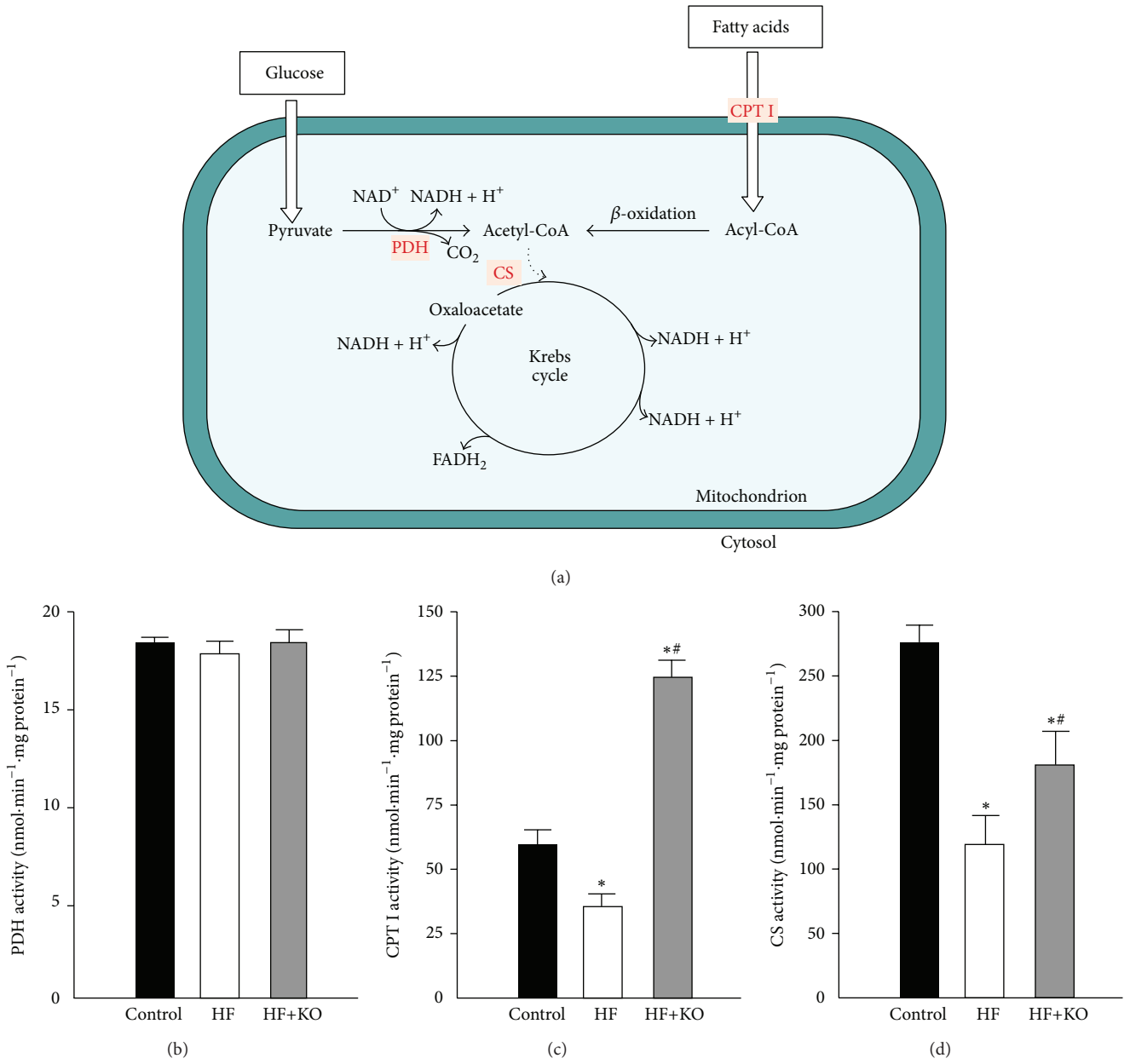

FIGURE 2: Mitochondrial metabolic pathways from glucose and fatty acid oxidation. Schematic picture of the metabolic pathways from glucose and fatty acid oxidation (a). Effect of KO on the activity of mitochondrial enzymes PDH (b), CPT I (c), and CS (d) in control, HF, or HF+KO-fed rats liver. The values reported in the figure represent the means ± SD ($n = 5$). $^{*}P < 0.05$ versus rats fed a control diet; $^{\#}P < 0.05$ versus rats fed a HF diet.

Table 3: Mitochondrial respiratory efficiency.

		Control	HF	HF+KO
Palmitoyl-L-carnitine + malate (β oxidation)	V_3 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	85.8 ± 7.8	59.7 ± 8.5*	114.0 ± 11.2*#
	V_4 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	14.3 ± 2.4	22.9 ± 1.5*	18.5 ± 5.2*#
	RCR	6.0 ± 0.4	2.6 ± 0.3*	6.1 ± 0.8#
Pyruvate + malate (Complex I)	V_3 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	17.0 ± 0.3	15.9 ± 0.4*	19.9 ± 0.9*#
	V_4 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	5.4 ± 0.1	7.6 ± 0.2*	6.6 ± 0.6*#
	RCR	3.2 ± 0.3	2.1 ± 0.3*	3.0 ± 0.4#
Succinate + rotenone (Complex II)	V_3 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	61.5 ± 3.3	53.1 ± 3.7*	78.3 ± 4.0*#
	V_4 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	10.1 ± 0.3	25.0 ± 1.2*	15.0 ± 1.9*#
	RCR	6.1 ± 0.9	2.1 ± 0.8*	5.2 ± 1.0#
Ascorbate + TMPD + rotenone + antimycin A (Complex IV)	V_3 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	55.1 ± 5.3	47.5 ± 6.5*	68.6 ± 7.8*#
	V_4 (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$)	9.3 ± 0.7	17.2 ± 0.9*	12.8 ± 0.8*#
	RCR	5.9 ± 0.6	2.8 ± 0.3*	5.4 ± 0.9#

Respiratory control ratio (RCR) was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (V_3) to the rate observed when added ADP had been completely phosphorylated to ATP (V_4).
*$P < 0.05$ versus rats fed control diet; #$P < 0.05$ versus rats fed HF diet.

condensation with oxaloacetate to synthesize citrate (Figure 2(a)). A significant decrease (about 55%) in the activity of the CS was found in HF animals (Figure 2(d)), while only a moderate decrease (about 35%) was observed in HF+KO animals in comparison to the control groups. The partial reversal of CS inhibition observed in HF animals after KO supplementation could be explained by a compensatory response of this enzyme to sustain oxidation of acetyl-CoA generated from fatty acid oxidation.

3.3. Mitochondrial Oxygen Consumption, Respiratory Chain Complex Activities, and ATP Synthesis. The increased fatty acid oxidation observed in HF+KO animals produces higher levels of reducing equivalents, which are normally addressed towards the mitochondrial oxidative phosphorylation for ATP production. We therefore analyzed the respiratory efficiency of freshly isolated mitochondria by oxygraphic methods. Mitochondrial oxygen consumption was measured with various substrates in the presence (state 3) or the absence (state 4) of ADP (Table 3). According to the results shown in Figure 2(c), when we used palmitoyl-L-carnitine and malate as substrates, V_3 (mitochondrial respiration state 3) decreased in HF animals thus indicating lower mitochondrial β-oxidation. On the other hand, the increase in the V_4 (mitochondrial respiration state 4) values suggests that the excess of dietary fat most probably is the cause of a partial uncoupling between respiration and phosphorylation in mitochondria. Interestingly, the dietary KO supplementation is able to efficiently abolish this effect, keeping the mitochondrial respiratory efficiency unaltered.

When we added respiratory substrates for mitochondrial complexes I, II, and IV (Table 3), a little yet significant decrease in V_3 was observed in the HF groups in comparison to control animals. In the HF+KO animals a significant increase in the V_3 values was found in comparison to HF and control animals. V_4, also known as the resting state of respiration, showed an increase in the HF and HF+KO group, even if the increase observed after KO supplementation was less evident than that found in HF animals. As a consequence, the RCR values were profoundly and significantly lower in HF animals with respect to those calculated for control animals. The KO supplementation of the HF diet reversed this effect, thereby keeping RCR values similar to those calculated for control group.

In a more selective approach for investigating the functionality of the mitochondrial oxidative phosphorylation we assayed the activity of single components of the respiratory chain: complexes I–IV and ATP-synthase (Figure 3(a)). A significant decrease (about 30%) in the activity of all respiratory chain complexes was found in HF animals. On the contrary, the values of complexes activity of the HF+KO rats were practically identical to those measured in control animals.

Surprisingly, western blotting analysis of the OXPHOS proteins showed significantly higher protein levels in both HF and HF+KO animals compared to the control group (Figures 3(b) and 3(c)). Such an increase was more evident for complexes I, III, and IV, which are associated as supercomplexes in the mitochondrial inner membrane [21]. This effect could be determined by a compensatory mechanism related to the reduction of the activities of the respiratory complexes.

In order to prove biological relevance of alteration in mitochondrial respiratory chain activity, we measured ATP concentration and oxidative damage in liver samples from the three groups of animals (Figure 3(d)). ATP quantification demonstrated that a HF diet caused a slight but not significant decrease in ATP content, an effect that was rescued by KO. Interestingly, in parallel experiments, we found a significant increase (about 30%) in the LPO levels in HF animals and this effect was efficiently reversed by KO supplementation of the HF diet.

3.4. Expression of Proteins Related to the Respiratory Chain Function. Uncoupling protein 2 (UCP2) uncouples respiration from oxidative phosphorylation. We therefore analyzed the expression profile of this carrier protein in the three groups of animals (Figure 4(a)). After 4 weeks of HF dietary

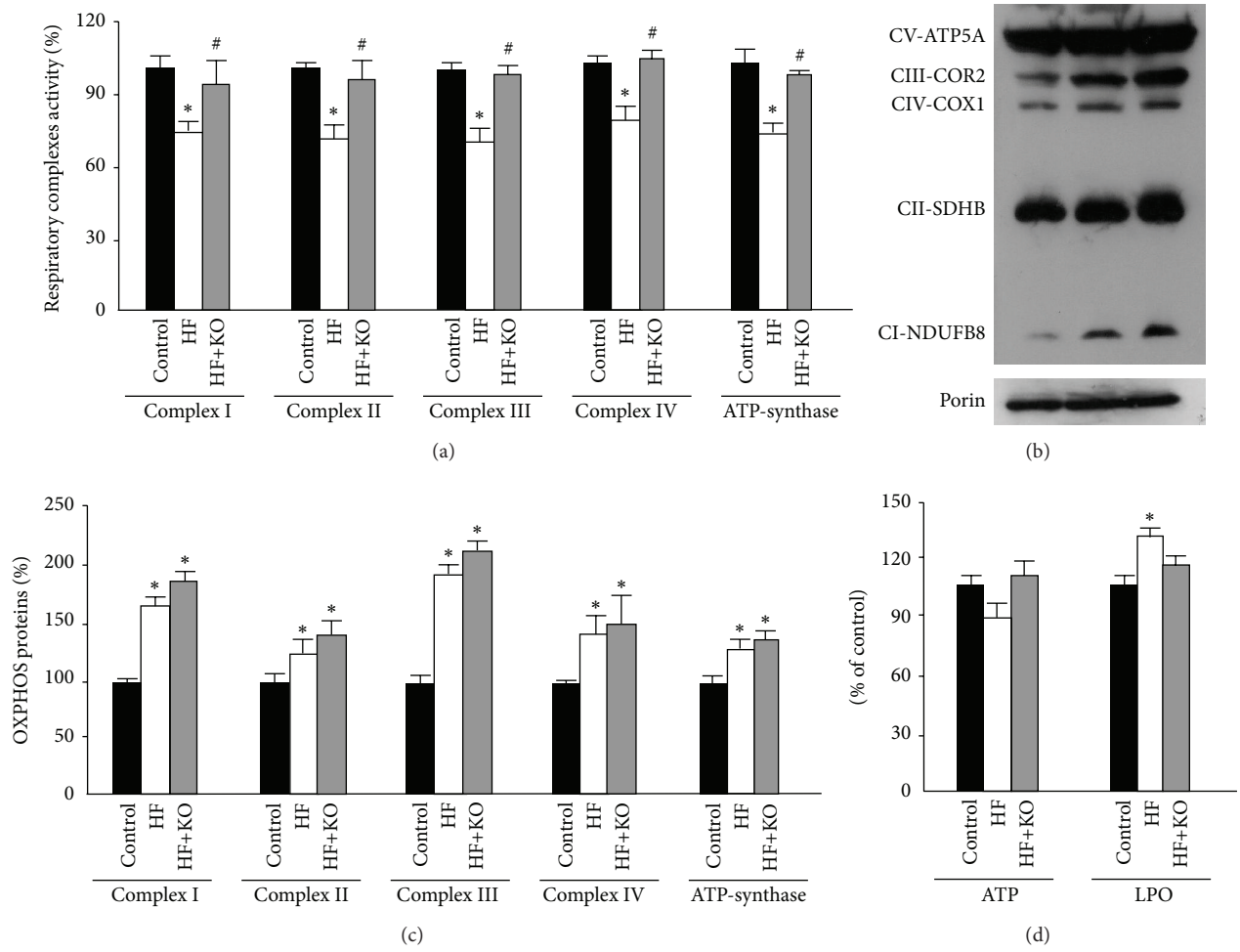

Figure 3: Effect of KO on respiratory complexes activity, expression, and function. Effect of KO on respiratory complexes activity (a). Enzymatic activities measured in the control group were set to 100%. Effect of KO on OXPHOS proteins levels (b). Liver mitochondrial proteins from control, HF, or HF+KO-fed rats were separated by SDS-PAGE, transferred to nitrocellulose, and then immunodecorated with a cocktail against NDUFB8, SDHB, COR2, COX1, ATP5A, or the mammalian porin as a control. The amount of OXPHOS proteins revealed by immunodecoration in the control group was set to 100% (c). ATP and LPO quantification of liver samples isolated from animals fed a control diet, a HF diet, or a HF+KO (d). The amount of ATP or LPO (expressed as nmoles per mg protein) in the control group was set to 100%. The values reported in the figure represent the means ± SD ($n = 5$). $^{*}P < 0.05$ versus rats fed control diet; $^{\#}P < 0.05$ versus rats fed HF diet.

treatment the amount of this protein increased about 5-fold, compared to the control group. Interestingly, a significant smaller increase in the expression of UCP2 was found after KO supplementation. In fact, in the presence of KO in the HF diet the level of this carrier protein was about 50% of that found in HF rats.

The AAC (ADP/ATP carrier) is another mitochondrial carrier which is able to regulate the basal proton conductance. This protein exchanges ADP for ATP across the mitochondrial inner membrane and may also play an important role in the mitochondrial permeability transition pore. When we analyzed the expression of this carrier protein (Figure 4(b)), we found an increase of about 2.2-fold in HF animals compared to the control group. AAC levels in the HF+KO animals, even if higher than those found in the control rats, were significantly lower than those found in HF group. The amount of porin, an outer membrane protein tested as a control, did not change in any group of animals (Figure 4(c)).

These results suggest that the KO supplementation is able to modulate the expression of mitochondrial carrier protein involved in the regulation of proton conductance, reducing the uncoupling effect caused by a HF diet.

4. Discussions

In animal experiments, a diet rich in fat or carbohydrates has been shown to induce hyperglycemia and hyperinsulinemia and to contribute to the development of obesity and NAFLD [2, 6, 7, 22]. This last condition is recognized as the most common chronic liver disease in the Western world. NASH is a more severe form of NAFLD that is broadly defined by the presence of steatosis with inflammation and progressive

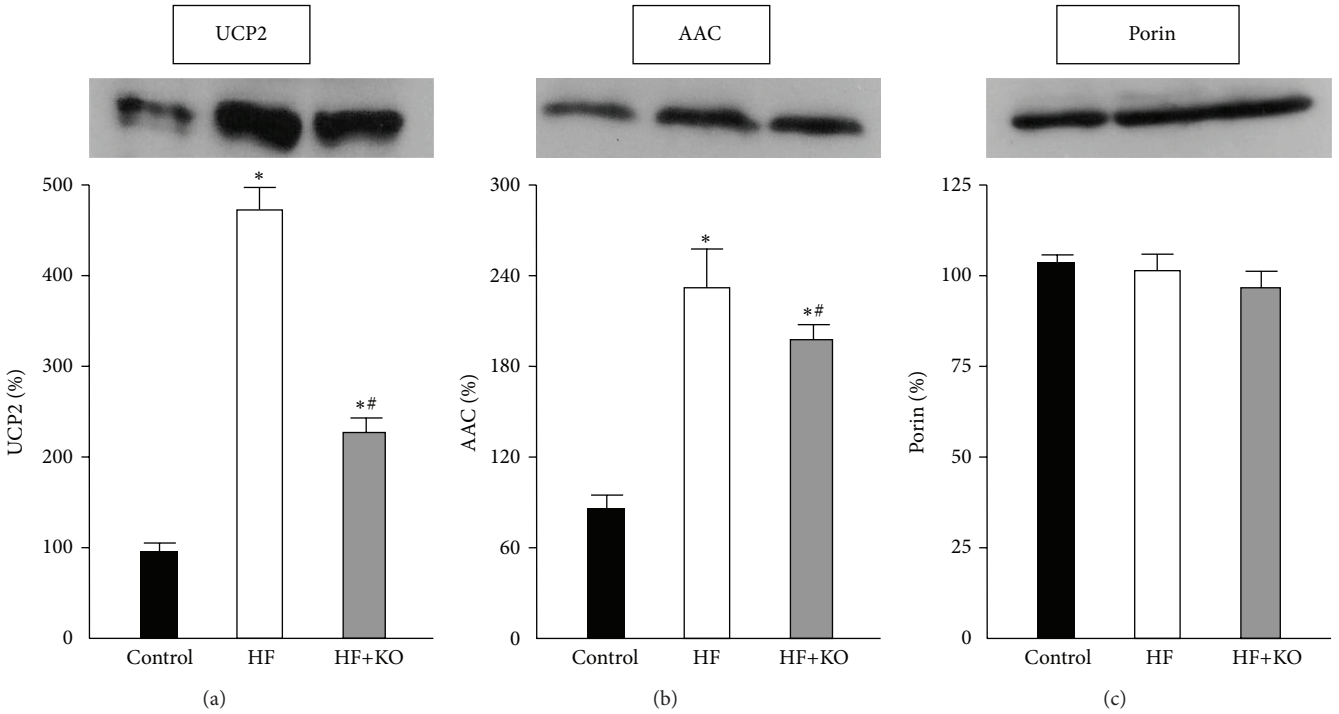

Figure 4: Effect of KO on proteins related to the respiratory chain function. Effect of KO on proteins levels of UCP2 (a) and AAC (b). The amount of porin, an outer membrane protein, was tested as a control (c). Liver mitochondrial proteins from control, HF, or HF+KO-fed rats were separated by SDS-PAGE, transferred to nitrocellulose, and then immunodecorated with antisera against UCP2, AAC or the mammalian porin. The amount of proteins revealed by immunodecoration in the control group was set to 100%. The values reported in the figure represent the means ± SD ($n = 5$). $^{*}P < 0.05$ versus rats fed control diet; $^{\#}P < 0.05$ versus rats fed HF diet.

fibrosis, leading then to cirrhosis and hepatocellular carcinoma. Some patients with NAFLD develop NASH through poorly understood mechanisms. Since NAFLD and NASH are considered lifestyle-associated diseases, diet intervention is an important approach to their treatment.

In this context it is important to underline that the "obesogenic" Western diet has a ratio of fatty acids and carbohydrates of approximately 50/50. Moreover, it is characterized by an insufficient n-3 PUFAs consumption and a high n-6/n-3 ratio, which are associated with the progression from simple steatosis to NASH [8] along with modifications of gut microbiota [23].

Although the mechanisms responsible for increased lipid accumulation in the liver are not yet fully elucidated, decreased capacity to oxidize fatty acids, increased transport of fatty acids to liver, and increased hepatic fatty acid synthesis seem to be important factors involved in the pathogenesis of NAFLD [2, 6, 7, 24]. Moreover, abnormalities in mitochondrial morphology and functions seem to be associated with fatty liver [1–3, 25–27]. This finding suggests that regulation of mitochondria function and of molecular mechanisms involved in lipid accumulation in the liver could provide useful targets to prevent the development of NAFLD.

In recent years, the use of dietary supplements has rapidly increased [2, 6, 10, 24, 28] with the aim to prevent NAFLD. KO, a dietary supplement of n-3 PUFAs, has become increasingly popular because of its capability of reducing hepatic lipogenesis and of stimulating catabolization of excess fat introduced by a HF diet [2, 10]. The analysis of hepatic transcriptome in mice demonstrated that KO supplementation was able to regulate genes involved in hepatic energy metabolism, including glucose metabolism, lipid biosynthesis, fatty acid metabolism, cholesterol biosynthesis, and mitochondrial electron transport, more efficiently than fish oil [10, 12]. These differential results were tentatively ascribed to the influence of the structural form of n-3 PUFAs, esterified to either phospholipids or triglycerides, on a biological response. Moreover, PUFAs as phospholipids (KO) or triglycerides (fish oil) may differentially influence gut microbiota or synthesis of bacterial metabolites.

A previous study by our group [2] suggested that KO supplementation to a HF diet resulted in a retention of normal mitochondrial respiration efficiency, which was impaired in HF animals. The aim of the present study was therefore to analyze the molecular mechanisms by which dietary fats influence mitochondrial energetic metabolism. This new and intriguing aspect was evaluated in hepatocytes from rats fed a HF diet or a HF diet supplemented with 2.5% KO, with the aim to investigate the role of mitochondria in the modulation of nutrient metabolism exerted by KO.

No change in PDH activity, which essentially produces acetyl-CoA from carbohydrate, was seen after administration of HF or HF+KO diets. Nevertheless, fasting serum glucose and insulin were higher after feeding a HF diet, suggesting

an altered insulin sensitivity as indicated by HOMA index changes. In fact, an excess of energy supplied by fat corresponds to a scarce tissutal utilization of glucose, thereby causing hyperglycemia and hyperinsulinemia. Insulin resistance was reduced after KO supplementation.

CPT I activity, which is involved in fatty acid oxidation, was inhibited by a HF diet and stimulated after KO supplementation. The increase in CPT I activity observed in HF+KO group could be explained by the lower levels of malonyl-CoA, a metabolic intermediate of hepatic lipogenesis, which, in this case, is instead strongly inhibited [2, 10]. Interestingly, the capability of KO to stimulate fatty acid oxidation seems to be associated to the presence of a high lipid content (35%) in the experimental diet used in this study. In fact, when KO was added to a standard diet, the expression of enzymes involved in mitochondrial fatty acid β-oxidation significantly decreased [12].

The modulation of the CPT I activity, observed in HF and HF+KO animals, seems to be accompanied by a parallel modulation of the activity of CS, a Krebs cycle enzyme that catalyzes the synthesis of citrate from oxaloacetate and acetyl-CoA. Recently, a strong and significant correlation of CS with obesity was noted, with a reduced enzyme activity in mitochondria of human omental adipose tissue in the state of obesity [28]. According to this observation, the increase in CS activity revealed after KO supplementation could be explained as a compensatory response to maintain the entry of acetyl-CoA produced by fatty acid oxidation into the Krebs cycle. It is interesting to underline that citrate generated by CS continues in the Krebs cycle. In fact, in animals fed a HF diet supplemented with KO a reduction in the activity and in the expression of citrate carrier protein (CIC), which transports citrate from mitochondria to the cytosol for fatty acid synthesis, was observed [2, 10, 24].

NADH and $FADH_2$ generated by β-oxidation and Krebs cycle are used in the final common oxidative phosphorylation system (OXPHOS) to generate ATP. This process is coupled with the transfer of electrons along the respiratory chain. Our data indicate that a HF diet caused a strong decrease in the mitochondrial respiratory efficiency and this effect could be due to a possible uncoupling effect exerted by the excess of fatty acids present in this diet. In fact, we found a little decrease in V_3 and a strong increase in V_4 values in liver mitochondria isolated from HF animals. V_4 is the resting state of mitochondrial respiration and an increase in this value suggests a stimulus of mitochondrial respiration independent of ADP phosphorylation. In other terms, during HF feeding, a lower coupling between mitochondrial respiration and ATP synthesis occurs. Interestingly, the addition of KO to the HF diet almost completely reversed this effect, thereby leading mitochondria respiration efficiency to be comparable to that of control rats.

When we assayed, in a more selective approach, the activity of single components of the respiratory chain, we observed a significant decrease in the activity of all respiratory chain complexes in HF animals, according to a recent study [29] in which an impairment of mitochondrial function and, in particular, of complex I activity was found. After KO supplementation, the values of complex activities were identical to those measured in control animals. Expression of OXPHOS proteins significantly increased in HF animals compared to the control group and this evidence was accompanied by the presence of ATP levels in HF animals that were only slightly decreased in comparison to those found in control animals. According to this result, previous studies [30, 31] demonstrated that a high fat content in the diet induced higher level of these proteins as a compensatory response to the reduced activity of the respiratory chain complexes. When we performed western blotting analysis in mitochondria isolated from HF+KO animals liver, we found a higher level of the OXPHOS proteins compared to the control and the HF groups, probably caused by the synergistic effect of the HF diet and the KO supplementation. Indeed, it is already known that genes involved in mitochondrial OXPHOS proteins expression were upregulated in mice consuming KO as revealed from the transcriptomic analysis [12]. Overall, this means that KO is able to induce the burning of excess of fat introduced by diet stimulating fatty acid oxidation, Krebs cycle, and respiratory chain complex activity and preventing a condition of oxidative damage.

Uncoupling between mitochondrial respiration and ATP synthesis, observed in HF animals, could be caused by a different expression of mitochondrial carrier proteins involved in the regulation of proton conductance. It is known that uncoupling proteins (UCPs) homologues uncouple mitochondrial respiration from oxidative phosphorylation, increasing thermogenesis while reducing the efficiency of ATP synthesis. In a previous study [32], an increase in the expression of the UCP2 isoform was found in rats with NAFLD. Interestingly, in this study we found a massive increase of this carrier protein in HF animals, but the addition of KO to the diet was able to strongly reduce the expression of hepatic UCP2. Therefore, it is reasonable to assume that the addition of KO to the HF diet can increase the coupling between mitochondrial respiration and ATP synthesis by a downregulation of UCP2 expression.

Not only are UCPs involved in mitochondrial uncoupling, but AAC is another carrier protein of particular interest in the understanding of the mitochondrial energy coupling process [33]. This carrier catalyzes the exchange of ADP^{3-} for ATP^{4-} and therefore utilizes, as its driving force, the electrochemical potential generated across the inner membrane by the respiratory chain. The exchange reaction is electrogenic and driven by the electrical component of the proton gradient, so that entry of ADP into mitochondria and exit of ATP are favored. We can therefore speculate that, if mitochondria are uncoupled and proton gradient was dissipated by the entry of H^+ into the matrix by UCP2, an upregulation of the expression of AAC could compensate a reduced flux of ATP from mitochondria when membrane potential was reduced. This hypothesized compensatory mechanism was observed in animals fed a HF diet. The increase in the expression of AAC was less evident in HF+KO animals, in which liver mitochondria respiratory efficiency was unaltered in comparison to the control animals.

In conclusion, the present study indicates that KO counteracts the negative effects of a HF diet. In particular, our

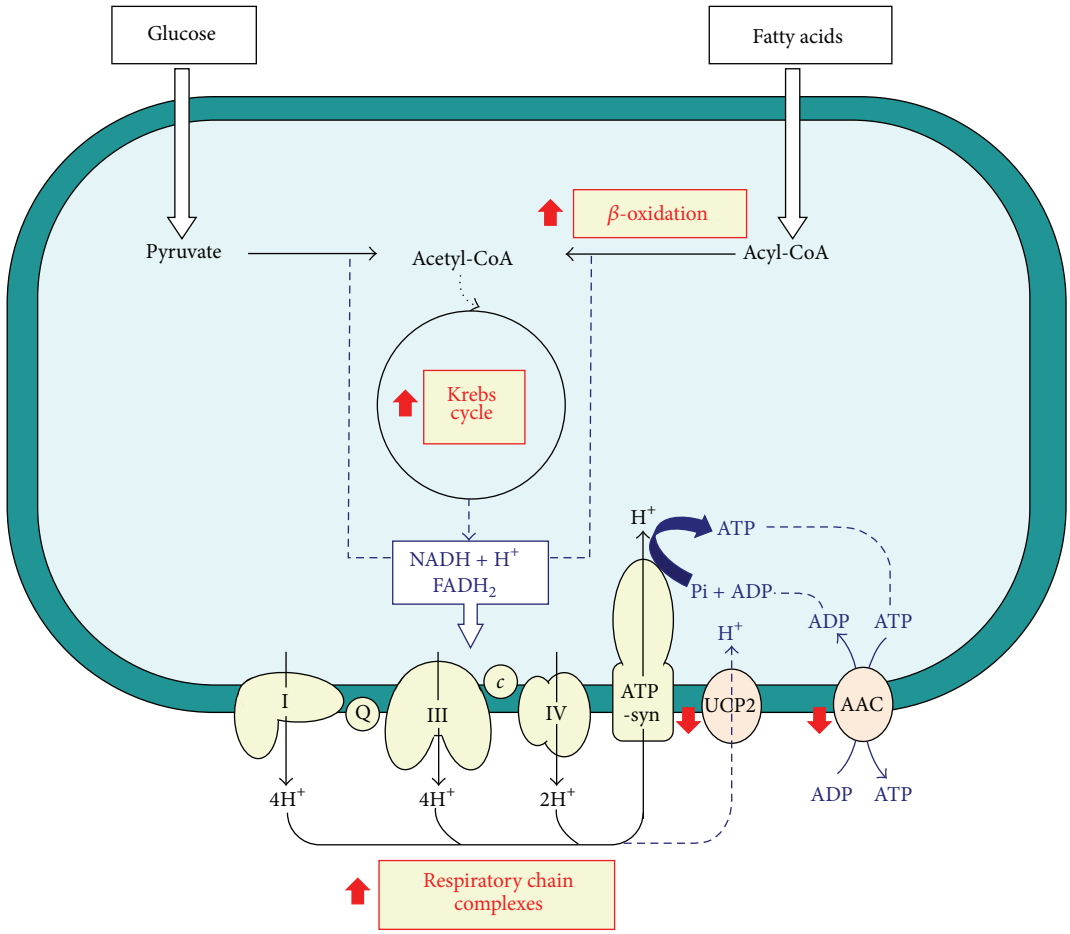

Figure 5: Mitochondrial pathways influenced by the addition of KO to a HF diet. *c*, cytochrome *c*; Q, ubiquinone.

results contribute to elucidate the molecular mechanism (Figure 5) by which KO positively influences many metabolic steps and ameliorates mitochondrial dysfunctions caused by the administration of a hyperlipidic diet, which often characterizes the nutritional habits of western populations. Nevertheless, additional studies in humans are required to confirm this view, since rodents may respond to the dietary macronutrients composition in a different manner than human subjects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Omega-3 Fatty Acids in Early Prevention of Inflammatory Neurodegenerative Disease: A Focus on Alzheimer's Disease

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Alzheimer's disease (AD) is the leading cause of dementia and the most common neurodegenerative disease in the elderly. Furthermore, AD has provided the most positive indication to support the fact that inflammation contributes to neurodegenerative disease. The exact etiology of AD is unknown, but environmental and genetic factors are thought to contribute, such as advancing age, family history, presence of chronic diseases such as cardiovascular disease (CVD) and diabetes, and poor diet and lifestyle. It is hypothesised that early prevention or management of inflammation could delay the onset or reduce the symptoms of AD. Normal physiological changes to the brain with ageing include depletion of long chain omega-3 fatty acids and brains of AD patients have lower docosahexaenoic acid (DHA) levels. DHA supplementation can reduce markers of inflammation. This review specifically focusses on the evidence in humans from epidemiological, dietary intervention, and supplementation studies, which supports the role of long chain omega-3 fatty acids in the prevention or delay of cognitive decline in AD in its early stages. Longer term trials with long chain omega-3 supplementation in early stage AD are warranted. We also highlight the importance of overall quality and composition of the diet to protect against AD and dementia.

1. Introduction

Alzheimer's disease (AD) was first described in 1906 by German psychiatrist Alois Alzheimer, who observed abnormal clumps and tangled bundles of protein in the brain of a patient who experienced memory loss, language difficulties, and abnormal behaviour [1]. The risk of developing AD increases exponentially with age and is the leading cause of dementia and the most common neurodegenerative disease in the elderly; prevalence rates in 65–74 year olds are estimated to be 3%, rising to 19% for 75–85 year olds, and nearly 50% in those aged over 85 [2]. AD is more common among older people but it is not a normal part of ageing. As the global population ages, the prevalence of AD is expected to rise from 36 million to 115 million sufferers by 2050 [2].

Dementia imposes a huge economic burden, both through direct (medical and social care) and indirect (unpaid caregiving by friends and family) costs. In 2010, the estimated worldwide cost of dementia to society was US$ 604 billion, of which 89% was incurred in high income countries [2]. A UK study commissioned by the Alzheimer's Research Trust identified that the societal costs of dementia (£23 billion) were almost equivalent to those of cancer, heart disease, and stroke combined; yet dementia research attracted only 2% of the funding that cancer and heart disease research attracted [2]. Given that the dementia epidemic will continue to rise worldwide alongside diabetes and cardiovascular disease, the economic burden of care will become untenable. Therefore, a focus on prevention or early intervention is urgently needed. Although the recent National Institutes of Health State-of-the-Science conference on Alzheimer's prevention reported that "firm conclusions cannot be drawn about the association of any modifiable risk factor with cognitive decline or AD," there was a clear message that the evidence was insufficient, trials were focused on older participants with late stage disease, and further research was urgently needed in early onset disease where the impact is likely to be greater [1]. This review specifically focusses on the evidence supporting the role of long chain omega-3 fatty acids in the prevention or delay of progression of AD in its early stages.

2. Currently Known Risk Factors for Alzheimer's Disease

The nonmodifiable risk factors for AD are well established and include advancing age, genetic factors (such as the presence of the APOEe4 allele), and family history [24]. To date, there is no high level evidence to support or confirm that any modifiable risk factor (such as nutritional supplements, herbal preparations, diet, prescription and nonprescription medications, social or economic factors, medical conditions, and toxins or environmental exposures) is associated with reduced risk of AD [1]. Although multiple studies have shown that diet and lifestyle factors are associated with risk for AD, the scientific evidence is lacking and further studies are needed. Chronic diseases such as obesity, diabetes, hypertension, hyperlipidaemia, and depression have been associated with AD [24].

Due to an increasing body of evidence suggesting an association between diabetes and increased risk of neurodegenerative diseases such as AD (with AD itself being suggested as type 3 diabetes), it is essential to explore potential therapeutic interventions. Although the underlying pathophysiology and mechanistic aspect of diabetes-induced cognitive impairment still remain poorly understood, therapeutic interventions to delay or prevent the onset of cognitive impairment due to disease conditions, such as diabetes, need to be explored. It is evident from the existing literature that onset of cognitive impairment in diabetes and neurodegenerative conditions such as AD could have major underlying processes that cause neuronal death: these include defective antioxidant defence mechanisms, inflammatory processes, and reduced mitochondrial energy production. A nonpharmacological treatment approach is preferred because of the nature of its safety and efficacy. Trials that have used long chain polyunsaturated fatty acids (PUFAs) demonstrate an array of beneficial effects, particularly improving cognitive function in patients with early onset mild cognitive impairment [15, 16, 22].

At the recent International Conference on Nutrition and the Brain (Washington DC, July 2013), an expert panel developed dietary and lifestyle guidelines for the prevention of AD based on the best available evidence [24]. The panel agreed on seven guidelines which include minimising intake of saturated fats and trans fats; increasing intake of vegetables, legumes, and fruits; sourcing Vitamin E from foods not supplements; increasing intake of B12 through fortified foods and supplements; choosing multivitamins without iron; avoidance of cookware or other products containing aluminium; and including aerobic exercise of 40 min three times per week.

Interestingly, this consensus report by Barnard and colleagues [24] on diet and lifestyle guidelines did not address the importance of omega-3 fatty acids, or intake of fish, on AD risk. Multiple consensus documents and recent reviews [1, 2, 25] have identified that the most consistent evidence on nutrition and AD risk is for longer chain omega-3 fatty acids, primarily obtained through regular fish consumption; however recent supplementation trials are promising. Long chain omega-3 fatty acids, including eicosapentaenoic acid (EPA; 20:5n-3) and Docosahexaenoic acid (DHA; 22:6n-3), are predominantly sourced from marine fish [26]. EPA and DHA can also be synthesised from α-Linolenic acid (ALA; 18:3n-3), which is present in a number of green leafy plants, seeds, nuts, herbs, and oils, such as flaxseeds, walnuts, soybean oil, canola oil, and hempseed oil [27]. A low conversion efficiency of ALA into EPA and DHA, which varies between individuals, has been reported in a number of studies (e.g., see review [28]). The conversion efficiency of ALA to EPA varies between 0.2% and 21%, and that of ALA to DHA varies between 0% and 9% [29, 30]. This suggests a minor role for ALA in reducing AD risk and, therefore, the emphasis of our review is on EPA and DHA omega-3 fatty acids.

3. What Is Alzheimer's Disease?

3.1. Pathophysiology of Neurodegenerative Diseases. Neurodegenerative diseases represent major unmet challenges for therapeutic intervention. Neurodegenerative disorders can be considered in 3 main groups: (1) protein misfolding disorders, (2) mechanical injury and ischemia-reperfusion injury, and (3) myelin and lipid storage disorders [31]. These disorders arise from inflammatory, neurodegenerative, metabolic, or ischemic primary insults [32]. Generally, the risk of developing a neurodegenerative disease increases with aging. They result from failure in brain connectivity, which is formed by neuronal-neuronal, neuronal-glial, and glial-glial contacts [33]. This review focuses on Alzheimer's disease, a protein pathology disease.

3.2. Genetics, Clinical Trajectory, and Major Histopathology of Alzheimer's Disease. The exact etiology of AD is unknown, but environmental and genetic factors are thought to contribute. Genetic factors play a significant role in familial AD. This rare autosomal, dominant disease with early onset (<65 years) is caused by mutations in the genes encoding amyloid precursor protein (APP) and presenilin (PSEN1), both linked to amyloid-β metabolism [34]. Familial AD accounts for about 1–5% of all AD cases [35]. For late onset AD (>65 years; representing 95–99% of all AD cases), the relevance of specific genetic mutations is not clear. For this sporadic form of AD, it is generally accepted that epigenetic components (ageing, genetic, and environmental risk factors) play a more dominant role [35].

AD is characterized by cognitive alterations, memory loss, and behavioural changes which affect daily living. Table 1 records the well-characterised stages of slow but progressive AD development. The predominant theory for the degenerative process in AD (summarised in Figure 1) subscribes that the deposition in the brain of highly insoluble amyloid-β occurs early in AD and suppresses synaptic plasticity. Disruption of dendritic spine formation thereby interferes with memory consolidation. The formation of amyloid plaques also activates glial cells to augment inflammation in the brain. Subsequent signalling events trigger abnormal intraneural formation of hyperphosphorylated tau protein (pTau), resulting in neurofibrillary tangles years/decades later [36].

Table 1: Disease progression in Alzheimer's disease (AD).

Stage	Clinical trajectory
Early preclinical AD (changes begin 10–20 yrs before symptoms)	(i) Degeneration in hippocampus (where short-term memory is converted to long-term memory) (ii) Neuronal loss leads to shrinkage (iii) Memory loss is the first sign of AD
Mild-moderate AD	(i) Mild: memory loss, confusion, poor judgment, mood changes, anxiety (ii) Moderate: increased memory loss and confusion, problem recognizing people, difficulty with language, repetitive statements
Severe AD	(i) Extreme shrinkage of brain (ii) Patients completely dependent on others for care (iii) Weight loss, seizures, skin infections, moan/groaning, loss of bladder, and bowel control (iv) Death usually occurs from aspiration pneumonia or other infections

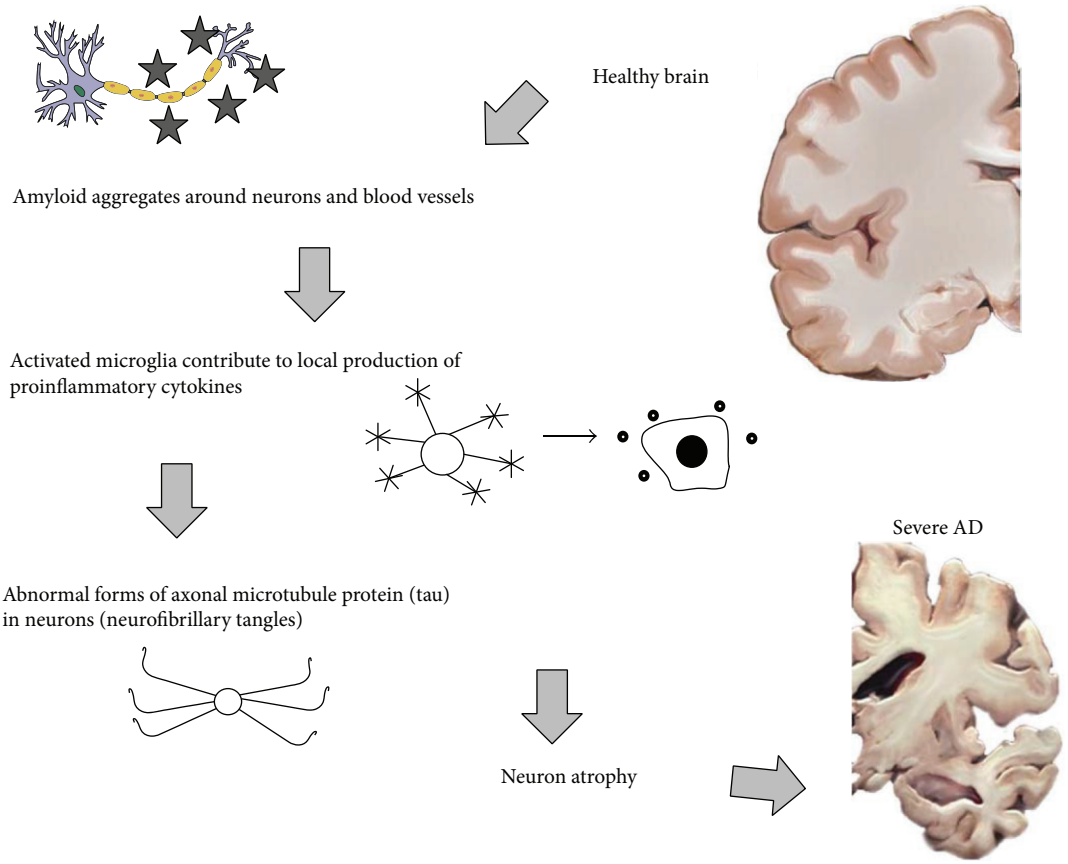

Figure 1: Schematic representation showing key pathological features of the degenerative process in Alzheimer's disease (AD). The common characteristics are (1) amyloid-β plaques and (2) neurofibrillary tangles. Neuroinflammatory changes (3) have been identified as the third important component of the disease. Microglia migrate to the plaques and enhance amyloid-β deposition with chronic activation. *Brain and neuron images from Wikimedia Commons (*http://upload.wikimedia.org/wikipedia/commons/c/cc/Alzheimers_brain.jpg. http://upload.wikimedia.org/wikipedia/commons/b/be/Derived_Neuron_schema_with_no_labels.svg*).*

At autopsy, AD postmortem brains share a number of common features. These include significant cortical atrophy and secondary ventricular enlargement. Neuropathogenic assessment reveals the highly insoluble neuritic plaques (amyloid-β; extraneuronal) and neurofibrillary tangles (tau; intraneuronal). AD brains are also characterized by prominent reactive astrogliosis due to destruction of nearby neurons [37].

4. Role of Inflammation in Neurodegenerative Diseases

4.1. Acute versus Chronic Inflammation in the Brain. The brain is populated by 4 types of cells: microglia, astrocytes, neuron, and endothelial cells. Behind the blood brain barrier, there is an absence of leukocytes and antibodies which

constitute key defence cells in peripheral immune system. Instead, microglia are the brain's resident immune cells capable of phagocytosis. They mount localized inflammatory response and have both apoptotic and neurotoxic actions. Initial triggers for neurological diseases may differ considerably, but the subsequent pathways that involve inflammatory processes and involve brain damage often share crucial pathological mechanisms [38]. In an acute insult/stress scenario, pathogens, protein aggregates or damaged neurons may activate inflammatory microglia and, appropriately, they take on the morphology of activated macrophages to remove the threat [39]. In contrast, sustained low grade brain inflammation (chronic microglial activation) may contribute to the ongoing pathology of neurodegenerative disease (e.g., Alzheimer's disease, Parkinson's disease, Multiple sclerosis). Various proinflammatory mediators are increased in the brains of patients with neurological disorders and have been shown experimentally in animal models. These inflammatory factors are thought to contribute to the damage and subsequent loss of neurons [32, 40]. Since prolonged low grade inflammation can lead to loss of neurons in the brain, one hypothesis is that early prevention or management of inflammation could delay the onset or reduce the symptoms of AD.

4.2. Inflammation in Alzheimer's Disease. Among the protein misfolding disorders, AD has provided the most positive indication to support that inflammation contributes to this neurodegenerative disease. In AD, there is no obvious accumulation of activated immune cells in the central nervous system. However, potent inflammatory molecules such as cytokines/cytokine receptors are detected in cerebral spinal fluid and plaques from AD patients [37, 38]. The amyloid-β plaques have been demonstrated to increase proinflammatory cytokines and reactive oxygen species, in addition to neurotoxic secretory products [37]. Changes in microglia morphology have been described (from ramified (resting) to amoeboid (active)) and astrogliosis has been observed surrounding senile plaques [41]. Moreover, long-term use of anti-inflammatory drugs (NSAIDS) reduces the risk of developing AD [42] (decreased plaque burden pathology/increased cognitive function/linked to reduced microglial activation), supporting therapeutic immunemodulatory approaches against AD progression. An important note is that these benefits of NSAID treatment cannot be obtained in established AD [43]. This implies that inflammation precedes neuronal loss in the disease. We have previously shown that DHA supplementation reduces markers of inflammation in the hippocampus [44].

5. Is Alzheimer's Disease Type 3 Diabetes?

For decades, AD has been commonly attributed to amyloid-β and pTau aggregation. An emerging body of evidence, led by neuropathologist de la Monte [45], suggests that people that have insulin resistance, in particular those with type 2 diabetes, have an increased risk of suffering from AD, estimated between 50% and 65% higher. Whether primary or secondary in origin, brain insulin/insulin-growth-factor resistance initiates a cascade of neurodegeneration that is propagated by metabolic dysfunction, increased oxidative and endoplasmic reticulum (ER) stress, neuroinflammation, impaired cell survival, and dysregulated lipid metabolism. Lack of insulin not only impairs cognition but seems to be implicated in the formation of the amyloid plaques [45]. Additionally, insulin resistance with subtle cognitive impairments is seen even in normal adults with prediabetes, suggesting a higher risk of developing AD in later life [46]. Insulin resistance in the brain has been shown to compromise survival of neurons, metabolism, and neuronal plasticity which are critical for memory formation and normal cognitive function [47].

The largest epidemiological studies conducted so far, the Rotterdam [48] and Hisayama [49] studies, indicate that patients suffering from diabetes have increased likelihood of developing cognitive impairment as seen in dementia. The relationship between diabetes and dementia has been clearly established by Velayudhan and coworkers [50], where diabetic patients progressed to dementia after a 4-year followup period. These results indicate a clear need for early screening of mild cognitive impairment (MCI) in diabetes patients, as MCI has been suggested to be the earliest detectable stage of AD [51]. An early screening for MCI in diabetic patients would also be beneficial in modifying lifestyle-related factors and creating the potential for dietary intervention or supplementation such as with omega-3 fatty acids to ameliorate AD disease progression.

6. Existing Pharmacological and Herbal Treatments for Alzheimer's Disease

There are currently over 1400 registered clinical trials investigating the effect of drugs or single nutrient supplements on the development/progression of AD [52].

In early stage AD, current approved drug therapies include the cholinesterase inhibitors (e.g., Donepezil (Aricept)). In moderate to advanced stage AD N-methyl D-aspartate receptor antagonists (e.g., Memantine (Ebixa)) are commonly used. Although these drugs improve symptoms in the short term they invariably do not inhibit progression of AD and are often associated with multiple side effects including nausea, diarrhoea, insomnia, tremor, muscle cramps, and occasionally hallucinations [53].

Over the last decade there have been an excess of 28 systematic reviews published on the Cochrane database investigating the evidence for the beneficial effects of supplementation with B-group vitamins (folate, B-12, thiamine, B-6), Vitamin E, or herbal medicines such as ginkgo biloba and ginseng on cognitive decline in AD. The evidence for the potential benefit of these nutraceuticals on dementia in AD has been systematically reviewed and evaluated against the National Health and Medical Research Council (NHMRC) of Australia levels of evidence by Kotsirilos and colleagues [54]. The authors conclude that there is Level 1 evidence (equivalent to meta-analyses and systematic reviews) for the benefits of fish oil and the Chinese herb ginko biloba; Level 2 evidence (equivalent to findings from randomized controlled trials (RCTs)) for the Ayurvedic herb brahmi (Bacopa Monnieri), sage, lemon balm, turmeric, ginseng, CoQ10 (Coenzyme Q10), Vitamin D, and magnesium. These

nutraceutical therapies require careful evaluation of the patient's health status and medication as drug-nutrient and drug-herb interactions can occur [54].

Patients with AD are often malnourished and present with multiple nutrient deficiencies. Therefore, correcting nutrient deficiencies, particularly the B-group vitamins and Vitamin D, and improving nutritional status are clearly important in prevention of AD progression. As noted previously, normal physiological changes to the brain with ageing include depletion of major long chain omega-3 fatty acids, which constitute 30–35% of the brain, and this process is accelerated in neurodegenerative conditions such as AD [55]. The long chain omega-3 fatty acids are concentrated in the phospholipid membrane of the brain, particularly at the synapses [56], and brains of AD patients have lower DHA. Therefore, there appears to be a strong physiological rationale to supplement patients with AD with the long chain omega-3 fatty acid DHA.

7. Epidemiological Evidence for the Role of Dietary Intake and Alzheimer's Disease

7.1. Evidence from Observational Studies. Cross-sectional studies provide evidence of a relationship between diet quality and prevalence of AD [3–8]. Lower intake of dietary nutrients was reported using food records and/or 24-hour diet recalls in "early stage" AD patients ($n = 36$) compared to age-matched cognitively intact subjects ($n = 58$) [3]. Nutrient intakes identified as being significantly lower in patients with AD included energy, all macronutrients, calcium, iron, zinc, Vitamin K, Vitamin A, dietary fibre, omega-3 fatty acids, and omega-6 fatty acids [3]. Dietary patterns were also assessed in 2,148 elderly subjects, of which 253 developed AD. A dietary pattern which exhibited a lower risk on development of AD was identified and was comprised of higher intakes of salad dressing (not specified, but presumably olive oil and/or vinegar-based), nuts, fish, tomatoes, poultry, cruciferous vegetables, fruits, and dark and green leafy vegetables; the diet also contained a lower intake of high-fat dairy products, red meat, organ meats, and butter [4].

Some epidemiologic studies suggest that higher dietary intake of antioxidants, vitamins B6, B12, and folate, unsaturated fatty acids, and fish are related to a lower risk of AD, but reports are inconsistent [57]. Modest to moderate alcohol intake, particularly wine, may be related to a lower risk of AD [7]. The Mediterranean diet may also be related to lower AD risk [8]. The traditional Mediterranean diet is characterised by an abundance of bioactive phytonutrients, with antioxidant and anti-inflammatory potential, derived from extra virgin olive oil as the main added fat, fresh fruits and leafy vegetables, legumes, wholegrain cereals, nuts and seeds, fish and red wine, with moderate portions of meat and dairy [58]. This diet provides a model that delivers a rich anti-inflammatory diet in a palatable cuisine.

A recent systematic review of 11 prospective studies worldwide examined the link between a Mediterranean-type diet and cognitive decline (including AD) [9]. Amongst 4 prospective studies that focussed on AD incidence, the review reported a 28–48% reduced risk of developing AD in response to higher level of adherence to a Mediterranean-type diet. Furthermore, higher adherence to a Mediterranean-type diet was associated with a 73% lower risk of dying of the disease in participants who already had clinically diagnosed AD [9]. A recent meta-analysis on 133,626 people within 3 prospective cohort studies also showed that closer adherence to a Mediterranean diet led to a 13% lower incidence of neurodegenerative diseases such as Parkinson's disease and AD [10].

Whilst dietary patterns are of interest, this review is focused on dietary fats in relation to prevention of AD. Dietary fat alone has been reported to have a role in the prevention of AD onset. Laitinen and colleagues [5] found that polyunsaturated fats were associated with decreased rates of dementia and AD. This data was derived from a 21-year followup of 1,449 individual original study participants. Saturated fats were associated with an increase in rates of dementia and AD [5]. In relation to the role of polyunsaturated fats in particular, epidemiological evidence exists showing a role for omega-3 fatty acids in reducing the onset of AD. A case-cohort study showed a 72% reduction in the odds of developing AD in those with the highest tertile of dietary DHA. Fish intake was also associated with lower odds of developing AD, but this did not reach statistical significance [6].

7.2. Evidence from Dietary Interventions. Whilst there are a number of observational studies assessing the association between diet and the risk of neurodegenerative diseases such as AD [3–6, 57], there are no published dietary intervention RCTs investigating the effect of diet, including omega-3 rich food such as oily fish, and the onset of AD. Dietary intervention studies have instead assessed markers of AD.

Dietary intervention RCTs previously conducted in relation to the development of AD (see Table 2), include a study comparing a diet high in saturated fat and high glycemic index (GI) carbohydrates to a diet low in saturated fat and GI [11]. Bayer-Carter and colleagues [11] measured cerebrospinal fluid markers of AD and tested cognition in 20 healthy adults and 29 adults with amnestic mild cognitive impairment (aMCI). Participants receiving a diet low in saturated fat and low GI for 4 weeks exhibited a decrease in markers associated with disease risk of AD, including central nervous system levels of amyloid-β 42 (Aβ-42), compared to the high fat and high GI intervention. Lipoproteins, oxidative stress, and insulin levels were also decreased in response to the low saturated fat and low GI diet [11]. A similar finding in relation to acute effects of dietary fat was reported by Karczewska-Kupczewska and colleagues [12]. These researchers showed that a single high fat meal (Calogen), in which energy comes almost in total from fat (450 kcal/100 mL), decreased circulating brain-derived neurotrophic factor (BDNF), which plays a crucial role in modulating synaptic plasticity for hippocampal-dependent cognitive functions, in 20 healthy male subjects (mean age 22.7 ± 2.3 years; mean BMI 24.9 ± 1.5 kg/m^2) [12]. These findings suggest benefits of reduced fat intake on markers of AD; however, the research team did not investigate particular fatty acids such as the omega-3s.

Table 2: Summary of observational and dietary intervention studies in patients with Alzheimer's disease.

Reference	Study design Duration Population (*n*, mean age/age range)	Dietary intake measures	Measures of cognitive decline	Outcome
[3]	Longitudinal study 18 mths "early stage" AD (36, ≥65 yrs) cognitively intact (58, >65 yrs)	Food records 24-hr diet recalls	MMSE score or Reisberg Global Deterioration Scale	Nutrient intakes significantly lower in patients with early stage AD included omega-3 fatty acids and omega-6 fatty acids
[4]	Longitudinal study 3.9 yrs cognitively intact (2148, ≥65 yrs)	FFQ	Neuropsychological battery and evidence of cognitive deficit	Dietary pattern exhibiting a lower risk on AD incidence included higher intake of nuts and fish
[5]	Longitudinal study 21 yrs healthy participants (1449, 65–79 yrs)	FFQ	DSMMD, 4th edition	Polyunsaturated fats associated with decreased rates of dementia and AD. Saturated fats associated with an increase in rates of dementia and AD
[6]	Case-cohort study (266, 65–100 yrs) 42 with dementia 30 with possible/ probable AD	FFQ	MMSE, Trail making test–part B, HVRT, CFT, BSRT	Reduction in odds of developing AD in those with the highest tertile of dietary DHA. Fish intake associated with lower odds of developing AD, but did not reach statistical significance
[7]	Prospective cohort 4 yrs nondemented (980, >65 yrs)	FFQ	DSMMD, 4th Edition	Consumption of up to three servings of wine daily was associated with a lower risk of AD in elderly
[8]	Longitudinal study 4 yrs nondemented (2258, 77 yrs)	FFQ	DSMMD, Revised 3rd Edition	Higher adherence to the MD was associated with lower risk for AD
[9]	Systematic review 6 studies various (16995, various)	FFQ	Various	Higher adherence to the MD associated with a lower risk of dementia or AD than subjects in the lowest tertile of adherence
[10]	Meta-analysis 3 studies various (133626, various)	Various	Various	Adherence to MD led to a 13% lower incidence of neurodegenerative diseases such as AD
[11]	Dietary intervention 4 wks healthy participants (20, 69 yrs) aMCI (29, 68 yrs)	Daily food diary	Immediate and delayed memory test, executive function test, motor speed test	Diet low in saturated fat and GI decreased markers associated with risk of AD compared to the high fat and GI intervention
[12]	Dietary intervention single meal healthy males (20, 23 yrs)	Single meal delivered	Not measured	High fat meal caused a decrease in circulating brain-derived neurotrophic factor (BDNF)
[13, 14]	Dietary intervention 12 wks headache patients (56, 41 yrs)	24-hr diet recalls	Not measured	Lowering dietary linoleic fatty acids significantly reduced levels of plasma oxidated linoleic acid metabolites (OXLAMs)

aMCI = amnestic mild cognitive impairment; BSRT = Buschke-Fuld Selective Reminding Test; CFT = Category Fluency Test; DSMMD = Diagnostic and Statistical Manual of Mental Disorders; FFQ = Food Frequency Questionnaire; HVRT = Heaton Visual Reproduction Test; MD = Mediterranean Diet; MMSE = Minimental State Examination.

A study on dietary polyunsaturated fats investigated the effect of a diet low in linoleic fatty acid on the plasma levels of oxidized linoleic acid metabolites (OXLAMs) [13]. Plasma OXLAMS are raised in AD and may relate to the disease progression [59]. Of interest, a separate study by this group showed a decrease in plasma OXLAMS in response to a 12-week intervention with a diet low in linoleic acid in chronic headache patients [14]. Further dietary interventions of longer duration should be conducted in at risk populations to clarify the effect of dietary fats, in particular omega-3 fatty acids, on risk of AD.

8. Role of Fatty Acids in Brain Function

8.1. Long Chain Omega-3 Fatty Acids and Brain Function. Dietary intake of DHA is required for normal neurodevelopment and brain health, particularly during prenatal brain development [60]. DHA is incorporated in large amounts into foetal brain through fatty acid transport protein-4 (FATP 4) [61]. DHA that is transferred from the maternal circulation to foetal brain plays a crucial role in brain growth especially with regard to synaptogenesis [62, 63]. However, the amount of maternal DHA in the synapses and neural membranes depends on the dietary intake [64–66]. The essential DHA is selectively enriched in neuronal tissues especially in neuronal and synaptic membranes, oligodendrocytes, and also subcellular particles such as myelin and nerve endings [67–69]. With aging, and especially among patients with AD, DHA levels in the brain tend to decrease [70].

8.2. Molecular and Cellular Mechanisms Underlying DHA Effects. DHA has been shown to have a crucial role in regulating neural gene expression [71]. Previous studies have shown that DHA acts as an endogenous ligand for retinoic acid receptors (RAR) and retinoid x receptors (RXR) [72]. RAR and RXR have been shown to decrease with age and these receptors are associated with age-related memory deficits. Dyall and colleagues [72] suggest that a reversal in the decrease of RAR and RXR following DHA supplementation could alleviate the memory deficits and increase neurogenesis. In patients diagnosed with AD, significantly lower DHA levels were detected in blood plasma and brain [73, 74]. This not only could be due to lower dietary intake of omega-3 fatty acids, but it also could be attributed to increased oxidation of PUFAs [75, 76].

While preclinical evidence suggests that a diet enriched with DHA reduces amyloid formation in dementia with AD [77, 78], clinical trials have yielded limited or negative results to date. Dietary supplementation of DHA has been shown to increase the levels of hippocampal BDNF [79]. Akbar and coworkers [70] provided additional evidence that DHA is highly enriched in neuronal membranes and that it facilitates the activation of protein kinase B (PKB), also known as Akt, via an increase in phosphatidylserine. Akt signalling is a critical pathway in neuronal survival. Activation of Akt, thus, can cause an increase in BDNF which further strengthens synaptic plasticity and cell survival. Furthermore, Calon and colleagues [80] found that a diet rich in DHA activates Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). This signalling cascade is critical for learning and memory and plays a crucial role in induction and maintenance of long-term potentiation in hippocampus [80, 81].

Studies also suggest that DHA modulates multiple cellular functions including enhanced membrane fluidity of amyloid precursor protein (APP) and a shift towards non-amyloidogenic processing, which inhibits α and β secretase, thereby reducing amyloid-β release [82]. Because of the influence of DHA on membrane fluidity, it has been speculated that DHA has significant impact on neural membrane function. DHA is suggested to facilitate N-methyl-D aspartate (NMDA) responses [83] and block K^+ channels [84], which results in long-term potentiation, crucial for synaptic modification for long term memory and learning [83]. Omega-3 supplementation, DHA in particular, has also been shown to modulate gene expression at the transcription level, for example, by activating peroxisome proliferator-activated receptor (PPAR) family members [85] and the mRNA stability of several enzymes associated with glucose and lipid metabolism [86]. Studies in rodents indicate that treatment with fish oil resulted in overexpression of genes related to synaptic plasticity, signal transduction, energy metabolism, and regulatory proteins [87–89]. While interest in the underlying molecular and cellular mechanisms by which DHA exerts its beneficial effects in neurodegenerative conditions such as AD continues, the exact mechanisms are not clearly understood.

8.3. DHA Depletion and Cognitive Impairment. Studies in animal models of AD suggest that deficiency of DHA in neural tissue leads to behavioural deficits, ultimately leading to neurodegeneration and cognitive dysfunction similar to that in patients with AD [90–92]. Furthermore, experimental evidence suggests that DHA decreases with age, particularly in regions of the hippocampus which are crucial for higher brain functions such as memory formation and cognition [90, 93]. Decreased DHA levels are reported to detrimentally affect the major excitatory neurotransmitter, glutamate, which contributes to the integrity of brain function in learning memory performance [69].

9. Omega-3 Fatty Acid Supplementation Trials in Patients with Impaired Cognition and Alzheimer's Disease

In addition to health benefits of omega-3 supplementation in other settings, a number of studies report using omega-3 supplementation in (early) AD [94]. Epidemiological and preclinical studies indicate that consumption of long chain omega-3 PUFAs (omega-3 fatty acids) may slow cognitive decline and prevent the progression of mental health disorders such as AD. The relationship between mental health disorder and omega-3 fatty acids has been shown by lower levels of omega-3 fatty acids in the erythrocyte membrane or plasma of the patients suffering from neurodegenerative disorder as compared with healthy people [95–97]. Serum samples of patients with AD have also been reported with less than half the level of DHA compared to healthy individuals [98]. Importantly, however, very few randomized controlled

Table 3: Summary of DHA/EPA dietary intervention trials in patients with mild cognitive impairment (MCI) in the last 10 years.

Reference	Clinical trials with MCI patients (n, mean age)	Dosage of DHA/EPA per day	Trial duration and design	Measures	Outcome
[15]	Patients with MCI (23, 74 yrs)	0.72 g DHA + 1.08 g EPA or placebo	6 mths randomized double-blinded placebo-controlled trials	ADAS-cog.; CIBIC plus	Significant improvement in ADAS-cog; in patients with MCI after omega-3 supplementation
[16]	Patients with MCI (23, 68 yrs)	240 mg DHA + 240 mg AA or placebo	3 mths, placebo controlled trial	Japanese version of RBANS (5 cognitive domains)	Improvement of immediate memory and attention in omega-3 supplemented group
[17]	Elderly persons with MCI (36, 66 yrs)	1.3 g DHA + 0.45 mg of EPA or placebo	12 mths, randomized double-blinded placebo controlled trial	RAVLT, MMSE, CDT, WAIS-R	Significant improvement in cognitive function in omega-3 supplemented group
[18]	Elderly patients suffering from MCI (11, 85 yrs)	1.4 g DHA + 572 g EPA or placebo	3 mths, randomized double-blinded placebo controlled trial	MMSE	Significant improvement in MMSE, semantic verbal fluency, and olfactory sensitivity assessment in omega-3 supplemented group
[19]	Older people with MCI (100, 74 yrs)	180 mg DHA + 120 mg EPA or placebo	6 mths, randomized double-blinded placebo controlled trial	MMSE, AMT	Low prescription dose had no effect on cognitive function in omega-3 supplemented group

AA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; MMSE = Minimental State Examination; ADAS-cog. = Cognitive Portion of the Alzheimer's Disease Assessment Scale; CIBIC plus = Clinician's Interview-Based Impression of Change Scale; RBANS = Repeatable Battery for the Assessment of Neuropsychological Status; RAVLT = Rey's Auditory Verbal Learning Test; CDT = Clock Drawing Test; WAIS-R = Wechsler Adult Intelligence Scale; AMT = Abbreviated Mental Test.

trials have been conducted to ascertain the beneficial role of omega-3 fatty acids in prevention and progression of neurodegenerative diseases such as AD.

Controlled studies using omega-3 fatty acid supplementation in patients diagnosed with MCI (a precursor to early AD) or AD are few in number. To date, controlled studies conducted on patients with MCI and supplemented with omega-3 fatty acids (summarised in Table 3) suggest a positive effect on cognitive performance following supplementation ranging from 3 to 12 months. Kotani and colleagues [16] demonstrated that supplementation with 240 mg/day of DHA and 240 mg/day arachidonic acid significantly improved immediate memory and attention scores in adults with MCI, but not in 8 patients with AD who were given the same dose of supplementation for the same duration. These results are similar to the study conducted by Chiu and colleagues [15], who also reported improvements in MCI, but not AD, patients following omega-3 fatty acid supplementation over 24 weeks. It should be noted that one study reported no significant prevention of cognitive decline in older people with MCI over 6 months [19]. The researchers speculated that the lack of effect could be due to the low dose of DHA/EPA employed (180 mg DHA + 120 mg EPA of omega-3 PUFAs).

Most of the omega-3 supplementation studies in AD patients (summarised in Table 4) show no significant improvement in AD measures, except for Freund-Levi and colleagues [21], who conducted the largest trial to date ($n = 174$). These workers reported that supplementation with 1.72 g DHA + 600 mg EPA per day for 6 months did not show any improvement in cognitive decline in AD patients. However, in a very small subgroup of patients ($n = 27$) diagnosed with the mildest form of AD, a significant reduction in cognitive decline rate was observed compared to the placebo group. Another study conducted by Boston and coworkers [20] reported no difference in rate of cognitive decline between AD patients taking 1 g of ethyl-EPA daily for 3 months compared to placebo. A positive effect ($P = 0.02$) was seen in carer's visual analogue rating; however, the authors suggest that this result could be biased, as carer's were aware of the treatment regime. Nevertheless, the outcomes of this trial warrant further investigation in the form of a double-blinded study. Extending the duration of the supplementation period in AD patients has also been suggested.

In summary, results from controlled studies conducted over the last 10 years (summarised in Tables 3 and 4) suggest that nutritional intervention with omega-3 fatty acids is beneficial only in the earlier stages of cognitive impairment.

Table 4: Summary of DHA/EPA dietary intervention trials in patients with Alzheimer's disease (AD) in the last 10 years.

Reference	Clinical trials with AD patients (n, mean age)	Dosage of DHA/EPA per day	Trial duration and design	Measures	Outcome
[20]	Mild to moderate AD (22, 81 yrs)	1 g ethyl-EPA or placebo	6 mths; 12 wks without treatment, followed by 12 wks with treatment	MMSE, ADAS-cog.	NS difference between treatment and baseline, small improvement in carer's analogue rating ($P = 0.02$)
[16]	Patients with AD (8, 67 yrs)	240 mg DHA + 240 mg AA or placebo	3 mths parallel design	Japanese version of RBANS (5 cognitive domains)	NS improvement seen postsupplementation
[21]	Mild AD patients (178, 74 yrs)	1.72 g DHA + 600 mg EPA or placebo	6 mths parallel design	MMSE, ADAS-cog.	Positive effects of omega-3 supplementation seen only on patients with very mild AD
[15]	Mild to moderate AD (23, 74 yrs)	0.72 g DHA + 1.08 g EPA or placebo	6 mths, randomized double blinded placebo controlled trial	ADAS-cog.; CIBIC-plus	NS difference seen between placebo and omega-3 supplemented group
[22]	AD patients on acetylcholine esterase treatment (204, 74 yrs)	1.72 g DHA + 600 mg EPA or placebo	6 mths parallel + 6 mths cross-over to fish oil	DAD, CGB, MADRAS, NPI	NS effect between omega-3 supplemented placebo group on neuropsychiatric symptoms, positive effect on depressive symptoms
[23]	Mild to moderate AD (295, 76 yrs)	2 g DHA or placebo	18 months, randomized double blinded placebo controlled trial	ADAS-cog, CDR, MMSE; (brain MRI in sub pop. $n = 102$)	NS difference in rate of cognitive and functional decline, no effect on total brain volume

AA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; DAD = Disability Assessment for Dementia Scale; CGB = Caregiver Burden Scale; MADRS = Montgomery-Asberg Depression Rating Scale; NPI = Neuropsychiatric Inventory; MMSE = Minimental State Examination; ADAS-cog. = Cognitive Portion of the Alzheimer's Disease Assessment Scale; CIBIC plus = Clinician's Interview-Based Impression of Change Scale; RBANS = Repeatable Battery for the Assessment of Neuropsychological Status; NS = Nonsignificant; MRI = Magnetic Resonance Imaging; CDR = Clinical Dementia Rating.

Controlled studies on patients with well-established AD using both low and high doses of omega-3 fatty acids have not shown any improvement; this could be due to suboptimal levels of omega-3 fatty acids reaching the brain or the fact that the intervention is too late. The longest supplementation trial in AD patients reported to date is 18 months [23], although most have been for 6 months duration. Further studies of longer duration and with larger subject populations could provide more insight as to the therapeutic benefits of omega-3 fatty acids in slowing cognitive decline in AD patients. There is also good evidence from epidemiological studies that a Mediterranean-style dietary pattern (rich in plant derived ALA and LC-PUFA from fish and seafood) may be protective for neurodegenerative diseases such as AD and Parkinson's disease [99], and recent results from the Spanish PREDIMED study demonstrated improved cognition in high vascular risk participants who followed the Mediterranean diet compared with those on the low fat diet [100].

10. Conclusions and Future Directions

This review has highlighted the lack of consistent evidence for the potential of nutraceuticals and pharmacotherapies to delay the progression of AD. Evidence for a single nutrient therapy is inconsistent. Therefore, it appears that the overall quality and composition of the diet also contribute to protection against AD and dementia. The strongest evidence in support of nutrition preventing cognitive decline in AD is for long chain omega-3 fatty acids. Primarily, this is because long chain omega-3 has shown promising potential to ameliorate low grade inflammation in the early stages of this neurodegenerative disease.

Potential future directions for this field deserve attention in areas covering experimental design, dietary food guidelines, and targeting treatment for patients based on the stage of their disease. In particular, we highlight the need for the following: (1) Large, high quality randomised control trials with omega-3 fatty acid supplementation for a longer duration, possibly 18–24 months, to measure cognitive performance in Alzheimer's Disease Assessment Scale. Importantly, omega-3 doses need to achieve therapeutic concentration levels in the brain. These trials could offer long-term assessment of the effect of omega-3 on delaying the progression of cognitive decline associated with AD. (2) Standardized dietary guidelines for marine and plant-based omega-3 consumption are required and regular followup in patients with mild cognitive impairment is needed to ascertain if dietary intake could delay or reverse any deleterious effects that could progress to dementia or AD. (3) Finally, studies need to target people with mild cognitive impairment and early to moderate AD with low plasma levels of omega-3 at baseline.

Abbreviations

Amyloid-β:	Amyloid Beta (protein)
ALA:	Alpha-Linolenic acid
AD:	Alzheimer's disease
DHA:	Docosahexaenoic acid
EPA:	Eicosapentaenoic acid
MCI:	Mild cognitive impairment
PUFAs:	Polyunsaturated fatty acids.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Each author participated actively in all paper preparation stages.

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Research Article

Moderate Dietary Supplementation with Omega-3 Fatty Acids Does Not Impact Plasma Von Willebrand Factor Profile in Mildly Hypertensive Subjects

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Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) have blood pressure lowering and antithrombotic effects, which may benefit hypertensive patients. Increased plasma concentration of von Willebrand factor (vWF), a procoagulant glycoprotein, has been identified in patients with severe hypertension, with some, but not all studies showing an increase with mild hypertension. In this study, we determined the plasma concentration, multimer distribution, and collagen binding activity of vWF in subjects with mild hypertension and determined whether these parameters might improve after dietary supplementation with moderate amounts of LC n-3 PUFAs. Hypertensive and normotensive subjects were randomized to 12-week treatment with LC n-3 PUFAs (2.52 g/day) or placebo (canola oil). Home blood pressure measurements were recorded daily, and blood samples were collected every 3 weeks. LC n-3 PUFAs increased the n-3 index to cardioprotective levels (>8%). Plasma concentration, multimer distribution, and collagen binding activity of vWF were not reduced by LC n-3 PUFA treatment. We conclude that, at the concentration and duration used in this study, benefits of LC n-3 PUFAs in subjects with mild hypertension are not associated with a direct effect on vWF concentration or function. This trial is registered with the Australian New Zealand Clinical Trials Registry ACTRN12610000713099.

1. Introduction

Hypertensive disease is a risk factor for the development of thrombotic cardiovascular conditions and is a leading cause of death globally [1, 2]. For every 20 mmHg systolic or 10 mmHg diastolic increase in blood pressure, there is reported doubling of mortality from both ischemic heart disease and stroke [2]. The large procoagulant glycoprotein von Willebrand factor (vWF) is produced in vascular endothelial cells and is stored in specific storage granules called Weibel-Palade (WP) bodies [3]. Shear stress and shear-stress induced transcription factor (Krüppel-like factor 2) are associated with a small but significant reduction in number of WP bodies [4], consistent with their degranulation, and increase in basal and adrenaline-stimulated vWF secretion [5]. Hypertensive disease causes an increase in intramural pressure which leads to mechanical shear stress on the endothelium [6]. Accordingly, increased plasma levels of vWF have been reported for patients with stage 2 hypertension (≥160/≥100 mmHg) [7]. The capacity of mild (stage 1) hypertension (140–159/90–99 mmHg) to modulate vWF levels is inconclusive, with an increase in vWF concentration reported by some [8], but not by others [9].

vWF is a multimeric protein, where ultra-large multimers >10,000 kDa have greater thrombogenic potential compared to low molecular weight vWF multimers [10]. The greater thrombogenic potential of the ultra-large multimers is attributed to a large number of binding sites for interactions with platelets, extracellular matrix components and

endothelial cells. Since hypertension is linked to an increase in thrombogenic potential, we hypothesized that it might also be associated with a shift in distribution towards larger, more thrombogenic vWF multimers.

Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) have greater blood pressure-lowering effects in untreated hypertensive patients than in normotensive subjects [11]. We recently showed that LC n-3 PUFAs also modulate the release of vWF from human cultured umbilical vein endothelial cells in a cellular model of vascular inflammation [12]. It is not known whether dietary supplementation of mildly hypertensive subjects with LC n-3 PUFAs will also modulate vWF expression and function. To examine this question, we assessed the effect of 12 weeks of dietary supplementation of mildly hypertensive and normotensive subjects with LC n-3 PUFA (2.52 g/day) on vWF concentration, vWF multimer distribution, and vWF function. The findings showed that whilst the supplement produced cardioprotective levels of LC n-3 PUFAs in membrane phospholipids (n-3 index > 8%), the intervention was not sufficient to modulate plasma vWF concentration, multimer distribution, or collagen binding. It is possible that higher doses of LC n-3 PUFAs are required to modulate vWF in this population or that patients with more advanced hypertensive disease are required to see an effect.

2. Materials and Methods

2.1. Study Design. The study was conducted as a double-blinded, placebo controlled trial. Age matched normotensive subjects (blood pressure <120/80 mmHg, $n = 27$) and subjects with stage I hypertension (SBP 132–154 mmHg and/or DBP at 63–103 mmHg, $n = 15$) were recruited from March 2009 to September 2010. Each subject participated in the study for a 15-week period, with commencement of treatment from April 2009 to October 2010. Although registration of the trial occurred after commencement of the study (Australian New Zealand Clinical Trial Registry, ACTRN12610000713099), no changes were made to the study design or protocol in the time prior to and time after registration. Subjects were excluded if they were receiving antihypertensive treatment prior to or during the study. All subjects were assigned to placebo capsules (4 × 1 g; Blackmores canola oil) for the first 3 weeks and then randomised to placebo or LC n-3 PUFA supplementation (4 × 1 g; Blackmores Omega Heart, each containing 420 mg EPA and 210 mg DHA; 2.52 g/day) for a 12-week period. This study was conducted in accordance with the Declaration of Helsinki (1964). The protocol was approved by the University of the Sunshine Coast Human Research Ethics Committee (Ethics Approval Number A/08/167). All subjects provided informed, written consent for their participation.

2.2. Blood Processing. Blood samples were collected from subjects at 3-week intervals. EDTA and serum tubes were centrifuged (1500 ×g, 15 min, 15°C), and plasma or serum were stored at −80°C until further analysis. Erythrocytes from EDTA tubes were washed with an EDTA solution (1 mL; 0.15 M NaCl, 10 μM EDTA disodium salt, pH 7.4 with HCl), covered with nitrogen gas, and centrifuged at 4°C for 10 minutes (1500 ×g). The process was repeated twice. Aliquots of washed erythrocytes were covered with nitrogen gas and stored at −80°C until further analysis.

2.3. Omega-3 Index. To extract phospholipids from the subjects' erythrocytes, 600 μL of methanol containing butylhydroxytoluene (BHT, 20 mg/100 mL) was added to 300 μL of erythrocytes and cells were homogenised using glass rods for 1 min. Homogenates were covered with nitrogen gas and stored on ice for 30 min before adding 600 μL of chloroform. Cells were homogenised again for 1 min, stored on ice for 30 min, and then centrifuged (3000 ×g, 4°C, 5 min). The supernatant was withdrawn, covered with nitrogen gas, and stored on ice. The process was repeated twice using 300 μL methanol with BHT and chloroform, with 10 min storage on ice. To complete the extraction, 800 μL of chloroform and 460 μL of 0.05 M KCl were added to 1000 μL of the pooled lipid solution, mixed by vortex, and centrifuged (3000 ×g, 4°C, 10 min). The supernatant was discarded and the lipid fraction dried under nitrogen gas. To hydrolyse the extracted lipids, 500 μL of 9 M HCl : H_2O : acetonitrile (1 : 1 : 18) solution containing BHT (25 mg/50 mL) was added, and samples were covered with nitrogen gas and incubated overnight (65°C). Hydrolysed samples were dried under nitrogen gas and freeze-dried for 15 min before adding 250 μL of hexane and 10 μL of derivatising agent (1-tert-butyldimethylsilylimidazole). Samples were covered with nitrogen gas, incubated at 37°C for 2 h, and analysed using a Varian 3900 gas chromatograph (GC) coupled to a Varian Saturn 2100T mass spectrometer (MS). Samples were run on a Zebron ZB-5HT column (30 m × 0.25 mm ID × 0.25 mm film) with 70 eV ionization to determine cellular uptake of EPA and DHA. The n-3 index for each sample was calculated as a proportion of the combined integrated peak areas of DHA and EPA over the total peak area of fatty acids within the sample .

2.4. Von Willebrand Factor Plasma Concentration. Plasma vWF concentration was analysed using the IMUBIND vWF ELISA kit (Sekisui Diagnostics LLC, CT, USA).

2.5. Von Willebrand Factor Multimer Analysis. Plasma samples were defrosted at 37°C for 5 min and mixed at a 1 : 1 ratio with sample buffer (70 mM Tris base, 4 mM EDTA disodium salt, 9 M urea, 2.4% wt/vol sodium dodecyl sulphate (SDS) and bromophenol blue; pH 6.8). Samples were loaded onto 1% wt/vol horizontal agarose gels (SeaKem HGT-P) made up in gel buffer (100 mM Tris base, pH 8.8, 100 mM glycine and 0.4% wt/vol SDS) and run at 15 mA and 4°C using precooled HE33 mini horizontal agarose electrophoresis units (Hoefer, MA, USA) filled with running buffer cooled to 4°C (50 mM Tris base, 384 mM glycine and 0.1% wt/vol SDS, pH not adjusted; ~8.6) until the dye front reached the bottom of the gels (usually 6 h). Immobilon-P PVDF 0.45 μm membranes (Millipore Australia Pty. Ltd) were equilibrated in methanol

(1 min) and then transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, and 0.1% SDS, pH not adjusted; ~8.8; 15 min). Gels were equilibrated in transfer buffer for 15 min. A Hoefer TE 62 transfer electrophoresis unit was set up in an ice bath and filled with transfer buffer cooled to 4°C. The transfer was run at 200 Watts for 2 h, with constant stirring. Protein bands were detected using a Vectastain Elite ABC system staining kit, rabbit IgG (Vector Laboratories Inc., CA, USA) and the peroxidase substrate 3,3′-Diaminobenzidine tetrahydrochloride (DAB), with metal enhancer (SIGMA *FAST*). Sixteen bands were resolved, with unresolved very high molecular weight vWF (> band 16) also visible. Band intensity was quantified using ImageJ software (NIH, Bethesda, USA) after subtraction of background from an adjacent area of the membrane for each lane.

2.6. Collagen Binding Assay. Binding of vWF to collagen type I was assessed using the TECHNOZYM vWF:CBA ELISA Collagen Type I kit (Technoclone GmbH, Vienna, Austria).

2.7. Blood Pressure Measurements. Home blood pressure measurements were chosen for this study as they provide more accurate cardiovascular risk prediction than office-based clinical blood pressure measurements [13]. The threshold SBP and DBP defining stage 1 hypertension (135–137/86–89 mmHg) are lower for home compared to office blood pressure measurements (140/90) [14]. Subjects were provided with training in the correct method for acquisition of blood pressure data using a Medel Check automated blood pressure device. Four replicate measurements were collected each morning with exclusion of the first measurement. Subjects voided the bladder and abstained from caffeine prior to measurements. SBP and DBP were determined at baseline (7 consecutive days prior to randomization) and at week 12 (final 7 consecutive days of treatment).

2.8. Serum Lipid Analysis. Serum concentration of total cholesterol (TC), triglycerides (TG), and high density lipoprotein cholesterol (HDL-C) were determined using enzymatic Infinity kits (Thermo Fisher Scientific Australia Pty Ltd, Vic, Australia). Serum low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald Formula [15].

2.9. Statistical Analysis. Data were analysed using one-way ANOVA, with Tukey's post hoc analysis (IBM SPSS Statistics software, Version 22) or with *t*-tests (Microsoft Excel, Version 14.4.1).

3. Results

3.1. Subject Demographics. Subjects recruited to this study were matched for age and gender and, where possible, use of medication and smoking status (Table 1). The mean baseline BMI of subjects in the hypertensive LC n-3 PUFAs group ($n = 6$) was significantly higher when compared to subjects in the normotensive LC n-3 PUFAs treatment group (Table 1, one-way ANOVA, $P < 0.05$, $n = 9$). As expected, mean baseline SBP and mean baseline DBP were significantly higher in hypertensive subjects when compared to normotensive subjects (Table 1, one-way ANOVA, $P < 0.001$). Treatment of hypertensive subjects with the n-3 PUFAs had no significant effect on SBP or DBP (Table 1). There was no difference in serum lipid levels between the groups at baseline and after 12-week treatment with LC n-3 PUFAs (Table 1).

3.2. Omega-3 Index Calculations. The n-3 index was similar for hypertensive and normotensive groups at baseline. As expected, the n-3 index was significantly higher after 12-week n-3 PUFA supplementation when compared to baseline (Figure 1(a), one-way ANOVA, $P < 0.05$). Subjects allocated to the placebo capsules showed no enrichment of their erythrocyte membranes with n-3 PUFAs (Figure 1(a)). Treatment with LC n-3 PUFAs significantly reduced the proportion of arachidonic acid (AA) to all other FAs detected in the erythrocyte membrane phospholipids (% arachidonic acid) in normotensive subjects (Figure 1(b), one-way ANOVA, $P < 0.05$, $n = 13$). However, this was not observed in mildly hypertensive subjects (Figure 1(b)). Additionally, 12-week treatment with LC n-3 PUFAs significantly lowered the n-6 to n-3 PUFA ratio (Figure 1(c), one-way ANOVA, $P < 0.05$).

3.3. vWF Plasma Concentration. There was no significant difference in plasma vWF concentration between hypertensive and normotensive subjects at baseline (Figure 2(a)). 12-week supplementation with LC n-3 PUFAs or placebo had no effect on vWF plasma concentration in normotensive or hypertensive subjects (Figure 2(b)).

3.4. vWF Multimer Analysis. The change in pattern of subjects' vWF multimers throughout the 12-week study period was analysed. There was no significant difference in intensity of LMW (Figure 3(b), bands 1–5), IMW (Figure 3(c), bands 6–10), or HMW (Figure 3(d), bands >10) vWF bands after 12 weeks when compared to baseline within each treatment group (presented as fold change, Figure 3). Exposure to LC n-3 PUFAs or placebo for 12 weeks had no effect on the fold change of LMW (Figure 3(b)), IMW (Figure 3(c)), or HMW vWF band intensity (Figure 3(d)).

3.5. Collagen Binding Assay. Physiological function of vWF was assessed using a collagen type I binding assay. No difference in vWF function was observed between hypertensive and normotensive subjects at baseline (Figure 4(a)). 12-week treatment of subjects with LC n-3 PUFAs or placebo had no effect on vWF binding to collagen (Figure 4(b)).

4. Discussion

Treatment of normotensive and hypertensive subjects with LC n-3 PUFAs resulted in a significant increase in incorporation of EPA and DHA into erythrocyte membranes. Epidemiological studies conducted in the USA and Japan showed that an n-3 index < 4% is associated with a 10-fold increase in risk of sudden cardiac death compared to an n-3 index of >8% [16]. In the current study, 12-week treatment

Table 1: Subject demographics and clinical data for the different treatment groups.

Measurements	Treatment groups			
	Hypertensive n-3 PUFAs	Hypertensive placebo	Normotensive n-3 PUFAs	Normotensive placebo
Gender (F/M)	1/5	3/6	6/7	9/5
Age (years; mean ± SD)	45.7 ± 11.27	57.8 ± 10.29	51.1 ± 8.84	48.8 ± 8.84
BMI (kg/m^2; mean ± SD)	33.5 ± 3.64*	28.6 ± 5.01	25.9 ± 4.04	27.0 ± 3.97
Medications				
None	$n = 6$	$n = 4$	$n = 10$	$n = 9$
NSAIDs		$n = 1^\wedge$		$n = 1$
Antidepressant		$n = 3$	$n = 1$	$n = 2$
HRT		$n = 1^\wedge$	$n = 1$	
Contraceptive pill		$n = 1$		
Antiallergy			$n = 1$	$n = 1$
Paracetamol		$n = 1^\#$		
Thyroxine				$n = 1$
Glucosamine		$n = 1^\#$		
Pygeum		$n = 1^\#$		
Methotrexate		$n = 1^\#$		
Smoking status				
Current	$n = 2$	$n = 2$	$n = 2$	$n = 1$
Never	$n = 3$	$n = 4$	$n = 8$	$n = 5$
Previous	$n = 1$	$n = 3$	$n = 3$	$n = 8$
Blood pressure (mmHg)				
SBP, baseline	142.92 ± 3.14**	142.69 ± 2.35**	115.20 ± 2.07	116.60 ± 2.50
SBP, week 12	138.43 ± 3.63**	139.94 ± 3.58**	112.38 ± 2.59	115.27 ± 2.76
DBP, baseline	91.44 ± 3.74**	85.75 ± 3.47**	74.07 ± 1.29	73.88 ± 1.59
DBP, week 12	90.68 ± 3.28**	85.68 ± 3.03**	73.01 ± 1.72	73.21 ± 2.15
Serum lipids (mM)				
TC, baseline	4.35 ± 0.32	4.78 ± 0.43	4.93 ± 0.31	4.96 ± 0.33
TC, week 12	4.49 ± 0.34	4.69 ± 0.45	5.21 ± 0.33	5.04 ± 0.29
TG, baseline	1.33 ± 0.18	1.26 ± 0.34	1.06 ± 0.17	0.88 ± 0.10
TG, week 12	1.05 ± 0.13	0.95 ± 0.24	0.90 ± 0.14	0.99 ± 0.11
HDL-C, baseline	1.11 ± 0.13	1.48 ± 0.26	1.54 ± 0.17	1.67 ± 0.14
HDL-C, week 12	1.26 ± 0.12	1.66 ± 0.28	1.55 ± 0.18	1.70 ± 0.15
LDL-C, baseline	0.87 ± 0.20	0.93 ± 0.17	1.06 ± 0.13	1.10 ± 0.15
LDL-C, week 12	0.99 ± 0.15	0.95 ± 0.12	1.25 ± 0.13	1.07 ± 0.12

n-3 PUFAs: omega-3 polyunsaturated fatty acids; BMI: body mass index; NSAIDs: nonsteroidal anti-inflammatory drugs; HRT: hormone replacement therapy; $^{\wedge,\#}$multiple medications taken by the same subject; BP: blood pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; SL: serum lipids; TC: serum total cholesterol; TG: serum triglycerides; HDL-C: serum high density lipoprotein cholesterol; LDL-C: serum low density lipoprotein cholesterol; $^*P < 0.05$ (hypertensive n-3 PUFA versus normotensive n-3 PUFA treatment group; one-way ANOVA); $^{**}P < 0.001$ (hypertensive versus normotensive at baseline and week 12; one-way ANOVA). Data are expressed as mean ± SEM unless indicated otherwise.

increased the n-3 index to cardioprotective levels (>8%). The n-6 : n-3 PUFA ratio was significantly reduced after 12-week treatment with LC n-3 PUFAs. The modern western diet contains high amounts of n-6 PUFAs resulting in a high n-6 to n-3 ratio (~15 : 1), which may promote the development of cardiovascular disease [17]. Increased consumption of n-3 PUFAs lowers the n-6 to n-3 ratio and a dietary ratio of 4 : 1 can reduce mortality from cardiovascular disease [17].

The effect of mild hypertension on vWF levels is controversial. Whilst one study reported an increase of vWF in patients with mild hypertension [8], another did not [9]. We report here that subjects with mild hypertension did not present with higher plasma vWF levels.

Endothelial cells contain granules (WP bodies) that store large amounts of ultra-large vWF multimers. Increased fluid shear stress can induce WP body degranulation [4, 5] and it is therefore possible that endothelial cells exposed to high shear stress during hypertension release this vWF into the vascular lumen [18]. The ultra-large vWF multimers are prone to cleavage, which could result in changes to the distribution of multimeric vWF. In the current study we observed no significant difference in intensity of LMW,

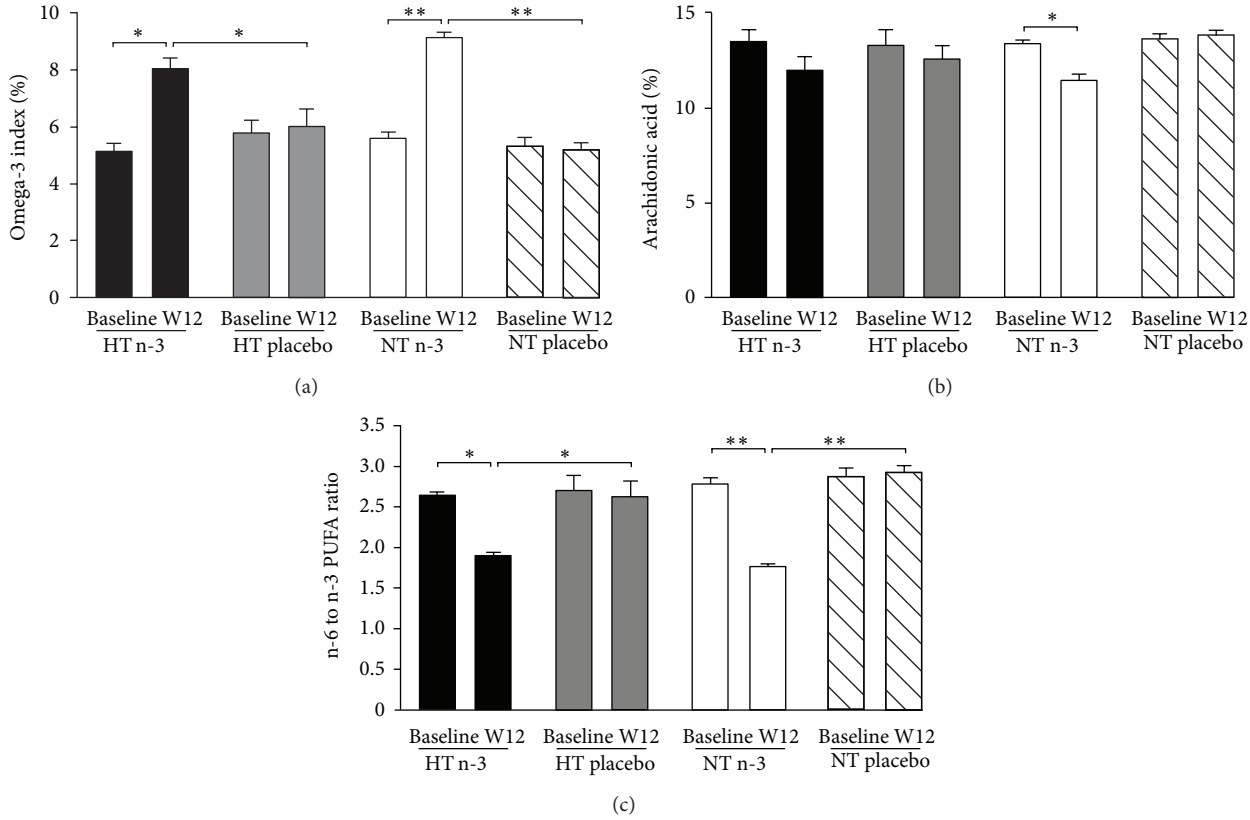

Figure 1: Measurement of the incorporation of long chain polyunsaturated fatty acids (LC PUFA) into erythrocyte membrane phospholipids at baseline and after 12 weeks of treatment with 2.52 g/day n-3 PUFAs. Parameters measured were the omega-3 index (a), the percent arachidonic acid (b), and the n-6 to n-3 PUFA ratio (c) (one-way ANOVA, *: $P < 0.05$; **: $P < 0.001$). Data are expressed as mean ± SEM. W12, Week 12; HT, hypertensive; NT, normotensive; n-3, omega-3 LC PUFA.

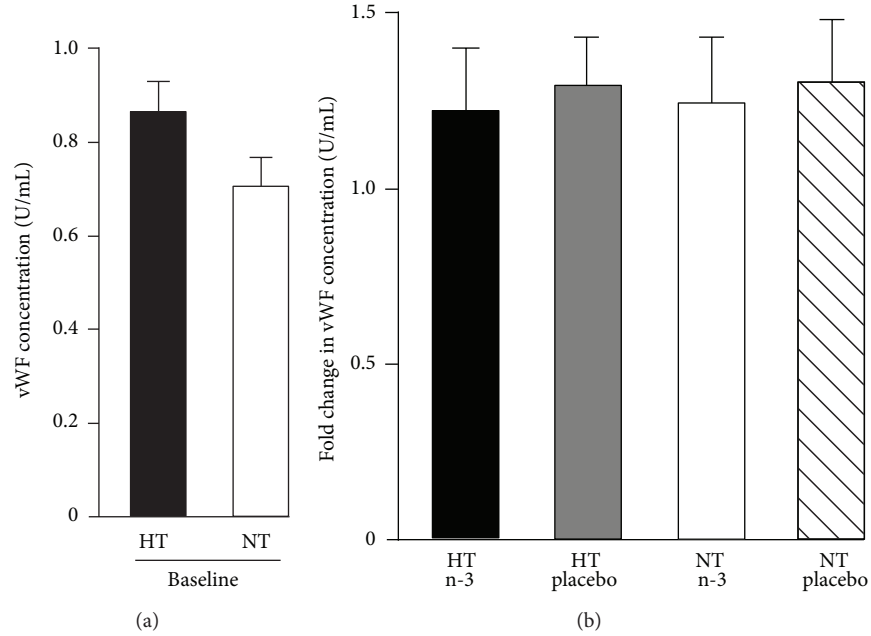

Figure 2: Plasma von Willebrand factor (vWF) concentration at baseline and after 12 weeks of treatment with 2.52 g/day long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs). Initial vWF concentration (international units/mL) was not different for hypertensive and normotensive subjects (a). The 12-week supplementation protocol with LC n-3 PUFAs had no effect on plasma concentration of vWF in normotensive and hypertensive subjects (b). Data are expressed as mean ± SEM. HT, hypertensive; NT, normotensive; n-3, LC n-3 PUFAs.

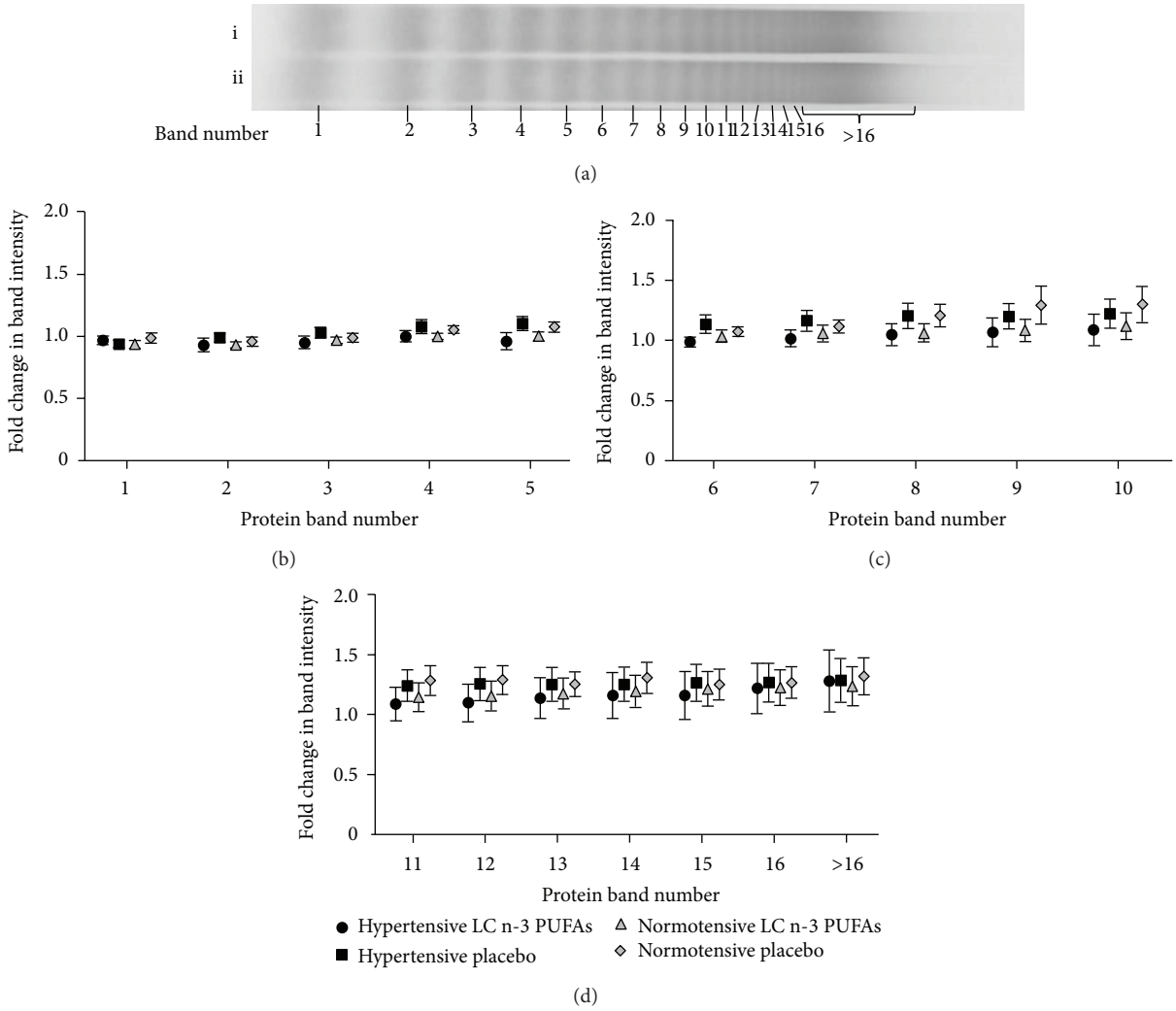

Figure 3: Fold change in von Willebrand factor (vWF) multimer band intensity after 12 weeks of treatment with LC n-3 PUFAs. Representative blots show protein bands at baseline (a)i and after 12 weeks (a)ii. Treatment with placebo or LC n-3 PUFAs had no effect on band intensity of low molecular weight (LMW) vWF (b, bands 1–5), intermediate molecular weight (IMW) vWF (c, bands 6–10), or high molecular weight (HMW) vWF (d, bands > 10) in hypertensive or normotensive subjects. LC n-3 PUFAs had no effect on the fold change of band intensity. Data are expressed as mean ± SEM.

IMW, or HMW vWF bands after 12-week LC n-3 PUFA treatment when compared to baseline. Although change in vWF concentration and change in vWF multimer profile were similar across treatment groups and intragroup variability was very small, it might be of interest in future studies to investigate larger cohorts of subjects or a population of subjects with more severe hypertension. It is noteworthy however that severe hypertension is typically managed with blood-pressure lowering medications and that this affects vWF profile [7, 19].

HMW vWF has greater thrombogenic potential than IMW or LMW vWF. vWF function can be assessed using a collagen type I binding assay. No difference in vWF function was observed between hypertensive and normotensive subjects at baseline. 12-week treatment of subjects with LC n-3 PUFAs or placebo had no effect on vWF binding to collagen. Whilst we excluded subjects who were taking antihypertensive medications and subjects who had a high dietary intake of LC n-3 PUFAs, we made no further exclusions based on other medications that subjects might be taking. It is possible that some of these might have impacted on vWF expression or function.

Blood pressure lowering effects of n-3 PUFAs have been reported in some, but not all studies. Responsiveness of blood pressure to n-3 PUFAs is likely influenced by disease state (n-3 PUFAs have greater blood pressure-lowering effects in untreated hypertensive patients than in normotensive subjects; [11]), dose (Higher doses of n-3 PUFAs are required for responses such as blood pressure reduction, whereas lower doses are sufficient for responses such as improved myocardial oxygen efficiency; [20]), and formulation (n-3 PUFAs, but not their ethyl ester derivatives, activate BK

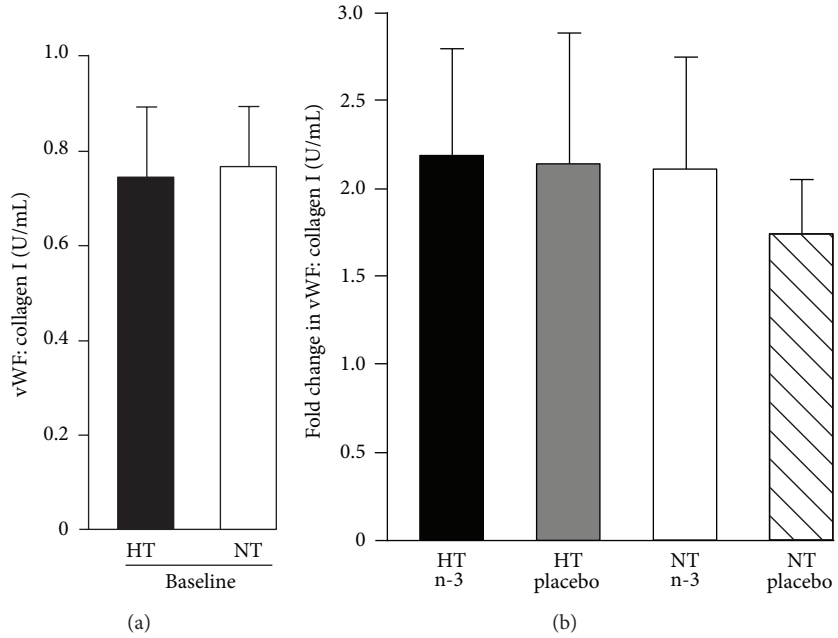

Figure 4: Effect of long chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA) supplementation on von Willebrand factor (vWF) binding to collagen type I. There was no difference in vWF binding (international units/mL) to collagen between hypertensive and normotensive subjects at baseline (a). Twelve-week treatment of subjects with LC n-3 PUFAs or placebo had no effect on vWF binding to collagen (b). Data are expressed as mean ± SEM. HT, hypertensive; NT, normotensive; n-3, LC n-3 PUFA.

channels to reduce blood pressure; [21]). In this study, a nonsignificant trend for reduced SBP (4.5 mmHg) was detected in our cohort of mildly hypertensive subjects.

In conclusion, mildly hypertensive patients did not present with increased plasma vWF levels or vWF function measured by collagen binding capacity. This questions the suitability of vWF levels as a marker for endothelial dysfunction at an early disease state and indicates that, at the early onset of hypertension, vWF is not a target of LC n-3 PUFAs to mediate their cardioprotective effects at the concentration and duration used in this study.

Conflict of Interests

The authors declare that there is no conflict of interests in their submitted paper.

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Review Article

Omega-3 PUFAs Lower the Propensity for Arachidonic Acid Cascade Overreactions

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A productive view of the benefits from omega-3 (n-3) nutrients is that the dietary essential omega-6 (n-6) linoleic acid has a very narrow therapeutic window which is widened by n-3 nutrients. The benefit from moderate physiological actions of the arachidonic acid cascade can easily shift to harm from excessive pathophysiological actions. Recognizing the factors that predispose the cascade to an unwanted overactivity gives a rational approach for arranging beneficial interactions between the n-3 and n-6 essential nutrients that are initial components of the cascade. Much detailed evidence for harmful cascade actions was collected by pharmaceutical companies as they developed drugs to decrease those actions. A remaining challenge is to understand the factors that predispose the cascade toward unwanted outcomes and create the need for therapeutic interventions. Such understanding involves recognizing the similar dynamics for dietary n-3 and n-6 nutrients in forming the immediate precursors of the cascade plus the more vigorous actions of the n-6 precursor, arachidonic acid, in forming potent mediators that amplify unwanted cascade outcomes. Tools have been developed to aid deliberate day-to-day quantitative management of the propensity for cascade overactivity in ways that can decrease the need for drug treatments.

1. Introduction to Cascade Control

Elucidation of the arachidonic acid cascade [1] began 50 years ago with the simultaneous publication of evidence for enzymatic formation of prostaglandin E2 (PGE2) from the n-6 5,8,11,14-eicosatetraenoic acid, arachidonic acid [2, 3]. The reports were soon accompanied by evidence that two other prostaglandins, PGE1 and PGE3, were formed from the n-6 8,11,14-eicosatrienoic acid and the n-3 5,8,11,14,17-eicosapentaenoic acid, respectively [4]. Another 1964 report described different relative potencies of PGE1 greater than PGE2 greater than PGE3 when controlling the hormone-activated mobilization of fatty acids from adipose tissue [5]. Further research discovered many enzymes in a metabolic cascade that synthesizes a diverse family of bioactive molecules called eicosanoids, including multiple forms of prostaglandins, thromboxanes, and leukotrienes [1]. These potent agents act through selective receptors [6, 7] present on nearly every tissue of the body, giving the cascade an ability to influence nearly every major physiological and pathophysiological event.

Scientific curiosity about molecular mechanisms in the formation and action of the cascade components [1, 8] partnered with the pharmaceutical industry's interest in developing, patenting, and marketing agents that can diminish unwanted pathophysiology. The alliance generated over 175,000 scientific reports cited by PubMed with nearly 5,000 new reports appearing annually for the past 30 years. Understanding the enzymes, cofactors, and receptors that create the health outcomes of the cascade helps identify new targets for drug development [8]. Newly developed agonists and antagonists of the selective receptors [7] continue to attract attention as researchers gain broader understanding of arachidonic acid cascade actions in inflammation and immune function, arthritis, asthma, COPD, cardiovascular diseases (including atherogenesis and thrombosis), metabolic syndrome, back pain, headache, bone density loss, cancer proliferation, Alzheimer's disease, brain development, learning and memory, behavioral disorders, oppositional behaviors, depression, and suicide. This large number of physiological and pathophysiological events indicates the wide scope of conditions in which the balance between

benefit and harm from arachidonic acid cascade products constitutes a narrow therapeutic window that this review recognizes as a situation we must manage.

2. A Narrow Therapeutic Window for NSAIDs

The first major insight for developing drugs to control the cascade was the recognition that aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase-catalyzed conversion of arachidonic acid into prostaglandins [9]. After discovery of the cyclooxygenase product, thromboxane, as a potent derivative that mediates thrombosis [10], aspirin was increasingly used to give benefits by lowering the risk of heart attacks. Small amounts of aspirin gave benefits by irreversibly inhibiting platelet cyclooxygenase. However, adverse side effects and iatrogenic deaths from larger doses of aspirin illustrate the need to recognize how narrow the therapeutic window can be for antithrombogenic agents like aspirin, warfarin, and Plavix. While small doses give benefits by decreasing risk of thrombosis, larger doses cause harm as they impair other needed actions of cyclooxygenase products. Decreasing the risk of unwanted blood platelet activity is now widely achieved by use of "low-dose" aspirin to avoid adverse events that come from blocking beneficial actions of the arachidonate cascade. After a second form of cyclooxygenase was discovered, tens of billions of dollars were spent in developing and marketing various selective "COX-2 inhibitors" with the hope of avoiding the adverse events linked to aspirin use. However, the new agents also had a limited therapeutic window [11], and some were withdrawn from the market.

A challenge for drug developers attempting to control harmful outcomes of the cascade is to find selective agents that will balance the remarkably diverse harmful and beneficial actions of the eicosanoids. The general strategy is to focus on specific events downstream from the initial phospholipase-catalyzed release of eicosanoid precursors and the oxygenase-catalyzed formation of reactive intermediates with the hope of creating more selective inhibition of specific unwanted receptor-mediated outcomes with fewer adverse iatrogenic effects caused by inhibiting beneficial cascade actions. The continuing search for new agonists and antagonists is enhanced by powerful techniques of cloning specific receptors and creating genetic "knock-out" animals [7] to identify explicit downstream effectors that give either beneficial or harmful outcomes.

While the pharmaceutical industry continues searching for drugs to treat and control unwanted actions of the arachidonic acid cascade, an alternate approach is suggested by the evidence that some populations seem less predisposed than others for some cascade-related health disorders [12, 13]. Traditional ethnic food habits may unintentionally provide a lower propensity for harmful cascade outcomes. The 1964 recognition that three different essential fatty acids form three different PGE compounds with different potencies [5] set the stage for considering a strategy of altering the "upstream" precursors of the cascade. Evidence of omega-3 acids competitively inhibiting conversion of n-6 arachidonic acid to prostaglandin [14] showed that the balance of precursor acids in the cascade can control formation rates as well as giving mediators with different receptor responses [5]. Rather than blocking harmful events in the cascade with drugs, people might deliberately create a predisposition for less harmful outcomes and thereby decrease the need for treatments. This review examines the degree to which balancing the n-3 and n-6 20- and 22-carbon highly unsaturated fatty acids (HUFA) that are maintained in the phospholipids of cell membranes can control beneficial and harmful outcomes of the arachidonic acid cascade.

3. Precursor-Based Propensities and Predispositions

The relative intensity of action by the n-3 eicosapentaenoic acid (20:5n-3) relative to that for the n-6 eicosatetraenoic acid (20:4n-6) in the prostaglandin part of the cascade is shown in Figure 1 as a ratio next to the enzymatic step or specific receptor. The initial step of phospholipase release of the precursor does not discriminate between the n-3 and n-6 structures, giving ratios of 1.0 for both cytosolic (cPLA2) and secretory (sPLA2) hydrolase actions. In contrast, both cyclooxygenases, COX-1 and COX-2, react more slowly with the n-3 than the n-6 substrate, giving cascade-mediated actions that are less intense with the n-3 precursor [15]. Different proportions of n-3 and n-6 precursors in cellular membrane lipids give different intensities of n-6 agonist action, and weak n-3 agonist action inevitably becomes an antagonist of the more potent n-6 agonist.

This difference in actions occurs also with the synthases that form PGD and PGE, ensuring that supplies of those prostaglandins at their specific cell-membrane receptors will tend to be less intense for n-3 than n-6 eicosanoids. Finally, the selectively lower responses by EP and FP receptors with n-3 mediators moderate even further any predisposition or propensity for excessive actions of the arachidonic acid cascade. The consequence of different cascade responses to n-3 and n-6 acids was summarized in a 2014 review in terms of a therapeutic window for dietary essential n-6 nutrients that is very narrow, and its margin of safety is widened by eating n-3 nutrients [16].

For the leukotriene portion of the cascade shown in Figure 2, the first two steps, PLA2 and 5-lipoxygenase (5-LO), are relatively indiscriminate, whereas the leukotriene C synthase (LTCS) forms cysteinyl leukotrienes 10-fold [17] to 20-fold [18] less vigorously with n-3 than n-6 LTA intermediates. The lower intensity of LTC formation allows a higher proportion of n-3 in HUFA to lower the intensity of chronic bronchopulmonary events seen in asthma and chronic obstructive pulmonary disease (COPD). Additionally, the LTB receptor (BLT-1) that mediates important immune-inflammatory events responds 50-fold less vigorously to n-3 LTB5 than the potent n-6 LTB4 [19], making the development of severe inflammatory loci less intense when the HUFA balance has more n-3 HUFA.

The examples provided above illustrate how both n-6 and n-3 precursors give active mediators in the arachidonic acid cascade. However, the more intense responses from n-6 mediator actions increase the propensity for cascade overreactions that shift conditions from normal physiology

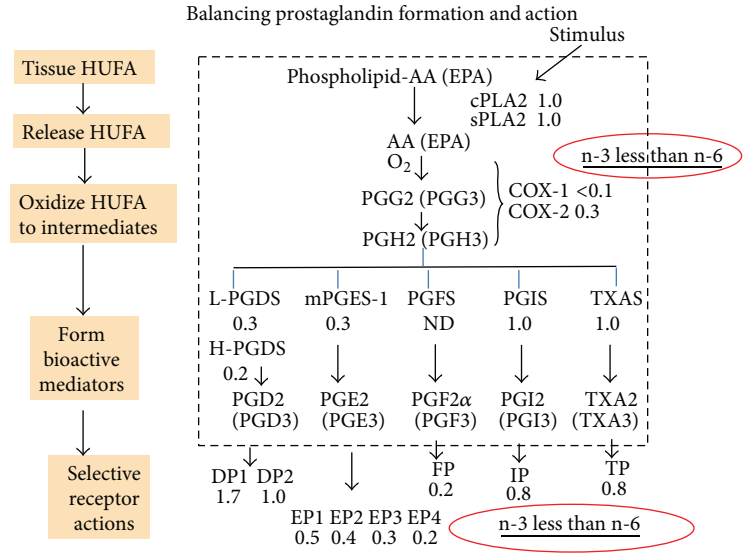

Figure 1: Balancing prostaglandin formation and action. The prostaglandin part of the arachidonic acid cascade begins with a stimulated phospholipase A2 releasing precursor HUFA from membrane phospholipids. The relative intensity of reaction for n-3 and n-6 mediators is shown as a ratio next to the interacting enzyme or receptor.

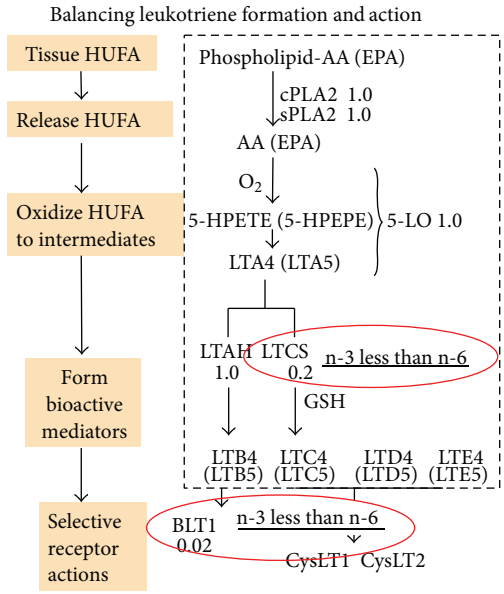

Figure 2: Balancing leukotriene formation and action. The leukotriene part of the arachidonic acid cascade begins with a stimulated phospholipase A2 releasing precursor HUFA from membrane phospholipids. The relative intensity of reaction for n-3 and n-6 mediators is shown as a ratio next to the enzyme or receptor.

to pathophysiology when the n-6 precursor is the dominant HUFA available. As a result, arranging for n-3 HUFA to be a greater proportion of the HUFA can moderate cascade-mediated events and widen the therapeutic window for dietary n-6 precursors. Of course, the cascade is not the only way that HUFA-based events affect human health. Endocannabinoids, arachidonyl glycerol and arachidonoyl ethanolamide, are HUFA-based bioactive lipids that act through selective receptors, CB1 and CB2. While some evidence suggests that arachidonoyl glycerol may be the biologically relevant mediator [20], results with fatty acid amide hydrolase inhibitors [21] indicate that both n-3 and n-6 HUFA ethanolamides may have important beneficial actions.

Although some studies show that CB1 and CB2 receptors have high structural specificity for the n-6 structure with a double bond at position 5 [20, 22, 23], the degree to which competing n-3 and n-6 forms of the ester and amide types of mediators influence health outcomes remains uncertain. Similarly, n-6 lipoxins [24] formed from arachidonic acid by multiple lipoxygenase actions bind the ALX/F receptor [25] and counteract some unwanted events mediated by the cascade. Further research studies will assign the degree to which various endocannabinoid and lipoxin actions contribute to major human health conditions and whether the balance of n-3 and n-6 HUFA influences their actions. Another group of n-3 HUFA-based bioactive mediators includes resolvins, protectins, and maresins [26] which counteract and help resolve inflammatory events promoted by the n-6 cascade mediators. With all of the mediator actions noted above, the same strategy of arranging for n-3 HUFA to be a greater proportion of tissue HUFA will moderate unwanted cascade-mediated health conditions caused by overabundant n-6 precursor availability and action. The degree of dominance of n-6 HUFA among the HUFA available for tissue responses can be called "HUFA balance," and it provides a useful biomarker that can be expressed as either the %n-6 in HUFA or the %n-3 in HUFA. Because the n-3 and n-6 acids are dietary essentials, accumulating a desirable HUFA balance in tissues inevitably depends on the average proportions of essential n-3 and n-6 nutrients provided by personal food choices. Examples of the impact of ethnic food habits on HUFA balance and health outcomes are noted below.

4. Food Habits Affect the Intensity of Cascade Outcomes

The 1979 report [12] relating food choices to the balance in actions of prostacyclin (PGI) and thromboxane (TXA) described a striking difference in the relative amounts of n-3 to n-6 precursors in blood phospholipids of Danes (0.2 n-3 to 8.0 n-6) and Eskimos (7.1 n-3 to 0.8 n-6). Subsequent increasingly detailed analyses have described a HUFA balance of 32% n-6 in HUFA in Greenland [27] and 44% [28] or 41% to 38% in Canadians at Nunavik [29]. A more recent survey at Nunavik showed different age groups with blood HUFA balance of 52% n-6 in HUFA for 45–74 years of age, 61% for 35–44, 68% for 25–34, and 72% for 18–24 [30]. The biomarker differences reflect the rapidly changed lifestyles among Arctic people as they acquire access to commercially prepared foods not typical for their traditional lifestyle [12]. The long-recognized low incidence of heart attacks among these people is not attributable to low blood cholesterol [31]. Rather, it likely is due to high tissue proportions of the moderating omega-3 precursors of the arachidonic acid cascade that blunt the predictive role of the food energy biomarker, blood cholesterol (see Figure 1 in [16]).

Japanese people also experienced a change in lifestyle in the past 50 years as "Western-style" processed foods displaced traditional foods and shifted the balance of arachidonate cascade precursors in their tissues [13]. The proportion of n-6 in HUFA has been progressively higher for younger Japanese generations until values for the youth approach those of American youth. The %n-6 in HUFA was near 35% for Japanese born in 1938, 42% for 1950, and 60% for 1974 [32]. The long-recognized low death rates from prostate cancer among Japanese octogenarians [33] rose steadily from 50 to 200 (per 100,000 people) between 1960 and 2000 while prostate cancer death rates for Americans (with a consistently higher %n-6 in HUFA near 78%) remained relatively unchanged at 500.

During this time, corresponding Italian prostate cancer mortality rates rose from 200 to 400 [33] while changes from traditional Mediterranean food habits were observed [34, 35]. In 2013, biomarker values near 84% were reported for 2- to 9-year-old Italian children, whereas the average was 78% for 40–59-year-old adults [36] and 79-80% for Italians in a multicenter study [37]. Residents of northern Italy had values of 80% n-6 in HUFA, whereas those on the islands of Sardinia and Sicily had values near 73% [38]. The biomarker was 67% n-6 in HUFA for residents of Barcelona on the Mediterranean coast [39], and it was 72% when inland Spanish communities were included [40]. Definitions of the "traditional Mediterranean Diet" become increasingly imprecise when children in Portugal have 78% n-6 in HUFA compared to 76% in Germany [35].

The biomarker of %n-6 in HUFA indicates the propensity for arachidonate cascade overreactions and is a useful indicator for health risk assessment. For example, the incidence of heart attacks for the quintile of Americans maintaining about 62% n-6 in HUFA was nearly one-half of that for those with median values near 80% [41]. Cross-cultural comparisons showed a very close association of CHD mortality with values from 32% to 80% n-6 in HUFA [41]. Knowing about n-6 mediators of inflammatory atherogenesis and of platelet-mediated thrombosis makes a change of 10% to 20% in this biomarker an important aspect in health conditions. Blasbalg et al. described the dramatic rise in n-6 linoleic acid consumption in the USA during the late 20th century [42]. The change reflects a widespread insertion of vegetable oils into many "modern" processed foods. That change was assessed by one research group [43] in these terms: "The widespread consumption of diets with more than 2% energy as LA should be recognized for what it is—a massive uncontrolled human experiment without adequate rationales or proven mechanisms." A constructive alternative to this situation would be to apply quantitative knowledge of the metabolic dynamics of dietary n-3 and n-6 nutrients to moderate the current propensity for unwanted overactive arachidonic acid cascade events.

5. Metabolic Dynamics in Balancing Tissue HUFA Proportions

The 18-carbon n-3 linolenic and n-6 linoleic acids in foods are major precursors of the n-3 and n-6 HUFA that accumulate in tissue phospholipids and eventually form eicosanoids. The n-3 and n-6 nutrients compete for the same enzymes with similar hyperbolic dynamics during the desaturation and elongation steps that form the HUFA [44–46]. Steep dose-response curves for n-3 and n-6 18-carbon essential nutrients providing tissue 20- and 22-carbon HUFA (see Figure 4 in [47]) describe the response when those nutrients are supplied in the range of 0 to 1 percent of daily food energy (en%). When supplied in amounts above 1 or 2 en%, a blunted dose-response relationship has the hyperbolic "plateau" characteristic of all saturable enzyme-catalyzed metabolic processes. The lack of a continuing linear response does not indicate that the precursor does not form the product. Rather, it is a reminder that dose-response dynamics need to be interpreted in terms of the range of substrate supply that is provided. Interpretations of results from diet-tissue studies can be improved by acknowledging that the sensitive dose-response range for accumulating n-6 HUFA from linoleic acid is below 0.5%en [16].

Another caution arises when n-3 linolenate is ingested in mixtures with severalfold greater amounts of n-6 linoleate. In this case, the indiscriminate enzymes inevitably react in proportion to the substrate supplied and make the n-3 nutrient appear less productive than the competing n-6 nutrient in forming and accumulating tissue HUFA [45]. This effect was evident in a large meta-analysis [48] that was limited to only additions of an n-3 fatty acid to diets that had much more n-6 than n-3 nutrient in all cases evaluated. The limited data led to limited interpretations. However, the seldom described or discussed converse condition of eating more n-3 than n-6 precursor clearly gives more accumulated n-3 than n-6 HUFA [45, 49]. The n-3 linolenic acid is not a poor substrate for forming HUFA. A 2014 review of the dynamics of essential fatty acid actions and tissue HUFA formation [16] noted that dietary intakes of linoleic acid between 0.3 and 0.5 percent of food energy (en%) prevent

signs of essential fatty acid deficiency and maintain tissue arachidonate above 50% of HUFA [44, 45, 49]. Importantly, lower values for the %n-6 in HUFA are associated with less inflammatory conditions [50] and cardiovascular events [41]. Lower values for HUFA balance are made possible by competing dietary n-3 nutrients that keep the tissue HUFA balance below 50% n-6 in HUFA. In the absence of n-3 nutrients, very small amounts (0.5 percent of daily calories) of the essential n-6 nutrient, 18:2n-6, can give a HUFA balance above 50% n-6 in HUFA, approaching values that are clearly associated with greater harm. Thus, the n-6 nutrient has a very narrow to almost nonexistent therapeutic window. Fortunately, the window of safety is widened by competing n-3 nutrients [16].

Some foods contain already formed n-3 and n-6 HUFA that compete with newly formed HUFA for storage in cellular phospholipids. The nonlinear competing interactions were described in 1992 by an empirical hyperbolic equation that fit data for rats, mice, and humans [51]. The general equation estimated well the observed balance of tissue n-3 and n-6 HUFA that was maintained by known daily intakes of n-3 and n-6 nutrients for nearly 4,000 people in 92 subject groups in 34 different studies from 11 different countries [52]. The similarity of the relatively indiscriminate dynamics for lipid metabolism among mammals reflects a well-recognized biological phenomenon of similar enzymes coded by similar housekeeping genes that did not vary much in structure or function during the evolution of diverse species.

Nevertheless, genetic adaptation has occurred for a human-specific haplotype defined by 28 closely linked single-nucleotide polymorphisms (SNPs) which affects the biosynthesis of HUFA from the 18-carbon precursors [53]. Haplotype D gives faster fatty acid desaturase activities (FADS1 and FADS2), making the newly synthesized HUFA more closely reflect the proportions of the 18-carbon precursors (and reflect less the dietary HUFA proportions). Interestingly, the "fast" haplotype D shows evidence of positive selection in African people and is less frequent outside of Africa [53, 54]. As a result, many African Americans living in a current food environment with high intakes of n-6 linoleic acid may now accumulate a higher %n-6 in HUFA [55, 56] than occurring in the African environment that favored selection of the alleles that form the D haplotype. With a genetic propensity to convert the USA high proportion of n-6 to n-3 PUFA into higher proportions of arachidonate in tissue HUFA, African Americans illustrate more clearly than Caucasians the need to lower intakes of n-6 PUFA and decrease the HUFA balance of %n-6 in HUFA to avoid excessive actions of the arachidonate cascade.

6. Choosing Foods That Lower the Propensity for Cascade Overreaction

The balance of tissue HUFA maintained by daily food habits is readily measured by simple gas chromatographic analysis of a finger-tip blood-spot [57, 58]. As noted above, values for the HUFA balance range from 25% to 85% n-6 in HUFA depending on a person's average daily food habits. This health risk assessment biomarker relates predictably to the average daily balance of n-3 and n-6 nutrients (expressed as en%) in the foods that are routinely eaten [16, 51]. Several tools were developed to show people how the n-3 and n-6 nutrient supply relates to the tissue HUFA precursors of the arachidonate cascade. A simple spreadsheet calculator [59] embeds the empirical competitive hyperbolic relationship [60] to help clinicians design dietary interventions that can create sufficient differences in HUFA balance between intervention and control groups.

The equation [60] is also combined with specific nutrient data from the USDA Nutrient Database [61] to give an interactive computer program, KIM-2 [62], to give estimates of the likely HUFA balance that would result from continued ingestion of foods in the plan. Tissue lipid pools need weeks and months to equilibrate, and nutritionists advise having many different daily plans during that time to maintain palatability. Examples of daily plans that give HUFA balances of 15%, 26%, 35%, 50%, 63%, 71%, and 91% n-6 in HUFA are described (chapter 19 in [63]).

To see more quickly the likely impact that a food item will have on the eventual HUFA balance, eleven different n-3 and n-6 nutrients in a food item (expressed as mg/Cal) are combined into a single Omega 3-6 Balance Score for each food item [64]. Foods with positive scores increase the proportion of n-3 in HUFA, whereas foods with negative scores increase the proportion of n-6 in HUFA. The scores range in values from +100 to −200, and many common vegetables, fruits, and dairy products have values near 0. The calorie-weighted average score for a day's food relates linearly to the health risk assessment biomarker predicted by the software [62]. As average daily food scores range from +3 to −7, the corresponding blood biomarker value ranges from 25% to 85% n-6 in HUFA [64]. Deleting foods with high negative scores and adding foods with positive scores lower the %n-6 in HUFA. Significant positive health benefits came in a randomized controlled clinical trial that used this approach for three months and lowered the biomarker value from 77% to 61% n-6 in HUFA [65]. Such results parallel the finding that CHD deaths were nearly one-half for the quintile of Americans maintaining about 62% n-6 in HUFA compared to those with median values near 80% [41].

Lowering the propensity for arachidonate cascade overreaction is important for most large USA employers that have large financial losses due to employees' health-related absenteeism, presenteeism, and medical and pharmacy expenses [66]. Many health conditions that cause major financial losses [67] are made worse by n-6 mediators in arachidonate cascade overreactions (Table 1). A simple wellness plan that informs employees of their health risk status from finger-tip blood-spot assays and informs them of Omega 3-6 Balance Scores of common foods may help employees voluntarily shift their HUFA balance toward the lower values of %n-6 in HUFA which have a lower propensity for arachidonate cascade overreactions [16]. An Omega 3-6 Balance Score App [68] lists over 5,000 foods in a searchable format.

The ease with which a voluntary shift can be made is illustrated with the 100 foods most frequently eaten by Americans [69] which have an average Omega 3-6 Balance Score near −6 [16, 64]. Removing the ten foods with the

Table 1: Prevalence of health conditions causing major USA health care costs. The 25 most prevalent conditions in a large occupational medicine study [67] are shown with the ten having the most annual cost ranked in order. The overall annual costs include expenses from medical, pharmacy, absenteeism, and presenteeism aspects.

Health condition prevalence	Rank	Cost Rank
Depression	6	1
Obesity	2	2
Arthritis	4	3
Back and neck pain	9	4
Anxiety	7	5
GERD	5	6
Allergy	1	7
Other cancers	19	8
Other chronic pains	17	9
Hypertension	3	10
Asthma	8	
Migraine	10	
Sleeping problem	11	
Irritable bowel	12	
Fatigue	13	
Headache	14	
Diabetes	15	
Bladder/urinary	16	
Ulcer	18	
Coronary heart disease	20	
Osteoarthritis	21	
Skin cancer	22	
Bronchitis/emphysema	23	
Congestive heart failure	24	
COPD	25	

most negative scores gave 90 items with an average value near −3 (a value associated with traditional Mediterranean meals). The removed foods were not typically eaten 100 years ago in Mediterranean households, and their recent use is likely a reason for current measures of HUFA balance for Mediterranean people to be near 75% to 80% rather than 63%. Finding food combinations that shift the average daily Omega 3-6 Balance Score a few points more positive may decrease the HUFA balance to a 10% to 20% lower value and lower the unwanted propensity for arachidonate cascade overreactions.

7. Conclusion

Omega-3 nutrients play an important role in moderating the inherent propensity for arachidonic acid cascade overreactions when n-6 mediators dominate. The unintended consequences of eating foods that create such conditions can be prevented by combining knowledge about the explicit balance of n-3 and n-6 nutrients in each food item with knowledge of the dynamics of fatty acid metabolism and the different intensities of n-3 and n-6 eicosanoid actions. Tools are freely available for making informed food choices that can prevent serious health conditions caused by cascade overreactions.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

ω-3 PUFAs and Resveratrol Differently Modulate Acute and Chronic Inflammatory Processes

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ω-3 PUFAs and polyphenols have multiple effects on inflammation *in vivo* and *in vitro*. The effects of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and resveratrol (RV) were investigated in LPS-stimulated peripheral blood leukocytes (PBLs) (i.e., acute inflammation) and IL-1β activated human chondrocytes (i.e., chronic inflammation). Inflammatory mediators including chemokines, cytokines, interleukins, and PGE_2 were measured by multiplex analysis and gene expression was quantified by RT-PCR. In PBLs, RV decreased the secretion of PGE_2, CCL5/RANTES, and CXCL8/IL-8 but increased IL-1β, IL-6, and IL-10. In contrast to RV, ω-3 PUFAs augmented the production of PGE_2 and CXCL8/IL-8. EPA and DHA similarly affected the pattern of inflammatory mediators. Combination of RV and ω-3 PUFAs exerted synergistic effects on CCL5/RANTES and had additive effects on IL-6 or CXCL8/IL-8. Both ω-3 PUFAs and RV reduced catabolic gene expression (e.g., MMPs, ADAMTS-4, IL-1β, and IL-6) in activated chondrocytes. The data suggest that ω-3 PUFAs and RV differ in the regulation of acute inflammation of peripheral blood leukocytes but have common properties in modulating features related to chronic inflammation of chondrocytes.

1. Introduction

Cells and tissues respond to changes in physiological milieu as well as to external insults. Acute inflammatory processes are part of the normal response of the organism and are indispensable to restoring homeostasis. This is achieved by multiple mediators that initiate and modulate the extent and duration of and also resolve inflammatory processes. When these feedback mechanisms fail or are dysregulated, inflammatory mediators might remain in a status of low-grade or chronic inflammation as observed in obesity, cardiovascular diseases (CVD), diabetes, and arthritis. Cells of the immune system including peripheral blood leukocytes (PBLs) have a key role in the regulation of acute and chronic inflammation. They are continuously exposed to various nutrients, which can therefore influence their metabolic and functional status. Food components like ω-3 polyunsaturated acids (PUFAs) or micronutrients such as vitamins and antioxidants were found to beneficially modulate inflammatory processes and diseases [1–3]. Specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) modulate eicosanoid metabolism and are precursors of mediators that are essential for the resolution of inflammation [4]. The anti-inflammatory effects of ω-3 PUFAs have been demonstrated in cellular systems and human studies [5–16] (see also [17] for review). On the other hand, *in vitro* activated chondrocytes are an adequate cellular model to analyse potential effects of nutrients on cells such as chondrocytes at conditions of chronic inflammation.

In this study we investigated the effects of ω-3 PUFAs on a panel of inflammatory mediators (cytokines, interleukins, and chemokines) produced by activated PBL from healthy individuals and by stimulated human chondrocytes and compared them to the natural phenolic compound resveratrol (RV) that has multiple effects on the inflammatory response [18]. We show that the substances have distinct effects on leukocytes and chondrocytes, which reflect acute and chronic inflammatory processes, respectively.

2. Materials and Methods

2.1. PUFAs, Resveratrol, and Cell Culture Reagents. Free fatty acid eicosapentaenoic acid (EPA), docosahexaenoic acid

(DHA), and resveratrol were from Sigma/Aldrich (Saint Louis, MO). *E. coli* lipopolysaccharide (LPS, serotype 055:B5) and fetal bovine serum (FBS) were from Sigma/Aldrich (Saint Louis, MO). Cell culture reagents (RPMI 1640, 2-mercaptoethanol, and MEM nonessential amino acids (NEAA)) were from Invitrogen (Carlsbad, CA). IL-1β was purchased from PeproTech EC (London, UK). Substances were dissolved in DMSO and added to cells shortly before treating the cells with biological stimulators. Final concentrations of PUFAs and resveratrol were 10–50 μmol/L and 1.25–50 μmol/L, respectively.

2.2. Cell Culture. Human peripheral blood leukocytes (PBLs) were isolated from buffy coats obtained from healthy individuals and treated with inflammatory stimuli as described [19]. Briefly, PBLs were cultured in phenol red-free RPMI 1640 (containing 0.25% FBS, 0.1 mM NEAA, 50 U/mL penicillin, and 50 μmg/mL streptomycin) and stimulated with 1 μg/mL LPS in the presence of graded amounts of substances. Human *in vitro* PBL experiments were approved by the Swiss Federal Office of Public Health (number A050573/2) and the Ethical Commission of the Kanton Aargau, Switzerland. Cells were lysed in RLT buffer (Qiagen, Hilden, Germany) after 12 h of culture and total RNA was extracted. Alternatively, cells were cultured for 24 h; and secreted mediators were analyzed in culture supernatants.

Normal human articular chondrocytes from knee (NHAC-kn), prepared from different individuals, were cultured in chondrocyte growth medium (Lonza, Walkersville, MD) and used for experiments between passages 3 and 6 [20]. Cells (0.5×10^6 per well) were seeded into 6-well plates and confluent cells were activated with IL-1β (10 ng/mL) in the presence of graded concentrations of test substances for 4–24 h [20].

2.3. RNA Isolation, cDNA Synthesis, and RT-PCR. The isolation of total RNA, synthesis of cDNA, and quantitative RT-PCR have been detailed previously [19, 20].

2.4. Multiparametric Analysis of Cytokines, Chemokines, and Interleukins. Multiparametric kits were purchased from Bio-Rad Laboratories (Hercules, CA) and used in the LiquiChip Workstation IS 200 (Qiagen, Hilden, Germany) to measure the amount of secreted proteins. Data evaluation was done using the LiquiChip Analyser software (Qiagen). PGE_2 were measured as described previously [20].

2.5. Statistical Analysis. Data were evaluated by statistical tools described previously [20]. A p value <0.05 (calculated by using Student's t-test or one-way ANOVA) was considered to reflect statistically significant differences.

2.6. Calculation of Combination Effects. The algorithm developed by Chou and Talalay has been used to calculate synergism of inhibitory effects [21, 22]. Interactions were quantified by the combination index (CI) as described by Pappa et al. [23]: Using CalcuSyn software (Biosoft, Ferguson, MO), a CI was computed for every fraction affected. CI < 1 reflects synergistic inhibition of the respective inflammatory parameter; if CI = 1 the substances have additive interactions; when CI > 1 the interaction of substances reflects antagonism.

3. Results

3.1. Resveratrol and ω-3 PUFAs Modulate the Production of Cytokines and Chemokines in Leukocytes. In order to investigate the effects of nutrients on acute inflammatory responses, PBLs from different individuals were activated with LPS in the presence of graded amounts of substances. LPS triggered substantial secretion of cytokines, chemokines, and PGE_2; some of these differed considerably between individuals with respect to IL-1β, TNF-α, and CCL5/RANTES showing the largest interindividual variations (Table 1). The effects of resveratrol (RV) and ω-3 PUFAs on the inflammatory mediators of activated PBLs are shown in Figure 1. In order to correct for interindividual variations, the data are expressed as a ratio [(substance + LPS-treated cells)/LPS-treated cells]. RV drastically reduced the secretion of PGE_2, which is dependent on the LPS-induced expression of COX-2 in monocytes/macrophages (Table 1). Conversely, interleukin- (IL-) 1β, IL-6, and the anti-inflammatory IL-10 were increased in the presence of RV (6.25–25 μmol/L). We further investigated the impact of RV on chemokine secretion. CCL5/RANTES, which recruits activated T lymphocytes [24], was augmented by high concentrations of RV (25 μmol/L). The neutrophil recruiting chemokine CXCL8/IL-8 was blunted by increasing RV concentration. CCL2/MCP-1, which is involved in targeted migration of resident monocytes [24] and macrophage polarization [25], was not significantly altered. In contrast, RV enhanced production of CCL4/MIP-1β, a chemokine for subtypes of monocytes [26].

DHA modulated the production of inflammatory mediators of LPS-activated leukocytes in a different manner (Figure 1). It enhanced PGE_2 production (at 5–20 μmol/L). IL-1β and IL-6 were produced to larger extents when DHA was included in the cellular assay. Similarly, DHA markedly enhanced CXCL8/IL-8 secretion, whereas it mitigated CCL5/RANTES. The production of CCL2/MCP-1 and CCL4/MIP-1β by activated leukocytes was only influenced by high concentrations of DHA. EPA shared with DHA a similar activity pattern on the production of inflammatory mediators. It should be noted that the extent of the response of PBLs to LPS was subject to large interindividual variations that presumably mirrored the differing immune status of the donors (Table 1).

3.2. Altered Gene Expression in Activated PBLs. By using quantitative RT-PCR, we investigated the impact of the substances on the transcription of inflammatory genes in PBLs after 12 h of LPS-stimulation, when many LPS-responsive genes were still upregulated [19, 27]. RV had only a minor influence on IL-1β mRNA levels, whereas it significantly augmented IL-6 transcription (Figure 2), consistent with the increased IL-6 secretion (Figure 1). Similarly, IL-10 mRNA levels of RV-treated cells matched the higher secretion of

Table 1: Secreted proteins of unstimulated and stimulated PBLs obtained from different subjects.

Parameter	Unstimulated cells (pg/mL)	LPS-stimulated cells (pg/mL)	Range (pg/mL)	$^{(2)}R$ = (RV$^{(3)}$ + LPS)/LPS	$^{(2)}R$ = (EPA$^{(3)}$ + LPS)/LPS
IL-1β	0 ± 0$^{(1)}$	7889 ± 1250$^{(1)}$	2025–18000	2.62 ± 0.30	2.76 ± 0.94
IL-6	1 ± 1	111644 ± 19519	51300–209000	1.76 ± 0.30	2.29 ± 0.51
IL-10	1 ± 1	1279 ± 230	388–2530	1.29 ± 0.35	1.06 ± 0.21
IL-12 (p70)	0 ± 0	14 ± 7	0–73	—	—
TNF-α	0 ± 0	3499 ± 1300	418–12750	0.86 ± 0.15	1.44 ± 0.65
CCL2/MCP-1	1 ± 1	724 ± 163	363–2065	0.77 ± 0.26	0.95 ± 0.14
CCL3/MIP-1α	1 ± 1	14718 ± 2698	2720–28250	1.51 ± 0.56	1.27 ± 0.47
CCL4/MIP-1β	182 ± 31	68950 ± 12844	33300–162500	1.75 ± 0.46	0.81 ± 0.17
CCL5/RANTES	536 ± 117	6013 ± 1978	670–20300	1.55 ± 1.57	0.77 ± 0.14
CCL11/Eotaxin	28 ± 8	448 ± 82	78–878	1.16 ± 0.11	1.04 ± 0.09
CXCL8/IL-8	311 ± 35	298980 ± 50712	108800–614000	0.79 ± 0.35	2.10 ± 0.60
PGE_2	121 ± 15	3437 ± 499	1680–6249	0.11 ± 0.17	2.10 ± 0.64

$^{(1)}$Mean ± SEM of values obtained from PBLs of 8 different subjects (each done in duplicate).
$^{(2)}$Ratio: (metabolites produced by PBLs treated with substance + LPS)/metabolites produced by PBLs treated with LPS. Mean ± SD of triplicate values obtained from PBLs of 4 different subjects.
$^{(3)}$25 μmol/L RV, 20 μmol/L EPA.

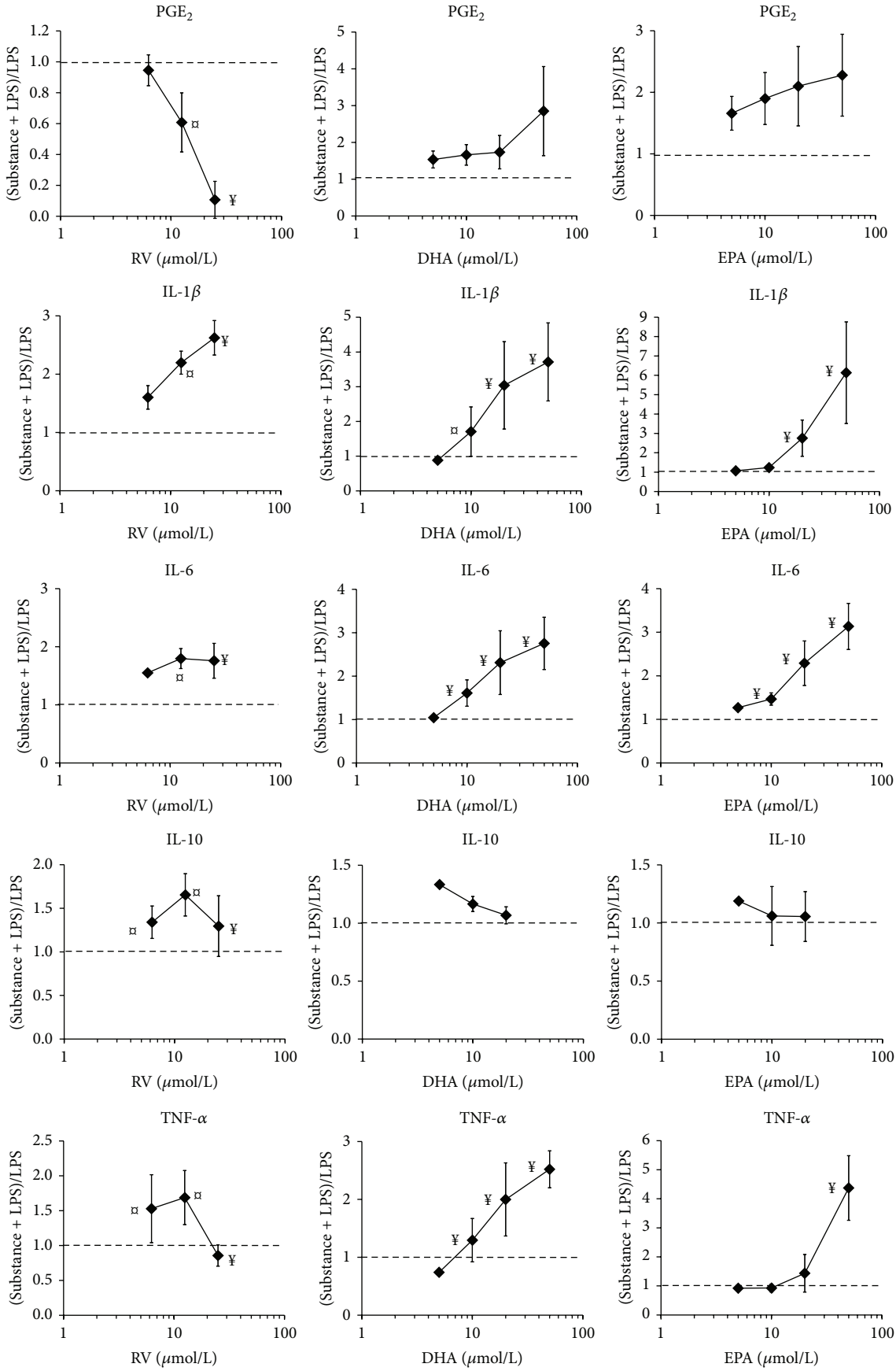
IL-10. We observed a significant decrease of CXCL8/IL-8 expression by RV (at 25 μmol/L) (Figure 2). Conversely, CCL5/RANTES gene expression was not induced by LPS-stimulation nor changed by concomitant RV treatment. DHA altered gene expression patterns in a similar way, as it changed the secretion of the respective proteins: IL-6 expression and, to a lesser extent, IL-1β and IL-10 expression were augmented by DHA (at 20 μmol/L) (Figure 2). CXCL8/IL-8 expression was enhanced, when DHA was included at 20 μmol/L in the assay. Collectively, the data indicate that RV and ω-3 PUFAs regulate cytokine and chemokine production at the level of transcription.

3.3. Effects of ω-3 PUFAs and RV Measured in PBLs. Since we observed similar and opposite effects of substances, we investigated the pattern on inflammatory parameters produced when PBLs were treated with a combination of substances. To this aim, cells were activated in the presence of different concentrations and ratios of individual substances and the secreted mediators determined (Figure 3). PGE_2 production was dominated by the inhibitory effect of RV, which partially counterbalanced the enhancing effect of DHA (Figure 3). CXCL8/IL-8 production was controlled by DHA, since the combined treatment with RV did not result in an intermediate production. Combinations of RV and DHA synergistically inhibited CCL5/RANTES secretion, as computed by the Chou-Talalay algorithm (Figure 3). Both RV and DHA concentration-dependently enhanced IL-6 secretion and combinations thereof had additive effects. IL-1β, however, appeared to be synergistically enhanced by RV and DHA, since the effect of combined substances largely exceeded the sum of RV and DHA applied individually.

3.4. Effects of ω-3 PUFAs and RV on IL-1β Activated Chondrocytes. Following activation with IL-1β, human chondrocytes (normal human articulocytes from knee, NHAC-kn) expressed various pathophysiological markers of osteoarthritis (OA) and enzymes that degrade the extracellular matrix (ECM) [28–30]. We treated normal human chondrocytes with IL-1β, the pathophysiological inducer of OA, and investigated the effect of RV and PUFAs on biological markers of OA. MMPs and ADAMTS, interleukins, and chemokines were upregulated in IL-1β treated chondrocytes [20]. In the presence of ω-3 PUFAs or resveratrol, gene expression of OA markers was significantly altered (Figure 4). Both substances had no significant impact on gene expression of unstimulated NHAC-kn (not shown). ω-3 PUFAs markedly blunted IL-1β and IL-6 expression in stimulated NHAC-kn cells. In general, EPA and DHA induced similar alterations of gene expression in IL-1β activated chondrocytes. Also, MMP-3 and ADAMTS-4 gene expression was mitigated by EPA or DHA, whereas MMP-1 expression was unaltered. MMP-13 was only slightly upregulated in IL-1β treated chondrocytes. Under these conditions, ω-3 PUFAs had no significant impact on MMP-13 mRNA levels. Conversely, expression of CCL5/RANTES and CXCL8/IL-8 was drastically upregulated in activated NHAC-kn cells. Concomitant treatment with EPA or DHA led to a mitigated expression of CCL5/RANTES, but they had no effect on CXCL8/IL-8 (Figure 4). EPA positively influenced chondrocyte anabolism, since the expression of Col2A and therefore the synthesis of ECM elements were increased. RV substantially altered the expression of MMP-3 and ADAMTS-4 in IL-1β-activated NHAC, whereas its impact on chemokine gene expression was not significant. Therefore, in comparison with ω-3 PUFAs, RV is predicted to have a limited impact on cartilage erosion.

4. Discussion

In this study we investigated whether dietary constituents altered the *in vitro* inflammatory response of human leukocytes from healthy individuals. RV was anti-inflammatory,



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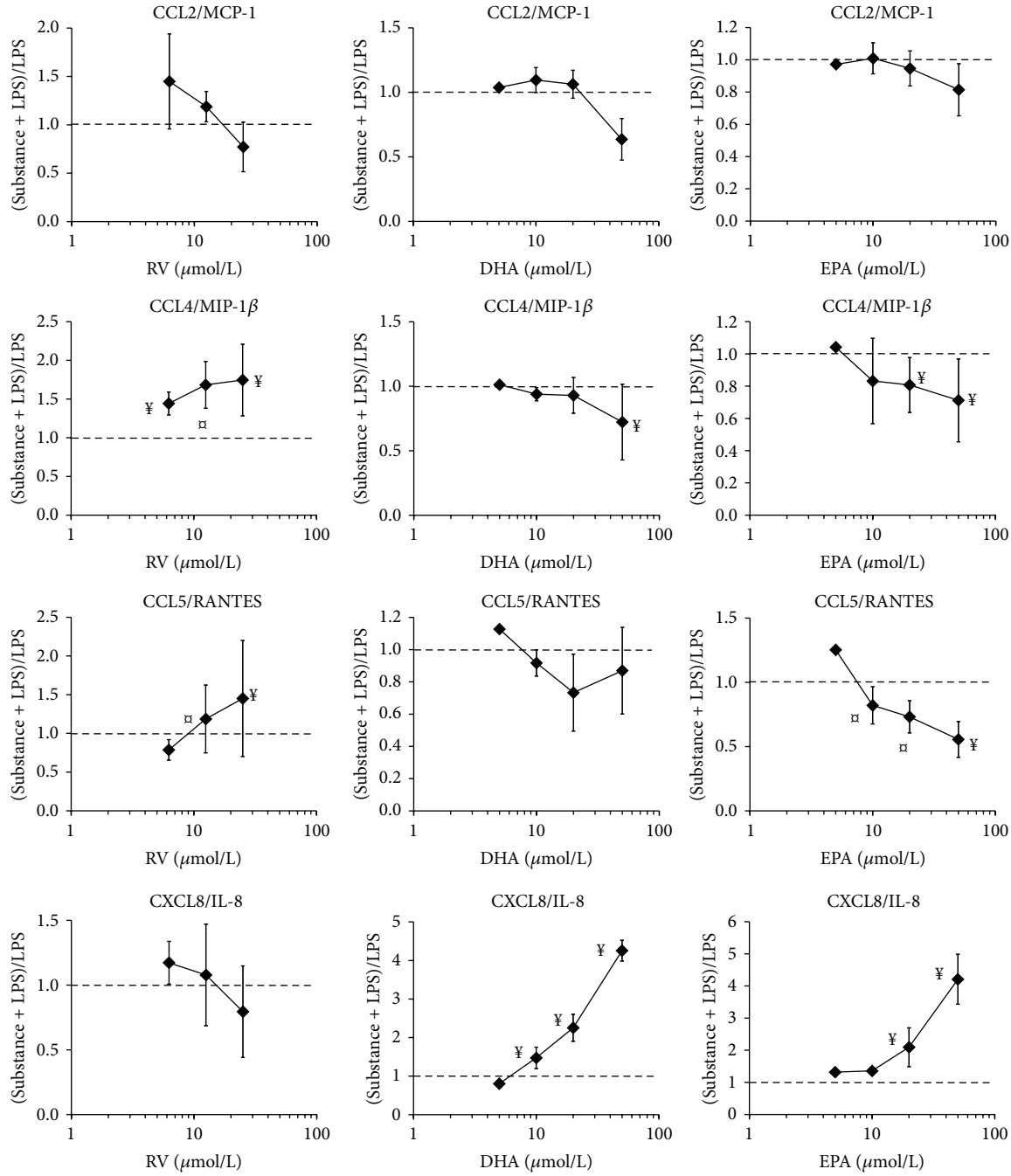


Figure 1: Production of inflammatory mediators by activated PBLs and its modulation by RV, DHA, and EPA. PBLs from healthy individuals were activated with LPS in the presence of the indicated substances (in μmol/L) and the inflammatory mediators in supernatants of 24 h cultures were determined in duplicate. The results are expressed as the ratio of substance + LPS-treated cells/LPS-treated cells. Data are given as mean ± SD of experiments obtained from PBL from at least four individuals. The dotted line (at ratio 1) indicates the no-effect level. ¤, ¥ indicate statistically significant differences observed in 50% and >75% of the donors, respectively.

since it reduced, for instance, PGE_2 and nitric oxide in human PBL [19] and macrophage cell lines. Yet, it had opposite effects on IL-6 produced by human PBL [9, 19, 31, 32]. RV and ω-3 PUFAs had similar effects on the production of IL-1β and IL-6 but markedly differed in the impact on, for example, CCL2/MCP-1, CCL4/MIP-1β, or PGE_2 production. ω-3 PUFAs induced striking changes in eicosanoid metabolites [33], modulated the inflammatory response in human PBLs via PPAR and NF-κB pathways [2, 8, 15, 34–36], and orchestrated the resolution of inflammation [4]. Since EPA and DHA are precursors of resolvins, they might accelerate the resolution of inflammation. The present *in vitro* study is the first report where effects of ω-3 PUFAs on a large panel of chemokines were investigated. EPA or DHA markedly

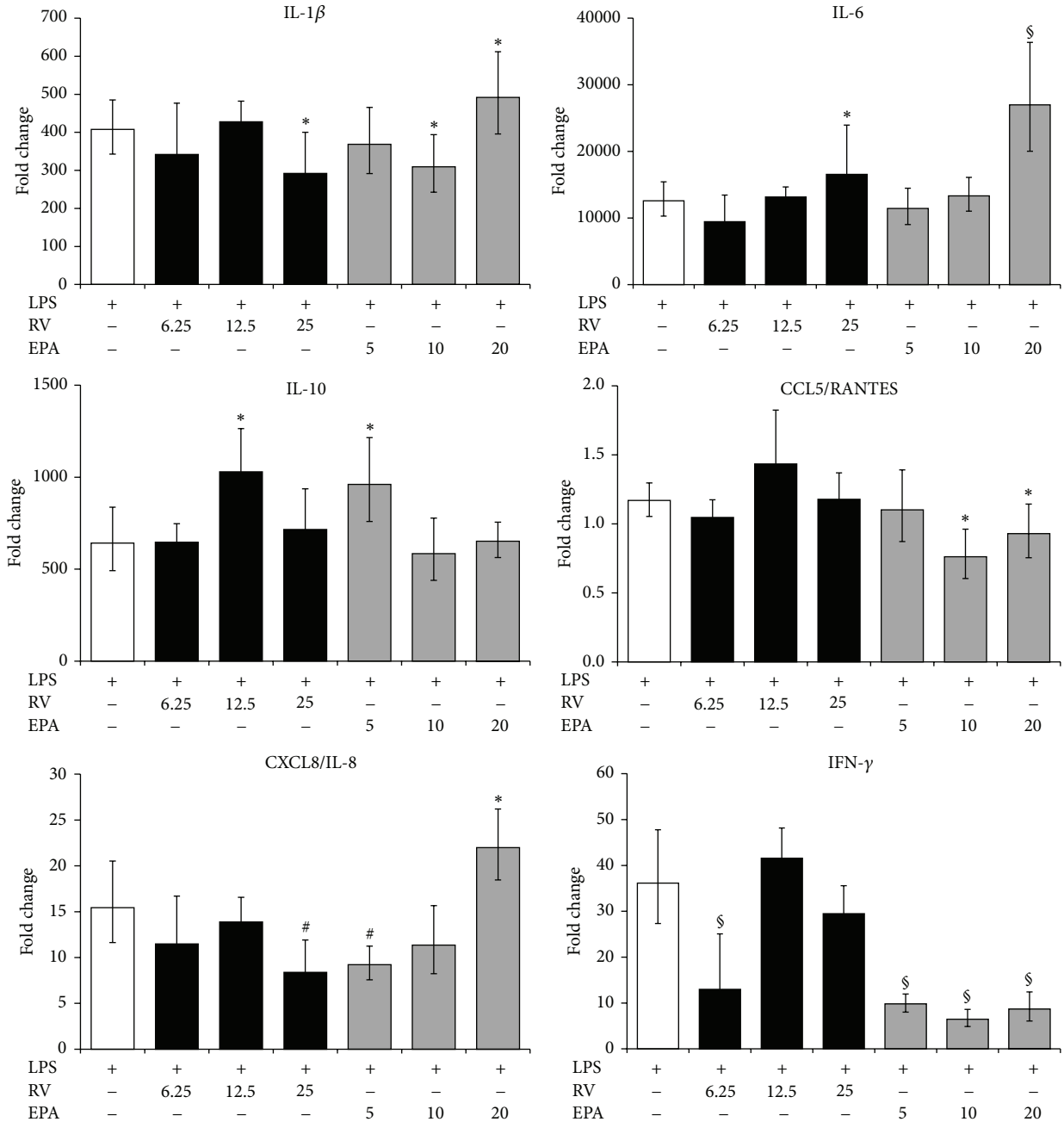


Figure 2: Expression of inflammatory genes in activated PBLs. Cells were activated with LPS with or without the indicated substances for 12 h. Gene expression was quantified by RT-PCR and the data expressed as fold change compared to levels observed in unstimulated cells. Mean ± standard errors of duplicates from three individuals are given. $^{*}p < 0.05$, $^{\#}p < 0.01$, and $^{\S}p < 0.001$ (LPS only versus LPS + substance).

increased CXCL8/IL-8 but blunted the secretion of CCL5/RANTES. Plausibly, ω-3 PUFA might increase neutrophil recruitment in the early inflammatory phase and attenuate migration of activated T lymphocytes during resolution. Previous studies showed modest effects of ω-3 PUFAs on inflammatory markers [37, 38]. A 12-week fish-oil supplementation did not significantly affect plasma cytokine and chemokine concentrations, although an overall trend for an increase of inflammatory markers was observed [39] and gene expression profiles of chemokines and cytokines in peripheral blood mononuclear cells (PBMC) were affected [5, 40]. DHA levels in PBMC were inversely related to IL-1β and IL-6 production [13] and diminished IL-1β and TNF-α secretion by activated PBLs *in vitro* [41]. Incubation of unstimulated PBLs with EPA upregulated the expression of IL-6 and CXCL8/IL-8 [42]. Since in the present study ω-3 PUFAs were added shortly before the stimulation of PBLs, they might alter the release of cellular arachidonic acid and the subsequent production of eicosanoid metabolites like PGE_2 and anti-inflammatory prostaglandins like PGD_2. These results

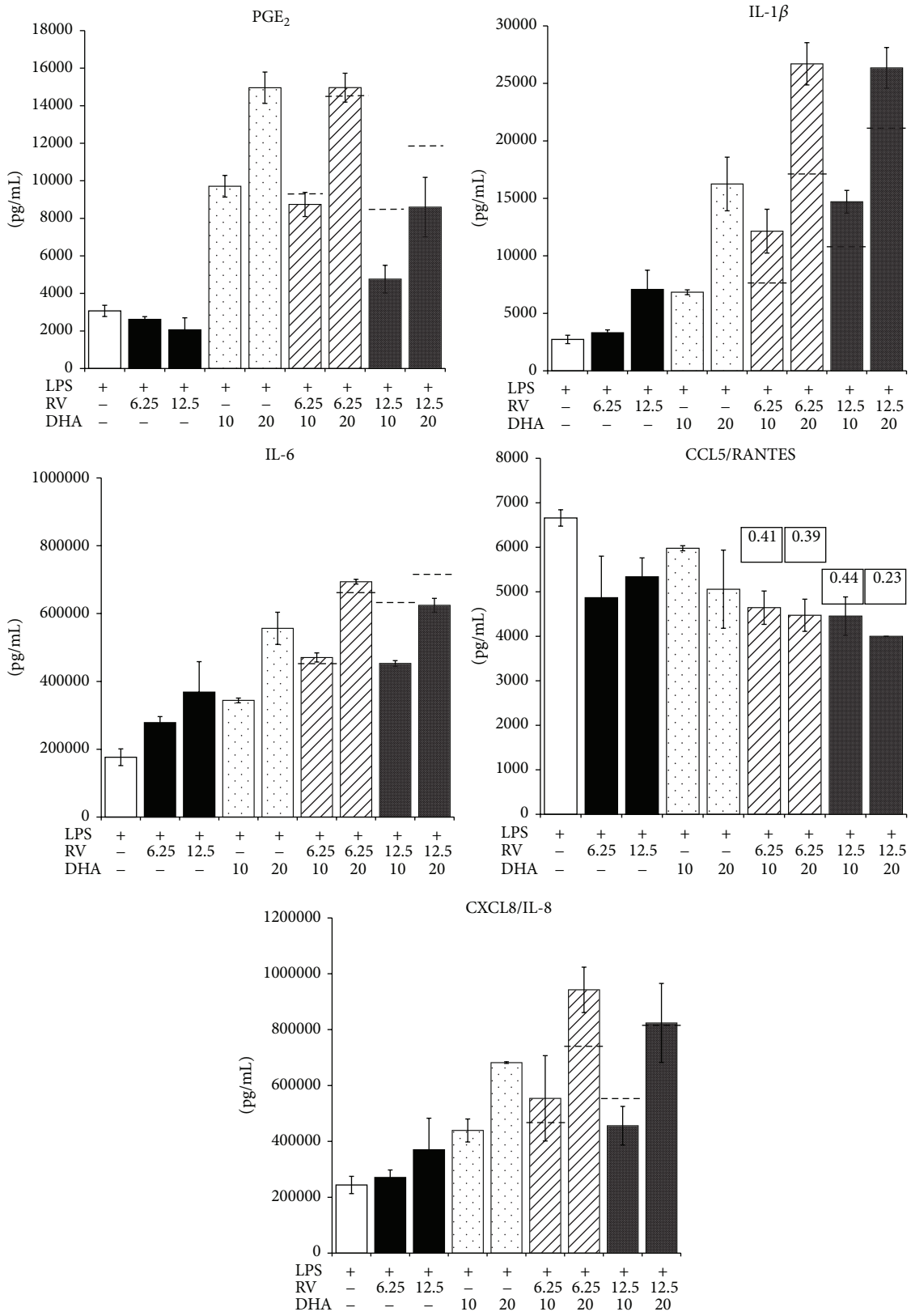
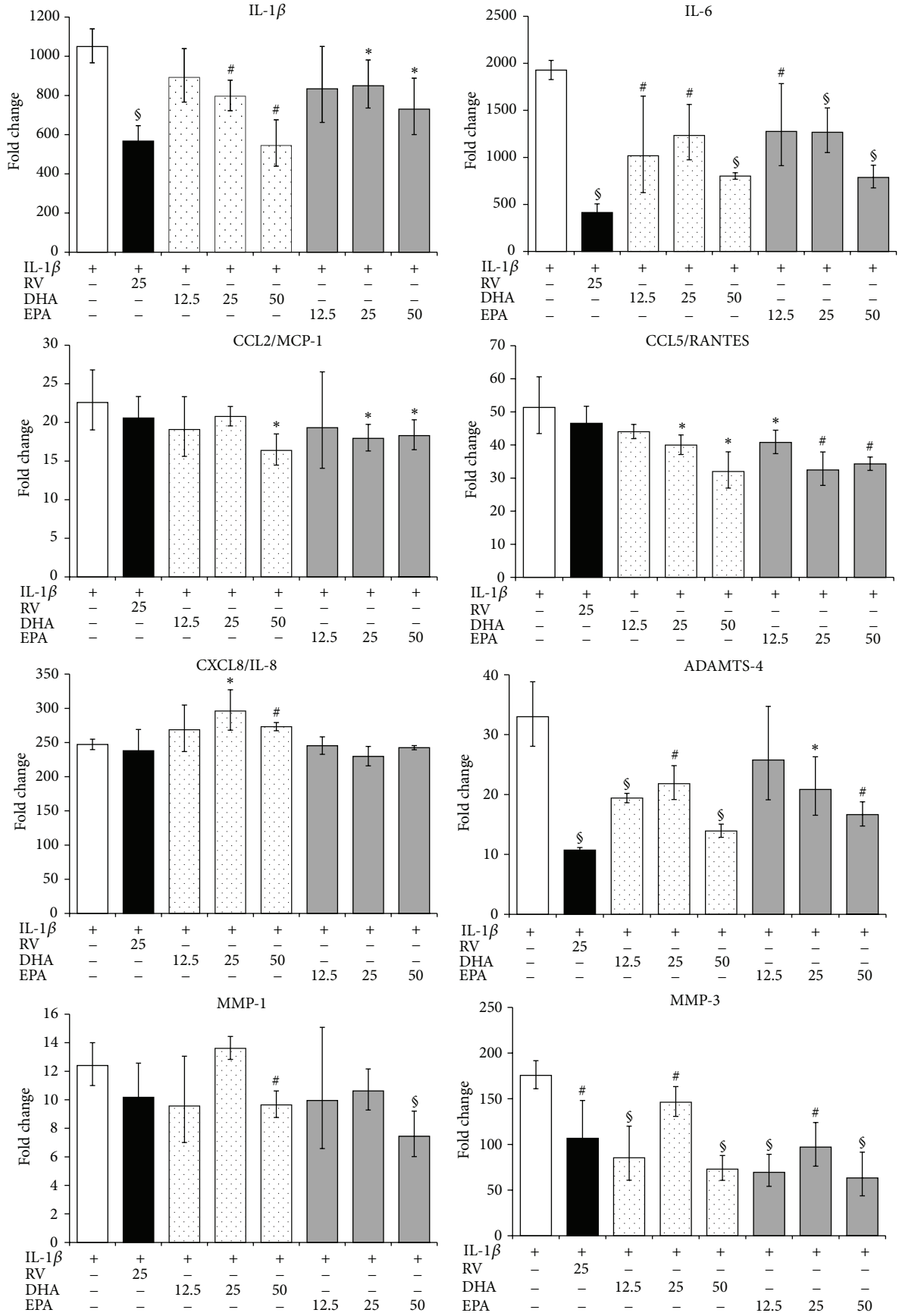


Figure 3: Effect of combinations of substances on inflammatory mediators produced by activated PBLs. PBLs were cultured for 24 h with or without the indicated substances and their combinations. In the case of *inhibition*, the combination index (CI) was calculated and is indicated in the figure. Dashed lines in the bar graphs indicate the computed sum of the respective single substance treatments.



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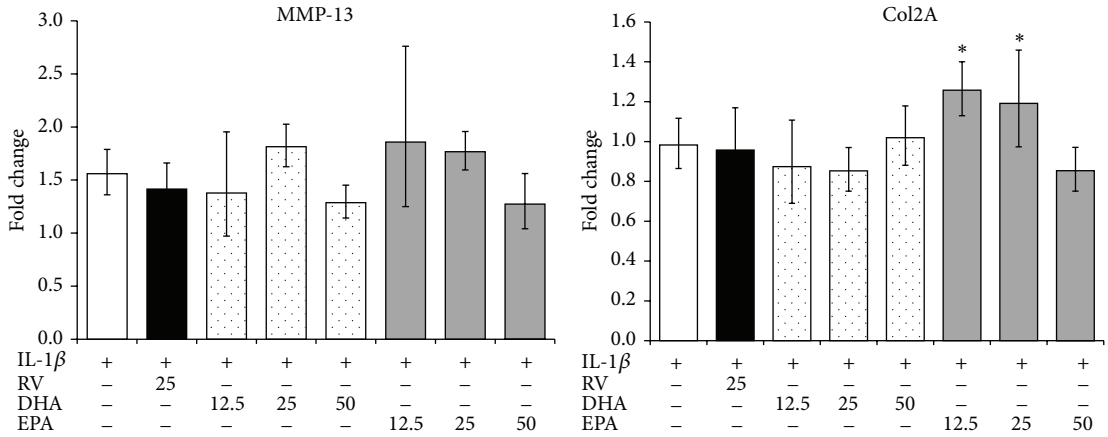

Figure 4: Modulation of gene expression in IL-1β activated human chondrocytes. Cells were activated with IL-1β with or without the indicated substances for 4 h. Gene expression was quantified by RT-PCR and the data expressed as fold change compared to levels observed in unstimulated cells. $^{*}p < 0.05$, $^{\#}p < 0.01$, and $^{\S}p < 0.001$ (IL-1β only versus IL-1β + substance).

suggest that acute supplementation with EPA and DHA might transiently disturb homeostasis in PBLs *in vitro* and *ex vivo*, whereas during long-term supplementations cells no longer sense subtle homeostatic changes. Cytokines and chemokines critically determine macrophage differentiation and function [43]. In addition, the T_h1 and T_h2 cell development is orchestrated by IL-6, IL-12, TNF-α, and chemokines and their receptors [44]. Since immune cells sense, and respond to, the presence of RV and ω-3 PUFAs by changes in cytokine and chemokine production, we hypothesize that these substances influence the differentiation of M2 macrophages and T_h2 cells [45]. For instance, an increase of IL-6 is expected to favour the differentiation of M2 macrophages [46].

In this study we also show that ω-3 PUFA modulated mRNA levels in human chondrocytes that were activated with the pathophysiological mediator IL-1β. These cells are used as an appropriate *in vitro* model for chronic inflammation, which is a typical feature of osteoarthritis. Notably, expression levels of MMPs, ADAMTS-4, and interleukins were reduced. Similar data were obtained when chondrocytes were incubated with rose hip that also contained significant amounts of free fatty acids including EPA or DHA [47]. Other studies in bovine chondrocytes and cartilage explants have identified that long-term *in vitro* treatment of chondrocytes with conjugated linoleic acids or EPA mitigated the production of PGE_2 and nitric oxide [48]. In bovine cartilage explants and chondrocytes, EPA affected ECM degradation, since it reduced glycosaminoglycan and collagen II release [49] and reduced gene expression of enzymes involved in OA [50]. Collectively, RV was less active on chondrocytes than ω-3 PUFAs.

From these data we infer that RV and ω-3 PUFAs modulate acute (in PBLs) and chronic inflammation (in chondrocytes) in different ways: RV mitigates early inflammatory events like the production of PGE_2. ω-3 PUFAs augment the amplitude and kinetics of inflammatory events in acute inflammation and its resolution. Yet, both substances diminish inflammatory processes during chronic inflammation.

5. Conclusions

ω-3 PUFAs and RV differ in the regulation of acute inflammation in leukocytes, but they have common properties in modulating biochemical events related to chronic inflammation of chondrocytes.

Conflict of Interests

All authors are employees of DSM Nutritional Products, a leading manufacturer of vitamins and PUFAs.

Authors' Contribution

Joseph Schwager and Nathalie Richard contributed equally to this work. Joseph Schwager, Nathalie Richard, Christoph Riegger, and Norman Salem Jr. designed the experiments. Nathalie Richard performed the experiments. Joseph Schwager and Nathalie Richard analysed the data. Joseph Schwager has written the paper. All authors read and approved the paper.

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Review Article

Omega-3 Polyunsaturated Fatty Acids: Structural and Functional Effects on the Vascular Wall

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Omega-3 polyunsaturated fatty acids (n-3 PUFA) consumption is associated with reduced cardiovascular disease risk. Increasing evidence demonstrating a beneficial effect of n-3 PUFA on arterial wall properties is progressively emerging. We reviewed the recent available evidence for the cardiovascular effects of n-3 PUFA focusing on structural and functional properties of the vascular wall. In experimental studies and clinical trials n-3 PUFA have shown the ability to improve arterial hemodynamics by reducing arterial stiffness, thus explaining some of its cardioprotective properties. Recent studies suggest beneficial effects of n-3 PUFA on endothelial activation, which are likely to improve vascular function. Several molecular, cellular, and physiological pathways influenced by n-3 PUFA can affect arterial wall properties and therefore interfere with the atherosclerotic process. Although the relative weight of different physiological and molecular mechanisms and the dose-response on arterial wall properties have yet to be determined, n-3 PUFA have the potential to beneficially impact arterial wall remodeling and cardiovascular outcomes by targeting arterial wall stiffening and endothelial dysfunction.

1. Introduction

Cardiovascular disease is the first cause of death in the developed world. Its main feature is the extensive presence of atherosclerosis, which is anticipated by morphologic and functional changes involving vessel wall and vascular endothelium. Impairment of functional properties of the arteries is strictly related to the morphologic changes in vessel structure and to the alteration in mechanical properties [1, 2]. Endothelial dysfunction is characterized by impaired endothelium-dependent vasodilation and "endothelial activation," which is associated with a proinflammatory and procoagulatory milieu that promotes development and progression of vascular disease [3, 4]. Cardiovascular risk factors are closely linked to the development of endothelial dysfunction and arterial wall stiffness, which are significant predictors of cardiovascular risk and mortality [5, 6]. A synergistic interplay exists among the anatomic structures of the vessel wall, the vascular endothelium, endothelial-derived factors, and circulating cytokines, and such interplay promotes the development of overt atherosclerosis.

Omega-3 polyunsaturated fatty acids (n-3 PUFA) have shown the potential to beneficially impact fundamental steps involved in the development of preclinical atherosclerosis [7]. By targeting arterial stiffness and endothelial dysfunction, administration of n-3 PUFA may prevent atherosclerosis and cardiovascular disease. A wide range of molecular and physiological pathways are affected by n-3 PUFA administration and are involved in the regulation of arterial stiffness and endothelial dysfunction.

This review will focus on the complex nature of arterial stiffness and endothelial dysfunction and on the translational potential of n-3 PUFA for treating vascular remodeling.

2. Structural and Mechanical Properties of the Arterial Wall

Arterial wall consists of a complex morphological organization, with multiple layers designed to maintain the

fundamental properties of blood carrying and blood pressure regulation. This structure is aimed at maintaining the elastic properties of the arterial wall, which are important for the physiological vascular function [8]. The distensibility of arterial vessels determines the amplitude of pulse pressure as well as the speed of the propagating pulse wave in the arterial system. The loss of elastic properties leads to arterial stiffness, a parameter that has been recognized in recent years as an intermediate endpoint for cardiovascular morbidity and mortality, independently of conventional risk factors for cardiovascular disease [9]. Arterial stiffening or arteriosclerosis, which is caused by the loss of the medial arterial load bearing components of the arterial wall, is pathologically distinct from the obstructive condition of arteries or atherosclerosis, usually defined as the deposition of lipids, white blood cells, and calcium in the arterial wall [10], although these two conditions are closely intertwined [11]. Arteriosclerosis could directly promote cardiovascular disease, by increasing pulsatile load on heart, reducing myocardial blood flow, damaging small vessels in kidney or brain, and by promoting atherogenesis through a reduction in shear stress rate. On the other hand, the presence of atherosclerotic plaques could mechanically alter the properties of the arterial wall.

The assessment of arterial stiffness has been increasingly used in clinical setting, considering its good predictive value for cardiovascular events. A large number of studies have been addressed to understand the mechanisms and factors influencing the development of arterial stiffness and to report interventions able to influence arterial wall properties [9]. Reducing arterial stiffness may be useful to reduce incidence of cardiovascular events and mortality; thus evidence-based treatments would be clinically important, but currently a specifically designed therapeutic strategy for this purpose has not yet been defined. Dietary habits are well-known determinants of the vascular changes occurring in the arterial wall with age, and many studies have focused on the effects of pharmacologic and nonpharmacologic interventions to modulate arterial elastic properties [12].

3. Effects of n-3 PUFA on Atherogenesis

A large body of evidence demonstrates the substantial benefits of n-3 PUFA in atherogenesis [13]. n-3 PUFA play several important roles in cellular molecular mechanisms, tissue metabolism and regulation, and act as pleiotropic agents on the cardiovascular system [14]. The mechanisms through which n-3 PUFA interfere with atherogenesis are therefore multiple. Their effect on endothelial dysfunction, oxidative stress, and inflammation, causing the onset of atherogenesis, will be discussed in the next section. The antiatherogenic effect of n-3 PUFA on serum lipid profile, with a reduction in both triglycerides and very-low-density lipoproteins, is well known and has recently been reviewed [15, 16]. However, it is unclear as to how many of cardiovascular benefits are related to n-3 PUFA lipid lowering effects and how many of them are due to lipid-independent effects.

Regarding the lipid deposition in the arterial layers, which is associated with atherogenesis [17], in a mouse animal model, n-3 PUFA were able to protect the arterial wall by decreasing the LDL uptake and by directing lipid deposition away from the aortic media, by decreasing the lipoprotein lipase expression [18]. This antiatherogenic effect is also associated with a reduction of macrophages and other proinflammatory markers and is enhanced by an incremental replacement of n-3 PUFA in the diet [19].

The proliferation of vascular smooth muscle cells and their lipid accumulation are associated with early lesion in the arterial wall and atherosclerosis promotion [20], highlighting the role of these cells in the pathophysiology of vascular remodeling [21]. An effect of n-3 PUFA on vascular smooth muscle cells activation has been reported in several studies. In culture cells, EPA and DHA were incorporated into phospholipids and slow down the progression of cell cycle, by inhibiting DNA synthesis and replication, thus suppressing vascular smooth muscle cells proliferation [22]. A similar inhibition in the proliferation of vascular smooth muscle cells was observed in human coronary arteries after consumption of fish oil, with a regulation of adhesion molecules on these cells [23].

A specific effect of n-3 PUFA on plaque stability has also been reported, in preventing the rupturing of vulnerable plaques, that leads to arterial thrombosis and obstruction. This effect could explain the reduction in cardiovascular endpoints observed in short-term trials conducted with n-3 PUFA. In patients undergoing carotid endarterectomy, atherosclerotic plaques revealed reduced macrophages infiltration and more stable morphology after n-3 PUFA administration [24]. In a more recent study, in plaques of patients supplemented with n-3 PUFA analyzed after carotid endarterectomy, reduced inflammation and significantly lower levels of mRNA for matrix metalloproteinases were observed [25].

The evaluation of intima-media thickness (IMT) has long been used as a marker of atherosclerotic involvement of arterial walls and as a surrogate endpoint of cardiovascular disease [26]. Although several observational studies reported an inverse association of n-3 PUFA administration, as diet consumption or fish oil administration, a systemic review of human intervention studies could not draw a firm conclusion on the effects of n-3 PUFA administration on IMT [27]. Also more recent trials were inconclusive: a positive effect on IMT was observed in patients with type 2 diabetes [28], although, in elderly men with hypercholesterolemia, a favorable effect on IMT progression was not confirmed, whereas n-3 PUFA imposed an improvement in arterial elasticity [29]. Recent cross-sectional studies reported that DHA levels, but not EPA, have an inverse association with IMT, suggesting that DHA may have a more potent antiatherogenic effect than EPA, independently of other risk factors [30].

4. Influence of n-3 PUFA on Arterial Wall Stiffening

As above mentioned, the alteration of mechanical properties of the arterial wall is strictly connected with atherosclerothic involvement. An increased plaque burden and a modification in the composition of arterial layers can hamper arterial elastic behavior. Nevertheless, although sharing some common

risk factors, these two processes should be considered separately. While effects of n-3 PUFA on atherogenesis, on atherosclerotic plaques stability, and on arterial restenosis have been previously extensively reviewed, we will focus on n-3 PUFA effects on hemodynamic properties of the large arteries. Arterial stiffness, while being firstly determined by traditional risk factors for cardiovascular disease, can be influenced by passive mechanisms that consider mechanical and elastic properties of the vessels, and active mechanisms, regulated by the cellular and molecular function of the endothelium, the vascular smooth cells, and the extracellular matrix [1]. Some of these mechanisms may be influenced by n-3 PUFA intake.

Arterial blood pressure is considered the main determinant of arterial stiffness [9]. A fundamental mechanical property of the arteries is that the arterial wall becomes stiffer when the distending pressure becomes higher. Hypertension can also increase arterial stiffness chronically, by inducing elastin fragmentation and arterial wall remodeling [31]. A large body of studies demonstrated that n-3 PUFA are able to reduce systemic blood pressure [32], and a recent meta-analysis confirmed that a consumption of >2 g/d of EPA + DHA can reduce systolic and diastolic blood pressure in humans [33]. Thus, blood pressure, a main factor associated with arterial stiffening, is influenced by n-3 PUFA intake, explaining part of the beneficial effect of fatty acids on the arterial wall.

Triglyceride levels are known to be affected by n-3 PUFA intake. A supplementation of 2-3 g/d of EPA + DHA can reduce triglyceride levels by 25–30%, although a slight increase of LDL levels was observed in some studies [34]. Lipid abnormalities are well-known determinants for the development of atherosclerotic vessels disease and related abnormalities, such the stiffening of large arteries. In large cross-sectional studies [35] triglyceride levels were strongly associated with arterial properties, although a specific benefit in arterial stiffness levels with therapies targeting triglycerides has not been demonstrated yet.

Elevated heart rate has been shown to be associated with an increased risk of cardiovascular events, and there is evidence that the heart rate is independently associated with the progression of arterial stiffness, both in animal models and in humans [36]. n-3 PUFA supplementation is able to reduce resting heart rate and recovery after exercise. Experimental studies suggested that heart rate lowering could result from direct effects on cardiac electrophysiology [37, 38]. Some studies also suggested that n-3 PUFA might improve neurogenic autonomic function of cardiovascular system, through a modulation of vagal and sympathetic balance [39], and an independent association between aortic stiffness and muscle sympathetic nerve activity has been reported [40].

The effect of n-3 PUFA on classical risk factors for cardiovascular disease may explain the favorable effect on arterial stiffness. Nevertheless other mechanisms, mediated through biochemical cellular signaling and through neurogenic and neuroendocrine pathways, have been explored. The association between endothelial dysfunction and increased arterial stiffness has been demonstrated in vitro [41] and in vivo, both in animals and in humans [42, 43]. Considering the known effect of n-3 PUFA on endothelial function, which is discussed later in this review, this could be a main explanation of the reduction of arterial stiffness observed in experimental condition of n-3 PUFA supplementation. An enhancement in endothelial-dependent vasodilation of the muscular arterioles leads to a decrease in arterial stiffness because mechanical stresses are transferred to elastin components of the wall and because there is a reduction in reflected pulse waves [44]. A direct vasodilatory effect and an inhibition of constrictor response of DHA have been demonstrated in humans [45]. An interrelation of this vasodilatory effect has been found with different endocrine pathways, as the vascular constrictor response to angiotensin [46] and norepinephrine [47] is attenuated by n-3 PUFA in humans.

Therefore, the improvement in arterial properties shown after n-3 PUFA supplementation is multifactorial and involves both passive and active mechanisms of arterial hemodynamics, mediated by multiple cellular and molecular pathways and influenced by some major cardiovascular risk factors (hypertension, blood lipids, and autonomic balance).

5. n-3 PUFA and Arterial Stiffness: In Vivo Studies

Many studies have focused directly on the evaluation of arterial stiffness after n-3 PUFA supplementation (Table 1). Considering animal models, Sato et al. [48] found that supplementation of EPA reduced aortic PWV in high-cholesterol-diet-fed rabbits. Masson et al. [49] reported that pulse pressure obtained from telemetry, an index of arterial stiffness, was reduced by n-3 PUFA in fructose-fed rats, a model of insulin-resistant state. Similarly, Engler et al. [50] demonstrated that DHA supplementation reduced pulse pressure and vascular wall thickness in spontaneously hypertensive rats. More recently our group demonstrated that n-3 PUFA supplementation prevents arterial stiffening [51] and other vascular changes, such as baroreflex sensitivity [52] induced by ovariectomy, in a rat experimental model of menopause.

A number of randomized and controlled clinical trials have been conducted to explore the effects of n-3 PUFA on various endpoints related to arterial stiffness. A well-conducted meta-analysis by Pase et al. in 2011 [53], considering 10 intervention trials of n-3 PUFA supplementation, reported that the 2 main outcomes examined (PWV and systemic arterial compliance) were favorably affected by the intervention, thus providing strong support to the use of n-3 PUFA as an evidence-based mean to reduce arterial stiffness. The randomized clinical trials considered in this meta-analysis considered mainly high risk patients, with cardiovascular risk factors ranging from dyslipidemia, hypertension, and obesity to type 2 diabetes. More recent trials confirmed this result with the validated endpoint of carotid-femoral PWV, actually considered the gold standard measure for arterial stiffness [9]. These studies, performed in special patient population such as healthy smokers [54] and metabolic

Table 1: Studies evaluating n-3 PUFA effects on arterial stiffness.

(a) Animal studies

First author	Year	Dose	Sample	Duration (w)	Study design	Outcome measure	Results
Sato [48]	1993	300 mg/kg/day EPA	High-cholesterol-diet-fed rabbits	12	AES-PG	cf-PWV	Prevent increase in cf-PWV
Engler [50]	2003	DHA-enriched oil	Spontaneous hypertensive rats	6	AES-PG	Wall thickness, PP	Reduce wall thickness and PP
Masson [49]	2008	diet enriched w. 16 g/kg n-3 PUFA	Fructose-fed rats	10	AES-PC	PP	Prevent increase in PP
Losurdo [51]	2014	0.65 g/kg/d versus placebo by gavage	Ovariectomized rats	8	AES-PC	cf-PWV	Prevent increase in cf-PWV

(b) Human studies

First author	Year	Dose	Sample	Duration (w)	Number	Study design	Outcome measure	Results
McVeigh [110]	1994	1800 mg EPA + 1200 mg DHA/d versus placebo (olive oil)	Type 2 diabetes	6	20	RCT-PC	Total AC	Increase in total AC
Nestel [111]	2002	3000 mg EPA/d versus 3000 mg DHA/d versus placebo (olive oil)	Dyslipidemic	7	38	RCT-PC	Total AC	Increase 36% with EPA, 27% with DHA
Tomiyama [62]	2011	1800 mg EPA/d versus control (diet therapy)	Dyslipidemic	52	84	RCT-PG	ba-PWV	Reduction of ba-PWV
Hjerkinn [29]	2006	2400 mg n-3 PUFA versus control diet	Dyslipidemic	156	563	RCT-PG	cr-PWV	Decrease in cr-PWV 4%
Hill [112]	2007	1560 mg DHA + 360 mg EPA/d versus placebo (6 g sunflower oil/d)	Overweight, hypertensive, dyslipidemic	6 to 12	38	RCT-PC	Small and large AC	Increase in small AC 26%.
Mita [28]	2007	1800 mg EPA/d versus control (no EPA)	Type 2 diabetes	6	64	RCT-PG	ba-PWV	Reduction of ba-PWV
Wang [113]	2008	540 mg EPA + 360 mg DHA versus placebo capsules	Overweight, hypertensive	8	52	RCT-PC	Small and large AC	Increase in large AC 21%
Satoh [114]	2009	1800 mg EPA/d + diet versus control (diet only)	Metabolic syndrome	12	92	RCT-PG	ca-PWV	Reduction of ca-PWV 6%
Ayer [115]	2009	32 mg EPA/d + 135 mg DHA/d + canola oil versus control diet	Healthy children	260	616	RCT-PG	Carotid artery distensibility, cb-PWV, Aix	No difference
Sjoberg [116]	2010	1560 DHA + 360 mg EPA/d versus placebo (sunola oil)	Overweight	12	67	RCT-PC	Small and large AC	Increase in large AC 14%
Dangardt [60]	2010	1200 mg n-3 PUFA versus placebo	Obese adolescents	12	25	RCT-PC	cf-PWV	No difference
Sanders [58]	2011	1800 mg n-3 PUFA versus placebo	Healthy subjects	52	312	RCT-PC	cf-PWV	Decrease in cf-PWV
Haiden [57]	2012	1800 mg n-3 PUFA versus placebo	Hypertensive, dyslipidemic	52	19	CT	ba-PWV, aortic strain rate	Decrease in ba-PWV 1%, strain rate 17%
Siasos [54]	2013	2000 mg n-3 PUFA versus placebo	Healthy smokers	12	20	RCT-PC	cf-PWV	Decrease in cf-PWV 6%
Root [59]	2013	1700 mg n-3 PUFA versus placebo	Overweight young	4	30	RCT-PC	cf-PWV	No difference

(b) Continued.

First author	Year	Dose	Sample	Duration (w)	Number	Study design	Outcome measure	Results
Wong [56]	2013	4000 mg n-3 PUFA + diet versus diet alone	Obese	12	13	RCT-PC	Small and large AC	Increase in large AC 20%, small AC 22%
Tousoulis [55]	2014	2000 mg n-3 PUFA versus placebo	Metabolic syndrome	12	29	RCT-PC	cf-PWV	Decrease in cf-PWV 5%

n-3 PUFA, omega-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PWV, pulse wave velocity; cf, carotid-femoral; ba, brachial-ankle; cr, carotid-radial; PP, pulse pressure; AC, arterial compliance; AES, animal experimental study; RCT, randomized clinical trial; CT, clinical trial; PG, parallel groups; PC, placebo controlled; d, day; w, week.

syndrome patients [55], confirmed a reduction in arterial stiffness. A large study conducted evaluating carotid-radial PWV in elderly men with hypercholesterolemia [29] and systemic arterial compliance in obese patients on a weight loss diet [56] confirmed a favorable effect in arterial stiffness. Considering innovative measurement methods of arterial stiffness, a small open-label study observed an improvement in regional aortic stiffness assessed by strain rate, using tissue Doppler imaging [57]. Three randomized clinical trials conducted with small doses of n-3 PUFA (<1.8 g/d) on healthy patients [58] and on young healthy patients with metabolic syndrome [59] or obesity [60] did not find any significant effect on arterial stiffness. In a trial evaluating patients with cardiovascular risk factors a fish oil diet was ineffective in reducing brachial-ankle pulse wave velocity, while the subsequent administration of pure EPA in the same population significantly reduced the arterial stiffness [61]. Considering cross-sectional studies, in general Japanese population, there was no relationship between serum omega-3 levels and arterial stiffness, evaluated as brachial-ankle PWV [62], while in a sample of 299 Korean men a regression analysis found a significant inverse association with total n-3 PUFA and carotid-femoral PWV [63].

Despite the few negative results in randomized clinical trials, current evidences generally agree that n-3 PUFA are effective in reducing arterial stiffness in humans. We can speculate that the negative results in these trials [58–60] are due to the small dose of active treatment or to the fact that in these trials a population with a low risk for cardiovascular disease was considered (young patients, healthy volunteers). The preferred use of n-3 PUFA only in high risk patients or in secondary prevention is supported by current guidelines and could be applied also for n-3 PUFA administration for the purpose of reducing arterial stiffness, although well-designed clinical trials considering high and low risk population are needed to support this evidence.

As arterial stiffness is a strong risk factor for cardiovascular disease, n-3 PUFA should be considered, among the wide range of cardiovascular drugs, as a safe and evidence-based choice to positively affect the mechanical properties of arterial wall. Which dose is the best for this outcome and which group of patients should be treated constitute an important area of future research.

6. Regulation of Endothelial Function and Endothelial Dysfunction

Classically the term "endothelial dysfunction" strictly refers to reduced endothelium-dependent vasodilation, which is notably associated with impaired bioavailability of the main endothelium-derived relaxing factor, nitric oxide (NO). In addition to promoting vasodilation, NO is a powerful antiatherosclerotic agent, since it reduces leukocyte adhesion, platelet aggregation, and smooth muscle cell proliferation [64]. In the endothelium NO is produced by the enzyme endothelial nitric oxide synthase (eNOS). Reduced nitric oxide bioavailability can be the result of either decreased production or increased scavenging. Several mechanisms, including downregulation of eNOS expression, posttranslational modifications of eNOS, inhibition of the enzyme catalytic activity, enzyme uncoupling, and circulating eNOS inhibitors result in decreased NO release and endothelial dysfunction [65, 66].

On the other hand, a number of studies have shown that reactive oxygen species (ROS), which are increased in many conditions associated with enhanced oxidative stress, determine endothelial dysfunction by quenching NO, reducing its bioavailability and leading to the formation of the highly toxic peroxynitrite [66, 67].

Perturbations of NO bioavailability are usually associated with signs of vascular inflammation and of a prothrombotic and procoagulable state [68]. Therefore, in a comprehensive sense, the term endothelial dysfunction encompasses a wide range of alterations of endothelial function preluding overt atherosclerosis.

Endothelial dysfunction is typically detected in conditions associated with vascular disease, such as hypertension, smoking, diabetes mellitus, hypercholesterolemia, and aging [69]. Clinically, endothelial dysfunction can be noninvasively assessed by measuring flow-mediated dilation (FMD), at the level of the brachial artery or of the coronary bed. This parameter allows determining the capability of the vessel to dilate in response to various stimuli (hyperemia following sphygmomanometer cuff inflation or infusion of muscarinic receptor agonists) [4, 70]. Importantly, several studies have demonstrated the prognostic value of endothelial dysfunction in terms of future cardiovascular events in both populations at low and high cardiovascular risk, its

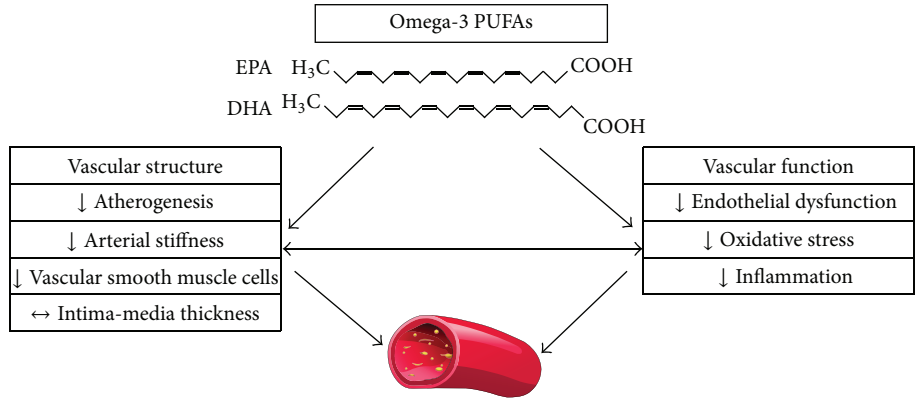

Figure 1: n-3 PUFA effects on vascular structure and function.

predictive value being not inferior to validated surrogate markers of vascular function [4, 71–73]. Therefore endothelial dysfunction can be considered an early marker of increased cardiovascular risk in patients with or without a previous history of cardiovascular disease.

7. Influence of n-3 PUFA on Endothelial Function

The mechanism by which n-3 PUFA influence endothelial function is mediated by their incorporation into biological membrane phospholipids; this allows modulation of membrane composition and fluidity. The importance of endothelial cell membrane composition has been documented by several studies (Table 2). The reason lies in the fact that endothelial cell membrane houses caveolae and lipid rafts where several receptors and signaling molecules crucial for cell function are concentrated [74]. Caveolae-associated receptor-mediated cellular signal transduction includes important pathways such as the nitric-oxide cGMP pathway, the NADPH oxidase and TNF-α –NFκB induced cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) activation pathway [75, 76]. By modulating the composition of caveolae, as described for other classes of lipids [77] n-3 PUFA may exert their beneficial effects, which include increased NO production and reduced production proinflammatory mediators.

Molecular evidence of enhanced eNOS activity/expression following administration of n-3 PUFA derives from experimental studies in endothelial cells in culture or in animals. Wu et al. [78] showed that in bovine aortic endothelial cells and in eNOS knock-out mice EPA induces NO production by stimulating AMP-activated protein kinase (AMPK) induced endothelial nitric oxide synthase (eNOS) activation. Similarly, Omura et al. demonstrated that EPA stimulates eNOS activation in endothelial cells by inducing its dissociation from the inhibitory scaffolding protein caveolin [79]. Likewise, Stebbins et al. reported that DHA promotes eNOS activity by increasing the interaction between eNOS and HSP-90, which activates PKB/AKt pathway finally resulting in eNOS phosphorylation and activation [80]. Finally, n-3 PUFA can enhance eNOS activity by reducing the circulating levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, which is increased in conditions as hypertension, renal failure, and aging [81].

Another mechanism by which n-3 PUFA increase NO production is by directly stimulating eNOS gene and protein expression. Improved vasodilation as a result of n-3 PUFA induced upregulation of eNOS gene/protein expression has been documented in a wide series of reports considering physiological and disease animal models including menopause, atherosclerosis, and diabetes mellitus by our and other groups [82–88]. Taken together these data indicate a strong potential of n-3 PUFA to potentiate NO availability by enhancing its production via different molecular mechanisms.

In addition to increasing NO production, n-3 PUFA decrease oxidative stress. This effect is controversial, since the prooxidant activity of long-chain n-3 PUFA especially at high doses has long been debated [89]. However experimental studies conducted so far in cell culture or in vascular beds of experimental animals have shown that relatively large doses of n-3 PUFA improve endothelial function by attenuating ROS production as a result of a direct modulatory effect on the sources of ROS formation, including the enzymes NADPH oxidase and iNOS, finally resulting in reduced peroxynitrite formation [82, 83]. In retinal endothelial cells in culture exposed to high glucose ALA directly reduces ROS information and increased superoxide dismutase (SOD) activity [90, 91]. A potentiation of endogenous antioxidant enzyme concentrations in plasma as a direct effect of n-3 PUFA oral administration has also been reported also by other reports [92].

Among the contributors to endothelial dysfunction, n-3 PUFA have shown the potential to attenuate cellular and systemic inflammation. In endothelial cells in vitro n-3 PUFA attenuate NF-κB activation, resulting in reduced VCAM-1 expression [90]. Additionally, n-3 PUFA exert systemic anti-inflammatory effects by raising the plasma levels of adiponectin [93] and suppressing the production of interleukin 6, interleukin 1β, soluble E selectin, and CRP [94]. These effects are dose-dependent, as relatively high doses of n-3 PUFA are required to achieve the anti-inflammatory effect and this cannot exclude the fact that

Table 2: Studies evaluating n-3 PUFA effects on endothelial function.

(a) Animal studies

First author	Year	Dose	Sample	Duration (w)	Study design	Outcome measure	Results
Nyby [84]	2005	Diet enriched with 60% fructose and 4.4% n-3 PUFA versus diet with 60% fructose or control diet	Hyperinsulinemic rats	8	AES-PG	EDD, oxidative stress	Improve EDD and oxidative stress
Matsumoto [88]	2009	300 mg/kg/day EPA versus control diet	Diabetic rats	4	AES-PC	EDD	Improve EDD
Zhang [83]	2013	Diet enriched with ALA 500 mg/kg/day versus control diet	Type 2 diabetic rats	5	AES-PC	EDD, oxidative stress	Improve EDD and oxidative stress
Gortan Cappellari [82]	2013	800 mg/kg/day by gavage versus control diet	Ovariectomized rats	8	AES-PC	EDD, oxidative stress	Improve EDD and oxidative stress

(b) Human studies

First author	Year	Dose	Sample	Duration (w)	Number	Study design	Outcome measure	Results
Woodman [117]	2003	3800 mg EPA or 3700 mg DHA versus olive oil	Hypertensive type 2 patients	6	30	RCT-PG	EDD, EID	Unchanged EDD and EID
Engler [118]	2004	1200 mg n-3 PUFA versus control diet	Hypercholesterolemic children	10	20	RCT-PC	EDD, oxidative stress, inflammation	Improved EDD, unchanged oxidative stress and inflammation
Ros [119]	2004	1100–1700 mg n-3 PUFA versus Mediterranean diet	Hypercholesterolemic patients	4	20	RCT-PG	EDD, oxidative stress and CRP	Improved EDD, unchanged oxidative stress and inflammation
Keogh [120]	2005	4700 mg mg n-3 PUFA versus isocaloric high carbohydrate, saturated or monounsaturated fat enriched-diet	Healthy subjects	4	40	RCT-PG	EDD, CRP, inflammation	Improved EDD in all groups except in saturated fat enriched diet
Prabodh Shah [121]	2007	500 mg n-3 PUFA versus placebo	Healthy subjects	2	26	RCT-PC	EDD, EID	Improved EDD and EID
Wright [122]	2008	3000 mg n-3 PUFA versus standard therapy	Systemic lupus erythematosus patients	24	56	RCT-PG	EDD, oxidative stress	Improved EDD and oxidative stress
Schiano [123]	2008	1700–2000 mg versus standard therapy	Intermittent claudication patients	13	32	RCT-PG	EDD, inflammation	Improved EDD, inflammation unchanged
Mindrescu [124]	2008	4500 mg n-3 PUFA + rosuvastatin 10 g versus rosuvastatin 10 g	Dyslipidemic patients	4	30	RCT-PG	EDD, EID	Improved EDD and EID
Rizza [125]	2009	1700–2000 mg n-3 PUFA versus placebo	Offspring of type 2 diabetic subjects	12	50	RCT-PC	EDD, inflammation	Improved EDD and inflammation
Wong [99]	2010	4000 mg n-3 PUFA versus control (olive oil)	Type 2 diabetes mellitus	12	97	RCT-PG	EDD, CRP, renal function	Improved renal function; no effect on EDD or CRP

(b) Continued.

First author	Year	Dose	Sample	Duration (w)	Number	Study design	Outcome measure	Results
Stirban [100]	2010	2000 mg versus control (olive oil)	Type 2 diabetes mellitus	6	34	RCT-PC	Postprandial EDD	Improved postprandial EDD
Sanders [58]	2011	450–900 or 1800 mg n-3 PUFA vresus placebo (refined oil)	Healthy subjects	51	310	RCT-PC	EDD	Unchanged EDD and EID
Skulas-Ray [101]	2011	850 or 3400 mg versus placebo	Moderate hypertriglyceridemia	8	26	RTC-PC	EDD, IL-6, CRP	No effect on EDD, IL-6, or CRP
Moertl [103]	2011	1000 or 4000 mg n-3 PUFA versus placebo	CHF	12	43	RCT-PC	LVEF, EDD, IL-6	Improved LVEF, EDD, and IL-6
Haberka [104]	2011	1000 mg n-3 PUFA versus control (standard diet and therapy)	Previous AMI	12	40	RCT-PG	EDD, EID	Improved EDD; EID unchanged
Din [105]	2013	2000 mg n-3 PUFA versus placebo	Cigarette smokers	6	20	RCT-PC	EDD, P-selectin, CD40L	Improved EDD and P selectin; CD40L unchanged
Din [108]	2013	2000 mg n-3 PUFA versus placebo	Previous AMI	6	20	RCT-PC	EDD, P-selectin, CD40L	No effect

n-3 PUFA, omega-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EDD, endothelium dependent dilation; EID: endothelium independent dilation; AMI: acute myocardial infarction; CHF: chronic heart failure; LVEF: left ventricular ejection fraction; IL-6: interleukin-6; CRP: C reactive protein; AES, animal experimental study; RCT, randomized clinical trial; CT, clinical trial; PG, parallel groups; PC, placebo controlled; d, day; w, week.

indirectly also the triglyceride-lowering effect contributes to improved endothelial function often observed in these conditions.

8. n-3 PUFA and Endothelial Dysfunction: In Vivo Human Studies

n-3 PUFA show the potential to improve endothelial dysfunction by activating NO production via different mechanisms and by reducing vascular oxidative stress and inflammation (Figure 1). Many studies have evaluated the effect of n-3 PUFA on human endothelial function and the results have been reported by two recent meta-analyses [95, 96], whose conclusions are not completely concordant. For review reasons, we will focus on studies published during the last 5 years. One issue that needs to be considered when evaluating the effect of n-3 PUFA supplementation on endothelial function is the poverty of data on n-3 PUFA basal enrichment in the patient population under consideration. The amount of n-3 PUFA in biological membranes can be directly extrapolated by measuring the omega-3 index in red blood cell membranes [97, 98] or by determining plasma concentrations of EPA + DHA which have shown a good correlation with their membrane levels [89]. This point is crucial as conditions characterized by n-3 PUFA depletion may mostly benefit from their supplementation. In a recent study conducted in an experimental model of menopause, deficiency of n-3 PUFA demonstrated by low omega-3 index was associated with endothelial dysfunction and increased oxidative stress, which were reversed by efficient n-3 PUFA supplementation, resulting in normalization of omega-3 index [82]. Having said this, clinical trials on the effects of n-3 PUFA on endothelial function are significantly heterogeneous in

(i) number of included participants;

(ii) inclusion criteria: age of participants, healthy or disease state, have been studied;

(iii) markers of endothelial function: in addition to flow-mediated dilation, at least 7 different classes of surrogate markers have been tested in the last 5 years. The most frequent categories tested, according to their different pathophysiological roles, are proinflammatory and anti-inflammatory cytokines, endothelial progenitor cells, markers of platelet activation, of fibrinolysis, of thrombosis, and of coagulation, and markers of oxidative stress [98–102];

(iv) dose and duration of treatment: doses ranging from 0.45 up to 4 grams have been tested as well as treatments ranging from 4 to 52 weeks [58, 103, 104];

(v) forms of n-3 PUFA: EPA, DHA, or ALA has been administered alone or in combination;

(vi) concomitant therapy: most of the studies on disease states do not provide accurate information on concomitant therapy, particularly on drugs known to improve endothelial function such as statins and ACE inhibitors/angiotensin receptor blockers. The presence of a robust concomitant therapy might improve endothelial function independently of n-3 PUFA (especially at low doses) in high risk patients.

So far, most studies have cautiously suggested that supplementation with n-3 PUFA might improve endothelial function. However, whether the amplitude of this effect depends on healthy or disease state or on the administered dose or whether the n-3 PUFA composition of supplementation differentially affects the outcome is currently unclear. In smokers, where the bias of concomitant therapies is not an issue, two recent studies have shown that n-3 PUFA supplementation for six and twelve weeks, respectively, improves endothelial function [54, 105]. In patients with moderate cardiovascular risk Seely et al. performed a meta-analysis where again quality and power of the available studies precluded any definite conclusion [106]. However, low-strength evidence seemed to suggest a benefit of n-3 PUFA in endothelial dysfunction. Similar results have been reported in high risk patients with previous myocardial infarction [104] although a recent comprehensive meta-analysis and a recent study in a similar population do not confirm these findings [107, 108].

When considering moderate/high risk patients assuming polytherapy, the issue of cost/benefit in terms of clinical efficiency and potential harms is important. Therefore, stronger evidence is needed before large scale prescription of n-3 PUFA in this population.

9. Endothelial-Independent Vasodilation and n-3 PUFA

Technically, flow-mediated dilation is the result of both endothelial-derived vasodilation (which is mainly NO-dependent) and endothelial-independent vasorelaxation. The latter depends on the ability of smooth muscle cells to respond to nitric oxide and therefore measures the integrity of arterial media. The hypotensive effect of n-3 PUFA can partly be explained by this mechanism. Therefore, when measuring FMD in vivo in humans, it is difficult to dissect the relative contribution of endothelium and smooth muscle cells unless a selective agonist is administered (muscarinic receptor agonist for EDD and NO donor for EID). A recent study addressed the physiologic mechanisms of EPA-induced relaxation in pulmonary arteries from an animal model [109] and showed that in these conditions the contribution of endothelium-derived NO release to vasodilation is prominent, while that mediated by endothelium-independent mechanisms is negligible.

These findings are in line with data from human studies, showing that when controlled trials assessing EID are considered, no significant effect of n-3 PUFA on EID is observed [96].

10. Conclusive Remarks

By targeting both arterial wall stiffness and endothelial dysfunction n-3 PUFA have the potential to beneficially impact arterial wall remodeling and cardiovascular outcomes. Their pleiotropic effects on systemic inflammation, modulation of lipid profile, and platelet aggregation contribute to the reduction of cardiovascular risk. Although dissecting the specific contribution of structural arterial remodeling to overall cardiovascular risk is difficult from experimental studies conducted in high risk populations, current results are encouraging. From here comes the need for large scale trials, advocated by most of the available literature. This process is likely to involve selection of homogenous patient populations in terms of target disease, endpoints, and modality of treatment.

Abbreviations

NO:	Nitric oxide
eNOS:	Endothelial nitric oxide synthase
iNOS:	Inducible nitric oxide synthase
NADPH:	Nicotinamide adenine dinucleotide phosphate
ROS:	Reactive oxygen species
TNFα:	Tumor necrosis factor alpha
IL-1β:	Interleukin 1 beta
IL-6:	Interleukin 6
COX-2:	Cyclooxygenase-2
PGE_2:	Prostaglandin E_2
SOD:	Superoxide dismutase
AMPK:	AMP-activated protein kinase
EPA:	Eicosapentaenoic acid
DHA:	Docosahexaenoic acid
ALA:	Alpha-linolenic acid
ADMA:	Asymmetric dimethylarginine
PWV:	Pulse wave velocity
IMT:	Intima-media thickness
EDD:	Endothelial-dependent dilation
EID:	Endothelial-independent dilation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publishing of this paper.

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Review Article

Omega-3 Polyunsaturated Fatty Acids: The Way Forward in Times of Mixed Evidence

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Almost forty years ago, it was first hypothesized that an increased dietary intake of omega-3 polyunsaturated fatty acids (PUFA) from fish fat could exert protective effects against several pathologies. Decades of intense preclinical investigation have supported this hypothesis in a variety of model systems. Several clinical cardiovascular studies demonstrated the beneficial health effects of omega-3 PUFA, leading medical institutions worldwide to publish recommendations for their increased intake. However, particularly in recent years, contradictory results have been obtained in human studies focusing on cardiovascular disease and the clinical evidence in other diseases, particularly chronic inflammatory and neoplastic diseases, was never established to a degree that led to clear approval of treatment with omega-3 PUFA. Recent data not in line with the previous findings have sparked a debate on the health efficacy of omega-3 PUFA and the usefulness of increasing their intake for the prevention of a number of pathologies. In this review, we aim to examine the controversies on the possible use of these fatty acids as preventive/curative tools against the development of cardiovascular, metabolic, and inflammatory diseases, as well as several kinds of cancer.

1. Introduction

Over the past three to four decades, a considerable body of literature has been published indicating possible health benefits of an increased dietary intake of long-chain omega-3 polyunsaturated fatty acids (LC-omega-3 PUFA). Their beneficial effects have been reported for a number of disorders, including cardiovascular [1, 2], neurodegenerative [3, 4], neuropsychiatric [5, 6], and inflammatory diseases [7], as well as for some cancer types (mainly colorectal, mammary, and prostatic cancer) [8–10]. During this time period, a large number of preclinical *in vitro* and *in vivo* studies have been performed which, rather unanimously, demonstrated the potential of omega-3 PUFA as preventive and therapeutic agents against hypertriglyceridemia, cardiac arrhythmia, inflammation, and proliferation, and many experimental studies have succeeded in elucidating and clarifying many of the biological and molecular mechanisms underlying these effects [11–17]. Also, for application in humans, consensus was reached on the efficacy of these fatty acids (FA) in

the secondary prevention of some cardiovascular diseases, since the results of a large and well performed clinical study [18] had clearly and strongly demonstrated beneficial effects linked to their intake. As a consequence, omega-3 PUFA have been approved as preventive and therapeutic tools in the management of several cardiovascular disorders, as well as for treatment of severe hypertriglyceridemia by a number of health agencies throughout the world, and a number of guidelines were published recommending omega-3 PUFA supplementation (see Section 3). Cardiologists and other physicians began to prescribe them routinely [19]. Moreover, as diet has always been an important topic for the popular press, recommendations have reached large segments of population, so that over-the-counter omega-3 PUFA supplements have become the most sold supplements worldwide [20].

In contrast to this remarkable story of research performance and dissemination, many human studies investigating the effect of an increased intake of omega-3 PUFA against noncardiovascular diseases were often not in keeping with data obtained in preclinical studies, and therefore consensus recommendations have not yet been made regarding the usefulness of omega-3 PUFA for the cure or prevention of inflammatory, neoplastic or neurologic disorders.

Some reports question the effectiveness of omega-3 PUFA in cardiovascular prevention [21–24], and others argue that an increased intake of these compounds could induce or exacerbate some neoplastic pathologies [25–27]. Where does this leave the field of omega-3 PUFA research and application? We will try to address this pertinent question exploring in Section 2 our body's need for omega-3 PUFA to satisfy physiological needs and to prevent the development of several kinds of diseases, in Section 3 the history and present status of omega-3 PUFA in cardiovascular diseases, in Section 4 inflammatory diseases, and in Section 5 colorectal and prostate cancer. We will describe in each of these sections the controversies arisen, and in Section 6 we will consider and critically analyze all the possible reasons for these discrepancies.

2. Omega-3 PUFA Insufficiency/Deficiency and Current Recommended Intakes

Alpha-linolenic acid (ALA), an 18-carbon omega-3 essential FA, is the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The term "essential" indicates that ALA cannot be synthesized by humans and therefore must be entirely acquired from exogenous sources. Evidence for the essentiality of ALA was first provided by a study showing that ALA supplementation reversed the abnormal neurologic signs observed in a 6-year-old girl who suffered from sensory loss and visual complications [28]. Following consumption, most of the ALA is catabolized via β-oxidation for energy generation, and a small proportion of it undergoes conversion to produce another two potent members of omega-3 PUFA family: EPA and DHA [29]. The conversion rates of ALA to EPA and DHA in humans are estimated at 8–20% and 0.5–9%, respectively [30]. Owing to the fact that EPA and DHA can be synthesized in the body from ALA, these two FA do not meet the definition of essential FA *per se*. However, as this conversion is not efficient enough to satisfy health requirements, EPA and DHA are also considered essential FA (or conditionally essential FA). In addition, although not conclusive, the benefits associated with ALA seem to stem mainly from EPA and DHA, and as major consequence of ALA deficiency it appears that EPA and DHA are not adequately produced [31, 32]. The clinical features of omega-3 PUFA insufficiency include impaired growth, skin lesions, infertility, kidney abnormalities, fatty liver, polydipsia, increased susceptibility to infections, reduced learning, and impaired vision [33, 34]. Yet, these symptoms are nonspecific and may also result from the dysregulation in omega-6 PUFA homeostasis [35]. For example, the skin lesions present in patients with atopic eczema have been associated with poor omega-6 PUFA intake [36].

An increased intake of omega-3 PUFA, especially the long-chain omega-3 PUFA EPA and DHA, could reduce the tissue omega-6/omega-3 ratio to a level that probably existed during millions of years of human evolution [37]. This ratio dramatically increased in recent millennia due to deep changes in dietary habits following the transition from the hunter-gatherer lifestyle to agricultural societies. This change could therefore be one of the crucial factors leading to the rise of the so-called diseases of civilization, further skewed towards omega-6 PUFA by the agricultural revolution in the 19th century and the massive use of corn (with its high omega-6 PUFA content) in western societies during the 20th century.

Dietary recommendations for omega-3 PUFA, previously focused on ensuring their sufficient intake to prevent deficiency, are increasingly seeking to define an "optimal" intake to reduce the risk of developing chronic diseases, in particular cardiovascular diseases (CVD). To date, there is no uniform scientific guideline on the ideal omega-3 PUFA intake. Nutritional guidelines have been published by several governments (France, Belgium, Netherlands, New Zealand, and Australia) and health organizations (Food and Agriculture Organization (FAO), American Dietetic Association). The most popular recommendations on omega-3 PUFA intake are those published by the American Heart Association (AHA), the UK Committee on Medical Aspects of Food Policy, the World Health Organization (WHO), and the European Food Safety Authority (EFSA). Recommendations for omega-3 PUFA (ALA, EPA, and DHA, individually or combined) for adults according to various recently published dietary guidelines are shown in Table 1, whereas Table 2 shows recommendations for infants and children [38–53]. Dietary recommendations for EPA + DHA range from 250 to 1000 mg/day for adults and from 40 to 250 mg/day for infants older than six months and for children and adolescents.

3. Omega-3 PUFA Efficacy in the Cardiovascular Field

The predominant field of omega-3 PUFA research in the past decades has been that of cardiovascular medicine and

Table 1: Recommended dietary intakes for omega-3 fatty acids in adults from national and international organizations.

Region/country	Organization, year, reference	ALA (g/day)	DHA (g/day)	EPA + DHA (g/day)
International	FAO/WHO, 2010 [38]			0.25–2.0
	ISSFAL, 2004 [39]			>0.5
	Eurodiet, 2001 [40]	2.0		0.2
	WAPM, 2008 [41]		0.2–0.3	
	EFSA, 2010 [42]			0.25
National				
UK	SACN, 2004 [43]			0.45
Netherlands	Health Council, 2006 [44]			0.45
France	ANSES, 2001 [46]		0.25 0.2	0.5
Spain	SENC, 2011 [48]			0.5–1.0
Australia	NHMRC, 2006 [49]	M: 1.3 F: 0.8		M: 0.61 F: 0.43
USA	IoM, 2005 [50]	M: 1.6 F: 1.1		
	American Diabetes Association, 2007 [51]	2.0		0.2
	ADA USA & Canada, 2007 [52]	M: 1.6 F: 1.1		0.5
	AHA, 2009 [53]			0.5–1.0

M: male; F: female.

Table 2: Recommended dietary intakes for omega-3 fatty acids in children from national and international organizations.

Region/country	Organization, year	Age range	ALA (mg/day)	DHA (mg/day)	EPA + DHA (mg/day)
International	FAO/WHO, 2010 [38]	6–24 mo		10–12/kg	
		2–4 y			100–150
		4–6 y			150–200
		6–10 y			200–250
	EFSA, 2010 [42]	7–24 mo		100	
		2–18 y			250
National					
Belgium	CSS, 2009 [47]	0–12 mo	500		
Netherlands	Health Council, 2001 [45]	0–5 mo	80/kg	20/kg	
		6 mo–18 y			150–200
France	ANSES, 2001 [46]	6 mo–3 y		70	
		3–9 y		125	250
		10–18 y		250	500
Australia	NHMRC, 2006 [49]	1–3 y	500		40
		4–8 y	800		55
		9–13 y	M: 1000 F: 800		70
		14–18 y	M: 1200 F: 800		M: 125 F: 85
USA	IoM, 2005 [50]	1–3 y	700		
		4–8 y	900		
		9–13 y	M: 1200 F: 1000		
		14–18 y	M: 1600 F: 1100		

M: male; F: female.

prevention. This is the field in which the hypothesis of a protective effect of omega-3 PUFA was founded [69], and a large clinical study establishing a benefit was the basis for approval of omega-3 PUFA as secondary prevention in patients after myocardial infarction [18]. The GISSI-Prevenzione trial was performed as an open-label trial in the 1990s in 11324 patients shortly after myocardial infarction. Participants in the omega-3 PUFA group received 1 g/d omega-3 PUFA (ratio of EPA/DHA 1 : 2, with a total amount of EPA + DHA of approximately 850 mg) for 3.5 years. There were no systematic fatty acid measurements performed in the participants, but, as an indication of omega-3 PUFA uptake, triglycerides were lower in the omega-3 PUFA group. Treatment with omega-3 PUFA significantly lowered the risk of death, including cardiovascular death [18]. This area has seen some good examples of translational research, particularly with regard to the antiarrhythmic effects attributed to omega-3 PUFA. Leaf [70, 71] was a pioneer in the field of omega-3 PUFA research showing that these FA can block proarrhythmogenic effects of adrenal agents, calcium, and other substances in isolated cardiomyocytes, continuing with studies in animals [72], and finally concluding his research in a large human multicenter trial [54]. The concept of cardioprotection through omega-3 PUFA supplementation has been firmly engrained into the canon of medical knowledge and clinical thinking, as is evidenced by the AHA guidelines recommending intake of omega-3 PUFA [73].

However, while preclinical data regarding the antiarrhythmic effect of omega-3 PUFA were strongly beneficial, the double-blind intervention study by Leaf et al. [54] does not show a clear benefit. A relevant problem of the study was the high noncompliance rate (35% of enrollees, similar in fish oil and olive oil groups), probably due to the high capsule load (2600 mg fish oil per day). Treatment with daily fish oil or olive oil for 12 months in patients with implanted cardioverter/defibrillators (ICDs) did not lead to a significant difference for the primary end point (time to first ICD event for ventricular tachycardia (VT) or death from any cause). However, there was a significant effect in participants who were at risk of fatal ventricular arrhythmias and, after staying on protocol for at least 11 months, showed a significant risk reduction of 38%. Phospholipid fatty acid content of red blood cells was compared in a subset of participants in the study, and omega-3 PUFA content increased significantly from 3.4% to 7.6% of total fatty acids, while it remained at 3.5% in the olive oil control group.

There are two more randomized, double-blind, placebo-controlled trials with omega-3 PUFA supplementation performed in patients with ICDs and at high risk of high-grade arrhythmic events. In one, 200 patients with an ICD and a recent episode of sustained VT or ventricular fibrillation (VF) received either 1300 mg/d omega-3 PUFA or placebo and were followed up for 2 years. While patients on fish oil had an increase in the mean percentage of omega-3 PUFA in red blood cell membranes from 4.7% at baseline to a steady-state maximum of 8.3% at 3 months, there was no protection from VT/VF events [59]. The third study did not find a significant advantage for omega-3 PUFA supplementation in ICD patients receiving 2 g/d of fish oil ($n = 273$) versus placebo ($n = 273$) for one year with the primary outcome of an ICD event or death occurring in 30% patients taking omega-3 PUFA versus 33% of those on placebo [60].

In addition to these high-quality data sets showing uncertainty regarding the antiarrhythmic effect of omega-3 PUFA, several large studies followed up on the initial positive GISSI-P results. The GISSI-HF investigated the effect of 1 g/d omega-3 PUFA in patients with heart failure in a randomised, double-blind, placebo-controlled trial with 6975 participants. Over approximately 4 years there was a small significant advantage in the omega-3 PUFA group for death from any cause and admission to hospital for cardiovascular reasons with a number needed to treat of 56 to prevent one death [55].

A multicenter, double-blind, placebo-controlled trial with 4837 patients after a myocardial infarction tested 40 months of fatty acid supplementation in the form of margarines, either adding a small amount of approximately 400 mg/d EPA + DHA, 2 g/d of ALA, both EPA + DHA and ALA, or placebo fat. Neither EPA-DHA nor ALA reduced the primary end point of a major cardiovascular event. Fatty acids were measured in plasma cholesteryl esters of subgroups, and supplementation with EPA + DHA increased EPA by 53.3% and DHA by 28.6%, as compared with placebo after 20 months. Unfortunately, fatty acid composition of red blood cell membranes was not measured in this study [21].

Another randomized, placebo-controlled, double-blind, multicenter trial in 3851 patients with 1 g/d omega-3 PUFA supplementation for one year in patients after acute myocardial infarction also demonstrated no benefit of omega-3 PUFA [58].

A third large study with 2501 participants found that administration of 600 mg/d omega-3 PUFA increased plasma concentrations of EPA + DHA by 37% compared with placebo but also had no significant effect on major vascular events in patients with ischaemic heart disease or ischaemic stroke [56].

Based on these and several smaller intervention studies assessing mostly relatively low-dose omega-3 PUFA supplementation, two thorough metastudies concluded that there is no convincing evidence for a preventive effect of omega-3 PUFA in patients with cardiovascular disease [22, 62].

The ORIGIN study [23] is the most recent large study that failed to show a clear benefit of omega-3 PUFA supplementation for cardiovascular health. This study, conducted in a patient population with dysglycemia, led many physicians to conclude that the relationship between dietary omega-3 and cardiovascular health needs to be viewed with caution. In this double-blind study 12536 dysglycemic patients at high risk for cardiovascular events were assigned to either 900 mg/d omega-3 PUFA or placebo. The primary outcome was death from cardiovascular causes during a median follow-up of 6.2 years, and there were no significant differences between the two treatment groups for this primary parameter as well as for a range of secondary parameters. It is noteworthy that the significant decrease of triglycerides is an indicator of effective omega-3 PUFA exposure in the verum group. However, unfortunately, fatty acid levels in the blood, particularly in the red blood cell fraction, were not evaluated.

Nutritional research, especially the clinical trials investigating different dietary regimes, has always been surrounded by controversy. The role of dietary fat in obesity and associated clinical conditions is a prime example [74], with ongoing uncertainty about the role of a low-fat diet in the battle against cardiovascular disease [61]. Recent recommendations by the AHA and the medical bodies in the US acknowledged also a protective effect of the omega-6 PUFA [53], and more studies have added complexity and controversy to the role of different FA classes (saturated fats, monounsaturated olive oil, polyunsaturated omega-6, and omega-3 PUFA), challenging not only the hypothesis of beneficial effects associated with unsaturated fatty acids but also the paradigm of the damaging effects of saturated fatty acids [61, 75].

What can we learn from all this regarding the potential cardiovascular benefits of omega-3 PUFA? It is difficult from today's perspective to adequately assess the conditions of the 70s observations regarding the omega-3 effects in the Greenland population, and the arguments of inadequate data validity in these studies made by Fodor et al. [24] are plausible giving the remoteness of many Greenland communities and problems with medical service and adequate documentation in these areas.

While the protective cardiovascular effect of omega-3 PUFA was not detected in several large recent high-quality studies, there are particularly two high-quality intervention studies, both of them open-label studies though, showing a clear benefit of omega-3 PUFA, the GISSI-P study [18] and the JELIS study demonstrating a protective effect of an EPA preparation combined with a statin medication [57]. This large open-label study performed on 18645 Japanese patients assigned them to randomly receive either 1800 mg/d of EPA daily plus statin or statin only. The primary endpoint of a major coronary event was significantly reduced in the EPA group with a 19% relative reduction. However, sudden cardiac death and coronary death did not differ between the groups. Plasma EPA was measured in this study and in patients still on supplement after 5 years of observation plasma EPA concentration was increased by 70% in the EPA group.

In conclusion, many high-quality preclinical (observational as well as interventional) and clinical studies have been performed to assess the potential cardiovascular benefit of omega-3 PUFA. A recent large metastudy has again analyzed and summarized these studies and found no clear benefit of omega-3 PUFA to protect from coronary artery disease in observational studies as well as in randomized, controlled trials [61]. This metastudy also explored the role of other fatty acids and concluded that the current evidence does not clearly support cardiovascular guidelines encouraging either high consumption of omega-3 or omega-6 PUFA, or even low consumption of saturated fats.

Cardioprotective effects of dietary omega-3 PUFA might be confounded by the use of cholesterol-lowering medications: in the GISSI-P study only 5% of participants were on a cholesterol-lowering drug [18], which is in strong contrast to the ORIGIN omega-3 PUFA study, where approximately half the patients were on a statin [23]. Statins have been hypothesized to interfere with the protective effect of omega-3 PUFA [76].

Modifications in the observed omega-3 effects might also be due to changes in the diet over the last 30–40 years, in which the hypothesis of omega-3 PUFA as beneficial dietary components has been propagated, and therefore background levels of omega-3 PUFA in the participants of more recent studies might be higher than several decades ago. This should be addressable by (comparable) measurements of omega-3 PUFA that, unfortunately, have been performed only sporadically in the studies carried out to date. Baseline omega-3 PUFA levels are lacking from most of the discussed large studies investigating omega-3 PUFA interventions. Several studies have suggested that erythrocyte EPA and DHA content (measured by a certified method as "omega-3 index") may better reflect the omega-3 PUFA status in patients than measurement in plasma and white blood cells [77, 78], and the low omega-3 index was described to be associated with higher mortality in patients with cardiovascular disease. However, so far, there has been no single well-accepted and uniformly performed blood test for the omega-3 PUFA status in human studies, complicating the comparison and interpretation of different studies. The current state of evidence for the effects of omega-3 PUFA in human studies regarding cardiovascular diseases and the contrasting outcomes have been summarized in Table 3. Additional possible reasons for the controversies in the field are reported in Section 6.

Last but not least, it should be noted that the effect of omega-3 PUFA in the cardiovascular field can also be viewed in the context of their role as precursors of highly potent physiologically beneficial lipid mediators [79, 80]. A cytochrome P450 epoxygenase-dependent metabolite of EPA, namely, 17(*R*),18(*S*)-epoxyeicosatetraenoic acid (17(*R*),18(*S*)-EETeTr), was found to exert negative chronotropic effects and can protect neonatal rat cardiomyocytes against Ca2+-overload [81]. Another EPA-metabolite, 18-hydroxyeicosapentaenoic acid (18-HEPE), was recently shown to inhibit macrophage-mediated proinflammatory activation of cardiac fibroblasts *in vitro*, while *in vivo* administration of 18-HEPE led to resistance to pressure overload-induced maladaptive cardiac remodeling in mice [82].

4. Omega-3 Efficacy in Inflammatory Diseases

Research data accumulated to date indicate that omega-6 PUFA play a significant role in the biology of inflammation. Work on the biochemistry of omega-6 led to the identification of prostaglandins and leukotrienes as key players in the physiology of inflammation [118]. Moreover, this work elucidated the cascade of the molecular mediator system arising from the omega-6 PUFA arachidonic acid and led to mechanistic understanding and further development of the most widely prescribed drug class worldwide, the cyclooxygenase-(COX-) inhibitors [119].

Early work on omega-3 PUFA identified the antagonism between omega-6 and omega-3 PUFA. It was postulated that, by competitive inhibition of COX and other enzymes, omega-3 PUFA could serve as anti-inflammatory agents. Indeed, it has been found that a high omega-3 intake was associated with a lower risk of inflammatory disease mortality [120].

Table 3: Current state of evidence for the effects of omega-3 PUFA in published human intervention studies regarding cardiovascular disease.

Type of study	Beneficial effect	Beneficial effect limited to subpopulation	Detrimental effect	No effect
Clinical trials	Lower risk of cardiovascular events and death with open-label omega-3 PUFA treatment (850 mg/d) [18]	High-dose omega-3 PUFA intervention in ICD patients at high risk of arrhythmia—significant protection in patients treated per protocol for 11 months [54]		Administration of 900 mg/d omega-3 PUFA in dysglycemic patients at increased cardiovascular risk had no protective effect [23]
	Lower risk of death in patients with heart failure on 1 g/d omega-3 PUFA [55]			No decrease in major cardiovascular events in patients on 500 mg/d omega-3 PUFA [56]
	Cardioprotective effect of open-label EPA supplementation in addition to a statin in hypercholesterolemic patients [57]			Administration of 1 g/d omega-3 PUFA in patients after a myocardial infarction showed no benefit [58]
				No reduction in cardiovascular events in patients after myocardial infarction with either 400 mg/d EPA + DHA or 2 g/d ALA, or both [21]
				No VT/VF protection in ICD patients on 1300 mg/d omega-3 PUFA [59]
				No arrhythmia protection in ICD patients on 2 g/d fish oil [60]
Meta-analyses				Current data do not support the concept of increasing omega-3 PUFA or omega-6 PUFA or decreasing saturated fatty acid intake, to reduce cardiovascular risk [61]
				No benefit of omega-3 PUFA supplementation in 14 randomized double-blind placebo-controlled studies [22]
				No benefit of omega-3 PUFA supplementation in randomized clinical trials assessing cardiovascular endpoints [62]

ICD: implanted cardioverter/defibrillator; VF: ventricular fibrillation; VT: ventricular tachycardia.

Many animal studies demonstrated the anti-inflammatory effect of omega-3 PUFA. As far as animal models of colitis are considered, EPA-ethyl or DHA-ethyl ester supplementations (1% w/w in the diet, given for 10 days prior to treatment) were reported to reduce colitis obtained in dextran sodium sulfate- (DSS-) treated mice [121], as demonstrated by their ability to inhibit the expression of several proinflammatory cytokines. Similar results were obtained also by supplementing a FO enriched diet (concentration not specified, dietary n:6/n:3 ratio: 1.5, for 7–11 weeks) in a severe combined immunodeficient mouse model of colitis [122]. In agreement with that, a FO enriched diet (containing 1% FO providing 0.5 g EPA + DHA g/day, for 5 weeks prior to infection) was shown to attenuate infection-induced colitis in mouse infected with *Citrobacter* [123]. Furthermore, also a parenteral lipid emulsion enriched in omega-3 PUFA (10% FO, 0.5 mL/h for 7 days) was found to reduce acetic acid-induced colitis in rats [124].

However, opposite results have also been obtained and extensively and recently reviewed by Fenton et al. [125]. In particular, dietary FO (8% w/w in the diet, for 5 weeks, starting one week prior to treatment) was shown to increase DSS-induced colitis [126]. Moreover, diets containing 2.25–6.00% FO (containing 59% DHA + EPA, for 8 weeks after infection) were recently found to induce severe colitis and epithelial dysplasia in mice infected with *Helicobacter hepaticus* [127]. Furthermore, FO (7% FO w/w in the diet, for 12 weeks) was found to exacerbate colitis and colitis-associated premalignant lesions [128] in IL-10 −/− transgenic mice, spontaneously developing enterocolitis [129].

In contrast, Chapkin et al. [130] showed that a FO enriched diet (4% FO w/w in the diet, for 10 weeks) fed to the same IL-10 −/− transgenic mice reduced the clinical score of both spontaneous and NSAID-induced colitis.

Interestingly, however, when at least the uncertainty related to variable intakes of FO was overcome by using the *Fat-1* mouse model of endogenously increased omega-3 PUFA [131], the inflammation-dampening effect of an increased omega-3 PUFA tissue content was consistently observed in the murine models of DSS-induced colitis [132, 133]. Moreover, when *Fat-1* mice were also treated with the carcinogen azoxymethane prior to DSS, an enhanced ability to resolve colitis and a reduced number of colonic adenocarcinomas per mouse were observed [134, 135]. In the *Fat-1* mouse model a reduced D-galactosamine/lipopolysaccharide- (D-GalN/LPS-) induced hepatitis [136] and cerulein-induced pancreatitis [137] were also observed.

As concluded by Fenton et al., it is particularly in the context of studies with infectious agents that deleterious effects of omega-3 PUFA were observed [125], and these authors argue that the same (auto)inflammation-dampening and cardioprotective properties of the omega-3 PUFA might be responsible for an ineffective immune response against pathogens and even decreased immune surveillance against tumor cells. However other data do not support this view of a uniformly immunosuppressant effect of omega-3 PUFA: a recent study shows that omega-3 PUFA can actually increase B-cell function and thus humoral immunity [138].

Also the clinical data from intervention trials with omega-3 appear to be controversial. Initial results in Crohn's disease and ulcerative colitis [63, 66] found a benefit. However, later studies showed a negligible impact of omega-3 PUFA on the relapse rate in Crohn's disease [65] as well as in ulcerative colitis; the studies performed in inflammatory bowel disease are reviewed in a recent metastudy, concluding that total data quality so far is insufficient for clear recommendations [68]. Data from acute pancreatitis in humans indicated some benefits [67]; however, there are no data from chronic pancreatitis, and the recently published Welcome Trial data regarding the use of omega-3 PUFA in nonalcoholic steatohepatitis (NASH) do not show an overwhelming benefit of supplementation in the intention-to-treat (ITT) analysis, even though there was a statistically significant linear correlation between decreased liver fat content and increased DHA enrichment [64]. The current state of evidence for the effects of omega-3 PUFA in human studies regarding inflammatory diseases and the contrasting outcomes have been summarized in Table 4. Possible reasons for the controversies in the field are reported in Section 6.

Experimental data from recent years are currently reshaping omega-3 PUFA research: since the initial description of resolvins [139] and the characterization of resolvin E1 [140], a wealth of data on this new class of omega-3 PUFA-derived lipid mediators has been published (reviewed in [80]). It is now clear that omega-3 PUFA not only play a role as competitive antagonists of the COX and lipoxygenase (LOX) enzymes, but also are substrates for potent lipid mediators themselves. It has been shown that a high abundance of these FA as substrates can significantly change the lipidome and eicosanome, given that they are efficiently metabolized as well [79]. For the DHA-derived D-resolvins, 17-HDHA has been characterized as a central pathway precursor/metabolite, and for the EPA-derived E-resolvins, 18-HEPE serves this role. Data from one of the authors have indicated that these might have anti-inflammatory properties themselves [141–143]. Other groups have described the effects of 17-HDHA as well [144–146], and very recently an independent role of 18-HEPE was also demonstrated in the context of a cardiac fibrosis model [82]. This is currently a very active field of research and will probably give us new tools for understanding the effects of omega-3 PUFA in the context of inflammation dampening, as well as a number of new leads for pharmacological compounds that could be used to mediate anti-inflammatory effects in the future. As these compounds can promote resolution of inflammation [147], this could lead to an entirely new approach to the treatment of chronic inflammation in the future.

Closely related to these effects regarding inflammation dampening are the experimental data regarding tumorigenesis. One of us and his group have validated a tumorigenesis inhibiting effect in the *Fat-1* mouse model with endogenously increased omega-3 PUFA for liver cancer [143] and colon cancer [135]; this fits well with data from other groups employing the *Fat-1* mouse model [134, 148, 149].

Particularly in gastrointestinal medicine, the paradigm of inflammation-associated carcinogenesis is central. It holds true for the development of esophageal carcinoma on the basis of Barrett's esophagus, stomach cancer on the basis of chronic *H. pylori* infection, liver cancer as a consequence of chronic hepatitis, cholangiocellular cancer in chronic cholangitis, pancreatic cancer as a consequence of chronic pancreatitis, and also in colorectal cancer. There are clinical data supporting the tumorigenesis inhibiting effect of omega-3 PUFA in the gastrointestinal tract, either with regard to colon cancer and the lowering of polyp formation in the colon [83, 90, 91] or with regard to hepatocellular carcinoma [150]. It is therefore conceivable that increasing omega-3 PUFA intake could be established as an easy and low-risk, high-gain intervention for the prevention of tumorigenesis. Given the very low risk of severe adverse events in comparison to interventions such as the use of the COX-inhibitor aspirin, for which similar antitumorigenesis effects are well established by now [151, 152], omega-3 PUFA supplementation becomes an attractive perspective. An ongoing study is even testing the combination of EPA and aspirin for the prevention of colon polyps [153].

5. Omega-3 Efficacy in Cancer

5.1. Mechanisms Involved in Omega-3 PUFA Anticancer Action. Most of the studies performed either *in vitro* or using animal models of cancer have demonstrated the possible preventive and therapeutic role of omega-3 PUFA against several types of cancer. These studies have also shed light on multiple molecular pathways modulated by these fatty acids in cancer cells and implicated in the regulation of several cell processes involved in cancer development and progression, such as cell proliferation, survival, differentiation,

Table 4: Current state of evidence for the effects of omega-3 PUFA in human studies regarding gastrointestinal inflammatory conditions.

Type of study	Beneficial effect	Beneficial effect limited to subpopulation	Detrimental effect	No effect
Clinical trials	Reduced rate of relapse with 2.7 g/d omega-3 PUFA in patients with Crohn's disease in a double-blind placebo-controlled study in 78 patients [63]	Decreased liver fat content with increased DHA enrichment in NASH patients [64]		Randomized, double-blind, placebo-controlled treatment with approx. 4 g/d omega-3 PUFA did not prevent relapses in patients with Crohn's disease [65]
	Improvements in histologic findings and weight gain in 18 ulcerative colitis patients on 5 g/d EPA + DHA in a randomized double-blind placebo-controlled crossover trial [66]			
	Shortened hospital stay in an open-label randomized prospective study administering 3.3 g/d enteral fish oil in patients with acute pancreatitis [67]			
Meta-analyses				Current data are not sufficient to support the concept of omega-3 PUFA supplementation for the treatment of inflammatory bowel disease [68]

NASH: nonalcoholic steatohepatitis.

invasion, and angiogenesis. Actually, it is quite astonishing how many signaling pathways and molecular targets have been so far implicated in the antineoplastic effects of these dietary compounds [8, 15, 154–156]. However, it has been hypothesized that these fatty acids may more directly act through just a few main direct routes and consequently influence the activity/expression/levels of plenty of cellular pathways and molecules more or less specifically altered in the different cancer tissues. Actually, the topic concerning the mechanisms underlying the omega-3 PUFA antineoplastic action is very complex and beyond the focus of this review. Several exhaustive reviews have been previously written by us and others that critically analyzed the possible cell signaling pathways involved in omega-3 antineoplastic action. The readers may refer to them to have a more complete view [8, 15, 154–159].

Nevertheless, the main direct routes through which omega-3 PUFA are hypothesized to more directly act are worth mentioning here. One of these routes is related to the omega-3 PUFA increased incorporation in cell membranes, as it happens consequently to their increased dietary intake. As a result, physical-chemical alterations of molecular lipid microenvironments (rafts) take place on cell surface [160]. These modifications may in turn lead to changes in the activity/expression of membrane constituents (receptors, channels, enzymes, adapter proteins, etc.) and multiple molecular pathways placed downstream. For instance, it was recently observed [121] that the incorporation of DHA in immortalized colonocytes *in vitro* altered the lateral organization of the Epidermal Growth Factor Receptor (EGFR), thus leading to increased ligand-induced receptor dimerization and phosphorylation along with its internalization and degradation. These changes, in turn, resulted in the disruption of the EGFR-Ras-ERK1/2 signaling cascade and the inhibition of cell proliferation. Accordingly, in chronically inflamed, carcinogen-injected mice, these authors found that a DHA-enriched diet induced the same effects observed *in vitro* on the EGFR signaling pathway and also inhibited tumor development. Similarly, we recently found [161] that DHA could dislocate from colon cancer cell surface the antiapoptotic Glucose Related Protein of 78 kDa (GRP78), abnormally expressed on cancer cells plasma membranes [162], where it acts as a signaling receptor promoting proliferation and survival. Through this mechanism, DHA inhibited the ERdj5/PERK/caspase-4 and caspase-7 pathway placed downwards and induced apoptosis [161].

A second main possible direct route for the omega-3 PUFA action is related to their high peroxidability that makes them optimal substrates for oxidants inside the cells [163]. Through this route, these fatty acids may induce alterations of the cell oxidative status and modulation of oxidative stress-dependent molecular pathways related to cell proliferation, apoptosis, or inflammation.

Moreover, a third main direct route for the omega-3 PUFA action is related to their possible metabolic conversion into bioactive molecules with powerful anti-inflammatory and proresolving action (i.e., resolvins, protectins, etc.). Receptors binding specifically to both these bioactive molecules (such as ChemR23, leukotriene B4 receptor 1, LTB4R1, and G protein coupled receptor, GRP32) [164] as well as to omega-3

PUFA (such as peroxisome proliferator activated receptor γ, PPARγ, and G protein coupled receptor, GPR120) have been recently identified [164, 165]. The signaling starting from these receptors may be translated downwards into the induction of specific cellular molecular pathways.

Finally, a very interesting possibility that may represent the field of future promising investigation in this research area is that omega-3 PUFA, through one or more of these main direct routes, may in turn affect DNA cytosine methylation, the covalent modifications of histones, or the expression of noncoding microRNA (miRNA). These effects may alter the epigenetic control of genes codifying proteins involved in cell proliferation and survival. It has been already hypothesized that alterations of epigenetic control of gene expression could be involved in the beneficial effects that omega-3 PUFA exert in immunological or neurodegenerative diseases [166–168] or in some kinds of cancer [157, 169–171].

5.2. Omega-3 PUFA in Colon Cancer. Dietary omega-3 PUFA have attracted considerable interest for their potential to prevent the development and progression of colorectal cancer (CRC) [8, 158, 172]. An impressive body of evidence has been obtained in preclinical studies using *in vivo* CRC models, consistently supporting the antineoplastic role of omega-3 PUFA [8, 154, 172], in spite of the high variability of the models and experimental conditions used [134, 135, 154, 173, 174]. Among all these data only very few were in conflict with a protective effect of the omega-3 PUFA [127, 175], but in these cases extremely high doses of LC-omega-3 PUFA were administered (about 3–7 g EPA + DHA/kg body weight in mouse or 12 g EPA + DHA/kg body weight in rat; for the calculation used, see [216]). These high doses may generate vast amounts of oxidized products with high prooxidant and carcinogenic activity [26].

Many *in vitro* studies [8, 154, 172] confirmed the antineoplastic activity of omega-3 PUFA and, as discussed in the previous section, allowed identification of possible biological and molecular mechanisms [154]. Remarkably, among the *in vitro* studies [176–178] are those that recently investigated the possible effects of omega-3 PUFA on colorectal cancer stem cells (CCSC). It is believed that CCSC may drive colon tumorigenesis, being principal targets of tumorigenic genetic alterations, due to their long lifespan and capacity for self-renewal. Moreover, they have also been related to cancer relapse, acquisition of chemotherapy resistance, and metastasis [179]. CCSC are characterized for lacking specific markers of colonic epithelium differentiation, such as cytokeratin 20 (CK20) or mucin 2 (MUC2), and expressing instead specific clusters of differentiation, such as CD133 or Lgr5 antigens (labeling undifferentiated cells) [180–182]. Moreover, most of cancer stem cells form spheres when cultured in serum-free conditions that are highly tumorigenic if injected in immunodeficient mice [176, 178]. Yang et al. [176] cultivated cancer stem-like cell spheres derived from SW620 colon cancer cell line and showed that both EPA and DHA (10–70 μM) inhibited their growth by inducing apoptosis and that the effect was markedly increased when they acted simultaneously. Moreover, EPA and DHA enhanced the efficacy of chemotherapeutics agents such as 5-fluorouracil (5-FU) and mitomycin C. Instead, de Carlo et al. [177] found that whereas 25 μM EPA inhibited the growth of the COLO 320 DM colon cancer cell line *in vitro*, it was unable to inhibit that of its CD133+ subpopulation. However, EPA upregulated CK20 and MUC2 and downregulated CD133 expression, thus indicating that it triggered the transition of CCSC to a more differentiated cancer cell phenotype. The authors hypothesized that the increased degree of CCSC differentiation induced by EPA could be strictly related to the EPA-induced sensitization of CD133+ cells to the anticancer agent 5-FU that they observed. Accordingly, Vasudevan et al. [178] found that EPA alone and especially in combination with 5-FU + oxaliplatin (FuOX) was effective in inhibiting cell growth and sphere formation, as well as in inducing apoptosis of (FuOX-) resistant HT-29 and HCT116 CRC, highly enriched in CCSC.

The confirmation in different experimental settings [176–178] that omega-3 PUFA can increase the sensitivity of colon CCSC to conventional antineoplastic drugs is very interesting, since, as reviewed by us and others previously [183–185], the most feasible potential anticancer application for these fatty acids in humans will be their possible use as adjuvants—to help increase nutrition status, as well as affort/modulate anticancer effects—in combination with conventional or single-targeted anticancer therapies.

If we turn to consider the interventional studies conducted on healthy volunteers, CRC patients, or subjects at high risk for CRC, the majority of them have concurred to demonstrate the beneficial antineoplastic effect of omega-3 PUFA (Tables 5(a) and 5(b)). The first studies were performed about twenty years ago by supplementing purified EPA + DHA or fish oil (FO). They demonstrated the efficacy of these FA in inhibiting abnormal CRC cell proliferation [83–85, 87], when given either in higher amounts (daily dose: 8-9 g EPA + DHA) for short (2 weeks) and longer periods (3–6 months) or in lower amounts (daily dose: 2.5–4 g EPA + DHA) after one month [85]. However, when the same treatment was performed in the presence of a basal high-fat diet and a low omega-3/omega-6 PUFA ratio of 0.25, no effect on rectal mucosa cell proliferation was observed [86], confirming that the efficacy of omega-3 PUFA is dependent on both the total lipid content and the omega-3/omega-6 PUFA ratio of the diet. Also Gee et al. [88] did not find any change in cell proliferation by administering 2.4 g/day EPA + DHA for 3 months, but in this case there is no available information on basal diet or dietary omega-3/omega-6 PUFA ratio. Instead, Cheng et al. [89], who treated patients at high risk for CRC with lower doses (as FO, about 500 mg EPA + DHA/day), but for a very long period (2 years), observed apoptosis induction in the mucosa adjacent to the resected CRC [89]. More recently, Courtney et al. [90] administered gastroresistant capsules containing EPA-free fatty acid (FFA, 2 g/day) and confirmed the inhibition of cell proliferation and induction of apoptosis in colonic mucosa of subjects at high risk for CRC cancer. The same treatment was recently shown [91] to reduce the number and size of rectal polyps in familial adenomatous polyposis (FAP) and to inhibit angiogenesis in patients carrying CRC liver metastases [92]. A large multicenter trial (seAFood Polyp Prevention Trial) [153] is now being

Table 5: Omega-3 PUFA antineoplastic effect in subjects at high risk for CRC or in CRC patients: completed interventional human studies.

(a)

Type of study	Enrolled subjects	Number of subjects	Omega-3 PUFA daily treatments	Control group daily treatment	Length of treatments	Antineoplastic effect observed in omega-3 PUFA-treated subjects	Reference
Randomized double-blind, placebo-controlled trial	High risk for CRC (sporadic adenomatous polyps)	10 (control group) 10 (omega-3 PUFA group)	OS: 4.1 g EPA-EE + 3.6 g DHA-EE	OS: 7 g olive oil	2 wk.–3 mo.	Inhibition of abnormal rectal mucosa cell proliferation	[83]
Double-blind crossover trial	Healthy volunteers	12 (control group) 12 (omega-3 PUFA group)*	OS: 11 g FO (providing 4.4 g omega-3 PUFA); omega-3/omega-6 ratio in the basal diet: 0.40	11 g corn oil	2–4 wk.	Inhibition of rectal mucosa cell proliferation; inhibition of PGE2 release by rectal biopsies	[84]
Randomized double-blind, placebo-controlled trial	High risk for CRC (sporadic adenomatous polyps)	10 (control group) 10 (omega-3 PUFA group)	OS: 2.5 g EPA-EE + DHA-EE	2.5 g olive oil	1–6 mo.	Inhibition of abnormal rectal mucosa cell proliferation (in subjects with abnormal baseline proliferation pattern)	[85]
Double-blind crossover trial	Healthy volunteers	12 (control group) 12 (omega-3 PUFA group)*	OS: 11 g FO (providing 4.4 g omega-3 PUFA); omega-3/omega-6 ratio in the basal high-fat diet: 0.25	11 g corn oil	2–4 wk.	No effect on inhibition of rectal mucosa cell proliferation	[86]
Randomized double-blind, placebo-controlled trial	Patients with resected CRC or severely dysplastic polyps	10 (control group) 17 (omega-3 PUFA group)	OS: 9 g omega-3 PUFA-EE concentrate (providing about 4.0 g EPA-EE and 2.2 g DHA-EE)	9 g omega-6 PUFA-EE concentrate (providing about 4.5 g LA-EE)	6 mo.	Inverse association between colon omega-3/omega-6 PUFA and cell proliferation (in subjects with abnormal baseline proliferation pattern); suppression of polyp development	[87]

CRC: colorectal cancer; EE: ethyl ester; FAP: familial adenomatous polyposis; FFA: free fatty acid; FO: fish oil; LA: linoleic acid; OS: oral supplementation; TG: triglycerides.
*The same subjects were treated with FO or CO in different periods.

(b)

Type of study	Enrolled subjects	Number of subjects	Omega-3 PUFA daily OS treatments	Control group daily OS treatment	Length of treatments	Antineoplastic effect observed in omega-3 PUFA-treated subjects	Reference
Randomized double-blind, placebo-controlled trial	Patients undergoing surgery for CRC	24 (control group) 25 (omega-3 PUFA group)	2 g FO (providing 1.4 EPA + 1.0 DHA g n-3 PUFA)	2 g safflower oil	12 days prior to surgery	No effect on frequency and spatial distribution of crypt cell mitosis	[88]
Single-blind (investigators) trial	Patients polypectomized for CR adenomas/tumors	20 (control group) 21 (omega-3 PUFA group)	FO (providing 0.1 g EPA + 0.4 g DHA) and DA*	No treatment; DA: reduction of fat consumption	2 years	Increased apoptosis in normal sigmoid colon mucosa	[89]

(b) Continued.

Type of study	Enrolled subjects	Number of subjects	Omega-3 PUFA daily OS treatments	Control group daily OS treatment	Length of treatments	Antineoplastic effect observed in omega-3 PUFA-treated subjects	Reference
Randomized single-blind (investigators) trial	Patients with one or more CR adenomas	15 (control group) 15 (omega-3 PUFA group)	2 g enteric coated EPA-FFA formulation	No treatment; DA: no fish intake	3 mo.	Reduced cell proliferation and increased apoptosis in normal colon mucosa crypts	[90]
Phase III randomised, double-blind, placebo-controlled trial	FAP patients	27 (control group) 28 (omega-3 PUFA group)	2 g enteric coated EPA-FFA formulation	2 g capric and caprylic acid medium-chain TG	6 mo.	Reduced polyp number and diameter	[91]
Phase II double-blind, randomised, placebo-controlled trial	Patients carrying CRC liver metastases	35 (control group) 36 (omega-3 PUFA group)	2 g enteric coated EPA-FFA formulation	2 g capric and caprylic acid medium-chain TG	12–65 days (median 30 days) prior to surgery	Reduced vascularity of CRC liver metastases	[92]

CRC: colorectal cancer; DA: dietary advice; FAP: familial adenomatous polyposis; FFA: free fatty acid; FO: fish oil; OS: oral supplementation; TG: triglycerides.
*DA: reduction of fat intake and increase in omega-3 PUFA/omega-6 PUFA dietary ratio.

conducted, supplementing the EPA-FFA formulation alone or in combination with aspirin to patients at high risk for developing colorectal adenomas. It would be interesting also to study the effect of DHA in the same formulation, since antineoplastic activities have also been reported for both fatty acids [155]. Moreover, even though EPA can be metabolically converted into DHA and DHA retroconverted into EPA, the *in vivo* efficiency of these two metabolic reactions may be substantially different [186].

The outcomes of the recent large multicentric interventional FISHGASTRO study [93, 94] showed that an extra 300 g/week fish serving for 6 months lowered significantly the serum C-Reactive Protein (CRP) concentration in all the subjects, but it did not alter the levels of several inflammation markers in fecal water and colonic biopsies or change the apoptotic rates in colonic mucosa. However, the mitotic rate decreased in the fish groups, even though the significance was not attained [93]. Still, it is possible to hypothesize that the small extra supplementation on top of an already large amount of fish consumed at baseline (corresponding to about 0.09 or 1.4 g EPA + DHA/day) was not sufficient to induce significant alterations in mucosal cell growth and survival. The reduction in serum CRP levels was confirmed in a recent trial conducted by Mocellin et al. [95] in CRC patients treated daily with FO containing 0.6 g EPA + DHA, supporting a general anti-inflammatory effect of omega-3 PUFA supplementation.

After more than twenty years of intense research, several meta-analyses and systematic reviews of the literature have been recently published on this topic. If we take into account only the most recent ones [100–102], their conclusions appear not to be in agreement. Gerber [102] updated the observations of the previous meta-analysis conducted by the FAO/OMS experts [101] to April 2011 and prudently concluded that overall, owing primarily to insufficient homogeneity of the observations, the studies analyzed provided only limited evidence suggesting a role for omega-3 PUFA in CRC prevention [102]. Wu et al. [100] considered in their meta-analysis 22 prospective cohort and 19 case-control studies performed until 2011. They more decidedly concluded that fish consumption was inversely associated with CRC. In particular, they found a significant inverse association between fish intake and rectal cancer and only a modest trend between fish consumption and colon cancer.

Since then, several other case-control and cohort studies were published. In their case-control studies, Murff et al. [96] and Habermann et al. [99] demonstrated an inverse association between an increased omega-3 PUFA dietary intake and the risk of developing colorectal neoplasms (adenocarcinomas or adenomas) [96, 99]. However, benefits were limited only to some of the subpopulations investigated. In particular, Murff et al. [96] showed that an increased omega-3 PUFA intake was associated with reduced risk of colorectal adenomas in women only, whereas Habermann et al. [99] found that an inverse association between low DHA intake and increase in CRC risk was confined to patients showing specific genetic variants that resulted in higher levels of proinflammatory mediators.

Recent cohort studies found associations only in subcohorts. Kantor et al. [97] found an inverse association between FO use and cancer risk, but only at the level of the colon (not rectum) and only in men (not in women). Moreover, the same authors [97] found that fish consumption or EPA/DHA intake was not associated with CRC risk overall but that associations were significantly modified by genetic risk, with inverse associations among low-moderate genetic risk groups and positive associations among high risk groups. The cohort study of Song et al. [98] found no association between fish/omega-3 PUFA intake and overall CRC incidence. In contrast with the Kantor et al. study [97], Song et al. [98] found an inverse association between a high LC-omega-3 PUFA consumption and rectal cancer (but not colon cancer) and even a positive association between LC-omega-3 PUFA increased intake and distal colon cancer.

The controversial findings in epidemiological studies now need clarification in the setting of large randomized clinical intervention trials, such as the one currently ongoing [153]. The current state of evidence for the effects of omega-3 PUFA in human studies regarding colorectal cancer and the contrasting outcomes have been summarized in Table 6. Possible reasons for the controversies in the field are reported in Section 6.

5.3. Omega-3 PUFA in Prostate Cancer. Recently, Brasky et al. [25] suggested a potential prostate cancer promoting effect of omega-3 PUFA, leading to a new round of discussions on the effects of omega-3 PUFA in prostate cancer [26, 187–195]. Even though many researchers hold the view that omega-3 PUFA intake is beneficial in this cancer type, the association between omega-3 PUFA and prostate cancer risk is not without controversy. On one hand, a case-control study with 3141 participants in Canada provided an inverse association between fish consumption and prostate cancer incidence and subsequently suggested that fish consumption may reduce the risk of developing prostate cancer [103]. Accordingly, a study focusing on aggressive prostate cancer also found that a high intake of omega-3 PUFA was strongly associated with a decreased risk of aggressive prostate cancer, and the odd ratio (OR) for prostate cancer comparing the highest with the lowest quartile of omega-3 intake was 0.37 (0.25–0.54) [106]. Moreover, Szymanski's meta-analysis showed an association between fish consumption and a significant 63% reduction in prostate cancer-specific mortality [116]. Chavarro et al. found that, compared to prostate cancer patients who consume fish less than once a week, patients who consume at least 5 times more fish per week had a 48% lower risk of prostate cancer death [108]. Torfadottir et al.'s study [110] on Icelanders found a negative correlation between risk of advanced prostate cancer and FO consumption in later life. Due to daily intake of omega-6 PUFA from the diet, the role of the omega-6/omega-3 PUFA ratio has also been considered. Williams et al. [104] found that the ratio of dietary omega-6/omega-3 PUFA was significantly associated with increased risk of high-grade, but not low-grade prostate cancer. Moreover, it has also been found [196, 197] that omega-3 PUFA or a low ratio of omega-6 to omega-3 PUFA could decrease the proliferation of prostate cancer cells in cell culture experiments. Based upon these

Table 6: Current state of evidence for the effects of omega-3 PUFA in published human studies regarding colorectal cancer.

Type of study	Beneficial effect	Beneficial effect limited to subpopulation	Detrimental effect limited to subpopulations	No effect
Clinical trials	Antiproliferative effect [83–85, 87, 90] Proapoptotic effect [89, 90] Reduced polyp number and size in FAP [91] Reduced angiogenesis [92] Reduced CRP levels in serum [93–95]			No antiproliferative effect [86, 88, 93, 94] No proapoptotic effect [93, 94] No anti-inflammatory effect in colony biopsies [93, 94]
Observational studies		Inverse association between increased dietary intake and risk of CR adenomas (only in women) [96] Inverse association between FO use and cancer risk (in men, not in women; in colon, not in rectum) [97] Inverse associations between FO use and CRC in low-moderate genetic risk groups, and positive associations among high risk groups [97] Inverse association between increased intake and RC (but not CC) [98] Inverse association between increased dietary intake and risk of CRC (only in specific genetic variants) [99]	Positive associations between FO use and CRC in high risk groups [97] Positive association between increased intake and distal CC [98]	
Meta-analyses	Significant inverse association between fish consumption and RC [100]			Limited evidence of a role in CRC prevention [101, 102]

CC: colon cancer; CRC: colorectal cancer; CRP: C-Reactive Protein; FAP: familial adenomatous polyposis; FO: fish oil; RC: rectal cancer.

association observations and considerable amounts of *in vivo* and *in vitro* experimental evidence [198, 199], several mechanistic hypotheses have been proposed to explain these phenomena. In particular, as already described in detail in Section 5.1, PUFA can be converted into many bioactive metabolites, which play various roles in modulating cell proliferation, apoptosis, and immune regulation. Many of the metabolites derived from omega-6 arachidonic acid (AA), including prostaglandin E2 and leukotriene B4, were shown to stimulate prostate cancer [200, 201].

On the other hand, several other studies did not provide evidence of a protective role of omega-3 against prostate cancer. Kristal et al.'s study [107] suggested that there was no association between omega-3 PUFA intake and prostate cancer risk. Brouwer's meta-analysis showed that the status of EPA or DHA did not associate with prostate cancer risk [117]. Park et al. [109] found no significant association between erythrocyte membrane omega-3 PUFA (EPA, DPA, or DHA) and total or advanced/high-grade prostate cancer risk. In Mannisto's study among male smokers [111], no association between serum EPA or DHA and prostate cancer risk was found. Harvei et al. [115] could not identify a clear association between prostate cancer risk and total serum phospholipid (PL) omega-3 PUFA either. A study based on the 2003–2010 National Health and Nutrition Examination Survey (NHANES) [114] found no association between omega-3 PUFA intake and prostate-specific antigen (PSA) level. Chua et al. [112] found that a high blood docosapentaenoic acid (DPA) level was negatively associated with total risk of prostate cancer. In contrast, a significant positive association between EPA or DHA and high-grade prostate cancer was shown [112]. Similarly, Sorongon-Legaspi et al.'s study [113] showed that DPA blood level was negatively linked with total prostate cancer risk and high EPA and DHA blood levels were associated with high-grade prostate cancer risk. Crowe et al. [105] described a positive association between EPA and risk of only high-grade prostate cancer, while DHA could not be linked to risk of any stages of prostate cancer.

Table 7: Current state of evidence for the effects of omega-3 PUFA in published human studies regarding prostate cancer.

Type of study	Beneficial effect	Beneficial effect limited to subpopulations	Detrimental effect limited to subpopulations	No or detrimental effects
Observational studies	Inverse association between fish consumption and cancer incidence [103]	Dietary omega-3/omega-6 PUFA ratio inversely associated with risk of high-grade cancer [104]	Positive association between EPA and risk of only high-grade cancer [105]	Dietary omega-3/omega-6 PUFA ratio not associated with risk of low-grade cancer [104]
	Inverse association between high intake and risk of aggressive cancer [106]		Positive association between high serum PL LC-omega-3 PUFA and cancer risk [25]	No association between fish or FO intake and cancer risk [107]
	Inverse association between higher fish intake and risk of cancer death [108]			No association between erythrocyte membrane EPA, DPA, or DHA and total or advanced/high-grade cancer risk [109]
	Inverse association between FO intake and risk of advanced cancer later life [110]			No association between serum EPA or DHA and cancer risk in male smokers [111]
	Inverse association between DPA level and total risk of cancer [112, 113]			No association between omega-3 PUFA intake and PSA level [114]
				Positive association between EPA or DHA and high-grade cancer [112, 113]
				No association between DHA level and risk of cancer at any stages [105]
				No association between total serum PL omega-3 PUFA and cancer risk [115]
Meta-analyses	Inverse association between fish intake and prostate cancer-specific mortality [116]			No association between FO intake or EPA/DHA blood level and cancer risk [117]

FO: fish oil; PL: phospholipids; PSA: prostate-specific antigen.

As mentioned above, in their recently published case-control substudy from the SELECT study population Brasky et al. [25] reported a positive association between high plasma phospholipid omega-3 PUFA content at baseline and prostate cancer. However, we should underline that the SELECT trial was not addressing omega-3 PUFA supplementation for the prevention of prostate cancer. The authors used data from two case-control studies [25, 202], nested within intervention trials which were unrelated to eating fish or taking omega-3 supplements. In these studies, the dietary intake of fish or omega-3 had not been analyzed, neither advice was given to eat fish or take fish oil supplement [194]. Moreover, omega-3 PUFA were measured only once at baseline and only in plasma phospholipids [27] and not in red blood cell membranes. In fact, it has been suggested that evaluating the "omega-3 index," that is, the incorporation of omega-3 PUFA in erythrocytes, may better reflect the omega-3 PUFA status of the subjects [77, 78].

The current state of evidence for the effects of omega-3 PUFA in human studies regarding prostate cancer and the contrasting outcomes have been summarized in Table 7, and additional possible reasons for the controversies in the field are reported in Section 6.

Overall, we can conclude that the literature on the role of omega-3 PUFA in prostate cancer is remarkably consistent among cell culture and animal studies, and controversies came mostly from association studies in human populations. However, the preventive effect of omega-3 PUFA on prostate cancer is still uncertain, and the controversy will probably remain until the mechanisms are clarified. However, while current human data are certainly not sufficient to recommend omega-3 PUFA to prevent prostate cancer, they are also far

from being sufficient to support a prostate cancer promoting effect of these FA [188, 190, 191, 193, 203, 204].

6. Possible Reasons for Controversies

Several reasons may be responsible for the discrepancies registered among the outcomes of human studies. First of all, it should be considered that even though omega-3 PUFA can be regarded as medication and high-dose preparations are approved as prescription drugs in many parts of the world, omega-3 PUFA are essential part of our diet. Their intake can vary widely from population to population and even within the same person, depending on actual dietary habits or short-term dietary changes (eating seafood during vacations on the coast). Often, the epidemiological observational studies, particularly the early studies, refer to Food Frequency Questionnaire and Diet Records to establish fish consumption. However, this methodology is prone to measurement errors [205], and a low correlation coefficient (0.11–0.18) has been shown to exist between fish intake frequency and direct measurements of FA in patient samples [205, 206], even after adjusting for confounding factors, such as age [207, 208]. A number of other variables may contribute to this scarce correlation, such as the individual capacity to adsorb and make the PUFA bioavailable at the serum level, the fat content of the diet, or the omega-3/omega-6 PUFA dietary ratio. Moreover, different methods are used to calculate dietary fats or specific FA in the diets [209]. Often the questionnaires refer only to fish servings per week, without considering the serving size or the kind of fish consumed (lean fish or fatty fish) [207]. It is known that there exists an extreme variability in fish intake among populations, and if we consider the amount of fish ingested by the "low fish consumers" among the populations at high fish intake, it may correspond to or be even higher than the amount consumed by the "high fish consumers" in the populations at low fish intake. As a matter of fact, positive association between dietary intake of omega-3 PUFA and health effects has been registered mainly among population eating fish at high levels [15]. Some intrinsic weaknesses of questionnaire studies could thus be addressed by actually measuring omega-3 PUFA in the participants. Some more recent studies have directly evaluated the levels of omega-3 PUFA in serum, in different plasmatic lipid classes, or in erythrocytes [210–212]. Whereas it has been argued that measuring the omega-3 PUFA level only in one class of plasma lipids may be misleading [27], it has been suggested that erythrocyte EPA and DHA content (named "omega-3 index") may better reflect the omega-3 PUFA status of the subjects [77, 78]. This is because the plasma-based measurements merely represent the short-term availability of omega-3 PUFA, being susceptible to artificial elevation following an acute omega-3 PUFA load [213]. Indeed, it has been recently reported that an acute single dose (3.4–4 g/d) of omega-3 PUFA, in the form of either prescription medication or dietary supplements, peaked plasma EPA + DHA levels as early as 5 h after administration, whereas the EPA plus DHA concentrations in erythrocyte membranes were only increased by approximately 10 orders of magnitude less than the concurrent plasma (3% versus 30%) from baseline at 24 h [214, 215]. Therefore, even if a single evaluation may be confounding, since serum FA are powerfully affected by feeding/fasting cycles and by the lipid content of the last meal consumed [27], one possibility of overcoming the discrepancies in omega-3 PUFA research is to call for actual correlation of questionnaire-based calculations of omega-3 PUFA intake with at least one actual measurement per participant in order to obtain some biochemical information regarding actual omega-3 PUFA levels.

Moreover, we ask if intervention studies administering defined amounts of omega-3 PUFA may solve this problem. At least these studies exactly define the amount of omega-3 PUFA administered. A rather big problem with these studies is the big range of doses of omega-3 PUFA administered on one hand and the variability of baseline diets on the other hand. In order to understand discrepancies and problems of omega-3 PUFA efficacy in intervention trials, we will have to regard the omega-3 PUFA as parts of diet and component of normal human body homeostasis. This is a concept obviously radically different from synthetic drug studies and something that has always to be taken into account when assessing intervention studies. In such studies, the animals/subjects have been treated with specific and controlled amounts of omega-3 PUFA. But most of these studies neither established baseline omega-3 PUFA body content in the participants nor monitored omega-3 PUFA during the study, making proper interpretation of the obtained results difficult. A recent study properly addressing these issues was the Welcome Trial in NASH patients, in which interpretation of the data focused on the correlation of measured DHA enrichment with liver fat content, as well as on pure ITT analysis of the treatment group. It demonstrated a statistically significant linear correlation between decreased liver fat content and increased DHA enrichment, but no significant effect in the ITT treatment group analysis [64]. Therefore, as in observational studies, intervention trials should perform biochemical analyses of omega-3 PUFA content to (1) define baseline levels in the different groups and (2) determine actual changes in the different groups due to the treatment as compared to the placebo group, where some of the participants might actually contaminate the results by starting to consume a high fish diet during the trial period.

Omega-3 PUFA dose is an issue as well. The omega-3 PUFA dose was very high in earlier animal studies, ranging from 2.0 to 8.0 (EPA + DHA g/kg body weight/day in mice) in most cancer studies [216]. But even the lower doses used for animals, after the interspecies conversion [217], appear rather high for humans (about 10–20 g/day in a person weighing 70 kg). However, it is remarkable that, at least in the few human interventional trials completed in the cancer field, the preventive effects were noticed with doses of only 2.0 g/day (corresponding to about 0.03 g/kg body weight in a person weighing 70 kg) [85, 91, 218, 219]. The positive anticancer effects were accompanied (if evaluated) by a comparable increase (ranging from 2-3- to 5-fold) in omega-3 PUFA incorporation in plasma or tissue lipids, in both animals and humans [216].

The source of the omega-3 PUFA ingested may also be important. Interventional studies often use highly purified

omega-3 PUFA. They may help to obtain more clear results than the use of fish/FO, since fish also represents a good source of selenium, various vitamins, taurine, and other compounds that have been shown to possess potential protective effects [220–222]. Moreover, high levels of contaminant and dangerous agents may accumulate in fish tissues and fat. Some of these agents (such as organochlorine pesticides or Hg) are environment-derived and may be present in the tissues of both farmed and wild fish (especially predator fish). Moreover, other cytotoxic and carcinogenic products of omega-3 PUFA may be formed in fish after cooking them at extremely high temperature (by broiling/frying fish) or preserving them by smoking procedures [26]. The omega-3 PUFA oxidative products may be formed in omega-3 fortified food and in omega-3 supplements that do not contain adequate levels of antioxidants and are stored at room temperature for a long time [26]. Furthermore, the general composition of the diet has to be taken into account, since the effect of increased omega-3 PUFA uptake might be different in the context of a high-carbohydrate diet as compared to a high-fat diet. This might change absorption and processing of the omega-3 PUFA and thus affect actual concentrations in the body. In addition, the highly critical factor omega-6 PUFA and the actual omega-6/omega-3 PUFA dietary ratio may deeply alter the effects of omega-3 PUFA supplementation.

Considerable attention has been drawn recently to the possibility that interactions may exist between the subject genetic background and the effects of nutritional interventions [223, 224]. Different observational studies have already demonstrated that the inverse association between omega-3 PUFA intake or plasma/tissue enrichment and the risk of several diseases may be limited only to subpopulations possessing specific genetic features/signatures [99, 106, 225–230]. Genetic differences or gender-specific effects [97] might be due to differences in the expression of effector proteins for the omega-3 PUFA and their lipid mediator products, but also due to differences in enzymes of lipid metabolism that might lead to differences in the processing of the omega-3 PUFA in the body. Both issues, assessing the source of omega-3 PUFA and dietary composition as well as genetic background affecting handling of the omega-3 PUFA, also call for more measurements to better understand controversial results.

7. Conclusions

Our review of the omega-3 PUFA research literature prompts us to conclude that the animal and *in vitro* data have been remarkably consistent in showing health benefits, particularly through mechanisms dampening inflammation and proliferation in different tissues. These results have established potential protective effects of omega-3 PUFA in diseases ranging from cardiac arrhythmia and inflammatory conditions (atherosclerosis to airway inflammation, colitis, pancreatitis, steatohepatitis, arthritis, etc.) to tumorigenesis (particularly colon and liver tumors, but also breast and prostate cancer). On the other hand, the outcomes of human studies have been so far quite controversial. Therefore, in order to shed more light on this point and better understand if the divergences were ascribable to possible methodological weaknesses in human studies or, alternatively, to different responses of human cells/tissues to the incorporation of omega-3 as compared to those of laboratory animal species, it is suggested that all future studies in this field should perform blood FA measurements in trial participants. These evaluations should be preferably performed in blood cells, and, whenever possible, at baseline as well as at different time-points during the study. This procedure would also be extremely helpful to (1) monitor the effect of the huge omega-3 PUFA supplement industry in the western world on omega-3 PUFA content in humans, (2) understand the effect of other FA in the context of omega-3 PUFA interventions, and (3) recognize variations in individual responses to omega-3 PUFA supplementation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Karsten H. Weylandt and Simona Serini contributed equally to this work.

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Research Article

The Omega-3 Fatty Acid Docosahexaenoic Acid Modulates Inflammatory Mediator Release in Human Alveolar Cells Exposed to Bronchoalveolar Lavage Fluid of ARDS Patients

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Background. This study investigated whether the 1 : 2 ω-3/ω-6 ratio may reduce proinflammatory response in human alveolar cells (A549) exposed to an *ex vivo* inflammatory stimulus (bronchoalveolar lavage fluid (BALF) of acute respiratory distress syndrome (ARDS) patients). *Methods*. We exposed A549 cells to the BALF collected from 12 ARDS patients. After 18 hours, fatty acids (FA) were added as docosahexaenoic acid (DHA, ω-3) and arachidonic acid (AA, ω-6) in two ratios (1 : 2 or 1 : 7). 24 hours later, in culture supernatants were evaluated cytokines (TNF-α, IL-6, IL-8, and IL-10) and prostaglandins (PGE_2 and PGE_3) release. The FA percentage content in A549 membrane phospholipids, content of COX-2, level of PPARγ, and NF-κB binding activity were determined. *Results*. The 1 : 2 DHA/AA ratio reversed the baseline predominance of ω-6 over ω-3 in the cell membranes ($P < 0.001$). The proinflammatory cytokine release was reduced by the 1 : 2 ratio ($P < 0.01$ to <0.001) but was increased by the 1 : 7 ratio ($P < 0.01$). The 1 : 2 ratio reduced COX-2 and PGE_2 ($P < 0.001$) as well as NF-κB translocation into the nucleus ($P < 0.01$), while it increased activation of PPARγ and IL-10 release ($P < 0.001$). *Conclusion*. This study demonstrated that shifting the FA supply from ω-6 to ω-3 decreased proinflammatory mediator release in human alveolar cells exposed to BALF of ARDS patients.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a form of acute diffuse lung injury associated with a predisposing risk factor, characterized by inflammation leading to increased pulmonary vascular permeability and loss of aerated lung tissue [1]. According to the Berlin definition, the acute phase of this syndrome is manifested by the early onset of respiratory failure (within 1 week of a known clinical insult or new/worsening respiratory symptoms) [2]. The main characteristic of the clinical syndrome is hypoxemia; specifically, each subcategory of ARDS (mild, moderate, and severe) is defined by mutually exclusive ranges of the ratio between partial pressure of arterial oxygen (PaO_2) and fraction of inspired oxygen (FiO_2) (200 mm Hg $< PaO_2/FIO_2 \leq$ 300 mm Hg, 100 mm Hg $< PaO_2/FIO_2 \leq$ 200 mm Hg, and $PaO_2/FIO_2 \leq$ 100 mm Hg, resp.) [2]. Mortality from severe ARDS in the 1970s was as high as 85–90% but, from 2000, it decreased to 20–40% [3].

In the ARDS early phase, the alveolar space is characterized by alveolar infiltration with neutrophils and macrophages, and both are able to release inflammatory cytokines with an accumulation of both proinflammatory and anti-inflammatory cytokines [4, 5]. Many cytokines were detected at elevated levels in bronchoalveolar lavage fluid (BALF) in patients with ARDS, that is, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8. The key role of a hyperinflammatory response, mainly characterized by the overproduction of proinflammatory cytokines, in the progression of the lung damage is well-documented [6, 7].

Moreover, many studies reported an increased mortality in patients who have elevated proinflammatory cytokine concentrations (TNF-α, IL-6, and IL-8) in the BALF at the onset of ARDS or persistent increased concentrations [8, 9]. Similarly, low levels of anti-inflammatory cytokines (i.e., IL-10 and IL-1 receptor antagonist) in the BALF in the ARDS early phase are associated with an increased mortality [10, 11]. Thus, the balance between proinflammatory and anti-inflammatory cytokines is of greater importance because the degree of cytokine imbalance is a contributing element to ARDS severity [12].

Over the last years, there has been an improving understanding of polyunsaturated fatty acid (PUFA) pathophysiology and several mechanisms for the interaction between PUFAs and inflammation or immune response have been demonstrated [13–16]. Indeed, after PUFA supply (diet or enteral and parenteral administration), many cell properties and related functions are modified, mainly the inflammatory and immunity responses [13]. Briefly, omega- (ω-) 3 PUFAs are more regarded as anti-inflammatory agents, whereas ω-6 PUFAs are regarded as proinflammatory agents. Recently, it was speculated that ω-3 PUFAs may be involved in the resolution of inflammation [14].

The discovered ability of ω-3 PUFAs to downregulate several different responses of the inflammatory process had suggested that these PUFAs might be used not exclusively as nutrients but mainly as pharmacological agents. Since the 1990s, many editorials have stressed the possibility to modulate the inflammatory response in acute lung injury (ALI) or ARDS patients using ω-3 PUFAs as drugs (the so-called "pharmaconutrition") [17–19]. Subsequently, a great number of studies with cell or animal models have been carried out with the aim to demonstrate the efficacy of fish oil or their main active components (i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in modifying the inflammatory responses [20–22]. Likewise, there have been several randomized controlled trials of enteral [23–28] or parenteral [5, 29–31] administration of fish oil-enriched nutrition formulas in mechanically ventilated patients with ALI, ARDS, or sepsis.

In a previous study [32], we demonstrated that shifting the PUFA supply from ω-6 PUFA (i.e., arachidonic acid (AA)) to ω-3 PUFA (i.e., DHA) significantly reduced the release of proinflammatory cytokines in human alveolar cells undergoing lipopolysaccharide (LPS) challenge. Moreover, we found that, in the presence of a DHA/AA ratio with a predominance of AA, there was a cytokine balance more oriented towards a proinflammatory response than with LPS alone. The aim of this study was to investigate whether in human alveolar cells the 1 : 2 DHA/AA ratio was effective in reducing proinflammatory response induced by an *ex vivo* inflammatory stimulus such as BALF of ARDS patients.

2. Materials and Methods

2.1. Bronchoalveolar Lavage Fluid Collection. The BALF was collected from ARDS patients—requiring mechanical ventilation and BAL for clinical purposes—within 24 hours after endotracheal intubation and stored at −80°C as previously described [33]. The selection of ARDS patients was carried out as previously described [34]. The institutional review board approved the study.

2.2. Cell Culture and Treatment. The human lung carcinoma cell line A549 (ATCC, Rockville, MD, USA) was used. A549 are alveolar epithelial cells with type II pneumocyte properties. The A549 were cultured in HAM-F12 K medium (Sigma-Aldrich, St Louis, MO, USA) and treated as previously described [35].

BALF was added 24 hours after seeding, while LPS (400 μg/mL) was used as positive control. After 18 hours, A549 cells were treated with 50 μM of 1 : 2 DHA/AA ratio (DHA 17 μM plus AA 33 μM) or 50 μM of 1 : 7 DHA/AA ratio (DHA 6.5 μM plus AA 43.5 μM) for 24 hours. PUFAs and LPS from *Escherichia coli* 055:B5 were obtained from Sigma-Aldrich.

2.3. ELISA. At the experimental end point (i.e., after 24 hours), cytokine contents were measured in BALF (i.e., IL-1β, TNF-α, IL-6, and IL-8) and in culture supernatants (i.e., TNF-α, IL-6, IL-8, and IL-10) via enzyme-linked immunosorbent assay (ELISA). Cytokine kits were purchased from Euroclone (Paignton-Devon, UK). Prostaglandins E_2 and E_3 (PGE_2 and PGE_3) release was measured in culture supernatants via ELISA. Prostaglandin kits were purchased from MyBioSource (San Diego, CA, USA). The assays were performed according to the manufacturer's instructions.

2.4. Fatty Acid Percentage Content in Membrane Phospholipids. At the experimental end point, the fatty acid (FA) percentage content was determined as previously described [36]. Briefly, total lipids were extracted by the method of Folch et al. [37] and separated by thin-layer chromatography using n-heptane : isopropylether : formic acid (90 : 60 : 3) as solvent. Phospholipids bands (deposition line) were scraped, extracted, and used for FA determination. FA methyl esters were prepared following the method of Metcalfe et al. [38] and separated by gas-liquid chromatography (CP 9002 Chrompack International B.V., Middelburg, Netherlands). Internal standard (methyl heptadecanoate) was added to each preparation to determine recovery.

2.5. Preparation of Total Cell Lysate for Western Blot Analysis. Total cells lysates were obtained as previously described [35]. Briefly, cells were sonicated in HCMF buffer containing 1% Triton, 0.1% SDS, 2 mM Calcium Chloride ($CaCl_2$), 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), and 1 μg/mL leupeptin at an intermediate setting (output ≅ 3) using a Branson Sonifier 250 (VWR Scientific, OH, USA). Lysates were cooled on ice for 3–5 min and the sonicating–cooling cycle was repeated for a total of 3 cycles.

2.6. Preparation of Cytoplasmic and Nuclear Extracts for Western Blot Analysis. At the experimental end point, culture media were collected and stored at −80°C until cytokine concentration evaluation with ELISA and cells were lysed for cytoplasmic and nuclear protein fractions extraction. Briefly, cells were lysed into buffer A (10 mM HEPES pH 7.9, 10 mM

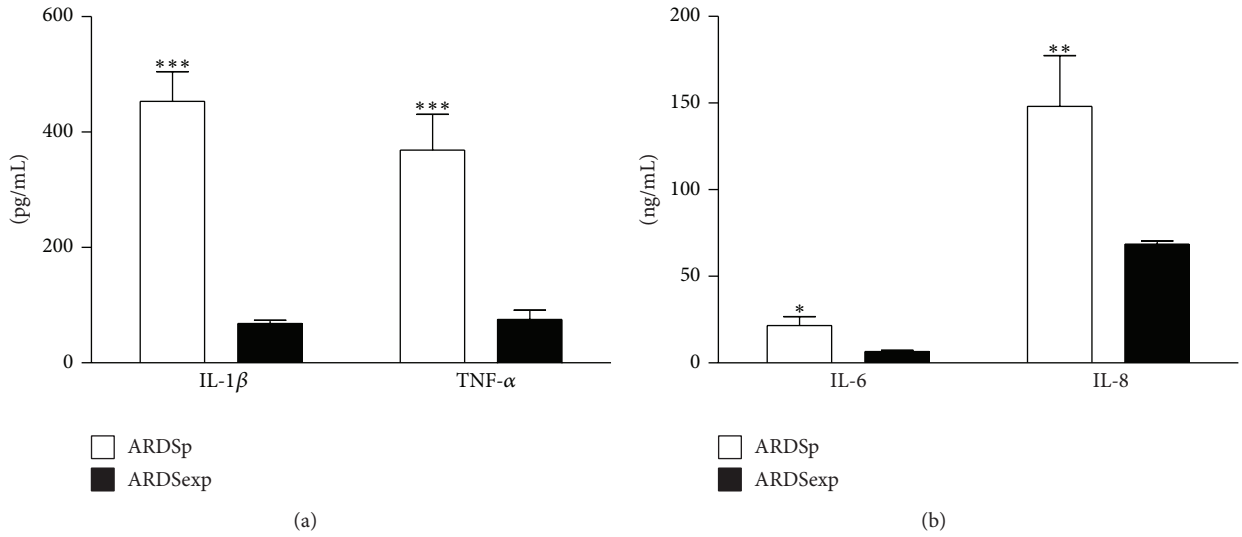

Figure 1: BALF cytokine pattern in ARDS. (a) IL-1β and TNF-α proinflammatory cytokine content in BALF collected from pulmonary (ARDSp, white bars) and extrapulmonary (ARDSexp, black bars) ARDS patients. (b) IL-6 and IL-8 cytokine content in BALF collected from pulmonary (ARDSp, white bars) and extrapulmonary (ARDSexp, black bars) ARDS patients. The results are expressed as picograms (pg) or nanograms (ng) of cytokines per mL as indicated. Data are presented as mean ± standard deviation of 12 independent determinations ($n = 7$ ARDSp and $n = 5$ ARDSexp). BALF, bronchoalveolar lavage fluid; ARDS, acute respiratory distress syndrome; IL, interleukin; TNF, tumor necrosis factor. $^{*}P < 0.05$ ARDSp versus ARDSexp. $^{**}P < 0.01$ ARDSp versus ARDSexp. $^{***}P < 0.001$ ARDSp versus ARDSexp.

KCl, 0.1 mM EDTA) added with 1 mM DTT, 0.5 mM PMSF, 5 μL of 10 μg/μL of aprotinin, leupeptin, and pepstatin A and 40 μL/mL of IGEPAL 10%. Cell lysates were centrifuged with a Microfuge at 15,000 rpm × 3 min at 4°C. Supernatants cytoplasmic fractions were collected and stored at −80°C until use. Nuclei pellets were lysated, for 2 h at 4°C, in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, and 10% glycerol) added with 1 mM DTT, 0.5 mM PMSF, 5 μL of 10 μg/μL of aprotinin, leupeptin, and pepstatin A. Nuclei lysates were centrifuged with a Microfuge at 15,000 rpm × 5 min at 4°C. Nuclei fractions were collected and stored at −80°C until use. HEPES, KCl, NaCl, EDTA, glycerol, DTT, PMSF, aprotinin, leupeptin, pepstatin A, and IGEPAL were purchased from Sigma-Aldrich.

2.7. Western Blot Analysis. Protein concentrations in the cell, nuclei, and cytoplasmic extracts were measured using the Protein Assay Kit 2 according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Thirty micrograms of proteins from the cell or nuclei fractions were separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gel. Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, MA, USA). The membranes were blocked overnight using 5% nonfat milk in 50 mM Tris/150 mM HCl (pH 7.5) containing 0.1% Tween 20 (TBS/Tween). After three 5-min rinses with TBS/Tween, membranes were probed with polyclonal anti-PPARγ, anti-COX-2, anti-p65 NF-κB, or anti-IκBα (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature. After three 5-min rinses with TBS/Tween, horseradish peroxidase- (HRP-) conjugated secondary antibodies goat anti-rabbit or goat anti-mouse IgG (Santa Cruz Biotechnology) were applied for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection system (Immun-Star HRP; Bio-Rad Laboratories, Hercules, CA, USA). To normalize protein signals, stripped PVDF membranes were reprobed with monoclonal anti-β-actin (Sigma-Aldrich) for cytosolic and cell fractions or with polyclonal anti-lamin A/C (Santa Cruz Biotechnology).

2.8. Statistical Analysis. Data were expressed as mean ± standard deviation. Multiple comparisons were carried out using one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test. SPSS statistical Software, version 14 (SPSS Inc, Chicago, IL, USA), was used for analyses. Significance was defined as $P < 0.05$.

3. Results and Discussion

3.1. BALF Cytokine Pattern in ARDS Patients. The BALF was collected from twelve adult ARDS patients (9 males and 3 females; age 56 ± 4 yrs; pneumonia $n = 7$ and sepsis $n = 5$; Simplified Acute Physiology Score (SAPS) II 42 ± 18). Proinflammatory cytokines IL-1 β and TNF-α were significantly higher in primary pneumonia patients (pulmonary ARDS, ARDSp) than in patients with ARDS originating from sepsis (extrapulmonary ARDS, ARDSexp) (Figure 1(a)). This was also observed for IL-6 and, in particular, IL-8 content (Figure 1(b)).

It has been extensively documented that in BALF of ARDS patients the content of proinflammatory cytokines is markedly elevated [12]. TNF-α and IL-1β are the main proinflammatory cytokines and they are important in driving the initial lung inflammatory response principally through the stimulation of other cytokines (i.e., IL-6 and IL-8). IL-6

occupies a critical place in modulating ARDS inflammatory response, while chemokine IL-8 is the major neutrophil chemotactic factor into the alveolar space and it is an early marker for the development of ARDS [10]. In particular, a high content of IL-6 and IL-8 is correlated with the progression of lung injury as well as with a poor outcome [8, 9].

There are many etiologic risk factors for ARDS, which are generally divided into those associated with direct injury (ARDSp) to the lung and those that cause indirect lung injury (ARDSexp) in the setting of a systemic process [17, 39, 40]. Indeed, experimental and clinical studies showed little overall differences in the inflammatory responses between direct and indirect lung injury categories. Thus, identification of the risk factor leading to ARDS in the single patient, regardless of its direct or indirect nature, is rather useful to guide treatment for the underlying disease causing ARDS [1]. Moreover, clinical data supported the theory that an overaggressive and persistent patient inflammatory response, rather than the condition causing lung injury, is the most important factor affecting survival in ARDS patients [41].

3.2. BALF of ARDS Patients Stimulates A549 Inflammatory Response. In a previous study, we demonstrated that human alveolar cells (A549) release proinflammatory cytokines (TNF-α, IL-6, and IL-8) in the culture medium when challenged with a proinflammatory stimulus such as LPS, suggesting that the alveolar epithelium has a role in the hyperinflammatory response associated with ARDS [32]. Since LPS is an artificial stimulus, in this study we challenged A549 with BALF of ARDS patients. In this study, BALF stimulation induced a proinflammatory response from A549 cells as demonstrated by release of inflammatory cytokines: TNF-α (Figure 2(a)), IL-6 (Figure 2(b)), and IL-8 (Figure 2(c)) while BALF did not elicit anti-inflammatory IL-10 release (Figure 2(d)). These results demonstrated that the pattern of cytokine release of A549 cells exposed to BALF is similar to that elicited by LPS in the previous study.

3.3. Opposite Effects of DHA/AA Ratios on the Cytokine Release from A549 Stimulated Cells. The nutrition support of ARDS patients includes lipids, usually soybean or safflower oil-based emulsions. These emulsions contain more than 50% of linoleic acid (ω-6), a precursor of AA [42], while are deficient, less than 10%, of ω-3 PUFAs (mainly, α-linolenic acid). Even though the beneficial effects of ω-3 PUFA have been extensively proven by plenty of experimental preclinical data, conflicting results have been obtained from both clinical trials and human intervention studies [15], in ARDS in particular [28]. Therefore, the debate in the scientific community is still open and a definitive accepted recommendation concerning the use of ω-3 fatty acids in ARDS is still lacking [42]. The observation that optimal ω-3 administration was not only dose-related but was also independently affected by the ω-3/ω-6 PUFA ratio has led many authors to focus the attention on the ω-3/ω-6 PUFA ratio in nutrition support to modulate inflammation responses. Different ω-3/ω-6 PUFA ratios, from 1 : 1 to 1 : 4, have been proposed, but the question of the most favorable ω-3/ω-6 PUFA ratio in ARDS patients is not definitely established [43].

In a previous study, we demonstrated that, by affecting ω-3/ω-6 ratio in phospholipids cell membranes with 1 : 1 and 1 : 2 DHA/AA (ω-3/ω-6) ratio supply, the balance between proinflammatory and anti-inflammatory cytokines was modulated, thus limiting the A549 LPS-induced hyperinflammatory response. Moreover, we found that ratios with a ω-6 prevalence (i.e., 1 : 4 and 1 : 7 DHA/AA) potentiated the effect of LPS stimulus [32]. In the present study, we investigated whether 1 : 2 DHA/AA ratio was similarly effective in reducing A549 proinflammatory response induced by an *ex vivo* inflammatory stimulus such as BALF collected from ARDS patients. The 1 : 2 ratio was preferred to 1 : 1 ratio because it could combine efficacy and decreased risk of immunosuppressive effects. The results clearly indicate that 1 : 2 DHA/AA treatment significantly reduced the release of proinflammatory cytokines induced by BALF challenge: TNF-α (Figure 2(a)), IL-6 (Figure 2(b)), and IL-8 (Figure 2(c)). Besides, we found that 1 : 2 DHA/AA treatment was also associated with an increased release of the IL-10 (Figure 2(d)), a potent anti-inflammatory cytokine, confirming that 1 : 2 DHA/AA was able to modulate the balance between proinflammatory and anti-inflammatory cytokines. Finally, the 1 : 7 DHA/AA ratio significantly potentiated the BALF inflammatory effects.

Several studies have identified two main key elements in the pathogenesis of ARDS: the occurrence of an imbalance between proinflammatory and anti-inflammatory cytokines [12] and the persistent elevation of proinflammatory mediators [8]. These conditions lead to additional nonpulmonary organ dysfunction which contributes to excess mortality rates in intensive care units [33, 42]. Therefore, strategies for limiting the intensity of lung inflammatory response are of major importance for prognosis and therapy. However, although some pharmacological strategies have proven to be successful in animal studies, human translation of these results has not been so effective on outcome [44].

3.4. Biochemical and Molecular Mechanisms Involved in Anti-Inflammatory Effects of ω-3 in BALF-Stimulated A549 Cells. Over the last 25 years, the pathophysiology and pharmacology of ω-3 PUFA have been continuously under scrutiny. These FA are able to partly inhibit a number of aspects of inflammation including eicosanoid and cytokine production and bioavailability. The PUFA capacity to modulate different signaling pathways involved in inflammation response has been extensively studied in both physiological and pathological conditions [14, 16]. The main mechanisms explaining the PUFA role in inflammation and investigated in this study were graphically represented in Figure 3: effects on phospholipid composition of A549 cell membranes, modulation of eicosanoid and cytokine biosynthesis and release, and effects on inflammatory signaling transcription pathways.

3.4.1. Eicosanoid Synthesis. The modulation of eicosanoid production is driven by modification of the FA composition of the phospholipids within cell membranes. PUFAs are rapidly incorporated into cell membrane phospholipids;

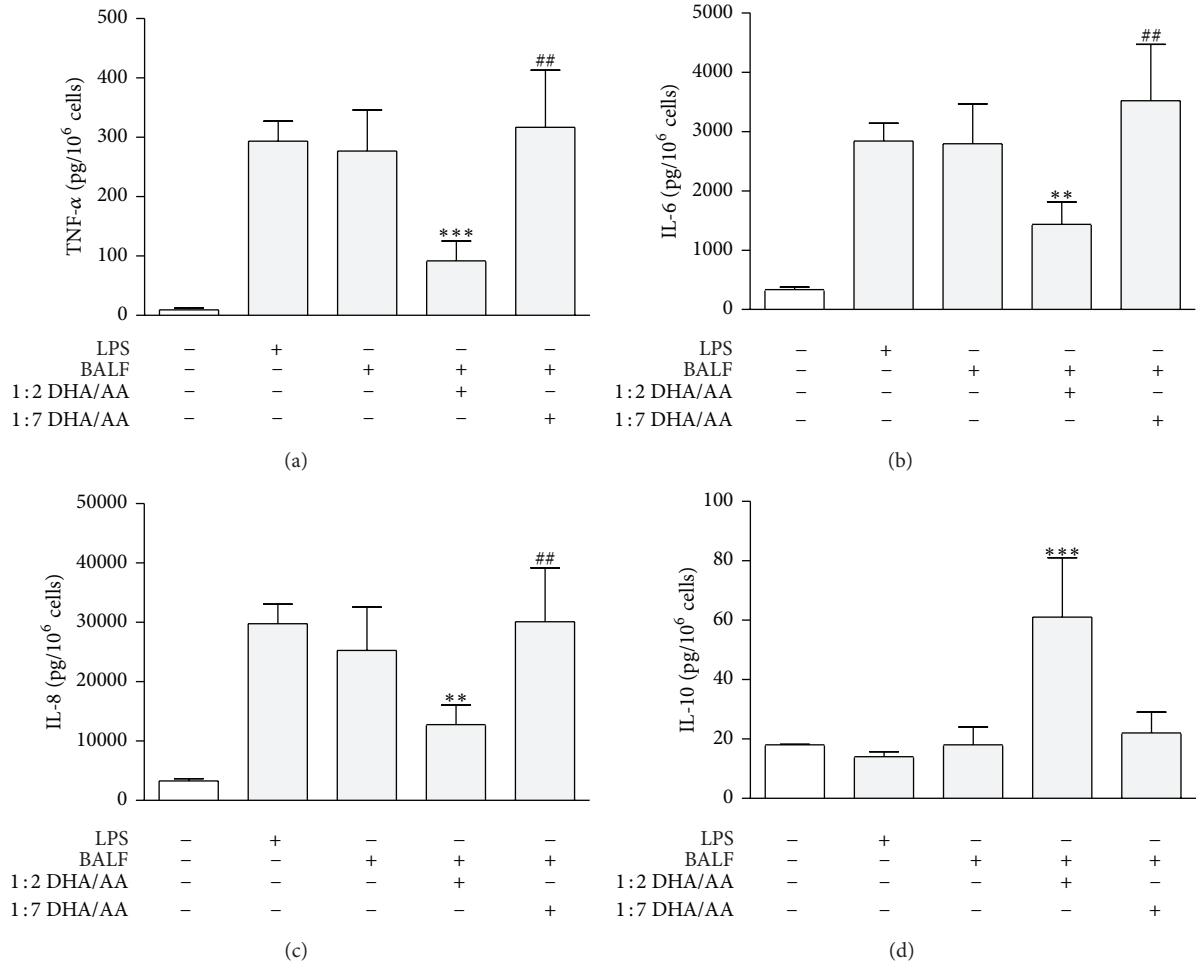

Figure 2: Effects of ω-3/ω-6 PUFA ratios on BALF induced cytokine release from A549 cells. (a) TNF-α proinflammatory cytokine release from A549 cells, stimulated with BALF and treated with 1 : 2 and 1 : 7 DHA/AA PUFA ratios. (b) IL-6 proinflammatory cytokine release from A549 cells stimulated with BALF and treated with 1 : 2 and 1 : 7 DHA/AA (ω-3/ω-6) PUFA ratios. (c) IL-8 proinflammatory cytokine release from A549 cells stimulated with BALF and treated with 1 : 2 and 1 : 7 DHA/AA PUFA ratios. (d) IL-10 anti-inflammatory cytokine release from A549 cells stimulated with BALF and treated with 1 : 2 and 1 : 7 DHA/AA PUFA ratios. In each panel, data are presented as picograms (pg) of the indicated cytokine per million cells. Data are presented as mean ± standard deviation of 12 independent determinations ($n = 12$). PUFA, polyunsaturated fatty acid; BALF, bronchoalveolar lavage fluid; TNF, tumor necrosis factor; DHA, docosahexaenoic acid; AA, arachidonic acid; IL, interleukin; LPS, lipopolysaccharide. $^{***}P < 0.001$ 1 : 2 DHA/AA versus all. $^{**}P < 0.01$ 1 : 2 DHA/AA versus all. $^{\#\#}P < 0.01$ 1 : 7 DHA/AA versus LPS and BALF.

moreover, the esterification of ω-3 FAs is mainly at the expense of ω-6 AA [14]. Membrane phospholipids PUFAs are precursors of eicosanoids, which are the biologically active lipid mediators of inflammation playing wide ranging roles in inflammation and in regulation of immune function [14]. Eicosanoids originated from AA ω-6 PUFA (2-series prostaglandins (PGs) and 4-series leukotrienes (LTs)) have proinflammatory properties while eicosanoids formed from EPA and DHA ω-3 PUFA (3-series PGs and 5-series LTs) are less active and potentially anti-inflammatory [14]. The balance between proinflammatory and anti-inflammatory eicosanoid synthesis determines the extent of inflammatory reaction [19].

In our previous study, we demonstrated that at the baseline the ω-3/ω-6 PUFA ratio in membrane phospholipids of A549 was 1 : 5 and that it can be changed by challenging the cells with 1 : 1 or 1 : 2 DHA/AA ratios [32]. Here, we examined the DHA and AA content in membrane phospholipids of A549 BALF-stimulated and challenged with 1 : 2 or 1 : 7 ratios DHA/AA (Figure 4). At baseline in A549 membrane phospholipids the ω-6 PUFA fraction is predominant, with a ω-3/ω-6 ratio of 1 : 5. Notably, cell treatment with both DHA/AA ratios reduced the AA content in A549 membrane phospholipids, but the 1 : 2 DHA/AA ratio also significantly increased the DHA percentage content. The supply of 1 : 2 DHA/AA ratio reversed the baseline predominance of ω-6 over ω-3 in the ω-3/ω-6 PUFA ratio in membrane phospholipids of A549 cells.

Eicosanoid biosynthesis begun when an inflammatory stimulus activates phospholipases, which are the enzymes that cleave the fatty acid precursors from the membrane phospholipids. Released PUFAs are then converted into

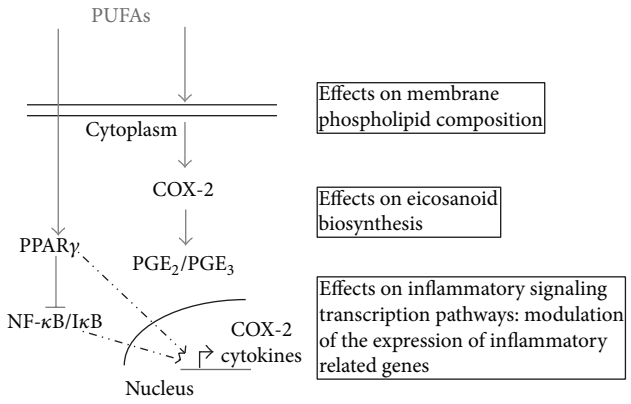

Figure 3: Schematic representation of PUFA mechanism of action in BALF-stimulated A549 cells. PUFAs, polyunsaturated fatty acids; COX, cycloxygenase; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; NF-κB, nuclear factor-kappa B; IκB, inhibitor of NF-κB.

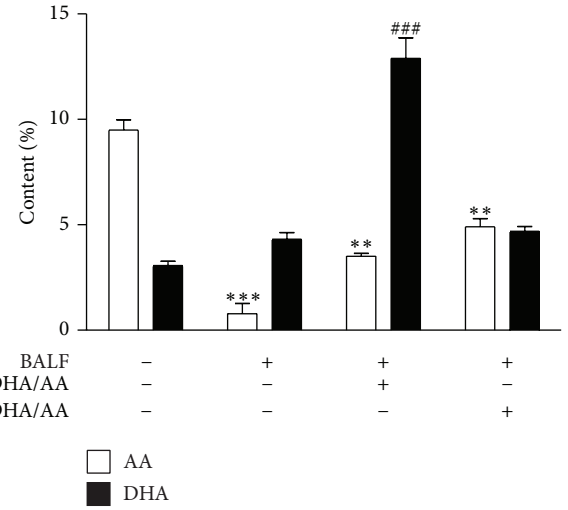

Figure 4: Effects of ω-3/ω-6 PUFA ratios on the percentage content of AA and DHA in A549 membrane phospholipids. Relative percentage content of AA (white bars) and DHA (black bars) in phospholipids of A549 cell membranes stimulated with BALF and treated with 50 μM 1 : 2 or 1 : 7 DHA/AA PUFA ratios. Data are presented as percentage content of AA and DHA in the membrane phospholipids of unstimulated or stimulated A549 cells as indicated. Data are presented as mean ± standard deviation of 4 independent experiments ($n = 4$). PUFA, polyunsaturated fatty acid; AA, arachidonic acid; DHA, docosahexaenoic acid; BALF, bronchoalveolar lavage fluid. $^{***}P < 0.001$ BALF versus unstimulated. $^{**}P < 0.01$ 1 : 2 and 1 : 7 DHA/AA versus all. $^{###}P < 0.001$ versus all.

eicosanoids, mainly PGs and tromboxanes by cycloxygenases (COXs) and LTs by lipoxygenases (LOXs) [14].

There are two COX isoforms: the COX-1 that is constitutively expressed in almost all cells and the COX-2 that is induced in many cell types by a broad range of proinflammatory agents [45]. The COX-2, also known as PG-endoperoxide synthase, is the key enzyme in PG synthesis from AA.

To verify whether in our experimental model there was a modulation of PG biosynthesis, we analyzed the content of COX-2. The results indicated that in response to BALF stimulus A549 cells increased by 2.5-fold the expression of COX-2 (Figure 5(a)) and produce a significant amount of PGE_2 (Figure 5(b)). Noteworthy, even if COX-2 is the inducible form of COX, A549 cells express the enzyme constitutively [46]. According to Yang et al. [46], we found that this level of COX-2 expression was not associated with a PG synthesis (Figure 5(b)) in unstimulated A549.

The 1 : 2 DHA/AA treatment significantly restored the expression of the enzyme at the level of unstimulated cells (Figure 5(a)). This effect was significantly associated with a reduction of PGE_2 release and an induction of PGE_3 synthesis (Figure 5(b)), suggesting that the enzyme was in an active form. The 1 : 7 DHA/AA ratio induced a less significant inhibitory effect on the COX-2 content and the PGE_2 synthesis. The differences in COX-2 content could be correlated with the PGE_2-dependent amplification of the enzyme [45]. These results indicate that both ω-3 and ω-6 PUFAs reduce the expression of COX-2 induced by inflammatory stimuli such as BALF but with a different extent. These effects on PGE_2 content well correlated with the availability of AA substrate into membrane phospholipids (Figure 4). Moreover, treatment with 1 : 2 DHA/AA also induced a significant increase in PGE_3 content, a less potent inflammatory PG. This finding well correlated with the increased DHA content in A549 membrane phospholipids (Figure 4). Notably, PGE_3 is a less potent inducer of COX-2 gene expression in fibroblasts and of IL-6 production by macrophages compared with PGE_2 [13]. In accordance with Yang et al.'s data [46], our results confirm that exposure of alveolar cells to ω-3 PUFA determines a decrease in the COX-2-mediated formation of PGE_2 and an increase in the level of PGE_3.

3.4.2. Inflammatory Signaling Transcription Pathways: NF-κB and PPARγ. The nuclear factor (NF-κB) is a key transcription factor involved in upregulation of inflammatory cytokines, adhesion molecules, and COX-2 genes [47]. Activation of NF-κB transcription factor has been implicated in a number of inflammation-related pathologies [14]. The p65 and p50 NF-κB heterodimers are maintained inactive in the cytosol by the binding with an inhibitory protein, namely, inhibitor of NF-κB (IκB) [48]. Proinflammatory stimuli induce phosphorylation, ubiquitination, and proteasome mediated degradation of IκB, allowing NF-κB translocation into the nucleus and NF-κB target gene transcription [48]. The inhibitory role of ω-3 PUFAs on NF-κB pathway has been demonstrated in several experimental models and pathological conditions [19]. Different authors demonstrated that ω-3 decreased TNF-α expression through the prevention of NF-κB activation by inhibiting IκB phosphorylation and consequently preventing NF-κB translocation into the nucleus [14]. The correlation between NF-κB activation and cytokine content in BALF of ARDS patients was previously demonstrated by Nys et al. [49]. In order to understand the role of 1 : 2 DHA/AA treatment on the NF-κB pathway, we analyzed the expression of p65 NF-κB both in the cytoplasmic and the nuclear fraction of A549 BALF-stimulated cells (Figures 6(a) and 6(b)).

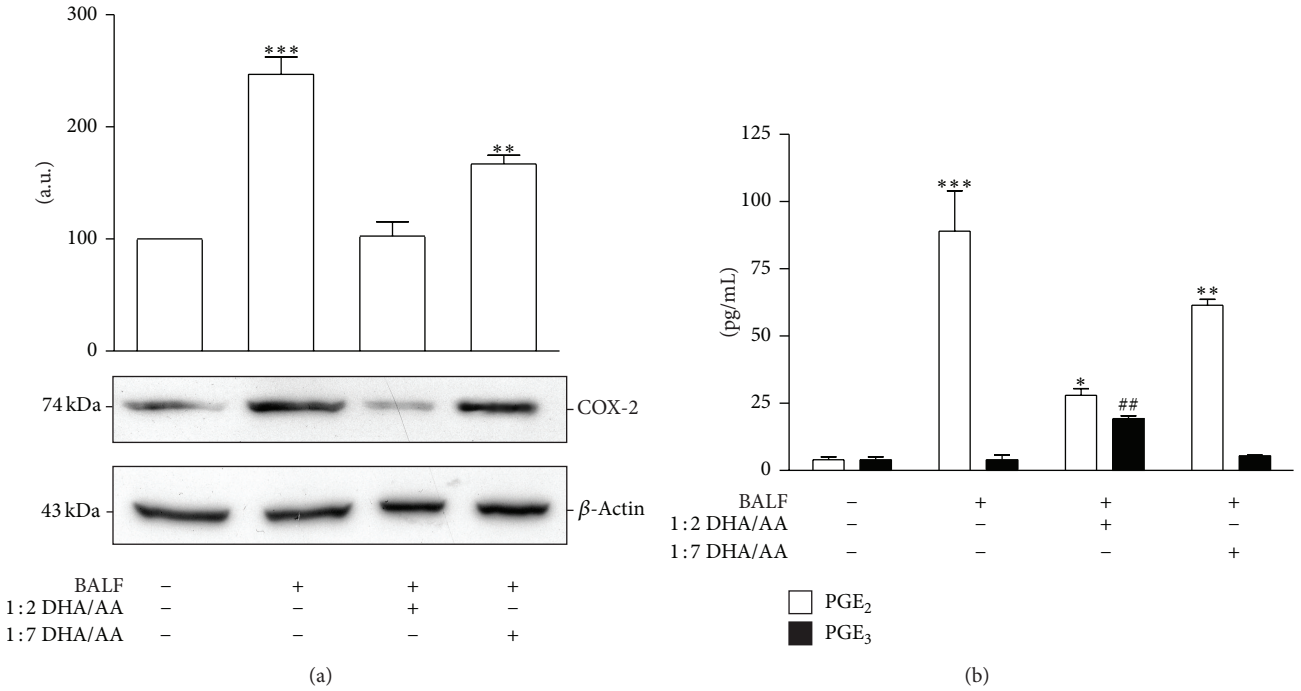

Figure 5: Effects of ω-3/ω-6 PUFA ratios on PGE_2 and PGE_3 synthesis and release. (a) COX-2 relative protein content in A549 cells, stimulated with BALF and treated with 50 μM 1 : 2 and 1 : 7 DHA/AA PUFA ratios. Data are expressed as "a.u." (arbitrary units) of the densitometric values, normalized on the corresponding β-actin. The value of unstimulated cells was arbitrarily set as 100. Data are presented as mean ± standard deviation of 6 independent determinations ($n = 6$). The image is representative of all the WB experiments. (b) PGE_2 (white bars) and PGE_3 (black bars) content in culture media of A549 cells, stimulated with BALFs and treated with 50 μM 1 : 2 or 1 : 7 DHA/AA ratio. Data are presented as picograms (pg) of the indicated PG per mL. Data are presented as mean ± standard deviation of 12 independent determinations ($n = 12$). PUFA, polyunsaturated fatty acid; PG, prostaglandin; COX, cycloxygenase; BALF, bronchoalveolar lavage fluid; DHA, docosahexaenoic acid; AA, arachidonic acid; WB, western blot. $^{***}P < 0.001$ BALF versus unstimulated cells and 1 : 2 DHA/AA. $^{**}P < 0.01$ 1 : 7 DHA/AA versus all. $^{*}P < 0.05$ 1 : 2 DHA/AA versus unstimulated cells. $^{\#\#}P < 0.01$ 1 : 2 DHA/AA versus all.

The results demonstrated that 1 : 2 DHA/AA treatment decreased NF-κB content, in the nuclear fraction in particular, indicating an inhibition of its activation. The results were confirmed by the finding that 1 : 2 DHA/AA treatment increased the content of IκBα, one of the isoforms of IκB (Figure 6(c)). These results suggested that the anti-inflammatory effects of 1 : 2 DHA/AA treatment were associated with the inhibition of the NF-κB inflammatory pathway. Since, genes of IL-1β, IL-6, and TNF-α, as well as COX-2, are regulated by NF-κB [50], we can speculate that the reduction of proinflammatory cytokine release and the inhibition of COX-2 expression were mediated by the inhibition of NF-κB transcriptional activity.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear transcription factors encoded by different genes. PPARs include 3 subtypes (α, β, and γ), which are characterized by unique functions such as ligand specificities and tissue distribution [51]. PPAR ligands encompass endogenous metabolites such as prostanoids and PUFAs, as well as synthetic drugs such as fibrates and thiazolidinediones. In macrophages, activation of PPARγ negatively influences the production of inflammatory cytokines like TNF-α, IL-6, and IL-1β [52]. It has been demonstrated that most of the effects of PPARs on cytokine expression result from crosstalk with other transcriptional factors and in particular with NF-κB [53]. To verify if also in our experimental model the anti-inflammatory effects of ω-3 were correlated with PPAR activation, we determined the PPARγ content (Figure 7). The results indicated that the proinflammatory stimulus was associated with the inhibition of PPARγ expression. Moreover, as we previously demonstrated [35], in the present study we found both PUFA ratios were associated with an increased PPARγ content but to a greater extent with the 1 : 2 DHA/AA treatment.

Summarizing, we speculate that in our experimental model the anti-inflammatory effects of 1 : 2 DHA/AA treatment could be mediated by reduction of COX-2 expression, decrease of NF-κB translocation into the nucleus, and PPARγ activation. Thus, the results presented herein give further insight into the mechanisms involved in the anti-inflammatory effect of ω-3 PUFAs.

3.4.3. Resolvins and Protectins. Recent studies have identified n-3 PUFAs as precursors of a distinct set of lipid mediators that probably act through distinct receptors to exert their anti-inflammatory effects. These new n-3 PUFA-derived, anti-inflammatory mediators have been named resolvins and protectins. For an understanding of resolvin and protectin

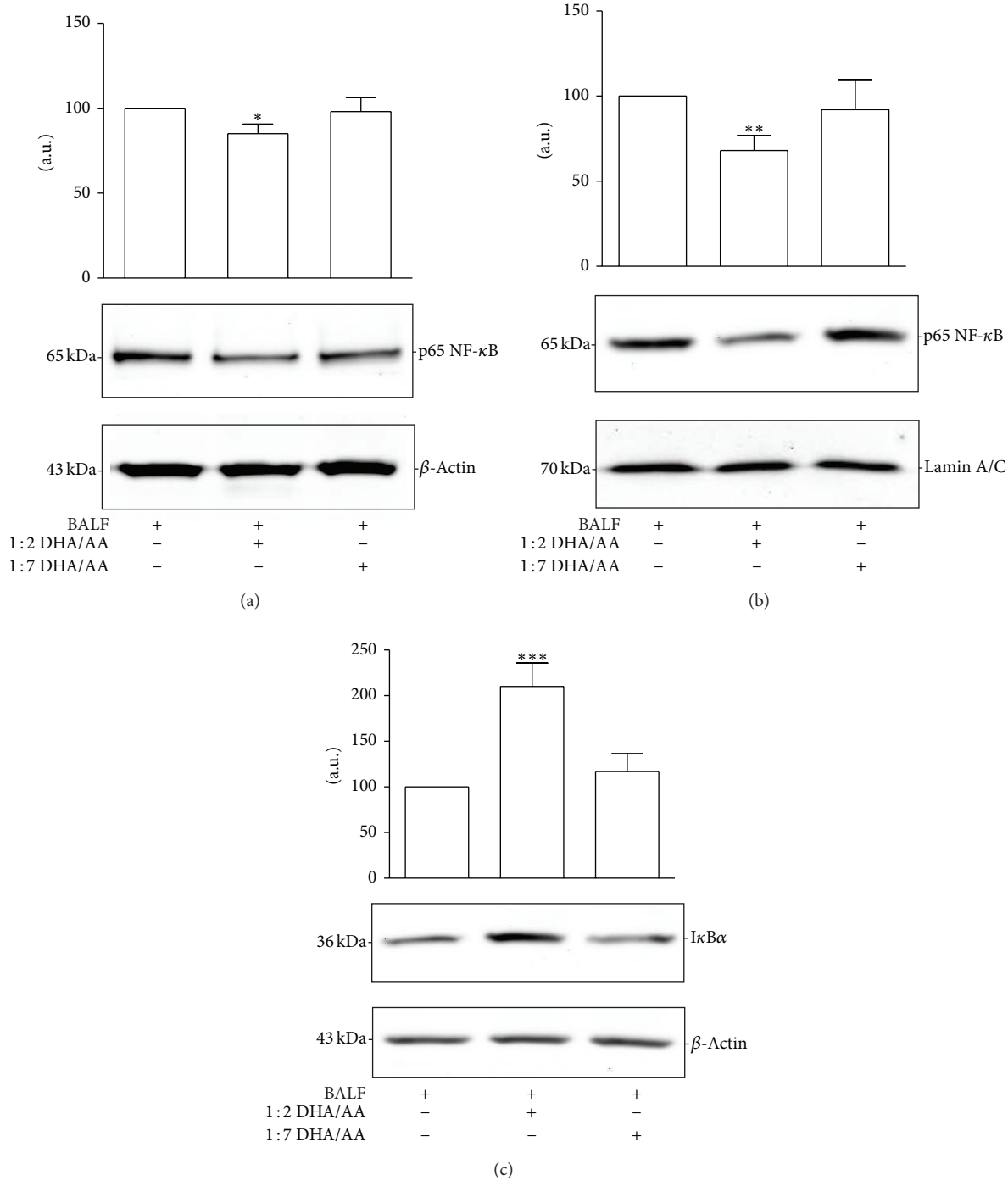

Figure 6: Effects of ω-3/ω-6 PUFA ratios on NF-κB. (a) p65 NF-κB relative protein content in the cytoplasmic fraction of A549 cells, stimulated with BALF and treated with 50 μM 1 : 2 or 1 : 7 DHA/AA ratios. Data are expressed as "a.u." (arbitrary units) of the densitometric values, normalized on the corresponding β-actin. The value of BALF was arbitrarily set as 100. Data are presented as mean ± standard deviation of 6 independent determinations ($n = 6$). The image is representative of all the WB experiments. (b) NF-κB relative protein content in the nuclear fraction of A549 cells, stimulated with BALF and treated with 50 μM 1 : 2 or 1 : 7 DHA/AA ratios. Data are expressed as "a.u." (arbitrary units) of the densitometric values, normalized on the corresponding lamin A/C. The value of BALF was arbitrarily set as 100. Data are presented as mean ± standard deviation of 6 independent determinations ($n = 6$). (c) IκBα relative protein content in the cytoplasmic fraction of A549 cells, stimulated with BALF and treated with 50 μM 1 : 2 or 1 : 7 DHA/AA ratios. Data are expressed as "a.u." (arbitrary units) of the densitometric values, normalized on the corresponding β-actin. The value of BALF was arbitrarily set as 100. Data are presented as mean ± standard deviation of 6 independent determinations ($n = 6$). The image is representative of all the WB experiments. PUFA, polyunsaturated fatty acid; NF-κB, nuclear factor-kappa B; BALF, bronchoalveolar lavage fluid; DHA, docosahexaenoic acid; AA, arachidonic acid; WB, western blot; IκB, inhibitor of NF-κB. $^{*}P < 0.05$ 1 : 2 DHA/AA versus all. $^{**}P < 0.01$ 1 : 2 DHA/AA versus all. $^{***}P < 0.001$ 1 : 2 DHA/AA versus all.

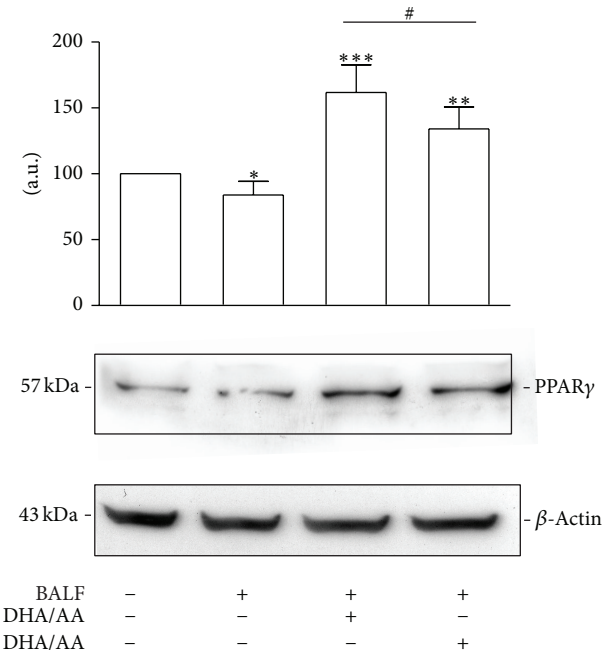

Figure 7: Effects of ω-3/ω-6 PUFA ratios on PPARγ expression. PPARγ relative protein content in A549 cells stimulated with BALF and treated with 50 μM 1:2 or 1:7 DHA/AA ratios. Data are expressed as "a.u." (arbitrary units) of the densitometric values, normalized on the corresponding β-actin. The value of unstimulated cells was arbitrarily set as 100. Data are presented as mean ± standard deviation of 6 independent determinations ($n = 6$). The image is representative of all the WB experiments. PUFA, polyunsaturated fatty acid; PPAR, peroxisome proliferator-activated receptor; BALF, bronchoalveolar lavage fluid; DHA, docosahexaenoic acid; AA, arachidonic acid; WB, western blot. $^{*}P < 0.05$ BALF versus unstimulated cells. $^{**}P < 0.01$ 1:7 DHA/AA versus BALF and unstimulated cells. $^{***}P < 0.001$ 1:2 DHA/AA versus BALF and unstimulated cells. $^{\#}P < 0.05$ 1:2 DHA/AA versus 1:7 DHA/AA.

formation see Weylandt et al. [54] for a complete review. Briefly, these lipid mediators might offer an important new concept to explain the protective effect of n-3 PUFAs in a wide variety of disease models. In particular, DHA constitutes the origin for the D-series resolvins—mainly, resolvin D1 (RvD1)—as well as (neuro-) protectin D1.

In this study, we did not investigate resolvins or protectins in our experimental model; however, previous experimental studies showed that RvD1 had potent anti-inflammatory effects in several disease models including lung injury. RvD1 could modulate the balance between proinflamatory and anti-inflammatory cytokines, alter the response of the host to pulmonary bacterial infection, and affect the early outcome of infection [55]. Wang et al. showed that RvD1 improved survival rate and attenuated ALI in mice induced by LPS; specifically, RvD1 inhibited increases in TNF-α and IL-6 production in the BALF, reduced expression of COX-2, and inhibited activation of NF-κB [56].

4. Conclusions

ARDS is an inflammatory disease whose clinical severity mostly depends on the grade of inflammation. Cytokines and eicosanoids are key elements in the pathogenesis and outcome of ARDS.

To the best of our knowledge, this is the first study reporting the role of ω-3 to ω-6 PUFA ratio in the modulation of release of four cytokines (TNF-α, IL-6, IL-8, and IL-10) and two prostaglandins (PGE_2 and PGE_3) in human alveolar cells exposed to BALF of ARDS patients. Differently from our earlier study, the present study has some original features. First, we challenged alveolar cells with an *ex vivo* inflammatory stimulus and not with LPS. Second, the number of experiments was greater (12 versus 4). Third, we also investigated the PG release as well as the content of COX-2. Finally, we examined the more important inflammatory signal transduction pathways (NF-κB and PPARγ) at work in this experimental inflammatory cell model.

The results of this study demonstrated that shifting the PUFA supply from ω-6 to ω-3 decreased the release of proinflammatory cytokines and PGE_2 in human alveolar cells challenged with BALF of ARDS patients. Moreover, these data confirmed our previous finding that a predominance of AA in PUFA supply determined a more aggressive proinflammatory response. Finally, these data provide a contribution to support the biochemical basis for current recommendations [57–60] to shift the lipid supply from ω-6 to ω-3 PUFA in the nutrition support of ARDS patients.

In conclusion, there are good experimental evidence and convincing rationale according to the ω-3 PUFA use in ARDS patients [61]; however, questions still remain to be answered regarding the *in vivo* effects of these PUFAs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Paolo Cotogni and Antonella Trombetta contributed equally to this work.

Acknowledgments

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