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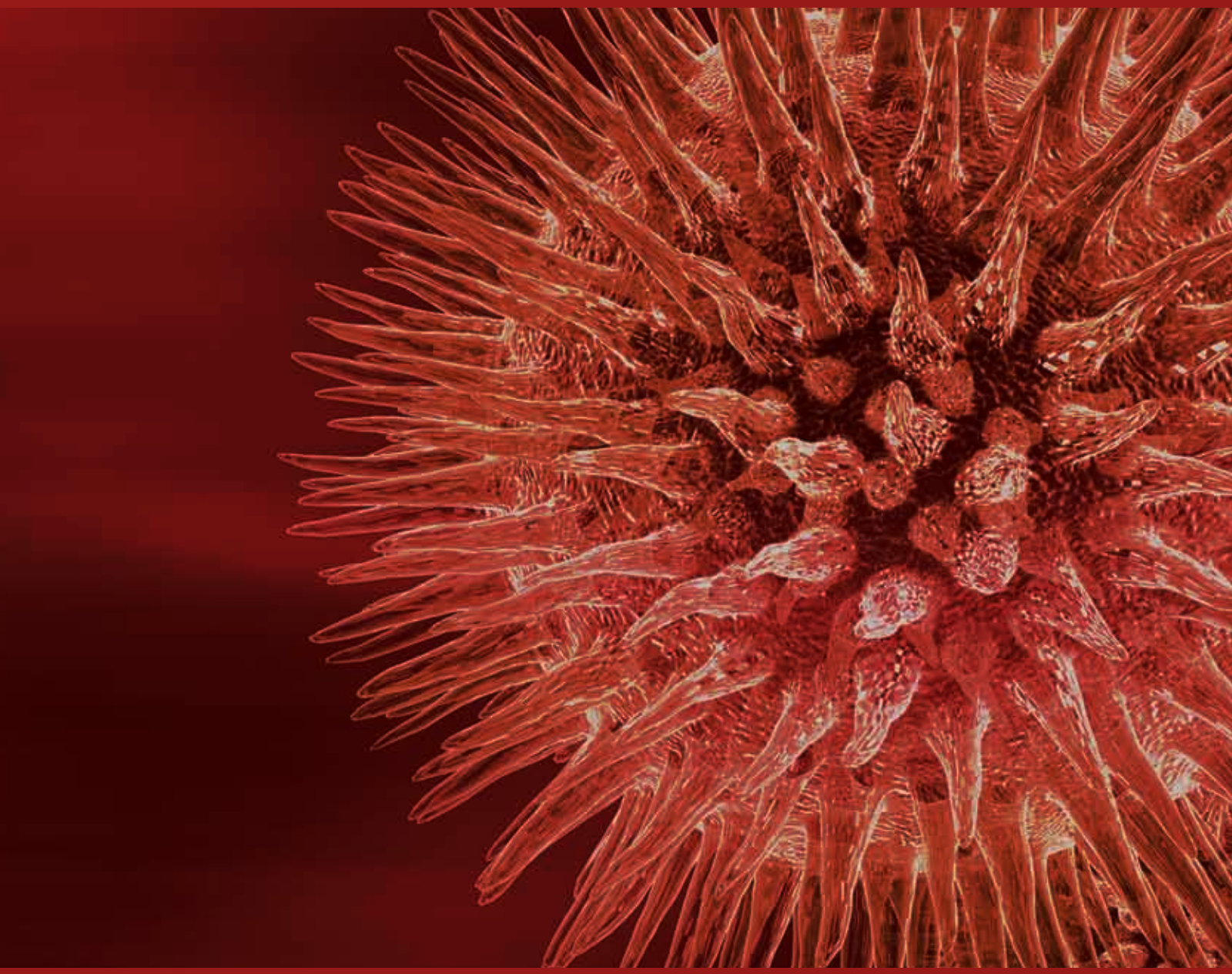
Guest Editors: Achille Cittadini, Gabriella Calviello, Hui-Min Su, and Karsten Weylandt



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Editorial

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

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The possibility of health benefits associated with dietary omega-3 polyunsaturated fatty acids (*ω*-3 PUFAs) has been described for several chronic conditions, including cardiovascular, neurodegenerative, and neoplastic diseases. A large body of evidence has emerged over the past years to show the critical role played by inflammation in the pathogenesis of these diseases, previously not considered inflammation related. Therefore, it has been recently hypothesized that *ω*-3 PUFAs' effects may be related, at least in part, to their direct anti-inflammatory activity as well as to that of their oxygenated metabolites (17-HDHA, 18-HEPE, resolvins, and protectins). In this special issue G. Calviello and collaborators summarize and comprehensively discuss the current knowledge regarding the modulating effects of *ω*-3 PUFAs on the production of inflammatory cytokines and proresolving or protective lipid mediators in the context of inflammatory, metabolic, neurodegenerative, and neoplastic diseases.

S. M. Lee and W. S. An present a review on the beneficial effects of *ω*-3 PUFAs on cardiovascular diseases and possible cardioprotective mechanisms of *ω*-3 PUFAs in chronic kidney disease (CKD) patients. They highlight their ability to reduce inflammation, decrease oxidative stress, inhibit platelet activity, exert antiarrhythmic effects, and improve triglyceride levels, both in the general population and in CKD patients.

Moreover, a research article by B. Parada et al. is presented showing that *ω*-3 PUFAs inhibit the development of premalignant and malignant lesions in a rat model of bladder cancer, possibly not only through their anti-inflammatory properties, but also by their ability to inhibit oxidative stress, proliferation, and angiogenesis.

It is known that local inflammation plays a central role in the development of colon cancer, and it has been suggested that its suppression may prevent the development of this malignancy. However, the role of nonsteroidal anti-inflammatory drugs (NSAID) in colitis is controversial, and aggravation of disease due to these compounds has been described. T. Köhnke et al. in their paper use the murine dextran-sodium-sulfate- (DSS-) induced colitis model and demonstrate that the treatment of animals with acetylsalicylic acid (ASA) decreases colitis activity and increases the formation of *ω*-3 PUFA-derived lipid mediators with high anti-inflammatory potential, such as 17-hydroxy docosahexaenoic acid (17-HDHA), a docosahexaenoic (DHA, 22:6 *ω*-3) oxygenated metabolite. This finding suggests that the metabolic transformation of DHA incorporated in colon cells in the presence of ASA could, at least in part, explain the colon cancer preventive activity of this drug, and it might argue for a synergistic effect of ASA and *ω*-3 PUFA in the prevention of colon cancer.

The hypothesis that dietary treatment with *ω*-3 PUFAs may potentiate the effect of antineoplastic drugs has been comprehensively examined by N. Merendino et al. Their review is focused on the current knowledge supporting the potential use of DHA as an adjuvant in tumor chemotherapy, since it has been reported that this fatty acid may enhance the uptake of anticancer drugs, regulate the oxidative status of tumor cells, and inhibit tumor cell invasion and metastasis.

Another research article is presented by S. Shin et al., showing that DHA induces simultaneously apoptosis and autophagy in prostate cancer cells expressing mutant p53. The authors find that DHA exerts such antineoplastic effects by inducing the generation of mitochondrial reactive oxygen species (ROS) and the ROS-mediated Akt-mTOR signaling.

Several other biological and molecular mechanisms have been so far described to explain the anticancer effects of ω-3 PUFAs in prostate cancer. A detailed overview of the possible mechanisms so far hypothesized is presented by Z. Gu and colleagues. The authors report that a large body of preclinical experimental work has demonstrated the high sensitivity of prostate cancer to the protective action of ω-3 PUFAs, while in contrast it has not been definitely established whether the ingestion of increased levels of these fatty acids may actually have a protective effect in prostate cancer patients. This represents a highly controversial issue, as outcomes of the epidemiological studies in patients bearing this and other kinds of cancer appear to be conflicting. Several reasons can explain these divergences, and these are discussed here by Z. Gu et al.

One possible reason for discrepancies in ω-3 PUFA studies, observed also among the interventional trials performed in cardiovascular patients, is highlighted by B. B. Albert et al. in this special issue. This paper discusses the possibility that the easy oxidation of the supplemented ω-3 PUFAs to lipid peroxides and other secondary oxidation products may make them ineffective or even harmful. The authors therefore recommend that all clinical trials investigating ω-3 PUFA harms or benefits report the results of simple assays determining the oxidative state of an oil. This recommendation appears appropriate also in view of the numerous observations [1] suggesting that very high doses of ω-3 PUFAs ingested by "high consumers" of fish and leading to very high incorporation of these fatty acids in plasma lipids, may result in high and harmful tissue levels of oxidized ω-3 PUFAs. This may result in a lack of health benefits or even in the induction of carcinogenic effects, such as those observed recently in some epidemiological studies.

This special issue, besides underlining the importance of identifying and ingesting an appropriate amount of nonoxidized ω-3 PUFAs, also drives attention to the importance of an optimal dietary balance between ω-3 and ω-6 PUFAs, that leads to an appropriate ω-6/ω-3 PUFA ratio in our tissues. The review by E. Murru et al. discusses the results that have so far demonstrated the relationship existing between the ω-6/ω-3 PUFA ratio present in tissues and the dietary form of these fatty acids. They focus on the findings that have so far shown a more efficient incorporation of eicosapentaenoic acid (EPA) and DHA into tissue phospholipids (PL), when they are mainly esterified to PL already in the diet.

Finally, the importance of establishing an appropriate dose of dietary ω-3 PUFAs, depending on the form of fatty acid supplementation and on the age and conditions of the subjects, is emphasized in the research article by J. Luo et al. They find that the same fish-oil-enriched diet given to dams breeding piglets or to weaning piglet causes a positive effect only on the growth of piglets breast-fed by the fish-oil-supplemented dams. That effect has been related to a reduced tissue production of proinflammatory cytokines. On the contrary, the piglets directly supplemented with fish oil show reduced growth and higher tissue levels of inflammatory cytokines.

On the whole, the papers contained in this special issue aim to examine the beneficial effects of ω-3 PUFAs in several chronic diseases and physiological conditions as well as to identify and examine the molecular mechanisms underlying these effects. Moreover, they highlight the importance of establishing and using in the studies a proper ω-3 PUFA supplementation, in terms of amount (daily dose), maximal content of oxidative products, and binding of ω-3 PUFAs to PL or triglycerides.

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Gabriella Calviello
Hui-Min Su
Karsten Weylandt

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Research Article

Acetylsalicylic Acid Reduces the Severity of Dextran Sodium Sulfate-Induced Colitis and Increases the Formation of Anti-Inflammatory Lipid Mediators

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The role of non-steroidal anti-inflammatory drugs in inflammatory bowel disease is controversial, as they have been implicated in disease aggravation. Different from other cyclooxygenase inhibitors, acetylsalicylic acid (ASA) enhances the formation of anti-inflammatory and proresolution lipoxins derived from arachidonic acid as well as resolvins from omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA). In this study, we examined the effect of ASA on murine dextran sodium sulfate colitis. A mouse magnetic resonance imaging (MRI) protocol and post mortem assessment were used to assess disease severity, and lipid metabolites were measured using liquid chromatography-coupled tandem mass spectrometry. Decreased colitis activity was demonstrated by phenotype and MRI assessment in mice treated with ASA, and confirmed in postmortem analysis. Analysis of lipid mediators showed sustained formation of lipoxin A4 and an increase of DHA-derived 17-hydroxydocosahexaenoic acid (17-HDHA) after treatment with ASA. Furthermore, *in vitro* experiments in RAW264.7 murine macrophages demonstrated significantly increased phagocytosis activity after incubation with 17-HDHA, supporting its proresolution effect. These results show a protective effect of ASA in a murine colitis model and could give a rationale for a careful reassessment of ASA therapy in patients with inflammatory bowel disease and particularly ulcerative colitis, possibly combined with DHA supplementation.

1. Introduction

The inflammatory bowel diseases (IBDs) ulcerative colitis (UC), confined to the colon, and Crohn's disease (CD), affecting the whole gastrointestinal tract, are chronic inflammatory disorders with considerable morbidity and, particularly for UC, mortality due to a high risk of colorectal cancer development. Current therapy for IBD focuses on drugs that inhibit inflammation [1–4]. Human data regarding the potential benefits and risks of COX inhibition in patients with IBD are conflicting [5–9]. This is also reflected in animal studies examining COX inhibition in experimental colitis models. While some recent studies found deleterious effects in animals with DSS colitis associated with inhibition of COX [10–13], others demonstrated a benefit of COX-2 inhibitor treatment [14, 15].

Recently, newly discovered lipid mediators have been implicated in the alleviation of colitis [16]. The lipoxins are

lipid mediators originating from the omega-6 polyunsaturated fatty acid (n-6 PUFA) arachidonic acid (AA) by enzymatic action of different lipoxygenases or by acetylated COX-2 [17]. Studies have demonstrated potent anti-inflammatory and inflammation dampening properties for lipoxins [18], and stable analogues of lipoxins were shown to alleviate experimental colitis [19, 20]. Similarly, protective lipid mediators (so-called resolvins) can be formed from omega-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Our previous work demonstrated formation of resolvins in mice with increased omega-3 fatty acid tissue content in the context of DSS colitis [21], and other studies have shown an anti-inflammatory effect of several of these compounds in the context of experimental colitis models [22–24].

In this study, we analyzed the effect of acetylsalicylic acid (ASA) in chemically induced acute DSS colitis in mice. DSS colitis is characterized by initial injury to the epithelial cells followed by inflammation and has been shown to be a good model to test therapeutics for human inflammatory bowel disease [25]. Many studies have used this model in the past as it is regarded as a good animal model of IBD, and particularly UC, with a predominant site of inflammation in the distal colon [26].

To evaluate disease activity in the different groups, we extended our analysis beyond the conventional phenotype and morphology markers and employed small animal MRI technology. Noninvasive imaging in murine models of colitis has been previously described using CT and MRI technologies [27–29]. For the study presented here, a modified protocol was devised in order to reliably measure and compare colon wall thickness and hyperemia in different mice.

To assess possible mechanisms responsible for the observed protection in ASA-treated mice, mass spectrometric measurements were then employed to determine hydroxylated lipid metabolites and mediators derived from AA (lipoxin A_4 and 15-epi-lipoxin A_4) and from 17-hydroxy-docosahex-aen-o-ic acid (DHA).

2. Materials and Methods

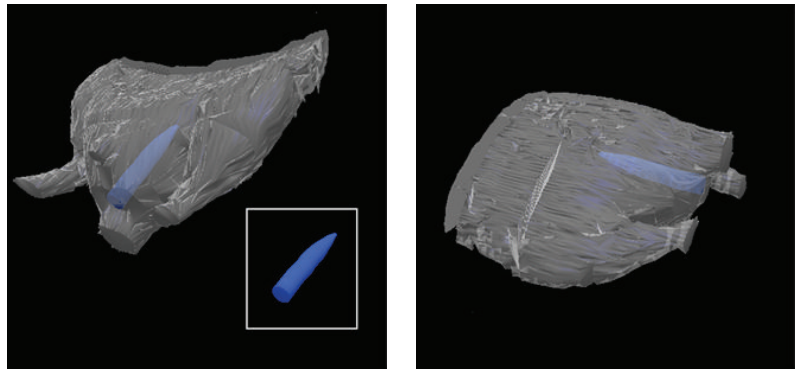
2.1. Induction of Colitis. Female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and held until the desired age (6 weeks) and body weight (19–21 g).

For testing of different treatment regimens, mice were divided into 3 groups. One group received 3% DSS dissolved in sterile drinking water and daily intraperitoneal (ip) injections of 0.5 mg acetylsalicylic acid (ASA, Sigma-Aldrich, St. Louis, MO) dissolved in 300 μL of vehicle (3.33% EtOH in 0.9% NaCl) for 5 days ($n = 5$). Another group received 3% DSS as well as ip injections of the vehicle alone for 5 days ($n = 6$). Finally, the last group received sterile drinking water and ip injection of vehicle alone and served as a control ($n = 3$). The first ip injection of ASA or vehicle was administered right before the mice were switched to DSS-containing drinking water. After 5 days, the DSS-containing water was replaced with sterile drinking water.

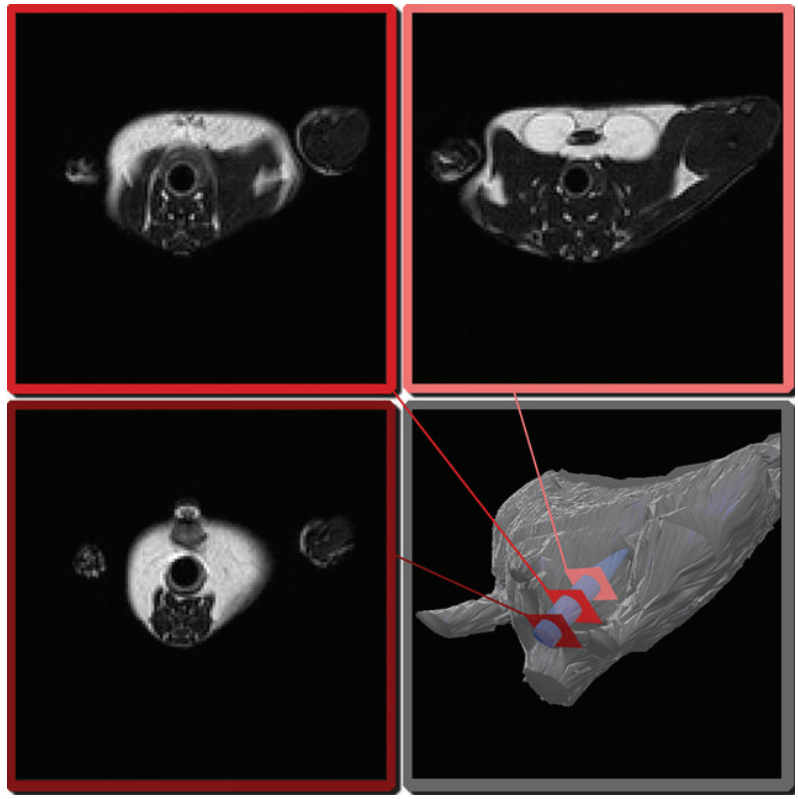
2.2. Evaluation of Colitis Severity. Body weight measurement (presented as percentage of body weight on day 0) as well as evaluation of stool status was carried out daily. Stool samples from individual mice were evaluated on a three-point scale using a test for occult blood (Hemoccult, Beckman Coulter Inc., Fullerton, CA) as well as macroscopic evaluation (0 = no occult blood; 1 = test for occult blood slightly positive; 2 = test for occult blood strongly positive; and 3 = bloody stool). On day 9, mice were sacrificed, colons were excised, and length was measured. All samples were snap frozen in liquid nitrogen and stored at −80°C for further analysis.

2.3. Histological Evaluation. Frozen tissue sections from the distal colon of all mice were mounted in OCT medium. Sections were cut at 5 μm thickness and mounted on glass slides. After air drying, sections were fixed in fixing medium (70% ethanol, 4% formaldehyde, and 5% glacial acetic acid in H_2O), washed in ethanol (70%) and water, followed by standard hematoxylin and eosin stain. Slides were evaluated in a blinded manner and scored according to severity of inflammation (0 = no inflammation, 1 = mild, 2 = moderate, and 3 = severe), thickness of inflammatory involvement (0 = no inflammation, 1 = mucosa, 2 = mucosa plus submucosa, and 3 = transmural), character of epithelial damage (0 = intact epithelium, 1 = disruption of architectural structure, 2 = erosion, and 3 = ulceration), and extent of lesions (0 = no lesions, 1 = punctuate, 2 = multifocal, and 3 = diffuse). This evaluation protocol is based on a previously described scoring system for DSS colitis [30].

2.4. Magnetic Resonance Imaging (MRI). Mice were scanned on day 3 and day 8 of the experimental protocol. MRI assessment of colitis was performed using a Bruker 4.7T Avance horizontal bore system (Bruker Avance, Karlsruhe, Germany) equipped with a 200 mm gradient set capable of 30 G/cm gradient strength. During the MR scan, the mice were mounted on a home-built nonmagnetic holding bar equipped with a nose cone for anesthesia delivery and were anaesthetized using 1.5%–2% (vol/vol) isoflurane (Baxter, Deerfield, IL) in 1 L/min oxygen flow. The breathing rate of the mice was monitored using a small animal monitoring and gating system (Small Animal Instruments Inc., NY). The isoflurane flow was adjusted to achieve a breathing rate of 20–30 breaths per minute during the scanning process. In order to standardize the comparisons of wall thickness as well as colon wall signal intensity between different animals, a non-magnetic (plastic) tube (outer diameter 2.1 mm) was placed approximately 9 mm into the rectum of the anaesthetized mice (Figure 1(a)). For each mouse, 24 T2 weighted 2D axial images were acquired to cover the colon using a fast spin echo sequence with a repetition time (TR) of 2683 ms, an echo time (TE) of 12 ms, a 128 × 128 matrix, and a 3.0 cm × 3.0 cm field of view (FOV). The slice thickness was set to 1 mm, and the number of averages was 8. After image acquisition, the plastic tube was removed, and the animals were transferred to a heating pad until they were fully awake. The images were analyzed using standard imaging software (Adobe Photoshop, Adobe Systems, San Jose, CA).



(a)



(b)

Figure 1: MRI analysis of colitis severity. (a) For MRI analysis of disease severity, a plastic tube with outer diameter of 2.1 mm was carefully placed approximately 9 mm into the rectum of the anaesthetized mouse. (b) Analysis was performed by comparing wall thickness as well as brightness between the different mice in three defined slices.

Colon wall thickness was then measured on the T2 weighted images from three slices of the distal colon with a standardized distance of 3 mm between slices (Figure 1(b)). For each slice, three measurements were performed, and the means for each animal were calculated. For signal intensity analysis, a region of interest (ROI) was drawn within the colon wall. A histogram tool was used to analyze the brightness of the pixels within the ROI. This was repeated for the same three slices that were used for the wall thickness measurements. The pixel brightness means were then normalized by comparison with the nearby fatty tissue around the ovaries of the mice.

2.5. Lipid Metabolite Analysis. 30 mg ground and frozen colon tissue was mixed with 500 μL water and 500 μL methanol, internal standard consisting of 10 ng LTB_4-d_4 was added, and the sample was then hydrolyzed with 300 μL of 10 M sodium hydroxide for 30 minutes at 60°C. The solution was neutralized with 60% acetic acid, and the pH was adjusted to 6.0 with sodium acetate buffer. A solid phase

extraction was performed as previously described [31]. For elution, an n-hexane:ethyl acetate extraction mixture (25 : 75) with 1% acetic acid was used. The eluate was evaporated on a heating block at 40°C under a stream of nitrogen to obtain a solid residue. Residues were then dissolved in 70 μL acetonitrile and measured using an Agilent 1200 HPLC system coupled to an Agilent 6410 Triple quad mass spectrometer with an electrospray ionization source. Analysis of lipid metabolites was performed with multiple reaction monitoring in negative mode.

2.6. Phagocytosis Assay. RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. All experiments were performed in a humidified atmosphere under 5% CO_2 at 37°C. Cells were plated in 96-well plates at a density of 1×10^5 cells/well and incubated overnight. To quantify phagocytosis, cells were incubated with fluorescein-labelled bioparticles (Vybrant Phagocytosis Assay, Invitrogen, Carlsbad, CA), diluted 1 : 2. Uptake was measured after 4 h in a fluorescence plate reader, essentially following the manufacturer's protocol. To evaluate the effect of 17-HDHA on phagocytosis, cells were pretreated with 1 μM 17-HDHA for 2 h. Positive controls with equivalent volumes of ethanol (vehicle) and negative controls were performed in parallel.

2.7. Statistical Analysis. Statistical analysis was performed with GraphPad Prism 3.02v Software (GraphPad, La Jolla, CA). Comparison was made using the Mann-Whitney U test or as indicated. All values are presented as the mean ± SEM. Statistical significance was set at $P < 0.05$.

3. Results

Induction of colitis resulted in marked changes in body weight, appearance of fecal blood, and general status. Mice treated with ASA showed significantly less body weight loss (Figure 2(a)) as well as a recovery of body weight within 3 days after the end of DSS exposure, while untreated mice exhibited only a stabilization of body weight after cessation of DSS.

Colitis disease activity was evaluated by MRI on day 3 and day 8 of the treatment protocol. The MRI results show a marked difference between the treated groups of mice with less wall thickness and a significantly lower T2 weighted signal intensity (brightness) in the mice treated with ASA, as signs of a lower inflammatory infiltration (Figure 2(b)). Due to technical limitations in the number of MRI scans we were able to perform, only 3 animals per group were assayed, which does not give enough values for statistical normality testing using, for example, the Kolmogorov-Smirnov test. Applying nonparametric Mann-Whitney U testing to these data led to nonsignificant findings. However, when hypothesizing normal distribution—and thus applying Student's t-test—for these data, the differences for thickness as well as brightness were significant on day 3 and day 8 with $P < 0.01$ when comparing the ASA + DSS group versus DSS alone.

The results of the MRI and body weight analyses confirmed the decreased disease activity in the mice treated with ASA. Furthermore, assessment of bloody stools in the animals showed a trend towards more severe and prolonged bleeding in the DSS group (Figure 2(c)). However, conclusive statistical evaluation of these data could not be performed, as it was not possible to do stool testing on every animal every day of the experimental protocol.

The *in vivo* disease activity assessments were validated in the postmortem analysis of the colons, with wall thicknesses similar to those observed in the MRI measurements (Figure 3(a)) and significantly less colon shortening in mice treated with ASA as compared to mice exposed only to DSS (Figure 3(b)). Microscopic grading of the changes in the colon revealed decreased inflammatory activity in mice treated with ASA (summarized scores are shown in Figure 3(c)). However, we were not able to document changes in inflammatory activity biochemically, as serum TNF-α levels, measured by ELISA, were not significantly different between the different treatment groups (data not shown).

As lipid mediators formation of AA-derived lipoxins and n-3 PUFA-derived resolvins can be increased by ASA-induced acetylation of the COX-2 enzyme [32], we assessed several of these mediators and their precursors in the colons of mice with DSS-induced colitis with and without treatment with ASA. Due to the presence of AA and DHA but not EPA in the colon tissue of the mice used in this study (data not shown), we focused on the assessment of AA-derived lipoxins and DHA-derived 17-hydroxydocosahexaenoic acid (17-HDHA). Lipoxin A_4 and 15-epi-lipoxin A_4 concentrations were decreased in colon tissue from mice three days after DSS exposure, but formation of 15-epi-lipoxin A_4 was significantly higher in mice treated with ASA as compared to the DSS alone treatment group (Figures 4(a) and 4(b)). Unexpectedly, the DHA-metabolite 17-HDHA was significantly higher in colon tissues from mice three days after DSS exposure (Figure 4(c)) and was increased even more in tissue samples from mice treated with ASA.

In order to further elucidate the mechanism by which 17-HDHA might contribute to inflammation dampening and resolution, the *in vitro* ability of 17-HDHA to increase phagocytosis was tested. In these experiments, an assay system with fluorescently labeled *E. coli* K-12 bioparticles was used as a tool to study overall phagocytotic activity of macrophages [33, 34]. A significant (1.25-fold) increase in phagocytosis activity after 1 μM 17-HDHA preincubation in the RAW 264.7 murine macrophage cell line was demonstrated (Figure 5(a)).

4. Discussion

The data presented here indicate that DSS-induced colon inflammation can be alleviated by acetylsalicylic acid. Although a number of previous studies have examined the effectiveness of COX inhibitors in the context of DSS colitis, the outcomes of these studies were inconsistent or conflicting [10–15], and, to our knowledge, ASA was never used in experimental DSS colitis. This may be due to the fact that ASA causes ulcerations, erosions, and gastrointestinal bleeding in the stomach as well as the small bowel, and many clinicians

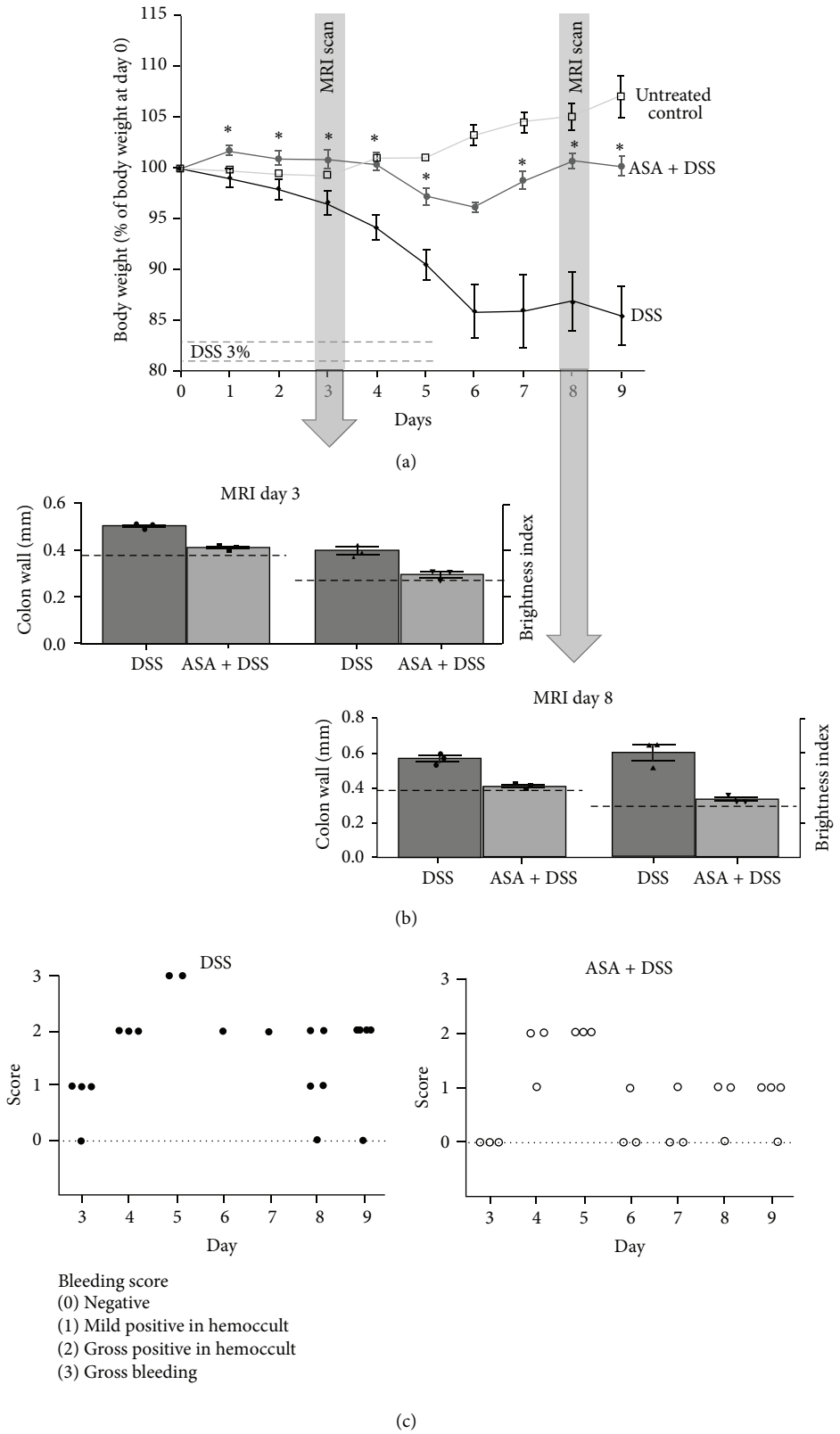


FIGURE 2: *In vivo* assessment of colitis severity. (a) Body weight course in the different groups, $n = 3$ for the control group, $n = 5$ for the ASA-group, and $n = 6$ for the DSS group; $^{*}P < 0.05$ versus DSS. (b) MRI-measured colon wall thickness and brightness on day 3 as well as day 8 of the experimental protocol, $n = 3$ for each treatment group. In dashed lines, the values of measurements in untreated control mice are shown. (c) Bleeding score evaluation in animals treated with DSS or ASA + DSS. There was no bleeding in control animals (not shown).

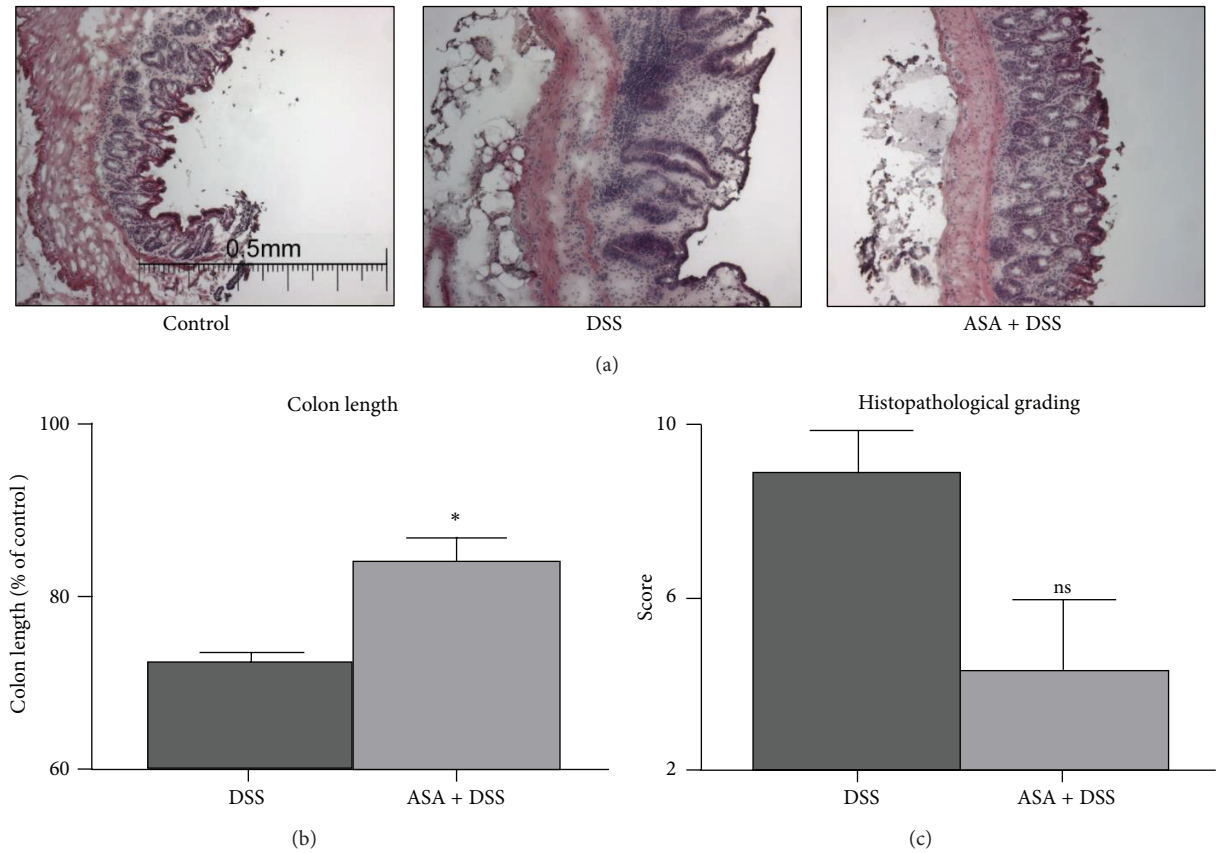


Figure 3: Postmortem assessment of colitis severity. (a) Hematoxylin and eosin stains of transmural colon walls. Representative images of control tissue as compared to animals treated with DSS and ASA + DSS. (b) Colon shortening as an indicator of disease severity was compared between the different experimental groups, $n = 5$ for the ASA-group and $n = 6$ for the DSS group; $^{*}P = 0.044$ versus DSS. (c) Histological grading score, $n = 5$ for the ASA-group and $n = 6$ for the DSS group; $^{ns}P = 0.054$ versus DSS. Untreated controls (not shown) reached a maximum of 2 points in this score.

believe it therefore to be too GI toxic to be of any value in the control of colonic inflammation.

At the same time, patients with IBD, and particularly UC, remain at increased risk for colorectal cancer [35, 36]. Long-term ASA treatment has been shown to be effective for colon cancer prevention [37–40]. In patients with UC, although data in this field is scarce, a similar risk reduction of colorectal cancer could be shown [41], arguing for ASA use also in these patients.

We set out to study the effect of ASA supplementation on the course of DSS-induced colitis in mice, hypothesizing an effect of aspirin treatment on the formation of hydroxylated lipid mediators such as the lipoxins as well as DHA-derived compounds. These were characterized as potent anti-inflammatory compounds also in the context of DSS colitis in previous studies [19, 23].

In our experiments, we aimed for an ASA dose comparable to low-dose ASA administration in humans. Previously published data on low-dose ASA administration in mice with intestinal or other tumors used doses of 40 mg/kg [42] and 50 mg/kg [43]. High-dose ASA dosage was at 400 mg/kg in a previous tumor prevention study in mice [44]. Two studies in intestinal tumor models used a dose of 25 mg/kg [45, 46], which the authors calculated to correspond to a dose of 80–110 mg/day in humans on a 2000 kcal diet following nutrient density calculations [47], and we decided to use this dose in our experiment to mimic low-dose ASA administration. A limitation of the data presented here is that no experiments with ASA only treatment were performed. However, the previous study by Mahmoud et al. has established the safety of long-term administration of ASA at a dose of 25 mg/kg in mice [45].

In the study presented here, we found significant changes in the tissue concentrations of anti-inflammatory lipoxins, with suppression of lipoxin A_4 and 15-epi-lipoxin A_4 in the mice with DSS colitis. As compared to this, the lipoxin levels in the ASA-treated animals were higher, which could contribute to the protection from colitis seen in these animals. In contrast, the amount of the DHA-derived 17-HDHA increased in animals after DSS treatment and even more so with concomitant ASA treatment. While higher levels of 17-HDHA were present in the colons from mice after DSS colitis induction as compared to controls, the treatment with ASA led to even higher 17-HDHA tissue concentrations.

17-HDHA is the precursor for the formation of anti-inflammatory D resolvins, and several previous studies from

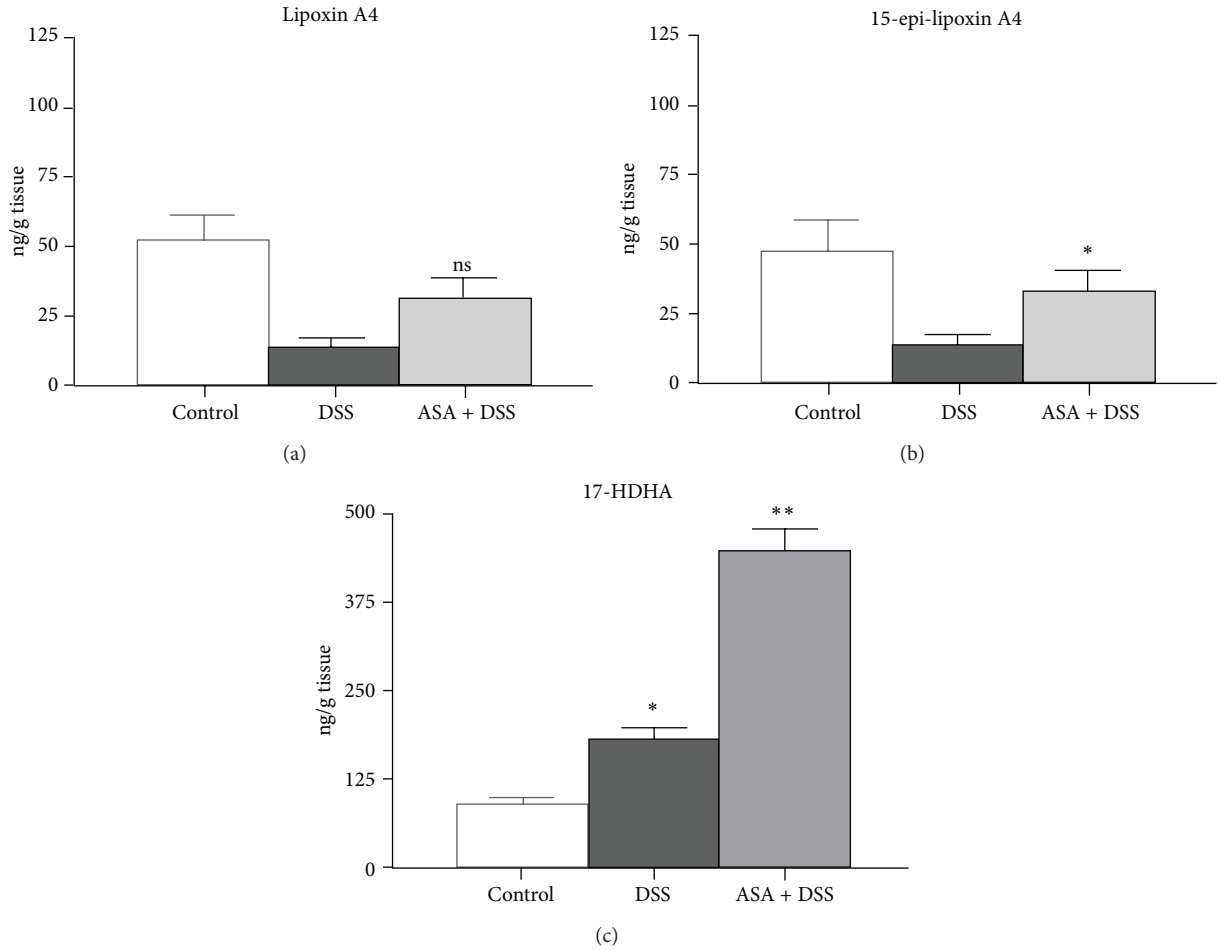


FIGURE 4: Assessment of lipid mediators. Shown are the arachidonic acid-derived lipoxin A_4 (a) and 15-epi-lipoxin A_4 (b) with $n = 3$ for the control group, $n = 5$ for both the DSS alone and the DSS plus ASA groups; $^{ns}P = 0.056$ versus DSS (not significant); $^{*}P = 0.016$ versus DSS. (c) The levels of 17-HDHA, with $n = 3$ for the control group, $n = 5$ for both the DSS alone and the DSS plus ASA groups; $^{*}P = 0.036$ versus controls; $^{**}P = 0.008$ versus DSS (b).

other groups have demonstrated biological effects of the different stereoisomers of this compound, as summarized in (Figure 5(b)). It was shown that TNF-α secretion from murine macrophages can be suppressed by 17S-HDHA [48]. Furthermore, it has been demonstrated that 17S-HDHA is a precursor of the D(S)-resolvins, which are potent anti-inflammatory mediators [49–51]. Similarly, the 17-HDHA epimer 17R-HDHA as well as the D(R)-resolvins were recently shown to be anti-inflammatory in the context of experimental colitis, peritonitis, and arthritis [23, 51, 52].

The liquid chromatograph tandem mass spectrometry (LC-MS/MS) setup used in the study presented here was not able to differentiate between the 17S- and 17R-HDHA epimers and could not reliably identify resolvin D1 in tissue samples. However, as both epimers of 17-HDHA are precursors of anti-inflammatory D-resolvins, the increase in total 17(R/S)-HDHA observed in this study is an indicator of significantly increased formation of these anti-inflammatory lipid mediators derived from DHA.

Indeed, our *in vitro* results presented previously showed a 17-HDHA-triggered decrease in TNF-α secretion [53]. Together with the increased phagocytosis activity by 17-HDHA shown here (Figure 5), this indicates that a mix of the 17R/S-HDHA epimers itself carries anti-inflammatory activity. Data for lipoxin A_4 [54, 55] and resolvin D1 [56] have established the concept of increased phagocytosis as a marker of inflammation dampening and resolution. Therefore, the data shown here for 17-HDHA add to the hypothesis that increasing the phagocytosis activity of macrophages is an important effect of the hydroxylated anti-inflammatory and proresolution lipid mediators derived from either AA or DHA.

Concerning the anti-inflammatory effect of ASA, the results presented here support additional anti-inflammatory mechanisms of ASA as compared to the mere inhibition of prostaglandin synthesis, by enhancing the formation of 17-HDHA in murine colitis. Together with the established effect of ASA in colon cancer prevention, the findings presented here might thus be able to contribute to interest in a careful reevaluation of ASA therapy in patients with UC with its high risk of colon cancer development, possibly in combination with DHA supplementation.

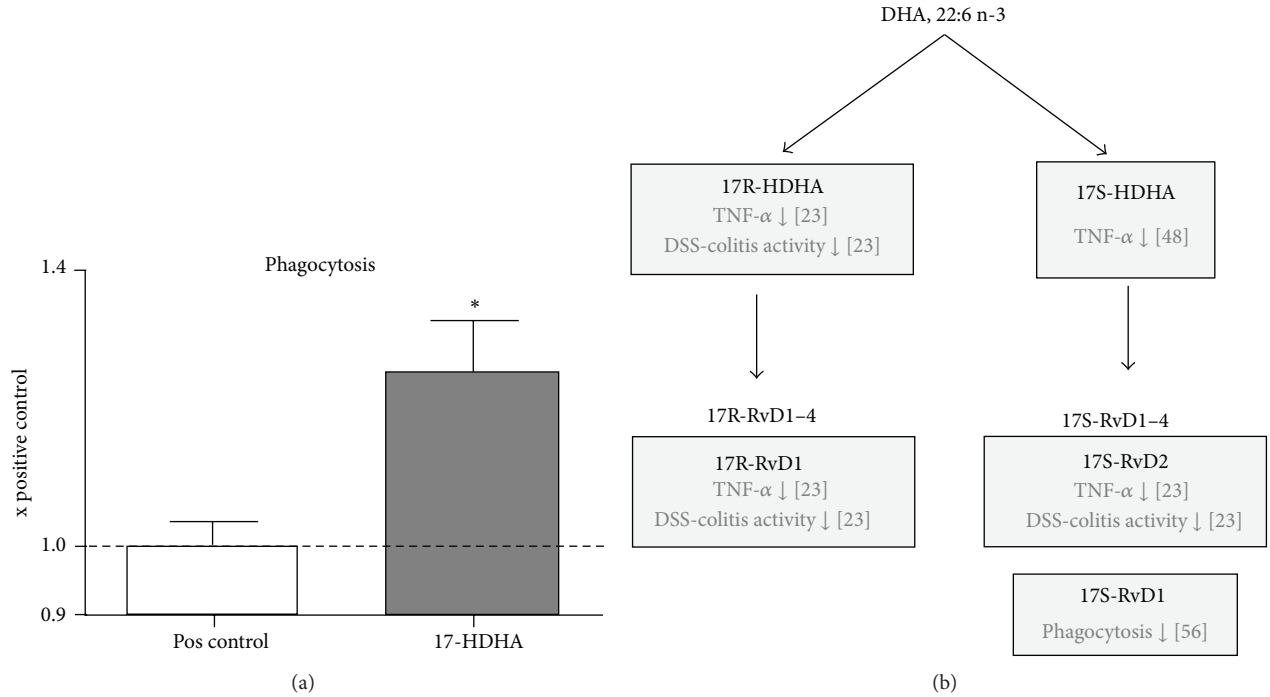


Figure 5: Effect of 17-HDHA on phagocytosis and overview of DHA-derived lipid mediators. (a) Effect of 17R/S-HDHA on phagocytosis in RAW 264.7 murine macrophages ($n = 12$, $^{*}P = 0.026$). (b) Synthesis pathways of DHA-derived anti-inflammatory lipid mediators arising from 17R- and 17S-HDHA, and the resulting mediators are called the resolvins D1-4. Included also are the references to the published effects of the DHA-derived mediators on TNF-α secretion from macrophages [23, 48], phagocytosis [56], and DSS-colitis activity [23].

Conflict of Interests

The authors have no relevant financial and personal interests to declare.

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Research Article

Responses of Growth Performance and Proinflammatory Cytokines Expression to Fish Oil Supplementation in Lactation Sows' and/or Weaned Piglets' Diets

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The study was conducted to investigate whether dietary fish oil could influence growth of piglets via regulating the expression of proinflammatory cytokines. A split-plot experimental design was used with sow diet effect in the main plots and differing piglet diet effect in the subplot. The results showed that suckling piglets from fish oil fed dams grew rapidly ($P < 0.05$) than control. It was also observed that these piglets had higher ADG, feed intake, and final body weight ($P < 0.05$) during postweaning than those piglets from lard fed dams. Furthermore, there was a significant decrease ($P < 0.01$) in the expression of interleukin 6 and tumor necrosis factor-α in *longissimus dorsi* muscle. In contrast, there was a tendency ($P < 0.10$) towards lower ADG and higher feed : gain in weaned piglets receiving fish oil compared with those receiving lard. Meanwhile, splenic proinflammatory cytokines expression was increased ($P < 0.01$) in piglets receiving fish oil during postweaning period. The results suggested that 7% fish oil addition to sows' diets alleviated inflammatory response via decreasing the proinflammatory cytokines expression in skeletal muscle and accelerated piglet growth. However, 7% fish oil addition to weaned piglets' diets might decrease piglet growth via increasing splenic proinflammatory cytokines expression.

1. Introduction

The growth rate of piglet is most rapid during the early postnatal stage, and it is very important to the subsequent growth, final body weight, and marketing time of pigs. Many researches revealed that fish oil could affect young piglets' growth [1–5]. It was reported that 3~5% of fish oil addition to sows' diets was beneficial to suckling piglet growth [1, 5]. However, lactation sow diet supplemented with 8~10% fish oil increased piglets' preweaning morbidity and decreased sows milk production and litter weight gain [2, 6]. Dietary supplementation with 2~3% fish oil promoted weaned piglets growth [3], whereas body weight and feed intake of weaned piglets fed 5% or more fish oil were lower versus corn oil [4].

Previous studies have demonstrated that proinflammatory cytokines could increase muscle protein degradation, reduce muscle protein synthesis, divert nutrients to the synthesis of components in the immune system, and suppress animal growth [7–9]. Fish oil has anti-inflammatory and immunomodulatory effect. Studies in animal models and in human subjects generally reported a decreased production of proinflammatory cytokines in immune cell in peripheral blood and spleen after fish oil supplementation [10–12], and the anti-inflammatory effect has also been shown for suckling piglets of fish oil fed sows [13]. Further study revealed that the immunomodulatory effect was caused by the (n-3) polyunsaturated fatty acids ((n-3) PUFA), especially, eicosapentaenoic acid (EPA, C20:5 (n-3)) and docosahexaenoic acid (DHA, C22:6 (n-3)) in the fish oil [14]. Remarkably, (n-3) PUFA could decrease the proinflammatory cytokines (interleukin 1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α)) expression and secretion in peripheral immune cells and

Table 1: Ingredients and composition of sow and piglet diets (%).

Ingredient	Sow diet		Piglet diet		Nutrients	Sow diet		Piglet diet	
	C	T	C	T		C	T	C	T
Corn	57.5	56.5	48	48	DE (Mcal/kg)[3]	3.45	3.44	3.36	3.35
Wheat bran	7	8	4	4	Crude protein	17.28	17.32	19.38	19.38
Dried whey	0	0	8	8	Calcium	0.80	0.79	0.79	0.79
Lard oil[1]	7	0	7	0	Total phosphorus	0.65	0.63	0.65	0.65
Fish oil[1]	0	7	0	7	Available phosphorus[4]	0.42	0.42	0.39	0.38
Fish meal	3	3	5	5	Lys[4]	1.03	1.03	1.25	1.25
Soybean meal	21.5	21.5	24	24	Met + Cys[4]	0.61	0.61	0.77	0.77
Sodium chloride	0.35	0.35	0.35	0.35					
Calcium carbonate	0.8	0.8	0.5	0.5					
Dicalcium phosphate	1.15	1.15	1.05	1.05					
L-Lysine HCl	0.20	0.20	0.25	0.25					
Methionine	0	0	0.1	0.1					
Vitamin mineral premix	1.5[2]	1.5[2]	1.75[3]	1.75[3]					

[1]The control diet contains 70 g/kg lard oil and test group diet contains 70 g/kg fish oil; 500 mg/kg ethoxyquin was added to oil as anti-oxidative.
[2]Provided following per kg of diet. Cu: 5 mg; Fe: 80 mg; Zn: 50 mg; Mn: 20 mg; I: 0.14 mg; Se: 0.15 mg; V_A: 2,000 IU; V_{D3}: 200 IU; V_E: 44 IU; V_{k3}: 0.5 mg; V_{B1}: 1 mg; V_{B2}: 3.75 mg; V_{B12}: 0.015 mg; biotin: 0.2 mg; folic acid: 1.3 mg; niacin: 10 mg; pantothenic acid: 12 mg.
[3]Provided following per kg of diet. Cu: 6 mg; Fe: 100 mg; Zn: 100 mg; Mn: 4 mg; I: 0.14 mg; Se: 0.3 mg; V_A: 11,000 IU; V_{D3}: 1,100 IU; V_E: 44 IU; V_{k3}: 0.5 mg; V_{B1}: 1 mg; V_{B2}: 3.5 mg; V_{B6}: 1.5 mg; biotin: 0.05 mg; folic acid: 0.3 mg; niacin: 15 mg; pantothenic acid: 10 mg.
[4]By calculation.

suppress animals' inflammatory response [5, 11, 15]. (n-3) PUFA could attenuate the growth inhibition effect by reducing the production of proinflammatory cytokines in several species [4, 16, 17]. Our previous study found that intake of n-3 PUFA leads to significant decreases in the expressions of proinflammatory cytokine genes in loin muscle and spleen, which may stimulate growth in growing-finishing barrows [18]. However, several studies have shown that high level of fish oil (30% or more) supplementation in mice diet increased TNF-α production in splenocytes [19, 20]. In the present study, we hypothesized that the inconsistent performance of piglets that ingested different levels of fish oil mentioned before was due to the different response of proinflammatory cytokines expression to (n-3) PUFA supplementation. Thus, piglets receiving lard directly from the postweaning diet or indirectly from the lactation sows' diets were used as the control. The aim of the present study was to investigate the effect of dietary supplementation of 7% fish oil in lactation sows' and/or weaned piglets' diets on growth performance of piglets and expression of proinflammatory cytokines including IL-1β, IL-6, and TNF-α in skeletal muscle and spleen.

2. Materials and Methods

2.1. Animals and Diets. This trial was carried out in accordance with Huazhong Agricultural University Animal Care and Use Committee guidelines. A split-plot experimental design was used with sow diet effect (L) in the main plots and differing piglet diet effect (PW) in the subplot. Landrace × large white sows ($n = 20$) at 10 d before parturition were assigned to 1 of 2 groups matched for parity and body weight. The test group (T) received a diet supplemented with 7% fish oil, while control group (C) received an isoenergetic, isonitrogenous, and isolipidic diet with 7% lard oil. Fish oil and lard were purchased from China National Cereals, Oils & Foodstuffs Corp. (COFCO Limited). The oil and fat were for food or feed-quality grade. And 500 mg/kg ethoxyquin was added to fish oil and lard as antioxidative. The two diets were formulated to meet NRC requirements of nutrient standards for lactation sow [21]. All diets were prepared weekly to keep them fresh. Two sows in the test group gave birth to less than 3 piglets and thus were dropped from the study. Upon farrowing, sows were fed their treatment diet twice daily (0800 and 1600 h). Sows were initially fed 2.0 kg, and this was increased daily by ~0.5 kg of feed until d 4 postpartum, depending on sows' feed consumption and recovery after parturition. After d 4 postpartum, sows had free access to their diets until weaning. The composition of the sow diets is shown in Table 1. Two sows of the fish oil fed group gave birth to less than 3 piglets and thus were rejected. At farrowing, litters were equalized within dietary treatments to the same number of piglets *per* litter (≈10). Prestarter feed was freely available to the nursing piglets from 7 d of age until weaning.

At 28 d of age, 56 piglets, 28 piglets (half females and half castrated males) *per* group of sows, were moved to cages and reared in nursery room. All piglets remained in the same treatment group defined by their dam; they were then subdivided into two groups of 14 piglets each (one female and one castrated male as a replication) such that a total of 4 experimental groups obtained: CC (control sows-control piglets), CT (control sows-treated piglets), TC (treated sows-control piglets), and TT (treated sows-treated piglets). Piglets from CT and TT groups were fed a starter diet supplemented with 7% fish oil, and an isoenergetic, isonitrogenous, and isolipidic diet supplemented with 7% lard was used as the starter diet fed to CC and TC piglets. The two diets were formulated to meet NRC requirements of nutrient standards for piglet [21]. The composition of the piglet diets is shown in Table 1. Weaned piglets were fed experimental diets from 35 d to 70 d

Table 2: Oligonucleotide polymerase chain reaction primers.

Gene[1]	Accession no.	Primer source	Primer sequences (5′ → 3′)	Orientation	Product size, bp	t_a[2] (°C)
IL-1β	M86725	Pig	ATTCGAGTCTGCCCTGTA	Forward	147	54
			TCTGGGTATGGCTTTCCT	Reverse		
IL-6	M80258	Pig	GCATTCCCTCCTCTGGTC	Forward	93	58
			ATAGTGTCCTAACGCTCAT	Reverse		
TNF-α	AY572787	Pig	CTCCCTCTTTGTCTCCTCC	Forward	77	54
			GCATTGGCATACCCACTCT	Reverse		
β-actin	SSU07786	Pig	GGACTTCGAGCAGGAGATGG	Forward	233	—
			GCACCGTGTTGGCGTAGAGG	Reverse		

[1] *IL-1β*: interleukin 1*β*, *IL-6*: interleukin 6, *TNF-α*: tumor necrosis factor-*α*.
[2] t_a: optimal PCR annealing temperature.

after farrowing. The feed intake of piglets was recorded daily. The body weight of piglets was measured at d 0, 21, 35, and 70 postnatal. The piglets' average daily gain (ADG) during 0~21 d and 35–70 d were calculated as body weight gain/days.

2.2. Collection of Milk and Tissue Samples. At d 21 of lactation, 30~40 mL of milk was collected from the functional glands of each sow after injection of 2 mL of oxytocin. The milk samples were immediately frozen at −20°C for later analysis. The milk samples were analyzed to determine fatty acid composition.

Sixteen piglets (4 *per* treatment, half females and half castrated males) were slaughtered at the end of the experiment. The pigs were deprived of feed for 12 h before humanely slaughter via electrically stun and exsanguination. The samples of the *longissimus dorsi* muscle were collected between the tenth and last ribs, and spleen samples were collected at cacumen. All the collected samples were snap frozen in liquid nitrogen and stored at −80°C for subsequent RNA isolation.

2.3. Fatty Acids Analysis. Fatty acids composition of milk (10 mL), diets (1 g), and diced muscle (2 g) were analyzed by gas chromatography. Lipids were extracted by chloroform: methanol (1 : 1) as described by Folch et al. [22]. Fatty acid methyl ester was prepared for gas chromatography determination using KOH/methanol (0.4 mol/L) [23].

The CP-3800 gas chromatography (Varian, Inc., USA) equipped with a 1177 injector, a flame ionization detector, and a capillary chromatographic column CPSil88 (Varian, Inc., USA) (50 m × 0.25 mm × 0.20 *μ*m) for fatty acid methyl ester was used in this experiment. The injector and detector temperatures were kept at 250°C and 270°C, respectively. Nitrogen was used as carrier gas with a flow rate of 1.0 mL/min, and the split ratio was 1 : 100. The column temperature was programmed as follows: 100°C at first, increased to 200°C (5°C/min), and held constant for 5 min; then, the temperature was increased to 225°C (2°C/min) and kept constant for 2 min. The total analysis time was 39.5 min. The fatty acids were identified by comparing the retention times of the peaks with those of known standards (Sigma Chemical Co., St. Louis, Mo). Response factors for the fatty acids were calculated using the same standard mixtures plus an internal standard [24].

Fatty acid results are presented as g/100 g fatty acids. Saturated fatty acids are the sum of C14:0, C16:0, and C18:0. The monounsaturated fatty acids are the sum of C16:1 (n-7) and C18:1 (n-9). The (n-3) PUFA are the sum of C18:3 (n-3), C20:5 (n-3), C22:5 (n-3), and C22:6 (n-3). The (n-6) PUFA are the sum of C18:2 (n-6) and C20:4 (n-6). The sum of the PUFA was calculated as the sum of (n-3) PUFA and (n-6) PUFA.

2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using the TRIzol regent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's specifications. The RNA samples were quantified spectrophotometrically at 260 and 280 nm. A two-step semiquantitative RT-PCR method was used to measure gene expression [25]. Oligo-$(dT)_{20n}$ (Toyobo, Osaka, Japan) was used as the primer in the first step of cDNA synthesis. Reverse transcription reaction solution (20 *μ*L) consisted of 2 *μ*g of total RNA, 100 U of MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (Toyobo, Osaka, Japan), 20 U of an RNAse inhibitor (Toyobo, Osaka, Japan), 0.5 mmol/L of deoxyribonucleotide triphosphates (dNTP) (Toyobo, Osaka, Japan), and 0.5 *μ*L oligo-dT primers. The cDNA stock was stored at −20°C. The yield of cDNA was measured according to the PCR signal generated from the internal standard housekeeping gene, *β-actin,* which was amplified from 25 to 35 cycles starting with 0.5 *μ*L of the cDNA solution. The volume of each cDNA pool was adjusted to give the same exponential-phase PCR signal intensity for *β-actin* after 25 cycles [26].

Relative RT-PCR [27] was performed to measure gene expression of *IL-1β*, *IL-6*, and *TNF-α* of *longissimus dorsi* muscle and spleen. Primer sequences and optimal PCR annealing temperatures (t_a) are listed in Table 2. PCR was performed on a 2720 Thermal Cycler (Applied Biosystems, USA). The PCR program began with a 94°C denaturation for 5 min, followed by 25~35 cycles denatured at 94°C for 30 s, annealing for 30 s, and extension for 30 s at 72°C, with a final extension at 72°C for 10 min. The linear amplification range for each gene was tested on the adjusted cDNA. The optimal cycle number was then considered to be two cycles lower than the highest cycle of linearity. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide (10 *μ*g/mL). The gel images were digitally captured with G:BOX (Syngene, Cambridge, UK) and densitometry values were measured using the Gene Tool software (Syngene, Cambridge, UK). RT-PCR values are

Table 3: Lipid concentration and fatty acid composition of sow and piglet diets[1].

	Sow diet		Piglet diet	
	C	T	C	T
Lipid, g/100 g diet	11.57	11.32	9.72	9.69
Fatty acid composition, g/100 g total fatty acid				
C14:0	1.13	4.37	1.38	4.56
C16:0	23.32	22.29	24.07	22.21
C18:0	11.77	4.92	9.53	4.02
∑SFA[2]	36.22	31.58	34.98	30.78
C16:1 (n-7)	1.72	6.14	1.73	6.22
C18:1 (n-9)	35.48	26.49	31.79	22.90
∑MUFA[2]	37.20	32.63	33.52	29.12
C18:2 (n-6)	20.09	14.00	20.76	15.80
C20:4 (n-6)	0.20	0.30	0.31	0.14
∑(n-6) PUFA[2]	20.30	14.30	21.07	15.94
C18:3 (n-3)	0.92	0.97	1.05	0.97
C20:5 (n-3)	0.13	6.55	0.04	5.90
C22:5 (n-3)	0.10	0.62	0.06	0.47
C22:6 (n-3)	0.22	3.87	0.05	2.94
∑(n-3) PUFA[2]	1.37	12.01	1.20	10.28
∑PUFA[2]	21.67	26.31	22.27	26.22

[1]Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The position of the first double bond relative to the methyl (n) end of the molecule is also indicated.
[2]SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids; ∑SFA: the sum of C14:0, C16:0, and C18:0; ∑MUFA: the sum of C16:1 (n-7) and C18:1 (n-9); ∑(n-6) PUFA: the sum of C18:2 (n-6) and C20:4 (n-6); ∑(n-3) PUFA: the sum of C18:3 (n-3), C20:5 (n-3), C22:5 (n-3), and C22:6 (n-3); ∑PUFA: the sum of ∑(n-6) PUFA and ∑(n-3) PUFA.

Table 4: Lipid concentration and fatty acid composition of milk of sows fed with and without fish oil[1,2].

	C	T	SEM	*P*-value
Lipid, g/100 g milk solid	5.69	5.81	0.27	0.767
Fatty acid composition, g/100 g total fatty acid				
C14:0	3.43	4.47	0.20	0.003
C16:0	32.44	33.69	0.68	0.218
C18:0	3.76	3.91	0.18	0.548
∑SFA[3]	39.63	42.07	0.73	0.035
C16:1 (n-7)	8.42	10.77	0.23	<0.001
C18:1 (n-9)	27.97	20.73	0.72	<0.001
∑MUFA[3]	36.40	31.50	0.72	<0.001
C18:2 (n-6)	13.56	10.78	0.44	<0.001
C20:4 (n-6)	0.39	0.29	0.03	0.008
∑(n-6) PUFA[3]	13.95	11.07	0.45	<0.001
C18:3 (n-3)	0.56	0.65	0.04	0.104
C20:5 (n-3)	0.04	2.71	0.07	<0.001
C22:5 (n-3)	0.10	1.12	0.04	<0.001
C22:6 (n-3)	0.07	2.53	0.06	<0.001
∑(n-3) PUFA[3]	0.77	7.00	0.11	<0.001
∑PUFA[3]	14.71	18.07	0.49	<0.001

[1]Values are means ± pooled SEM, control $n = 9$, treatment $n = 6$.
[2]Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The position of the first double bond relative to the methyl (n) end of the molecule is also indicated.
[3]SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids; ∑SFA: the sum of C14:0, C16:0, and C18:0; ∑MUFA: the sum of C16:1 (n-7) and C18:1 (n-9); ∑(n-6) PUFA: the sum of C18:2 (n-6) and C20:4 (n-6); ∑(n-3) PUFA: the sum of C18:3 (n-3), C20:5 (n-3), C22:5 (n-3), and C22:6 (n-3); ∑PUFA: the sum of ∑(n-6) PUFA and ∑(n-3) PUFA.

presented as a ratio of the specified gene signal in the selected linear amplification cycle divided by the *β-actin* signal. Data for each replicate represented the mean of three individual RT-PCRs.

2.5. Statistics. Experimental animals were assigned to different treatments in a completely randomized design. Pen was the experimental unit. Statistical analysis of the data was performed with the ANOVA procedure of SAS8.01 [28], and the residual error was used to test the main effect of dietary treatments. The multiple comparisons were preceded with DUNCAN procedure. Data were presented as means ± SEM. Growth performance and gene expression data of postweaning piglets were analyzed as 2 × 2 randomized ANOVA with lactation (L) and postweaning diet period (PW) as effects. The correlation between ADG and expressions of proinflammatory cytokine genes was performed with the CORR procedure of SAS8.01 [28]. Means were considered statistically different at $P < 0.05$.

3. Results

3.1. Fatty Acids Composition of Diets and Milk. Fatty acid composition of sow and piglet diets is shown in Table 3. Fatty acid composition of sow milk at d 21 of lactation is shown in Table 4. Fish oil diets and lard diets had the same levels of lipids, while the (n-3) PUFA were nearly 8~9 times higher in fish oil diets as compared to lard diets. Compared with lard oil, fish oil administration increased the concentration of PEA, DHA and total (n-3) PUFA in sows' milk ($P < 0.01$), whereas, the EPA, DHA, and total (n-3) PUFA contents in milk from fish oil fed sows were lower than those in fish oil added diets which fed to piglets during postweaning period (7.00 g/100 g total fatty acid versus 10.28 g/100 g total fatty acid). As a result, piglets from fish oil fed dams received fewer amount of (n-3) PUFA than weaned piglets fed 7% fish oil diet (0.41% versus 1.00%).

3.2. Fatty Acids Composition in Longissimus Dorsi Muscle. Fatty acid composition of *longissimus dorsi* muscle is shown in Table 5. The saturated fatty acids (C14:0, C16:0, C18:0, and C20:0), C20:4n-6, and n-6 PUFA contents in *longissimus dorsi* muscle of postweaning piglets from fish oil fed dams were significantly lower ($P < 0.05$) than those of piglets from control sows. The C20:1n-9 (0.78% versus 0.62%) and C22:5n-3 (0.84% versus 0.2%) contents were significantly increased ($P < 0.05$) in piglets from sows compared with those from control sows.

Table 5: Fatty acids composition of *longissimus dorsi* muscle.

Fatty acid	Diet[1]				SEM	*P*-value[2]		
	CC	CT	TC	TT		L	PW	L × PW
	Fatty acid composition, g/100 g total fatty acid							
C14:0	1.77	1.72	1.33	1.58	0.10	0.03	0.34	0.17
C16:0	25.07	23.36	22.66	21.62	0.45	0.01	0.02	0.49
C16:1n-7	4.01	4.07	3.02	4.08	0.16	0.11	0.01	0.02
C18:0	10.24	10.78	10.87	7.38	0.59	0.03	0.06	0.01
C18:1n-9	33.41	26.10	34.05	31.53	1.26	0.06	0.01	0.11
C18:1n-7	2.97	3.87	3.50	3.56	0.24	0.66	0.06	0.10
C18:2n-6	16.09	17.38	15.90	13.76	0.52	0.06	0.44	0.02
C18:3n-3	0.65	0.76	0.64	0.79	0.04	0.77	0.01	0.61
C20:0	0.11	0.09	0.09	0.06	0.01	<0.01	<0.01	0.44
C20:1n-9	0.44	0.79	0.56	1.00	0.04	<0.01	<0.01	0.35
C20:4n-6	2.09	1.80	1.49	0.68	0.24	0.01	0.06	0.31
C20:5n-3	0.07	1.43	0.19	1.34	0.10	0.87	<0.01	0.38
C22:5n-3	0.10	0.29	0.84	0.83	0.04	0.01	<0.01	0.04
C22:6n-3	0.22	1.19	0.45	1.57	0.15	0.13	<0.01	0.65
SFA[3]	34.29	31.95	32.96	30.55	0.60	<0.01	<0.01	0.08
MUFA[3]	40.83	34.82	41.14	40.17	1.28	0.05	0.02	0.07
n-6 PUFA[3]	18.18	19.18	17.39	14.43	0.61	0.02	0.16	0.02
n-3 PUFA[3]	1.03	4.22	1.57	4.54	0.27	0.24	<0.01	0.78
PUFA[3]	19.21	23.40	18.96	18.97	0.71	0.08	0.03	0.03
n-6/n-3[3]	17.71	4.55	11.24	3.30	0.65	<0.01	<0.01	0.01
P/S[3]	0.56	0.65	0.54	0.57	0.03	0.13	0.10	0.34

[1]Values are means ± pooled SEM, $n = 7$. CC: all period fed lard diet; CT: post-weaning period fed fish oil diet; TC: lactation period fed fish oil diet; TT: all period fed fish oil diet.
[2]Effects of lactation (L) and the post-weaning (PW) or the interaction between lactation and the starter diet period (L × PW).
[3]Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The position of the first double bond relative to the methyl (n) end of the molecule is also indicated. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids; ∑SFA: the sum of C14:0, C16:0, and C18:0; ∑MUFA: the sum of C16:1 (n-7) and C18:1 (n-9); ∑(n-6) PUFA: the sum of C18:2 (n-6) and C20:4 (n-6); ∑(n-3) PUFA: the sum of C18:3 (n-3), C20:5 (n-3), C22:5 (n-3), and C22:6 (n-3); ∑PUFA: the sum of ∑(n-6) PUFA and ∑(n-3) PUFA.

Table 6: Effect of fish oil supplementation during late gestation and lactation on the performance of suckling piglets[1].

Item	Diet		SEM	*P*-value
	C	T		
Litter weight at delivery, kg	15.41	15.72	0.62	0.723
Body weight at birth, kg	1.58	1.62	0.07	0.811
Body weight at 21 d, kg	5.91	6.35	0.15	0.080
Average daily gain, g	206	225	6	0.048

[1]Values are means ± pooled SEM, control $n = 10$, treatment $n = 8$.

The C16:0, C20:0, and SFA contents in *longissimus dorsi* muscle of piglets receiving fish oil were significantly decreased ($P < 0.05$) compared with those receiving control diets during the postweaning period. The C20:5n-3 (1.39% versus 0.13%), C22:5n-3 (0.56% versus 0.47%), C22:6n-3 (1.38% versus 0.34%), and total n-3 PUFA (4.38% versus 1.3%) contents in *longissimus dorsi* muscle of piglets receiving fish oil were significantly increased ($P < 0.01$) more than those receiving control diets during the postweaning period.

3.3. Growth Performance of Piglets. Growth performance of suckling piglets is shown in Table 6. The body weights at birth and litter weights at delivery were not different ($P > 0.05$) between groups. However, the average daily gain (ADG) of suckling piglets was significantly ($P < 0.05$) higher in the fish oil group than in the control group.

The growth performance of weaned piglets was also significantly ($P < 0.05$) affected by sow diet effect (L) (Table 7). The final body weights (21.29 kg versus 18.79 kg) of postweaning piglets from fish oil fed dams were significantly higher ($P < 0.05$) than those of piglets from control sows. The ADG (325 g/d versus 265 g/d) and average daily feed intake (ADFI) (615 g/d versus 510 g/d) were also significantly increased ($P < 0.05$) in piglets from sows compared with those from control sows, whereas the feed conversion rate (1.91 versus 1.96) was not different ($P > 0.05$) between groups.

The growth performance of weaned piglets was not significantly ($P > 0.05$) affected by piglet diet effect. However, there was a tendency ($P < 0.10$) towards lower ADG (270 g/d versus 321 g/d) and higher feed : gain (2.00 versus 1.87) in piglets receiving fish oil compared with those receiving control diets during the postweaning period, but the ADFI (534 g/d versus 591 g/d) and final body weights (19.18 kg versus 20.90 kg) were not significantly ($P > 0.05$) different between groups. Additionally, final body weight, ADG, and ADFI were significantly higher in group TC than those in group CT ($P < 0.05$).

Table 7: Growth performance of weaned piglets born to fish oil-treated and control sows and fed diets with and without fish oil supplementation in the post-weaning period.

Item	Diet[1]				SEM	*P*-value[2]		
	CC	CT	TC	TT		L	PW	L × PW
Body weight (35 d), kg	9.50	9.50	9.86	9.96	0.41		0.789	
Body weight (70 d), kg	19.66^{ab}	17.91^{b}	22.14^{a}	20.45^{ab}	1.20	0.047	0.163	0.979
Average daily gain, g	290^{ab}	240^{b}	351^{a}	300^{ab}	27	0.036	0.072	0.984
Average daily feed intake, g	533^{ab}	493^{b}	656^{a}	574^{ab}	44	0.028	0.179	0.631
Feed : gain	1.88	2.06	1.88	1.94	0.06	0.328	0.075	0.328

[1]Values are means ± pooled SEM, $n = 7$. CC: all period fed lard diet; CT: post-weaning period fed fish oil diet; TC: lactation period fed fish oil diet; TT: all period fed fish oil diet.
[2]Effects of lactation (L) and the post-weaning (PW) or the interaction between lactation and the starter diet period (L × PW).
[a,b]Means with different letters are significantly different ($P < 0.05$) among groups.

Table 8: The correlation coefficients between ADG and expressions of proinflammatory cytokine genes in *longissimus dorsi* muscle and spleen.

Item	*Longissimus dorsi* muscle			Spleen		
	IL-1β[1]	*IL-6*[1]	*TNF-α*[1]	*IL-1β*	*IL-6*	*TNF-α*
ADG						
Correlation coefficients (r)	−0.7692	−0.3276	−0.3956	−0.7595	−0.3028	−0.2871
P-value	0.001	0.233	0.161	0.001	0.293	0.300

[1]*IL-1β*: interleukin 1*β*, *IL-6*: interleukin 6, *TNF-α*: tumor necrosis factor-*α*.

3.4. Proinflammatory Cytokines Gene Expression in Longissimus Dorsi Muscle and Spleen. Figure 1 shows the results of cytokines expression in *longissimus dorsi* muscle and spleen of weaned piglets in C-C, C-T, T-C, and T-T groups. *IL-6* (Figure 1(b)) and *TNF-α* (Figure 1(c)) expression in *longissimus dorsi* muscle was significantly ($P < 0.05$) decreased in weaned piglets from fish oil fed dams compared with those of piglets from lard fed dams, while the *IL-1β* expression in *longissimus dorsi* muscle (Figure 1(a)) was not different ($P > 0.05$). Additionally, the *IL-1β* and *IL-6* (Figures 1(d) and 1(e)) expression in spleen was not different ($P > 0.05$). However, postweaning piglets from fish oil fed dams had higher ($P < 0.05$) *TNF-α* (Figure 1(f)) expression in spleen.

Proinflammatory cytokines expression of weaned piglets in *longissimus dorsi* was not affected by postweaning fish oil supplementation (PW) or L × PW interaction ($P > 0.05$) (Figures 1(a), 1(b), and 1(c)). However, postweaning fish oil supplementation resulted in significantly increased ($P < 0.01$) spleen *IL-1β*, *IL-6*, and *TNF-α* mRNA abundances (Figures 1(d), 1(e), and 1(f)).

3.5. The Correlation between ADG and Expressions of Proinflammatory Cytokine Genes. The mRNA expressions for proinflammatory cytokines in groups CC, CT, TC, and TT were pooled and compared with the ADG of the corresponding slaughtered piglets in order to evaluate a possible correlation between expressions of proinflammatory cytokines and growth performance. The correlation coefficients between ADG and expressions of proinflammatory cytokine genes are shown in Table 8. Proinflammatory cytokines' expressions in *longissimus dorsi* muscleor in spleen were negatively correlated with piglets' growth. There were statistically significant negative correlations between muscular *IL-1β* expression ($r = −0.7692$, $P < 0.01$) or splenic *IL-1β* expression ($r = −0.7595$, $P < 0.01$) levels and ADG.

4. Discussion

Daily supplementary low level of cod liver oil (50 mL/d, approximately 1%~2%) to sows from day 107 of gestation until weaning did not affect weight gain and overall morbidity of piglets in the study by Taugbøl et al. [29]. It was suggested that 1.75% (17.5 g/kg of diet) of fish oil to pregnant diet and 3.5% (35 g/kg of diet) of fish oil to lactation diet for sows improved growth of their progeny [1]. The previous results showed that the body weight and ADG of suckling piglets at 21 d postnatal were increased by 7% (70 g/kg of diet) fish oil supplementation to the late gestation and lactation sows' diets, which agreed with other researchers [1, 30]. We also found that weaned piglets from fish oil fed dams had a higher growth rate than those from lard oil fed dams during the d 35 to 70 postnatal period. These results revealed that 7% of fish oil supplemented to sows' diets could promote the growth performance of their progeny, and this effect even lasted during the postweaning period.

Interestingly, high level of fish oil supplementation was not beneficial for piglets' growth. It was found that 8% (80 g/kg of diet) fish oil addition to sow diet during late gestation and lactation period decreased litter weight gain of suckling piglets, compared with 8% animal fat (6). Similarly, 10% (100 g/kg of diet) fish oil addition to lactation sow diet resulted in increased piglets' preweaning morbidity and decreased sows milk production (2). We also found the same trend in the present study. The ADG and feed conversion rate

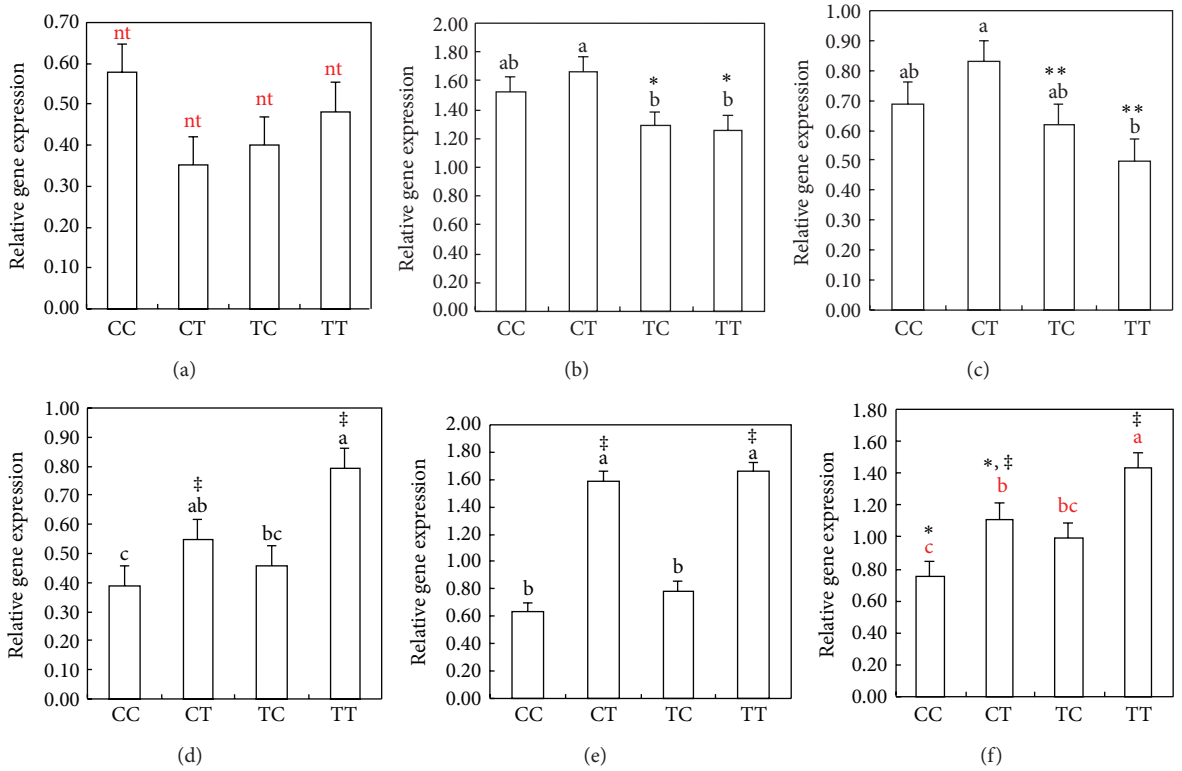

Figure 1: Relative *longissimus dorsi muscle* and spleen cytokines expression of weaned piglets born to fish oil-treated or control sows, and piglets fed diets with and without fish oil supplementation during the postweaning period. (a), (b), and (c) were *longissimus dorsi muscle* cytokines expression; (d), (e), and (f) were spleen cytokines expression. (a) and (d): *IL-1β* expression; (b) and (e): *IL-6* expression; (c) and (f): *TNF-α* expression. Gene signals were normalized to the expression of the housekeeping gene *β-actin* optical density signal. CC: all period fed lard diet; CT: postweaning period fed fish oil diet; TC: lactation period fed fish oil diet; TT: all period fed fish oil diet. Data shown are mean values ± SEM, $n = 4$. *,**: means lactation decrease effect with $P < 0.05$, $P < 0.01$; †,‡: means postweaning increase effect with $P < 0.05$, $P < 0.01$; ^{a,b}: means with different letters are significantly different ($P < 0.05$) among groups; ^{nt}: means have no significant difference.

(FCR) of postweaning piglets were tended to decrease after 7% of fish oil feeding. The results were in general agreement with another study showing that weaned piglets receiving 5% (50 g/kg of diet) menhaden fish oil + 1% (10 g/kg of diet) corn oil during the d 24 to 36 postnatal period had a lower body weight gain and ADFI than those fed 6% corn oil [4].

The three proinflammatory cytokines IL-1, IL-6, and TNF-α could induce great metabolic changes [31]. The cytokines appear to be primarily derived from macrophage-rich tissues, such as the liver and spleen, and various myelomonocytic cells as well. However, it was reported that cytokines are also produced by cells not traditionally considered to be part of the immune system such as adipocytes and myofibers [32, 33], which are effective sources and targets of cytokines [34, 35]. Proinflammatory cytokines mediate "reprogramming" of metabolism and shift the partitioning of dietary nutrients away from skeletal muscle accretion toward metabolic responses that support the immune system [7, 31]. Skeletal muscle as the biggest body tissue, amounting for 40~45% of the total body mass, is also the most quickly growing tissues during early postnatal period except for bone and nervous system. Collectively, these data suggest that the autocrine/paracrine actions of cytokines are of potential importance in muscle and thus in postnatal animal growth.

There was a paucity of data pertaining to the cytokines expression after (n-3) PUFA administration in skeletal muscle. Previous study in our laboratory showed that during normal physiological processes feeding linseed diet (rich in α-linolenic acid) suppressed the expression of proinflammatory cytokines in *longissimus* muscle of growing-finishing barrows and decreased serum level of TNF-α from 0.073 to 0.052 ng/mL during normal physiological condition. These results showed that the appropriate reduction of serum TNF-α levels that ranged from 0.073 to 0.052 ng/mL might be beneficial to increase the longissimus muscle mass under normal physiological condition [18]. EPA and DHA were considered to be more potent in regulating immune function [14]. Fish oil has been reported to decrease production of proinflammatory cytokines because of the high content of EPA and DHA [10–12]. The *IL-6* and *TNF-α* expression was significantly suppressed in muscle of weaned piglets from fish oil fed dams in the present study. We also found negative correlation between ADG and proinflammatory cytokines expression. Thus, suppression of cytokines expression in muscle of piglets may positively reduce the inflammatory response in skeletal muscle, attenuate the negative effect of the potent proinflammatory cytokines, and promote piglet growth.

Remarkably, it was not always consistent with the result which moderates fish oil supplementation decreased production of proinflammatory cytokines. The study in rodents suggested that mice fed as high as 30% (300 g/kg of diet) of fish oil for 4~6 weeks increased the TNF-α syntheses in splenocytes [19]. Barber et al. [20] also reported that TNF-α secretion was increased in splenocytes separated from 4% of EPA (>30% fish oil according to our data) fed mice after stimulation with LPS. We also found that all of the three proinflammatory cytokines expressions were significantly increased in spleen after 7% of fish oil feeding during post-weaning. High production of proinflammatory cytokines in spleen, the biggest immune organ, may increase proinflammatory cytokines levels of the whole body, suppress skeletal muscle protein accretion, and impact animal growth [7, 9, 36].

The results suggested that administration of 7% fish oil to lactation sows significantly increased the growth rate of their progeny during postweaning period. However, the addition of 7% fish oil to diets of postweaning piglets was likely to decrease the growth rate and FCR of piglets. The contrary effects of 7% fish oil supplementation to sows' or weaned piglets' diets may be due to the different content of (n-3) PUFA that the piglets received. The current results demonstrated that the EPA, DHA, and total (n-3) PUFA contents of milk were lower than those of piglets' diets (2.71% versus 5.90%, 2.53% versus 2.94%, and 7.00% versus 10.28%, resp.). As a result of the lower level of lipid content in milk compared with that in the diet, piglets only received ~40% of (n-3) PUFA from milk relative to that ingested from fish oil diets directly. Our previous study found that intake of n-3PUFA could increase the n-3PUFA content in *longissimus dorsi* muscle of growing-finishing pigs [37]. In the current experiment, C22:5n-3 contents in *longissimus dorsi* muscle of postweaning piglets from fish oil fed dams were increased (0.84% versus 0.2%) more than those of piglets from control sows. However, the C20:5n-3 (1.39% versus 0.13%), C22:5n-3 (0.56% versus 0.47%), C22:6n-3 (1.38% versus 0.34%), and total n-3 PUFA (4.38% versus 1.3%) contents in *longissimus dorsi* muscle of piglets receiving fish oil were increased more than those of piglets receiving control diets during the postweaning period. These results revealed that n-3PUFA contents in the muscle were higher in piglets fed fish oil directly than that in weaned piglets from fish oil fed dams. It was further observed that the *IL-6* and *TNF-α* expression either in the muscle or in spleen was lower in weaned piglets from fish oil fed dams than that in piglets fed fish oil directly. It could be concluded that moderate (n-3) PUFA intake was beneficial to piglets' growth by decreasing proinflammatory cytokines production and suppressing inflammatory response in skeletal muscle. However, high intake of (n-3) PUFA may promote splenic proinflammatory cytokines production and impact animal growth consequently.

In conclusion, fish oil might regulate piglet growth through modulating proinflammatory cytokines production in body tissues. Appropriate levels of fish oil supplementation to sows' diets may increase (n-3) PUFA content in milk and (n-3) PUFA ingestion of their progenies, decrease the proinflammatory cytokines expression and their unfavorable effects on skeletal muscle, and thus promote growth of piglets. However, high levels of fish oil supplementation to postweaning piglets' diets may increase splenic proinflammatory cytokines expression and thus negatively impact the growth of weaned piglets. Given that the limited replicate number of slaughtered animals in the present study may be not enough to generate a definitive conclusion, further investigation with more number of samples is required to determine the appropriate fish oil supplementation level, duration, and the precise mechanisms by which long chain (n-3) PUFA affect piglets' immunity and growth.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Jie Luo and Feiruo Huang contributed equally to this work.

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Review Article

Nutritional Properties of Dietary Omega-3-Enriched Phospholipids

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Dietary fatty acids regulate several physiological functions. However, to exert their properties, they have to be present in the diet in an optimal balance. Particular attention has been focused on tissue highly polyunsaturated fatty acids (HPUFAs) n-6/n-3 ratio, influenced by the type and the esterified form of dietary fatty acids. Dietary EPA and DHA when esterified to phospholipids (PLs) are more efficiently incorporated into tissue PLs and seem to possess peculiar properties through specific mechanism(s) of action, such as the capacity to affect endocannabinoid biosynthesis at much lower doses than EPA and DHA in triglyceride form, probably because of the above mentioned higher incorporation into tissue PLs. Downregulation of the endocannabinoid system seems to mediate the positive effects exerted by omega-3-enriched PLs on several parameters of metabolic syndrome. PLs are one of the major dietary forms of EPA and DHA we are exposed to with the everyday diet; therefore, it is not surprising that it guarantees an effective EPA and DHA nutritional activity. Future studies should address whether EPA and DHA in PL form are also more effective than other formulations in ameliorating other pathological conditions where n-3 HPUFAs seem to exert beneficial activities such as cancer and psychiatric disorders.

1. Introduction

Dietary fatty acids have a wide range of physiological functions, and, to fully exert their effects, they have to be present in the diet in a certain balance. Thus a proper amount of all families of fatty acids needs to be taken into account in making dietary recommendations. Particular attention has been paid to the ratio between highly polyunsaturated fatty acids (HPUFAs) n-6 (mainly arachidonic acid (20 : 4n-6; ARA)) and n-3 HPUFAs (mainly EPA and DHA) in tissues as a consequence of dietary n-6/n-3 ratio, since an inverse correlation between circulating n-3 HPUFAs and the occurrence of coronary heart disease has been observed [1].

In addition, dietary n-3 HPUFAs have been shown to prevent and modulate essential fatty acid (EFA) deficiency in infancy (retinal and brain development), autoimmune disorders, Crohn's disease, and cancers of breast, colon, and prostate. They have also been shown to exert beneficial effects in a wide range of psychiatric disorders [2]. However, even though they appear as the panacea for any pathological states, some epidemiological studies cast doubts on some of these beneficial effects [3]. It is therefore compelling to individuate the mechanism(s) of action and what are the conditions where n-3 HPUFAs maximise their nutritional activities. The metabolism and biological activities of marine n-3 PLs have been recently reviewed [4]; in the present paper, we therefore focused our attention on the putative mechanisms of action by which EPA and DHA in PL form exert their distinct effects with respect to the triacylglycerol (TAG) form.

2. Effects of EPA and DHA on Oxygenated Eicosanoid Biosynthesis

It has been shown that EPA and DHA, besides possessing antithrombotic properties [5], are able to decrease both the formation and tissue incorporation of ARA and, hence, to reduce the release of inflammatory acute-phase proteins [6, 7].

In fact, proinflammatory eicosanoids, including prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), are synthesized from ARA on phospholipase-mediated release from cellular phospholipids (PL) [8–10], while n-3 HPUFAs are prostaglandin and leukotriene precursors (PGE_3 and LTB_5), with a quite low conversion coefficient, with anti-inflammatory properties [11–13].

The daily endogenous formation of PGs is much lower than the daily ARA intake and the endogenous ARA production. EPA and DHA inhibit the in vitro production of ARA-derived eicosanoid [14, 15], but not in vivo, where eicosanoid formation seems to be less affected by EPA and DHA dietary intake [16, 17]. To observe a slight decrease of in vivo production of ARA-derived eicosanoids several grams per day of EPA and DHA for many weeks are needed therefore the beneficial effect of a relatively low dose of n-3 HPUFAs on death from coronary heart disease in the GISSI study [18] is unlikely to be associated with changes in eicosanoid production. In vivo PG production by dietary supplements with pure ARA (e.g., 6 g/day for 2-3 weeks in humans) can be slightly increased [19]. These studies suggest that eicosanoid formation is not promptly influenced by ARA dietary intake, at least within the range of traditional human diets [20, 21]. However, it has been shown that very high amounts of fish oil may decrease PG formation with consequent weak anti-inflammatory and analgesic effects [22].

Prescott showed that isolated human polymorphonuclear leukocyte neutrophil (PMNs) incorporated EPA, mainly in phospholipids (PLs), and were able to convert it to LTB5, suggesting an impairing replacement of ARA with EPA in PMN membranes [23]. These results are in contrast with the report of Lammi-Keefe and coworkers, who did not observe such an effect on neutrophils probably because of the low amount of EPA consumed with the dietary supplement in their experiments [24].

Therefore, these mechanisms may only explain in part the effects demonstrated in different experimental conditions [25–27].

3. Interaction with Other Dietary Fatty Acids

EPA + DHA supplementation, with or without different levels of gamma-linolenic acid (GLA, 18 : 3n-6), was shown to induce changes on fatty acid profile [28]. Intake of both GLA and EPA + DHA decreased ARA concentrations in animal tissues and cells [29, 30], not in humans supplemented with GLA + EPA (1 : 1) [31]. However a significant reduction in ARA concentrations was observed in serum PLs, likely induced by the competition with EPA and dihomo-γ-linolenic acid (DGLA) for esterification into cellular PLs and the attenuating effect of n-3 fatty acids on Δ5-desaturase, necessary for the conversion of DGLA to ARA [32, 33].

4. Does Dietary Form of PUFAs Affect Their Lipid Incorporation and/or Nutritional Activities?

It is often overlooked what is the most efficient dietary form to convey dietary fatty acids in terms of tissue bioavailability and biological effects. The choice of the form is mostly dictated by practical or economic reasons. However, there are several reports showing that PL-bound EPA and DHA have distinct effects with respect to TAG-bound EPA and DHA.

Fish oil supplementation improves lipoprotein profiles by reducing plasma LDL, cholesterol, and TAG levels and increasing HDL cholesterol [34–36] through inhibition of TAG and VLDL synthesis in the liver [37, 38]. Such effects have been generally obtained through prolonged supplementation of 1 or more g/day of EPA and/or DHA, marketed either as TAG or ethyl ester (EE). In a 5-week supplementation of 4 g EPA or DHA, as EE, added to the ordinary diet, EPA showed a more rapid and comprehensive increase in serum PLs than did DHA [39].

Interestingly, it has been shown that EPA and DHA as krill powder are able to significantly decrease plasma triglycerides (TAGs) at much lower doses, about 400 mg per day [40].

However, it cannot be ruled out if some of the effects may be related to different actions of EPA and DHA. In fact some authors reported that EPA accumulated faster than did DHA in plasma [41, 42], leukocytes [43], and erythrocytes [44]. Willumsen et al. reported that EPA decreased serum TAGs in rats, probably through an induction of FA mitochondrial oxidation, whereas DHA did not [45]; however, other authors reported that DHA had similar TAG-lowering effect to EPA [39, 46]. Conversely others reported a concomitant increase of total, LDL, and HDL cholesterol concentrations when n-3 HPUFAs, particularly DHA, have been administered in amounts of at least 1 g/day [47]. Therefore, when comparing the effects of the form, a similar EPA/DHA ratio should always be used [48].

In addition, it has also been suggested that the food matrices may affect the bioavailability. Visioli and coworkers observed that the administration of as low as 300 mg/day of EPA + DHA to healthy subjects, incorporated into milk, subdivided into smaller doses throughout the day, for 3–6 weeks, resulted in a significant increase in EPA and DHA levels in plasma lipids as well as in HDL concentrations, while TAG concentrations decreased without affecting total cholesterol concentration [49]. This is noteworthy since the same effects on TAG and HDL concentrations have been obtained with 1 to 7 g/day of EPA and DHA [50].

A recent paper by Galli et al. suggests that variable blood levels of n-3 HPUFAs are due to their presence as common dietary components and, possibly, are responsible, among other reasons, for the variability of the results observed; hence, it is recommended to apply a selection of subjects with uniform n-3 background levels before a trial begins [51].

Thus, it is quite difficult to make any recommendation about n-3 HPUFA doses since the matrices, the form, and the quality may greatly influence tissue bioavailability.

American Heart Association, AHA, dietary guidelines for n-3 HPUFAs and fish for primary prevention of coronary diseases, on a food-based approach [52], are two servings of fatty fish per week [53], 250–500 mg EPA + DHA per day [54], while it is suggested a larger daily intake, 1 g or more, for a TAG-lowering effect [55–57]. Indeed fish oils are major sources of n-3 HPUFAs, while other types of oil, especially vegetable oils, are a good source of ALA [58]. However, it

should be borne in mind that even though ALA shares with EPA and DHA the double bond at the third carbon from the methyl end that characterises the n-3 PUFA family, and it is their dietary precursor, possesses distinct metabolism and nutritional properties [59].

5. Digestion, Absorption, and Bioavailability of PL-Bound EPA and DHA

Dietary fat is mainly composed of TAG with a small portion of PLs (3–6%) [60]. The daily intake of PL/day is 2–8 grams. The most common PL in the intestinal lumen is phosphatidylcholine (PC) which is derived mostly from bile (10–20 g/day in humans) and also from the diet, while other PLs, such as phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), are present in much smaller amounts.

Fatty acid chain length and unsaturation number influence fat digestion, absorption, transport in the blood, and metabolism at cellular level. Medium-chain fatty acids (MCFA) are better absorbed than longer fatty acids because they can be dissolved in the aqueous phase and then be absorbed bound to albumin and transported to the liver directly by the portal vein [61]. Dietary TAGs structure influences the bioavailability of fatty acids. For example palmitic acid in fat human milk is well absorbed because of its incorporation in the sn-2 position of glycerol backbone [62]. On the other hand, whether fatty acids esterified in TAGs or in PLs impact on their absorption and tissue incorporation is still debated. TAGs and PLs are digested and absorbed in different ways in the small intestine. The first requires emulsification by bile salts while PLs can spontaneously form micelles and be conveyed in an aqueous environment. According to studies in humans and animals, oral administration of labeled PC leads to the rapid appearance of labeled PC in plasma, with rather weak formation of labeled TAGs [63]. In contrast to TAGs, PLs are not hydrolyzed by lingual or gastric lipases but only in the small intestine [64]. After oral administration, PC is more than 90% absorbed by the intestinal mucosa via conversion to LysoPC because PC is hydrolyzed by pancreatic phospholipase A2 (PLA2) which releases the fatty acid from the sn-2 position. After absorption by the enterocytes, this LysoPC can be reacylated into PC, while the previously released fatty acid can be used for TAG synthesis [65–67]. The absorbed PC is then incorporated into chylomicrons (CM) and, after degradation to the TAG-rich particles, is taken up by the high-density lipoprotein (HDL) fraction which occurs relatively rapidly, that is, within 5-6 hours of PL ingestion [66, 68]. A small proportion of this PC is taken up without prior hydrolysis. Therefore, dietary PLs may affect the lipoprotein composition and metabolism. It has been reported that lipoproteins secreted by the rats small intestine after the infusion of triolein were CM, whereas those secreted after egg PL infusion were VLDL-size particles [69]. Recently, piglets fed with HPUFA-TAG formula had higher HPUFA content in LDL phospholipids than those fed with HPUFA-PL formula. The opposite results were found in HDL PLs, indicating that dietary HPUFAs in form of TAG or PL differently affect the composition of HDL and LDL PLs [70]. Therefore, PLs in the lipoproteins can influence the distribution of lipoproteins in the body and fatty acid tissue incorporation.

Some studies in newborn infants have indicated that dietary PLs may be better absorbed than TAGs. In particular, a study on a group of preterm infants fed with different formulae has shown that the absorption of DHA was higher in those infants receiving the HPUFA-PL formula than in infants receiving breast milk or the HPUFA-TAG formula [71].

Maki et al. showed that EPA and DHA from krill oil (KO), mainly bound to PLs, were absorbed at least as efficiently as EPA and DHA from fish oil (FO), which are in the TAG form [72]. However, Schuchardt et al. [73] compared the uptake of three EPA + DHA formulations derived from fish oil (reesterified (rTAG), EE, and KO, mainly PL) and showed that the bioavailability of n-3 HPUFAs may vary according to their esterified form. The highest incorporation of EPA+DHA into plasma PLs was obtained by KO, followed by FO rTAG and then by EE. A study on the incorporation of labeled DHA into platelet and red cell PLs showed that, in platelets, [13C]DHA accumulated in both PC and PE, although a little faster in PC [74]. Also the availability of LysoPC and LysoPE has been well documented in platelets [75–77]. In contrast to platelets, [13C]LPC could be detected in erythrocytes when [13C]DHA started rising in PC [78]. This is in agreement with the hypothesis that DHA is preferentially esterified to LPC in erythrocytes [79] and is subsequently reacylated into PC [80]. Interestingly, DHA levels in erythrocytes may be considered, at least in part, as an index of that present in the brain, and LysoPC might be a preferential vehicle of DHA to the brain, as suggested in young rats [81, 82].

6. Biological Activities of PL-Bound EPA and DHA

n-3 HPUFAs in fish products are mainly bound to PLs, while in fatty fish, such as salmon, n-3 HPUFAs are bound to PLs and TAGs in a 40 : 60 ratio [83]. On the contrary, most of n-3 HPUFAs available as supplement, like FO, consist almost exclusively of TAG-bound n-3 HPUFAs. This seems like an important issue since dietary PLs have consistently been shown, per se, to affect plasma and liver lipid levels in experimental animals [84]. A recent work has demonstrated that the addition of dairy milk PLs to the diet at a level of 2.5% by weight (wt%) can reduce cholesterol and TAG levels in high-fat-fed C57BL/6 mice [85].

KO, extracted from Antarctic krill (*Euphavsia superba*) [86], is a relatively new source rich in n-3 HPUFAs in the form of PLs, mainly PC, rather than TAGs; the proportion of PLs in the total lipids of krill has been reported to vary between 30 and 60%, depending on krill species, age, season, and harvest time [87]. Moreover, KO contains a lipid-soluble antioxidant, astaxanthin, that may preserve KO from oxidation. The peculiar composition may influence the tissue bioavailability of n-3 HPUFAs and might be responsible, in part, for

the health-promoting effects of KO, such as its anti-inflammatory and hypolipidemic properties in humans [88, 89].

A daily intake of 3 g KO, containing 543 mg EPA + DHA, increased the plasma level of EPA and DHA to the same extent as dietary FO, containing 864 mg EPA + DHA [90], suggesting a comparable absorption for EPA and DHA after KO treatment, if not better than after FO treatment. There was, however, no significant difference across the treatments (KO, FO, and control) for TAG and lipoprotein response, confirming a previous study that also failed to detect any change in plasma lipids [91], probably because of the short period of treatment, the normolipidemic status of the subjects, or the low amount of EPA and DHA. On the other hand, in another study, a small increase in LDL cholesterol and no effect on HDL cholesterol were found after FO treatment [92]. Another controversial aspect is the hs-CRP, a marker of systemic inflammation, which showed no change among KO, FO, or placebo treatments [91, 93, 94], while in individuals with a proinflammatory status, an association among DHA/EPA and hs-CRP reduction has been reported [95].

In mice fed with high-fat diet, it has been demonstrated that n-3 HPUFA in PLs is more efficacious than TAG in reducing hepatic steatosis, low-grade inflammation in white adipose tissue [96], blood lipid levels, and glycaemia [97, 98]. Moreover, increased hepatic expression of fatty acid oxidation genes and downregulation of lipogenic genes were observed. The latter effect was stronger in the PL-treated mice, and a reduced plasma insulin and adipocyte hypertrophy was observed only with the PLs form [96]. It is likely that supplementation of n-3 HPUFA as PLs exerts stronger biological effects compared with the TAG form because (i) various PL species can also act as ligands for nuclear receptors involved in the transcriptional regulation of steroidogenesis and cholesterol metabolism [99, 100] and (ii) the PLs form has been shown to augment the bioavailability of DHA and EPA in both rodents [69, 101] and humans [71, 89, 102].

Other areas where EPA and DHA have been claimed to have an influence are neuropsychiatric disorders. Results from several epidemiologic studies [103, 104] suggest that dietary consumption of n-3 HPUFAs affects neuropsychiatric disorders, presumably because of their structural and neurochemical involvement in pathophysiological processes [105–107]. Interventions ranging from 1 to 6.2 g/d EPA and from 0 to 3.4 g/d of DHA were associated with a therapeutic effect in a broad spectrum of psychiatric disorders [108–111]. Children supplemented with n-3 HPUFA PLs and FO showed an increase in EPA, docosapentaenoic acid (DPA), and DHA concentrations and a decrease in ARA and adrenic acid concentrations in plasma PLs but not in TAGs and cholesterol ester fractions [112], as observed in previous studies [113]. A negative feedback mechanism on desaturase enzymes [114, 115] could be associated with these alterations. On the other hand, the same study showed limited effects on n-3 HPUFA concentrations and none on n-6 HPUFA concentrations in erythrocytes following n-3 HPUFA PLs and FO supplementation [112]; these observations are inconsistent with a previous study [116]. The controversial findings could be explained by the low n-3 HPUFA dose intervention and the kinetics of dietary EPA and DHA and/or the participants' metabolism.

In Vaisman study total test of variables of attention (TOVA) score increased in patients supplemented with n-3 HPUFA PLs and with FO with a limited extent, providing about 250 mg/d EPA/DHA. These findings differ from previous reports in which even larger amounts of DHA [117] or EPA + DHA [118] supplementation in attention-deficit hyperactivity disorder (ADHD) children for 2–4 months were shown to result in elevated plasma PLs DHA concentrations and in no effect on a continuous performance test (CPT) scores.

Although dietary manipulation of n-3 HPUFAs in the brain is complicated by the high concentrations in this organ, supplementation of DHA-containing PLs, such as the bovine brain cortex PS, to animal models was shown to attenuate neuronal effects of aging [119] and to affect behaviour as well [120, 121]. DHA supplementation of 345 mg/d or 3.6 g/body wt, in ADHD children, provided as EE [117] in capsules or as TAG in functional food [118], induced a pronounced increase in blood n-3 HPUFAs, but failed to affect ADHD symptoms. Results of recent studies, in which EPA- and DHA-enriched FOs were provided along with n-6 HPUFA-containing oils and/or vitamins to ADHD children, were conflicting [116, 122–124]. Interestingly, early observations suggested that dietary HPUFAs esterified to PLs rather than TAGs are more effective substrates for brain tissues accretion in term baboons [125].

7. PL-Bound EPA and DHA May Exert Their Biological Activity by Affecting Endocannabinoid Biosynthesis

Different studies support the hypothesis that the composition of dietary fatty acids can affect energy homeostasis via changes of endocannabinoid system (ECS). The endogenous cannabinoid system is an ubiquitous lipid signaling system that appeared early in evolution and which has important regulatory functions throughout the body in all vertebrates. The main endocannabinoids are molecules derived from ARA hydrolyzed from membrane PLs. In particular, anandamide (arachidonoylethanolamide or AEA) [126–128] derives from hydrolysis of membrane PLs at the sn-1 position, while 2-arachidonoylglycerol (2-AG) derives from hydrolysis at the sn-2 position [129–132]. They bind to a family of G-protein-coupled receptors, of which the cannabinoid CB1 receptor is widely distributed in different brain areas and peripheral tissues. However, by the same biosynthetic pathway of anandamide, other structure-related lipid messengers, palmitoylethanolamide (PEA) or oleoylethanolamide (OEA), are formed by palmitic acid or oleic acid, respectively, esterified in sn-1. Being the latter fatty acids preferentially incorporated in sn-1, PEA, and OEA are more abundant than anandamide. They exert their biological activity by interacting with other receptors. OEA, an anorectic mediator that affects lipid and glucose metabolism, activates PPAR-alpha [133]. PEA exerts anti-inflammatory actions via direct activation of PPAR-alpha [134], transient receptor potential of vanilloid type-1 (TRPV1), or PPAR-gamma [135].

From different studies it is reasonable to hypothesize that the tissue levels of endocannabinoids are in part regulated by the activity of the corresponding biosynthetic precursors and in part by catabolic enzymes, fatty acid amide hydrolase (FAAH) [136, 137], or monoacylglycerol lipase (MAGL) [138, 139]. ARA and possibly also glycerol and ethanolamine, produced from the hydrolysis of 2-AG and AEA, are rapidly incorporated into membrane PLs [140, 141]. The tissue levels of endocannabinoids may also depend on the availability of their biosynthetic precursors ARA in PLs [142–144]. In fact, the diet-induced changes were accompanied by changes in the corresponding fatty acids esterified to individual PLs. Watanabe et al. [145] found that mice fed with an n-3-PUFA-deficient diet exhibited higher brain 2-AG levels. Furthermore, short-term supplementation of DHA-rich FO reduced brain 2-AG levels as compared with the diet supplemented with low n-3 PUFAs. The authors observed a concomitant decrease in ARA levels and an increase in DHA levels in the major brain phospholipid species of mice fed with the FO diet as compared with those fed with the low n-3 PUFA diet.

The concentrations of endocannabinoids and their congeners could be modified by the dietary content of HPUFAs or by their essential biosynthetic precursors, mainly in peripheral tissues. A study has been carried out in vitro to determine whether incubation of cells with certain free fatty acids can affect locally produced AEA and 2-AG levels [146]. It has been shown that incubation of 3T3F442A mouse adipocytes with ARA strongly elevates 2-AG levels as well as the amounts of ARA esterified in TAGs and on the glycerol sn-2 position, but not on the sn-1, into PLs, whilst incubation with DHA decreased 2-AG and AEA levels and the amounts of ARA esterified on both sn-2 and sn-1 positions of PLs, but not on TAGs. This suggests that dietary HPUFAs might modulate fatty acid composition of adipocyte PLs that act as endocannabinoid precursors, and then it can be assumed that n-3 HPUFAs might have their beneficial effects in abdominal obesity, dyslipidemia, and insulin resistance by CB1-mediated lipogenic actions of endocannabinoids in adipocytes [147–149]. It has been shown that endocannabinoids can affect energy metabolism both by stimulating food intake and by affecting energy processing in the adipose tissue, liver, pancreas, and skeletal muscle [147, 149, 150]. Indeed, it has been shown that increase of peripheral endocannabinoid levels in both fasted and postprandial obese and overweight individuals correlates with intraabdominal obesity, glucose intolerance, dyslipidemia, and dyslipoproteinemia [150–154]. Therefore, dietary fatty acids by modulating ARA levels in tissue PLs may influence endocannabinoid biosynthesis and thereby downregulate an overactive endocannabinoid system.

In a recent study [49], the effects of dietary n-3 HPUFAs, in the form of either FO or KO, balanced for EPA and DHA content, on liver and heart fat and inflammation in Zucker rats (a model of obesity and related metabolic dysfunctions), were analyzed, as well amount of AEA and 2-AG in the abdominal and subcutaneous fat, liver, and heart. Rats fed with n-3 HPUFA diets had significantly lower liver TAGs and reduced peritoneal macrophage response to inflammatory stimulus than control rats; only in KO-fed rats, heart TAGs were significantly lowered. These effects were associated with a lower concentration of the endocannabinoids, AEA and 2-AG, in the visceral, but not subcutaneous, adipose tissue, and of AEA in the liver and heart; these decreased endocannabinoid levels were, in turn, associated with lower levels of ARA in membrane PLs. Since also CB1 antagonists can produce anti-inflammatory effects in macrophages [155], the observed n-3 HPUFA-induced reduction in endocannabinoid levels in the visceral adipose tissue might also be responsible for the dampened inflammatory response caused by FO and KO.

The dietary imbalance among macronutrients leads to metabolic derangement of glucose and lipid disposal characterized by a marked dyslipidemia, increased insulin resistance, and fatty liver, which are some of the characteristic features of the metabolic syndrome.

Two studies [49, 96] showed that dietary DHA and EPA in the form of PLs are superior to TAGs with respect to the preservation of glucose homeostasis and the reversal of hepatic steatosis, adipocyte hypertrophy, and low-grade inflammation. The higher efficacy of n-3 HPUFAs administered as PLs was associated with their better PL bioavailability and with a relatively strong suppression of the levels of major endocannabinoids in white adipose tissue and plasma, suggesting that modulation of the endocannabinoid system activity contributed to their greater efficacy when compared to TAG form of n-3 HPUFA. The pathophysiological role of the endocannabinoid system in the development of adipose tissue inflammation [146] or hepatic steatosis [147, 148] is well described; moreover, the anti-inflammatory effects of adipocyte-derived N-acyl ethanolamines EPEA or DHEA, that is, the amides of EPA and DHA, have recently been suggested to play a role [149, 156]. The increase in obesity prevalence in the United States may be associated with the increased consumption of linoleic acid (LA), the precursor of ARA, and hence the precursor of endocannabinoids [157, 158]. This hypothesis was supported by dietary experiments in mice fed with various diets differing in the LA content that was positively correlated with the levels of ARA, 2-AG, and AEA in PLs from liver and erythrocytes and also promoted accumulation of body fat [158]. On the other hand, addition of EPA and DHA to the diet resulted in a decrease of endocannabinoid levels in the liver and also in hypothalamus of mice fed with experimental diets with a high LA content [158]. The data further support the role of dietary LA as the key factor controlling the activity of the endocannabinoid system and the attenuation of this activity as the key mechanism underlying antiobesity effects of dietary n-3 HPUFA supplementation under these conditions. However, the role of various tissues in the metabolic impact of downregulated endocannabinoid system activity in response to n-3 HPUFA should be better characterized [159].

An association between elevated peripheral levels of endocannabinoids and the metabolic syndrome has been reported in a study of Piscitelli and coworkers [98]. They showed that 8 weeks of a high-fat diet increased endocannabinoid levels in all tissues except the liver and epididymal adipose tissue, while KO reduced AEA and/or 2-AG levels in all tissues but not in the liver, usually in a dose-dependent manner. KO was shown to affect levels of

endocannabinoids in part by reducing the availability of their biosynthetic precursors. KO supplementation was also accompanied by elevation of PEA levels, and, given its role as anti-inflammatory agent [160] and the previous observation that n-3 HPUFAs exert a protective effect against muscle damage induced by the proinflammatory cytokine TNF-alpha [161], the authors speculate that increased PEA levels might protect skeletal muscle from the damaging effect of TNF-α and contribute, together with KO-induced elevation of adiponectin levels, to the anti-inflammatory effects of KO. Since PEA is dysregulated in several tissues of obese Zucker rats [162], these data might suggest that KO can potentially produce beneficial metabolic effects against dysmetabolism and inflammation in obesity also by reequilibrating the activity of PPARalpha.

8. Influence of PL-Bound EPA and DHA on the Brain Endocannabinoid System

Various studies have explored the influence of dietary fatty acids on brain endocannabinoid concentrations, which, together with receptor CB1, are involved in the regulation of synaptic plasticity and other functions as the control of movement and sensory perceptions to mood and neurogenesis [163].

In piglets, milk formulations enriched in HPUFAs were able, after one month of administration, to significantly modify the levels of the corresponding N-acylethanolamines (NAEs) in various brain regions; in addition, feeding dietary ARA induced an increase in whole brain AEA levels in mice [164]. Of the two endocannabinoids, AEA seems to be the one most involved in adaptive mechanisms against stress and its consequences, while 2-AG is the one clearly involved in hyperphagia in animal models of obesity.

In mice brain, decreased levels of 2-AG, but not AEA, were induced by high dose of dietary n-3 HPUFA as FO, whereas the opposite effect was obtained with an n-3 HPUFA-deficient diet [145]. On the other hand, no changes have been observed in brain 2-AG and AEA concentrations of rats, to which EPA or DHA had been administered for one week [155].

Di Marzo et al. [165] observed that obese Zucker rats, fed for one month with low doses of n-3 HPUFA (0.5% of EPA + DHA, in the form of either FO or KO, equivalent to 0.8 en% in the rat diet and corresponding to 1.8 g/d in a 2000 kcal diet in humans), showed a rise in EPA and DHA levels in brain PLs of KO group, compared with FO group, and no changes in ARA levels. At the same time, levels of 2-AG in the brain were decreased in KO group but not in FO group, as previously observed by Watanabe et al. [145]. However, it is not clear if the increase of n-3 HPUFAs is sufficient to lower brain concentrations of 2-AG. Since these alterations were not associated with food efficiency and food intake, which are under the control of hypothalamus area, it was supposed that this area was not involved in such decreases or these were not sufficient to exert a significant effect on CB1 receptor activity in this brain area. Therefore, relatively low doses of KO to Zucker rats, which was previously shown to downregulate peripheral endocannabinoid levels, ameliorating some aspects of the metabolic syndrome [49], in the brain reduces only 2-AG levels, suggesting that the beneficial effect of KO on the metabolic syndrome is mostly exerted by modifying endocannabinoid levels in peripheral tissues.

These data indicate that both the amount of dietary n-3 HPUFAs and the dietary form, as FO or KO, may influence EPA and DHA incorporation into brain lipids and consequently either the biosynthesis of ARA or its incorporation into PLs via its partial replacement with EPA and DHA [166]. Thus, even though most of the nutritional effects of n-3 HPUFAs may occur through the modulation of the levels of PL-derived metabolites, such as oxygenated eicosanoids [167], and of endocannabinoid concentrations [159], it remains to clarify on whether and how modulation of the biosynthesis of these bioactive compounds may influence brain activities.

9. Nutritional Activities of PL-Bound EPA and DHA in Humans

There is plenty of evidence in humans that the endocannabinoid system is involved in the regulation of the homeostasis of body composition and food intake and that it is chronically activated both in the brain and in peripheral organs after high-fat feeding and/or in obesity [168–170]. An increase of AEA and 2-AG has been observed in overweight and obese subjects [153, 171, 172]; specifically, a study on visceral adipose tissue (VAT) in lean, subcutaneous obese, and visceral obese subjects has clearly shown that 2-AG plasma concentrations were increased predominantly in abdominal obese individuals. In addition, a significant correlation between 2-AG plasma levels and visceral fat mass was found [151]. This increase of 2-AG plasma levels was in agreement with previous findings in postmenopausal women [173]. Interestingly, it has been observed that the negative relationship between circulating 2-AG and insulin sensitivity was independent of any effect on fat mass, which may imply additional effects of the endocannabinoid system on peripheral tissues. Circulating 2-AG and AEA were also found increased in type 2 diabetes patients [174]. Higher 2-AG, but not AEA, plasma content has been described in VAT of obese patients [174], probably for the increased supply of endocannabinoid precursors and/or increased activity of enzymes involved in endocannabinoid synthesis [168] and/or a decreased endocannabinoid degradation. In fact a significant downregulation of FAAH gene expression in adipose tissue of obese compared with lean individuals was found [151]. However, it cannot be ruled out that a contribution of the 2-AG circulating levels may derive from TAG hydrolysis by hormone sensitive lipase in visceral adipose tissue, which is particularly active in obese individuals.

Activation of central CB1 receptors promotes food intake and thereby weight gain [175–177] by increasing de novo lipogenesis by upregulating the lipogenic transcription factor SREBP-1c [178] and lipoprotein lipase activity [179].

The pharmacological approach to downregulate the endocannabinoid system with a cannabinoid CB1 receptor antagonist failed because of the increased incidence of depression

and anxiety in obese subjects [180, 181]. Therefore a nutritional approach may represent an effective and devoid of adverse effects way to modulate the endocannabinoid system. Another effective strategy, combining supplements like n-3 HPUFA, with pharmaceutical treatment with thiazolidinediones, has been demonstrated to be very effective for obesity-associated disorders [182].

In humans, only few studies have been carried out on the potential benefits of a downregulation of the endocannabinoid system by n-3 HPUFAs PLs form on obesity-associated diseases. A recent study investigated the effect of relatively low doses of n-3 HPUFA, taken as 2 g/d of either KO or FO, 309 mg/d of EPA/DHA 2 : 1, and 390 mg/d of EPA/DHA 1 : 1, respectively, or olive oil for four weeks, on plasma endocannabinoids in overweight and obese subjects [183]. The results confirmed data in the literature [153, 171, 172], showing plasma AEA and 2-AG levels significantly higher in obese and overweight subjects and showed that KO, but not FO or olive oil, was able to significantly decrease 2-AG, although only in obese subjects. Even if the effects of the two n-3 HPUFA treatments in fatty acid PL plasma profile were similar, KO was more powerful than FO in inducing endocannabinoid changes. Interestingly, the decrease of 2-AG was correlated to the plasma PL n-6/n-3 HPUFA ratio, caused, probably, by the replacement of 2-AG precursor, ARA, with n-3 HPUFAs, as described in obese Zucker rats [49]. However, neither KO nor FO was able to affect metabolic syndrome parameters probably because of the short-period treatment. In fact, in a very recent study [41], where hypertriglyceridemic mildly obese subjects were treated up to 24 weeks with 4 g/d with krill powder containing about 400 mg of EPA + DHA, a significant decrease of triglyceridemia was associated with a steeply reduction of circulating levels of AEA. In addition, a significant decrease of the waist/hip ratio and visceral fat/skeletal muscle mass ratio was found.

Thus, it seems that dietary EPA and DHA in the PLs form may reestablish the physiological endocannabinoid tone at CB1 receptors, upregulated with visceral obesity, dyslipidaemia, insulin resistance, and atherogenic inflammation [180], through a decrease of the n-6/n-3 HPUFA ratio and thereby reduction of the endocannabinoid precursors.

10. Conclusions

Research on n-3 PUFAs has made important progress in different areas. Nevertheless, there are still some issues, such as efficacy according to the dietary form and putative mechanisms of action that should be better characterized. It is clearly emerging that dietary PL-bound EPA and DHA affect endocannabinoid biosynthesis at much lower doses than EPA and DHA in TAG form, probably because of the higher incorporation into tissue PLs, and positively modify several parameters of the metabolic syndrome.

EPA and DHA esterified to PLs are one of the major n-3 HPUFA dietary forms in our diet. We therefore have been exposed to this form throughout our evolution maximizing the ability to fully exploit the EPA and DHA nutritional properties.

Future studies should address whether the peculiar property of dietary EPA and DHA bound to PLs to modulate the endocannabinoid system is also effective in ameliorating other pathological conditions where dietary EPA and DHA seem to exert beneficial activities such as cancer and psychiatric disorders.

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Research Article

Omega-3 Fatty Acids Inhibit Tumor Growth in a Rat Model of Bladder Cancer

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Omega-3 (ω-3) fatty acids have been tested on prevention and treatment of several cancer types, but the efficacy on "*in vivo*" bladder cancer has not been analyzed yet. This study aimed at evaluating the chemopreventive efficacy of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) mixture in an animal model of bladder cancer. Forty-four male Wistar rats were divided into 4 groups during a 20-week protocol: control; carcinogen—*N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN); ω-3 (DHA + EPA); and ω-3 + BBN. BBN and ω-3 were given during the initial 8 weeks. At week 20 blood and bladder were collected and checked for the presence of urothelium lesions and tumors, markers of inflammation, proliferation, and redox status. Incidence of bladder carcinoma was, control (0%), ω-3 (0%), BBN (65%), and ω-3 + BBN (62.5%). The ω-3 + BBN group had no infiltrative tumors or carcinoma *in situ*, and tumor volume was significantly reduced compared to the BBN ($0.9 \pm 0.1\ mm^3$ versus $112.5 \pm 6.4\ mm^3$). Also, it showed a reduced MDA/TAS ratio and BBN-induced serum CRP, TGF-β1, and CD31 were prevented. In conclusion, omega-3 fatty acids inhibit the development of premalignant and malignant lesions in a rat model of bladder cancer, which might be due to anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties.

1. Introduction

Bladder cancer, the fourth most common tumor in men and the eighth in women, remains a huge concern for the medical community because of its incidence and prevalence rates, as well as high percentage of recurrence and progression [1–4]. Mortality rates in muscle-invasive disease are still very high, despite the growing efforts on earlier diagnosis and aggressive and multidisciplinary treatments [4, 5].

In this context, preventive strategies are crucial for the management of bladder cancer, but they still demand a better elucidation of the carcinogenetic process. Exogenous factors, such as cigarette smoking, which accounts for a huge percentage of cases, as well as occupational carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons, are important determinants of the disease appearance [6, 7]. However, apart from the genetic features already characterized [8, 9], the cellular and molecular mechanisms might involve inflammatory, proliferative, and oxidative stress phenomena that deserve further elucidation. In fact, the identification of promising drugs depends on continuous research concerning the molecular/cellular mechanisms underlying cancer appearance and progression.

The experimental model of rat bladder cancer induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) is an appropriate and validated model to study human cancer development. In fact, due to the histological similarities with the human bladder cancer, it has been the most used model for the study of tumor pathophysiology, as well as for the evaluation the efficacy of therapeutic strategies [10–12]. The urothelial carcinogenesis is a continuous and slow process that goes through molecular and morphological changes,

from benign to aggressive lesions, including initial dysplastic and proliferative epithelial abnormalities, preneoplastic changes, and malignant lesions (papilloma and carcinoma) [12–14]. Thus, an early treatment targeting these pathways could hypothetically prevent bladder cancer development and growth. Previous reports have demonstrated beneficial effects of preventive strategies, including our own studies using anti-inflammatory and anti-proliferative agents [15–19].

Polyunsaturated fatty acids (PUFAs) have many physiological roles in the body, including acting as sources of cellular energy, regulators of protein synthesis, building blocks of phospholipids and glycolipids required for cell membrane structure, and components of membranes that regulate the fluidity, permeability, and dynamics of cell membranes, as well as precursors for many hormones, inter- and intracellular messengers as well as their receptors [20, 21]. PUFAs nomenclature is based on the position of the first carbon-carbon double bond within the long hydrocarbon chain; two of the four families cannot be synthesized from the body's carbohydrate stores and are required from the diet and are, thus, named as essential fatty acids (EFAs) and include the omega-3 (ω-3) and omega-6 (ω-6) oils. Considering their various biological activities, such as antioxidant and anti-inflammatory properties, ω-3 fatty acids have been viewed as useful weapons against several conditions, including those of cardiovascular/atherogenic, neuronal/degenerative, and inflammatory and neoplastic nature [22–26].

There is a wide range of chronic inflammatory, oxidative, and proliferative conditions that make cells susceptible to neoplastic transformation. The omega-3 fatty acids, due to their antioxidant and anti-inflammatory properties have been tested as chemopreventive and/or therapeutic agents, *per se* and in combination with drugs and/or radiotherapy, in several cancer types [27–30], but the putative efficacy on bladder cancer remains to be elucidated. The purpose of this study was, thus, to evaluate the chemopreventive efficacy of ω-3 fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in a rat model of bladder cancer induced by a nitrosamine.

2. Material and Methods

2.1. Animals and Groups. Forty-four male Wistar rats obtained from Charles River Lab. Inc. (Barcelona, Spain), weighting around 250 g, were maintained in an air-conditioned room, subjected to 12 hours dark/light cycles, and given standard laboratory rat chow (SAFE A-04, Augy, France) and free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care. The animals were divided in four groups: control group ($n = 8$)—vehicle (orange juice); carcinogen (BBN) group ($n = 20$)—0.05% of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); ω-3 group ($n = 8$)—600 mg/kg BW/day of DHA + EPA (240 + 360, resp.); and ω-3 + BBN group ($n = 8$)—in the same conditions described for the previous treatments. The animals underwent a two-phase protocol: a first period of 8 weeks for tumor induction and pharmacological treatment (orange juice, BBN and/or ω-3) and a second one of 12 weeks for cancer expression and putative prevention. BBN was given in drinking water and ω-3 and vehicle were administered by gavage, using an esophageal cannula. All the animals completed the 20-week study protocol. Body weight (BW) was measured weekly and drink beverage was monitored during the experimental period at intervals of two days.

2.2. Sample Collection and Preparation. Blood: at the end of treatment, animals were subjected to a blood collection procedure: first, the rats were injected with intraperitoneal anesthesia with 2 mg/Kg BW of a 2 : 1 (v : v) 50 mg/mL Ketamine (Ketalar, Parke-Davis, Pfizer Lab., Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Vitória lab., Amadora, Portugal). Blood samples were immediately collected by venipuncture from the jugular vein in needles with no anticoagulant (for serum samples collection). Tissues: the rats were sacrificed by cervical dislocation, and the lungs, stomach, liver, kidneys, and intestines were immediately removed, weighted, and placed in formaldehyde for histological evaluation. Before removal, bladders were intraluminally injected with a buffered formaldehyde solution as prefixation for histological analysis.

2.3. Tumor Chemoprevention Analysis

2.3.1. Quantitative Analysis (Number and Tumor Volume). Each bladder pre-fixated in formaldehyde was carefully open; the lumen was inspected for grossly visible lesions and the number of tumors per rat and the tumor volume were reported in order to calculate the % of tumor per group and the mean volume per rat and per tumor.

2.3.2. Qualitative Analysis (Bladder Histology). The bladder was immersion fixed in 4% buffered formaldehyde and processed for paraffin sectioning. Three slices from each bladder were embedded. Three micrometer thick sections were stained with haematoxylin & eosin (H&E) and examined histologically by clinical personnel with expertise.

2.4. Proliferation, Inflammation, and Redox Status Markers. Serum levels of transforming growth factor beta-1 (TGF-β1) were measured through an ultrasensitive Quantikine ELISA kits (R&D Systems, Minneapolis, USA). Serum C-reactive protein (CRP) was measured by using an ELISA kit from Helica Biosystems, Inc. (Fullerton, CA, USA). Serum redox status was assessed by the thiobarbituric acid reactive species (TBARs) assay, measuring lipid peroxidation via malondialdehyde (MDA) content, and by the total antioxidant status (TAS) quantification, through ferric reducing antioxidant potential (FRAP) assay, as previously described [16].

2.5. Bladder Cancer CD31 Immunohistochemistry. Immunohistochemical (IHC) staining of CD31 in bladder cancer tissue was performed in paraffin-embedded tissue, which was

cut into 4 mm sections and mounted on polisinated slides, using standard staining procedures, as described previously [18]. Representative slides were selected for staining and histologic evaluation. Briefly, slides were deparaffinized and hydrated with water. Antigen enhancement was performed by pretreating with microwave heating in a citrate buffer, pH 6.00 (for three pulses of 5 min each at 250 W). The slides were washed three times, 2 min each, and then incubated with blocking serum for 10 min to block the nonspecific binding, and the excess of blocking serum was removed. Staining was performed using a primary monoclonal antibody. A negative control was obtained by omitting the primary antibody. Diaminobenzidine was used as chromogen. Standard procedures were used for visualization and the intensity and/or percentage of positive staining in the dominant pattern within the tumor was graded on a semiquantitative scale (0–3), in which 0 is very low intensity and <25% of staining; 1 is mild and between 25 and 50%; 2 is moderate and between 50 and 75%; and 3 is severe and >75% of staining. All slides were reviewed by expert investigators in a blinded data manner.

2.6. Safety Profile

2.6.1. Biochemical Assays. The following serum parameters were assessed on a Hitachi 717 analyser (Roche Diagnostics Inc., MA, USA) using standard methods: glucose, creatinine, aspartate and alanine aminotransferase (AST and ALT), total cholesterol (Total-c), and triglycerides (TGs).

2.6.2. Haematological Data. Red blood cell (RBC) count, haematocrit (Hct), haemoglobin (Hb) concentration, platelet (PLT) count, and plateletcrit (PCT) were assessed in an automatic Coulter counter (Beckman Coulter Inc., CA, USA).

2.7. Statistical Analysis. For statistical analysis, we used the GraphPad Prism version 5.00 software from GraphPad Software (San Diego, CA, USA). Results are means ± standard error of means (s.e.m). Comparisons between groups were performed using ANOVA with Bonferroni post hoc test. Significance was accepted at P less than 0.05.

3. Results

3.1. Tumor Chemoprevention Data

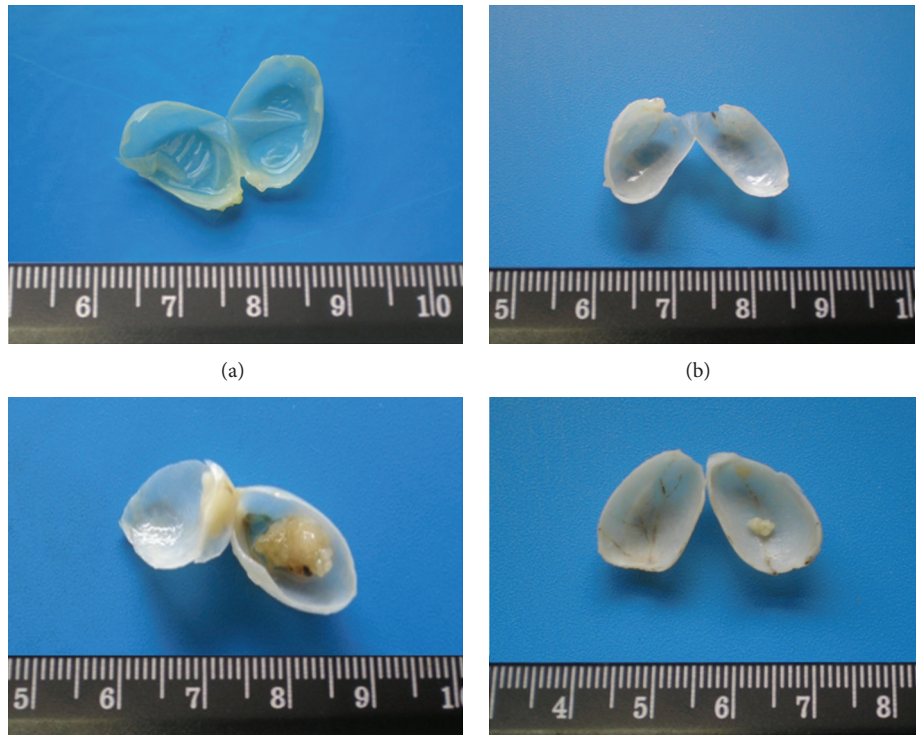
3.1.1. Body Weight and Beverage Consumption. No relevant changes were obtained between the groups during the study concerning BW and beverage consumption, despite a trend to lower BW in the two groups under ω-3 treatment (data not shown).

3.1.2. Macroscopic Evaluation. All formaldehyde pre-fixated bladders were opened and microscopically analysed for wall (urothelium) texture, thickness, and vascularization (Figure 1). The percentage of rats with tumor in each group, the number of tumors per rat with tumor, and the mean tumor volume per rat and per tumor were then evaluated (Table 1). All the bladders from the control animals have revealed a pattern of normality, with absence of any type of malignity. The wall texture, thickness, and vascularization were normal (Figure 1(a)). Similar profile was found for the ω-3 group, with limpid, translucent, and tiny bladders, without presence of any abnormal mass or vascularization (Figure 1(b)). In the BBN group, however, 65.0% (13 in 20) of the rats had bladder tumors, almost all easily seen by macroscopic analysis. The bladder walls were thicker, with new or enlarged small vessels, suggesting neoangiogenesis, and there was unequivocal formation of tumor, some of them of relevant volume, also visible in the whole bladder (Figure 1(c)). In the ω-3 + BBN group, the percentage of mice with tumor was 62.5%. In these, the bladder walls were thicker, with some visible small vessels, suggesting neo-angiogenesis and displaying preneoplasic lesions from hyperplasia but without high-grade dysplasia (Figure 1(d)).

3.1.3. Quantitative Evaluation. In the control group, as expected, no rat developed bladder cancer. The same profile was found for the ω-3-treated animals. In the BBN group ($n = 20$ rats), the percentage of rats with bladder cancer was 65.0% (13 in 20), with a mean of 1.2 ± 0.1 tumors per rat with tumor. Furthermore, the mean tumor volume per rat with tumor (in 13 rats) was $138.5 \pm 7.5\ mm^3$ and the mean tumor volume per tumor (in 16 tumors) was $112.5 \pm 6.4\ mm^3$ (Table 1). In the ω-3 + BBN group, there was no preventive action of this type of drug concerning the incidence of tumor appearance, since the percentage of mice with tumor was 62.5% (5 in 8), with an average of 1.2 ± 0.2 tumors per rat (6 tumors in 5 rats). The mean tumor volume per rat was $1.1 \pm 0.1\ mm^3$ and the mean tumor volume per tumor was $0.9 \pm 0.1\ mm^3$ (Table 1).

3.1.4. Qualitative Evaluation. Concerning the microscopic analysis of urothelium layer and urothelial tumors, the control and ω-3 rats have shown normal patterns (Figures 2(a) and 2(b), resp.). The bladder from control animals had no signs of pre-neoplasic lesions (neither hyperplasia nor dysplasia), as well as those from the ω-3 group (Table 1). In the carcinogen (BBN) group, there was evident malignant transformation, including hyperplasia (100%) and dysplasia (100%), present in all the animals, including those without tumor formation (Table 1 and Figure 2(c1)). Furthermore, there were also malignant lesions, papillary, infiltrative, and carcinoma *in situ* (*Cis*) (Table 1 and Figure 2(c2)). Omega-3 treatment has not reduced the incidence of cancer appearance (62.5%), but high-grade dysplasia was absent and there were no infiltrative tumor and no *Cis* (Table 1 and Figures 2(d1) and 2(d2)). The lungs, stomach, liver, kidneys, and intestines were normal on gross inspection and on histological examination for all the rats of all the groups.

3.2. Proliferation, Inflammation, and Redox Status Profiles. In the BBN group, there was a significant increment in serum TGF-β1 ($P < 0.01$) and CRP ($P < 0.001$) contents, when compared with the control group (Figures 3(a) and 3(b), resp.). In the ω-3 group, TGF-β1 and CRP serum concentrations remained unchanged when compared with control rats. In the ω-3 + BBN group, significant decrease of



(a) (b) (c) (d)

Figure 1: Macroscopic histomorphological evaluation of bladders. All the bladders from the Control (a) and ω-3-treated-rats (b) revealed a normal appearance. In the BBN group (c), 65.0% of the rats exhibited exuberant bladder cancer occupying almost the vesical lumen. In this group, the bladders showed a remarkable hypervascularization, suggesting neoangiogenesis, characteristic of tumor growth. In the preventive ω-3 + BBN group (d), identical percentage of tumors was found but with an outstanding reduction of tumor volume.

Table 1: Omega-3 effects on bladder cancer chemoprevention: macroscopic (quantitative) and microscopic (qualitative) evaluation of urothelial lesions.

Macroscopy (quantitative) & microscopy (qualitative)	Control ($n = 8$)	Omega-3 ($n = 8$)	BBN ($n = 20$)	Omega-3 + BBN ($n = 8$)
Tumor number				
Rats with tumor [% (n/n)]	0	0	65.0 (13 in 20)	62.5 (5 in 8)
Tumors/rat [mean (n/n)]	0	0	1.2 ± 0.1 (16 in 13)	1.2 ± 0.2 (6 in 5)
Tumor volume				
Mean/rat [mm^3 (n)]	0	0	138.5 ± 7.5 (in 13)	1.1 ± 0.1 (in 5)
Mean/tumor [mm^3 (n)]	0	0	112.5 ± 6.4 (in 16)	0.9 ± 0.1 (in 6)
Preneoplasic lesions [% (n/n)]				
Hyperplasia	0	0	100 (20 in 20)	87.5 (7 in 8)
High-grade dysplasia	0	0	100 (20 in 20)	0 (0 in 8)
Neoplasic lesions [% (n/n)]				
Papillary tumor	0	0	100 (13 in 13)	100 (5 in 5)
Infiltrative tumor	0	0	15 (2 in 13)	0 (0 in 5)
Carcinoma *in situ*	0	0	31 (4 in 13)	0 (0 in 5)

serum TGF-1 ($P < 0.05$) and CRP ($P < 0.001$) were obtained, versus the BBN group.

Serum MDA concentration showed a trend to higher values in the BBN group when compared with control rats. This value was accompanied by a significant increase ($P < 0.05$) in serum total antioxidant status (TAS). In agreement, serum MDA/TAS ratio, a redox status marker, was unchanged, compared with the control rats (Figures 4(a), 4(b), and 4(c)). In the ω-3-treated animals, there was a reduction in MDA and TAS levels, in relation to the control group, which was

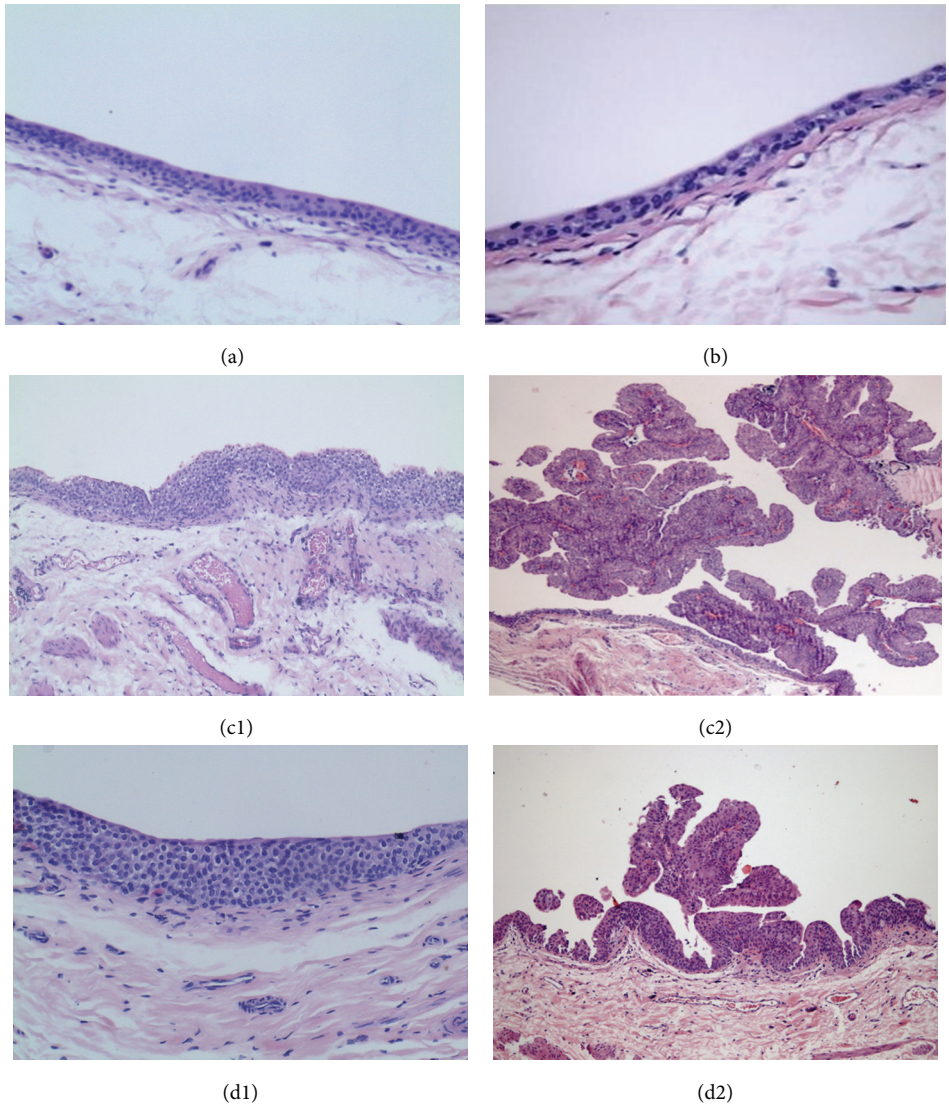


Figure 2: Microscopic histomorphology (H&E staining ×100). The bladders from Control (a) and ω-3-treated-rats (b) had no signs of preneoplastic or neoplasic lesions. In the BBN group (c1 and c2), all the bladders presented preneoplastic lesions, namely, hyperplasia and high-grade dysplasia (c1), even in the bladders without tumor growth. Neoplasic lesions like papillary tumors (c2), infiltrative tumors, and *Cis* were found in some bladders of the BBN-treated-rats. In the ω-3 + BBN rats, all the bladders also presented hyperplasia (d1), but high-grade dysplasia was absent, and the tumors were of papillary nature (d2), but no infiltrative tumors and *Cis* were found.

most significant in the case of MDA ($P < 0.001$). Thus, the MDA/TAS ratio, in the ω-3 group, was also significantly lower, showing a clear antioxidant sketch. Similar antioxidant profile was found for the ω-3 + BBN group ($P < 0.05$), when compared with the BBN one (Figures 4(a), 4(b) and 4(c)).

3.3. Bladder Cancer CD31 Immunohistochemistry. The bladders from control (Figure 5(a)) and Atorva (data not shown) treated rats presented low CD31 immunostaining intensity (grade 0). However, in the BBN group, both the hyperplastic (Figure 5(b)) and tumoral (Figure 5(c)) lesions showed elevated expression of CD1 (grade 1 and 2 intensity, resp.). In the ω-3 + BBN rats, all the bladders with tumor (Figure 5(d)) showed a reduced CD31 expression (grade 1) when compared with those of the BBN group with carcinoma and identical to the intensity found in the nontumoral hyperplastic regions of the BBN.

3.4. Safety Profile: Biochemical and Haematological Data. In the BBN group, serum glucose, creatinine, and lipidic profile data (Total-c and TGs) were unchanged; however, AST was significantly ($P < 0.001$) increased when compared with the control group (Table 2). Concerning the ω-3 group, there was a significantly decrease in serum creatinine ($P < 0.001$) and TGs ($P < 0.01$) levels when compared to the control group, while the other parameters were unchanged. Similarly, creatinine ($P < 0.001$) and TGs ($P < 0.01$) contents were significantly reduced in the ω-3 + BBN group when

Table 2: Safety profile: biochemical and hematological data.

Parameters	Control ($n = 8$)	Omega-3 ($n = 8$)	BBN ($n = 20$)	Omega-3 + BBN ($n = 8$)
Biochemical data				
Glucose (mmol/L)	9.90 ± 0.54	9.52 ± 0.48	10.22 ± 0.61	9.84 ± 0.52
Creatinine (mmol/L)	51.53 ± 1.68	35.36 ± 1.84^{aaa}	54.81 ± 1.72	32.90 ± 1.64^{aaabbb}
Total-c (mmol/L)	1.39 ± 0.06	1.21 ± 0.07	1.31 ± 0.05	1.43 ± 0.06
TGs (mmol/L)	1.73 ± 0.1	0.93 ± 0.03^{aa}	1.47 ± 0.1	0.96 ± 0.11^{aabb}
AST (IU/L)	51.57 ± 1.09	62.00 ± 1.08	77.18 ± 4.9^{aaa}	73.86 ± 3.45^{aaa}
ALT (IU/L)	30.86 ± 1.75	23.00 ± 0.91	36.17 ± 2.32	38.63 ± 1.86
Hematological data				
RBC count (10^{12}/L)	7.58 ± 0.29	7.79 ± 0.58	8.07 ± 0.11	7.47 ± 0.15
HCT (pp of 1.0)	0.40 ± 0.02	0.40 ± 0.03	0.42 ± 0.004	0.38 ± 0.009
HGB (g/L)	141.5 ± 5.24	139.5 ± 8.63	146 ± 1.47	137.3 ± 3.09
PLT ($\times 10^9$/L)	990.67 ± 46.21	760.8 ± 77.07	1008.13 ± 46.96	811.00 ± 37.13^{b}
PCT (pp of 1.0)	0.0057 ± 0.0002	0.004 ± 0.0004^{aa}	0.005 ± 0.0002	0.0047 ± 0.0002^{ab}

Values are mean ± SEM. $^{aa}P < 0.01$ and $^{aaa}P < 0.001$ versus control; $^{b}P < 0.05$, $^{bb}P < 0.01$ and $^{bbb}P < 0.001$ versus BBN.

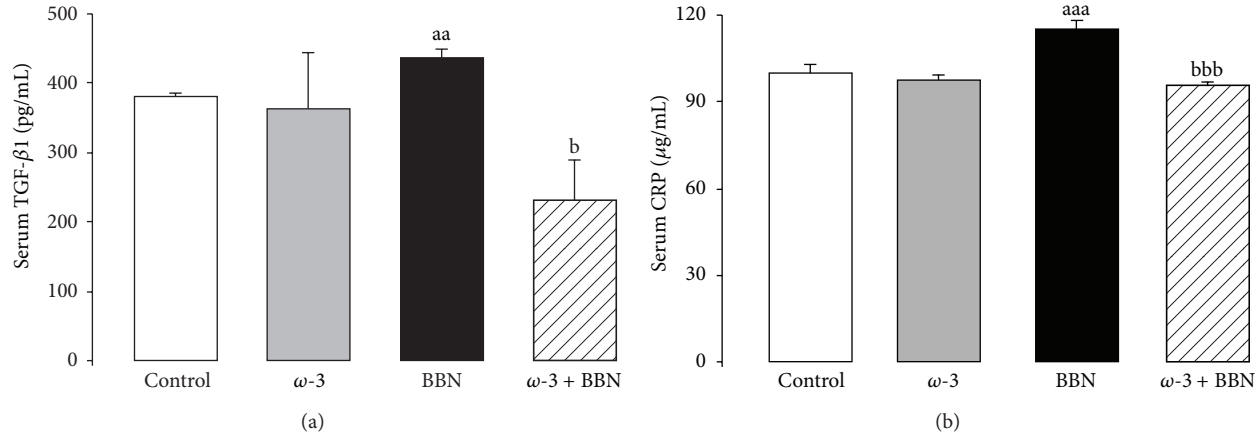


Figure 3: Serum markers of proliferation and inflammation: TGF-β1 (a) and CRP (b). Values are mean ± SEM. $^{aa}P < 0.01$ and $^{aaa}P < 0.001$ versus Control group; $^{b}P < 0.05$ and $^{bbb}P < 0.001$ versus BBN group.

compared to both the control and the BBN group, while the other markers remained unchanged when compared with the BBN-treated animals (Table 2). Concerning the hematological data, no significant changes were encountered for all the parameters, except a reduction of PCT for the groups under ω-3 treatment (Table 2).

4. Discussion

The bladder carcinoma is one of the most prevalent in the Western world. Although 75–80% are not invasive and exhibit high survival rates, they have a high risk of relapse and a significant proportion progresses to more invasive forms [31]. This recurring nature, together with the high morbidity and mortality of the invasive forms that justifies adjuvant therapeutics and aggressive multimodal treatment, makes it one of the cancers with higher socioeconomic costs. Effective methods in preventing relapses would have a role in addressing this neoplasm and become increasingly necessary.

Several types of new and old drugs have been tested during the last years as putative chemopreventive agents for bladder cancer [32]. Preventive and/or treatment options have been based on the possibility of targeting the mechanisms behind the carcinogenesis and tumor growth, including anti-inflammatory and antioxidant agents [16–19, 33, 34]. PUFAs, namely, omega-3 fatty acids, might meet these criteria, since they have demonstrated anti-inflammatory and antioxidant properties, among others, and are usually viewed as safe compounds. Furthermore, ω-3 fatty acids, besides their use in cardiovascular diseases, have already been tested in several types of tumors, including prostate, colorectal, and breast cancer [27–30, 35–37], but the data already available are insufficient and sometimes discrepant [38, 39]. Concerning the bladder cancer, in particular, the information available is very scarce. The study of Molinari et al. (2011) reported

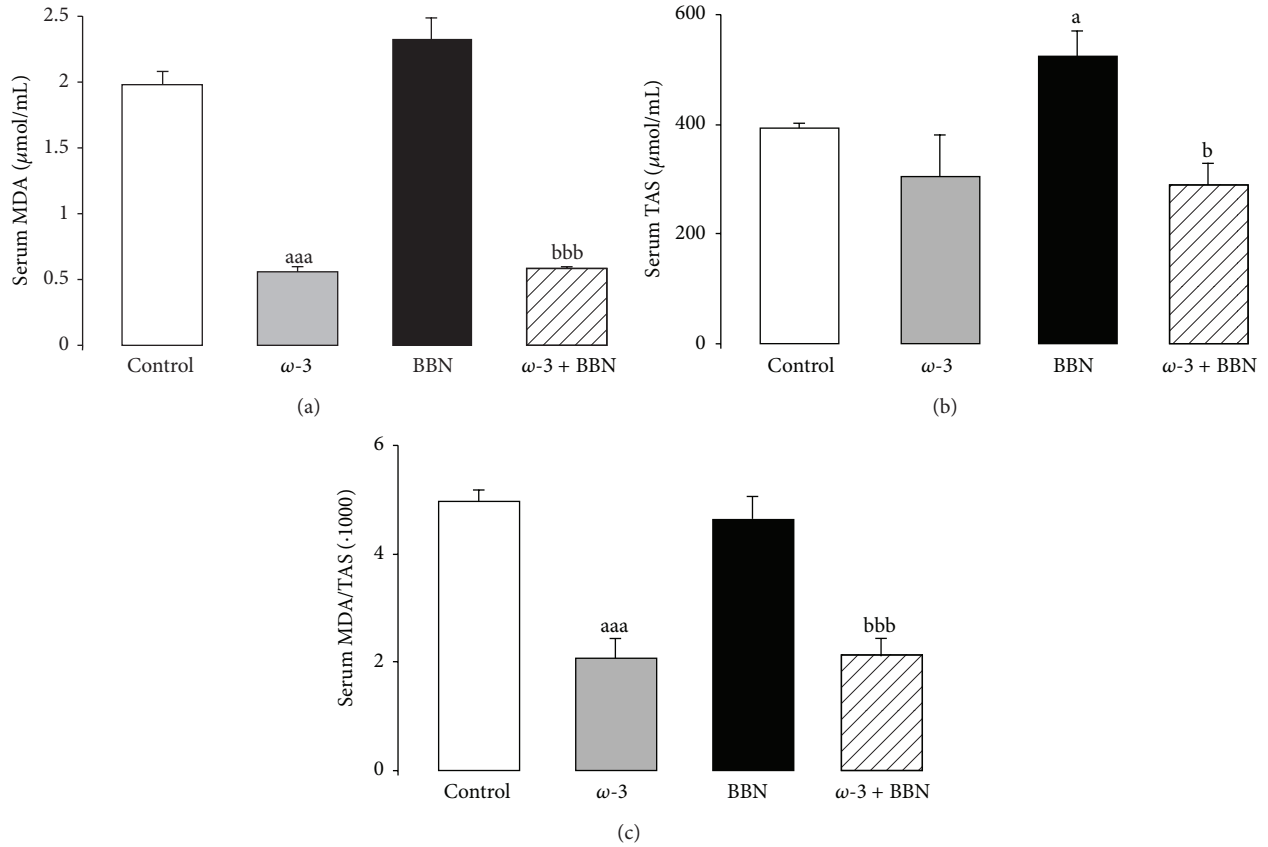


Figure 4: Serum redox status markers: lipidic peroxidation, via MDA content (a), TAS levels (b), and MDA/TAS ratio (c). Values are mean ± SEM. $^{a}P < 0.05$, $^{aaa}P < 0.001$ versus Control group; $^{b}P < 0.05$, $^{bbb}P < 0.001$ versus BBN group.

the ability of DHA to induce immunogenic cell death in a cell line of bladder cancer [40], while Mackie et al. (2006) have previously described the *in vitro* synergic effect between a soluble formulation of ω-3 fatty acids and epirubicin and mitomycin, used as intrabladder instillation adjuvants [41]. In rodents, the chronic deficiency of essential fatty acids induces transitional cell hyperplasia and augments the possibility of urinary tract carcinogenesis [42], which is in agreement with previous data showing lower serum levels in patients with bladder cancer [43]. In humans, a recent case control study reported that consumption of linolenic acid, an omega-3 fatty acid found in vegetable oils, may have a protective role against bladder carcinoma [44]. Despite some previous information on other types of carcinomas, sometimes divergent, the putative efficacy on bladder cancer remains to be elucidated and more experimental research is justified. The rat model of bladder cancer induced by a nitrosamine (BBN) has been used as a good tool to evaluate chemopreventive strategies, as it has histological similarities with the human bladder carcinoma [10–12]. Our results are in agreement with previous data from this model, concerning both % incidence of tumors and the type of lesions in the BBN rats [45, 46], confirming the credibility and value of this model to study preventive efficacy of drugs. Considering that omega-3 fatty acids are recognized as safe compounds and even present a positive impact on cardiovascular parameters, preventive strategies might be considered for this type of agents. However, in humans the exposure to carcinogens is chronic and we should recognize that even with preventive aims it is unexpected that a treatment could precede the exposure to carcinogens, which was the reason why we adopt the present protocol.

It is largely accepted that the mechanisms underlying cancer appearance and progression are multifactorial. Chronic inflammation and oxidative stress, through their mediators (cytokines, growth factors, and reactive oxygen and nitrogen species), seem to be great contributors for cancer growth [47], and justify the research using anti-inflammatory and antioxidant agents. Among them, omega-3 fatty acids could be viewed as good alternatives [20–30]. In our study, ω-3 fatty acids were unable to promote a significant reduction of incidence of bladder cancer, but the most aggressive premalignant and malignant lesions, such as high-grade dysplasia or *Cis* were absent and no infiltrative cancers were seen, which is of imperative interest. The remarkable reduction of tumor volume also deserves a special note. In fact, in the group under BBN beverage and ω-3 fatty acids treatment, the mean volume of tumor per rat was reduced from $138.5 \pm 7.5\,mm^3$ in the BBN group to $1.1 \pm 0.1\,mm^3$ in the ω-3 + BBN rats, accompanied by a similar outstanding reduction in the mean volume of tumor (from $112.5 \pm 6.4\,mm^3$ in the BBN rats to $0.9 \pm 0.1\,mm^3$ in the ω-3 + BBN ones). The inhibition of tumor

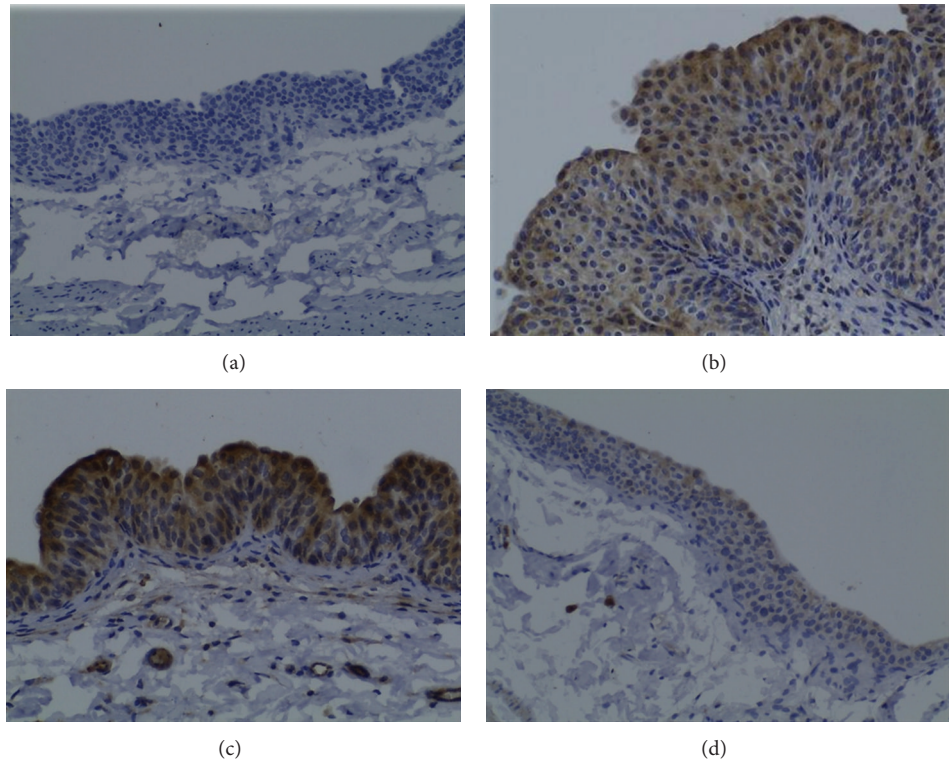


Figure 5: Bladder cancer CD31 immunostaining for the groups under study: Control (a); BBN, in tumoral (b) and nontumoral hyperplastic (c) regions, and ω-3 + BBN (d). Original magnification 200x.

growth is in agreement with other experimental *in vivo* and *in vitro* studies in hepatocarcinoma, breast, colon, and prostate cancer cells or animal models [48–51].

Given the potential anticarcinogenic mechanisms assigned to these compounds, which include the modulation of eicosanoid production, inflammation, angiogenesis, and susceptibility to apoptosis, similar to the pleiotropic activity of other compounds, such as statins, which have previously demonstrated a beneficial effect on this animal model of BBN-induced rat bladder cancer [18], there was the expectation of different results concerning tumor incidence, reinforced by other data from animal models [52]. The experimental knowledge concerning the preventive anticancer capacity of ω-3 fatty acids comes mainly from models of colorectal carcinogenesis. Studies in rats receiving diet supplements reported important reductions of 20 to 50% in the incidence of carcinoma and 30 to 70% in tumor multiplicity [36, 53, 54]. According to our view, the unchanged incidence of bladder cancer in the ω-3 + BBN group, when compared with the carcinogen group, might be due to two particular aspects. Firstly, the dose used might be viewed as a low/moderate ω-3 dose and higher DHA and EPA amounts might eventually be able to promote better impact on the cancer appearance. The doses tested vary between different studies of different carcinomas and there is no clear definition yet of the most effective dose, as well as the duration of treatment required to promote a significant effect on cancer prevention [36]. In our study, a dose of 600 mg/kg/day (240 of DHA and 360 of EPA) was used, which might be viewed as a low/moderate dose in terms of an experimental study in a rat model. Secondly, the most effective effect at this concentration of DHA and EPA was the remarkable beneficial impact on redox status, with a reduced MDA/TAS ratio, demonstrative of an antioxidant activity. Since the BBN group was unable to promote a significant impact on MDA/TAS ratio, the redox imbalance (oxidative stress) might not be the "fuel" for cancer appearance but eventually for cancer progression. In agreement, the outstanding positive effect of ω-3 on this aspect might have an importance for the reduction of tumor growth, but without impact on the percentage of tumor appearance, to which other mechanisms (genetic and nongenetic) should be better contributors and in which the ω-3 treatments are most probably less effective. However, and even more importantly, there was a remarkable inhibition of the development of more aggressive forms of tumor, such as high-grade dysplasia, *Cis*, and infiltrative cancers, which is of crucial interest.

Several molecular mechanisms have been proposed to explain the putative anticancer activity of ω-3 fatty acids [36, 55–57]. In our study, omega-3-treated rats were able to prevent the BBN-induced increment of CRP and of TGF-β1, suggesting that apart from a clear antioxidant action, there was an anti-inflammatory and anti-proliferative activities both of which might contribute to the inhibition of tumor growth. In fact, previous studies have suggested that the chemopreventive effect of ω-3 fatty acids on cancer growth might rely on anti-inflammatory, anti-proliferative, and anti-angiogenic activities [36, 55–58], and our preliminary data

of VEGF, bcl2 and P53 expression in bladder cancer tissue of omega-3 treated rats (data not shown) also suggest those properties, recommending additional research. In addition, we found a reduced CD31 expression in the bladders of the omega-3-treated rats when compared with the BBN samples. CD31 is a 130 kDa integral membrane protein, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), which mediates cell-to-cell adhesion, and is used as a marker of vascularization/angiogenesis. CD31 in bladder carcinoma has been correlated with the tumor grade and stage [59]. Further studies should also address the effect on COX-2 expression, which has been described as an important contributor for carcinogenesis [60] and able to be modulated by ω-3 fatty acids [61, 62].

One of the aspects of concern in the anticancer agents is the safety profile, often jeopardizing some drugs with good chemopreventive efficacy in cancer growth. Omega-3 fatty acids are recognized as safe compounds and even present a positive impact on cardiovascular parameters. In our study, ω-3 therapy was associated with a safe profile on biochemical and haematological data, with a reduction of TGs and unchanged values of renal and liver markers, which is in agreement with what would be expected for this type of compounds. Considering this feature, further studies should address the chemopreventive efficacy in bladder cancer with higher doses of ω-3.

5. Conclusions

Omega-3 fatty acids therapy was able to inhibit tumor growth in a rat model of bladder cancer, which might be due to anti-inflammatory, antioxidant, anti-proliferative, and anti-angiogenic properties. In addition, the development of more aggressive pre-malignant and malignant lesions was also inhibited. Given the safe profile demonstrated, and the benefits described in other inflammatory and cardiovascular conditions, omega-3 fatty acids might be viewed as potential interesting natural compounds for the prevention and/or therapy of carcinoma growth, eventually as adjuvants for other drugs, deserving further attention in this area of knowledge.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Belmiro Parada, Flávio Reis, Alfredo Mota, Arnaldo Figueiredo, and Frederico Teixeira conceived and designed the study protocol. Belmiro Parada, Flávio Reis, Raquel Cerejo, Patrícia Garrido, José Sereno, Maria Xavier-Cunha, and Paula Neto performed experiments. Belmiro Parada, Flávio Reis, Raquel Cerejo, Arnaldo Figueiredo, and Frederico Teixeira analysed the data and prepared the paper. All authors have read and approved the paper. Belmiro Parada, Flávio Reis and Raquel Cerejo equally contribute to the work.

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Research Article

The Omega-3 Polyunsaturated Fatty Acid DHA Induces Simultaneous Apoptosis and Autophagy via Mitochondrial ROS-Mediated Akt-mTOR Signaling in Prostate Cancer Cells Expressing Mutant p53

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Docosahexaenoic acid (DHA) induces autophagy-associated apoptotic cell death in wild-type p53 cancer cells via regulation of p53. The present study investigated the effects of DHA on PC3 and DU145 prostate cancer cell lines harboring mutant p53. Results show that, in addition to apoptosis, DHA increased the expression levels of lipidated form LC3B and potently stimulated the autophagic flux, suggesting that DHA induces both autophagy and apoptosis in cancer cells expressing mutant p53. DHA led to the generation of mitochondrial reactive oxygen species (ROS), as shown by the mitochondrial ROS-specific probe mitoSOX. Similarly, pretreatment with the antioxidant N-acetyl-cysteine (NAC) markedly inhibited both the autophagy and the apoptosis triggered by DHA, indicating that mitochondrial ROS mediate the cytotoxicity of DHA in mutant p53 cells. Further, DHA reduced the levels of phospho-Akt and phospho-mTOR in a concentration-dependent manner, while NAC almost completely blocked that effect. Collectively, these findings present a novel mechanism of ROS-regulated apoptosis and autophagy that involves Akt-mTOR signaling in prostate cancer cells with mutant p53 exposed to DHA.

1. Introduction

Prostate cancer is the second leading cause of male cancer-related death in the USA [1], and migration-associated changes in risk have provided evidence that genetic and environmental factors, such as p53 alteration and dietary fat, contribute to the disease [2–4]. Epidemiological data suggest that while high intake of saturated fatty acids is positively associated with prostate cancer risk, certain omega-3 polyunsaturated fatty acids (ω3-PUFAs), in particular docosahexaenoic acid (DHA), seem to prevent this type of cancer [5, 6]. The protective role of ω3-PUFAs against prostate cancer is also supported by evidence from in vitro and in vivo studies, and multiple mechanisms have been proposed to account for the association of ω3-PUFAs with reduced prostate cancer risk, including (a) modulation of phospholipase A_2 and oxygenases and synthesis of their corresponding metabolites [7–11], (b) alteration in cell membrane phospholipid composition and receptor function [11–15], and (c) regulation of gene expression and signal transduction [16, 17].

Autophagy and apoptosis are self-destructive processes that share many key regulators, such as reactive oxygen

species (ROS). Physiological levels of ROS lead to growth adaption and survival; however, excessive ROS cause irreversible cellular damage, thus provoking autophagy and/or apoptosis [18, 19]. Likewise, many pathways are commonly used by these two processes in response to a single stress. For example, inactivation of Akt-mammalian target of rapamycin (mTOR) signaling is responsible for the parallel occurrence of autophagy and apoptosis in human brain tumor cells exposed to hydrogen peroxide [20].

A recent study shows that ethanolamine derivatives of ω3-PUFAs reduce cell viability by inducing autophagy in MCF-7 breast cancer cells [21], which express wild-type p53. We previously reported that autophagy is involved in DHA-induced apoptosis via p53-mediated mTOR signaling in a number of cancer cells harboring wild-type p53 [22]. These observations indicate that the antitumor activity of ω3-PUFAs is tightly linked to their ability to trigger autophagy and apoptosis and set the stage for an effective treatment of tumors possessing functional p53; however, since p53 is frequently mutated in human cancers, it remains unclear whether simultaneous induction of autophagy and apoptosis is a universal ω3-PUFA antitumor mechanism regardless of p53 status.

To address this issue, we investigated the involvement of autophagy and apoptosis in DHA-treated prostate cancer cell lines with altered p53 status. The results indicate that DHA simultaneously induces autophagy and apoptosis in cancer cells expressing mutant p53 via mitochondrial ROS overproduction and that Akt-mTOR pathway inactivation mediated by ROS may play a key role in DHA-induced cytotoxicity in prostate cancer cells.

2. Materials and Methods

2.1. Cell Lines, Culture Conditions, and Treatment with Reagents. The human PC3 and DU145 metastatic adenocarcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 medium (Invitrogen, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin, and streptomycin at 37°C in a humidified 5% CO_2 incubator. When required, cells were allowed to grow to 60–70% confluence and were exposed to serum-free medium for 24 h before receiving any treatment.

Absolute ethanol was used to dissolve DHA, eicosapentaenoic acid (EPA), and arachidonic acid (AA) (Cayman Chemical, Michigan) and served as a control treatment (CN). The DNA staining dyes propidium iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Roche Applied Science (Indianapolis, IN). Unless indicated, all other reagents were from Sigma-Aldrich (ST Louis, MO). The lysosomal inhibitor chloroquine (CQ) and the ROS scavenger N-acetyl-L-cystein (NAC) were dissolved in H_2O. For exposure to DHA plus CQ or NAC, cells were pretreated with 2 μM CQ or 5 mM NAC for 1 h and then exposed to DHA.

2.2. Cell Viability Assay. The cytotoxicity of DHA (alone or in the presence of NAC) was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). PC3 and DU145 prostate cancer cells were seeded at 1×10^4 cells per well in 96-well plates. At the end of the assay, MTT was added to each well (final concentration 200 μg/mL) and the cultures were incubated at 37°C for 1 h. The supernatants were then removed and the formazan crystals were dissolved by adding dimethyl sulfoxide (200 μL). The absorbance was measured at 570 nm in a microplate spectrophotometer (Thermo Fisher Scientific), and cell viability was expressed as ratios versus untreated CN cells.

2.3. Measurement of ROS Production. PC3 and DU145 cell lines seeded in 6-well plates were exposed to DHA in the presence or absence of NAC for 2 h. The ROS probes dihydroethidium (DHE, 5 μM, Molecular Probes, Carlsbad, CA) or MitoSOX (2.5 μM, Molecular Probes, Carlsbad, CA) were then added and the cultures were further incubated at 37°C for 20 min. Direct imaging of ROS in probe-loaded cells was performed by fluorescence microscopy. Alternatively, fluorescence intensity was measured by flow cytometry. At least 10,000 events were analyzed using a FACScan instrument (FACS-Calibur, BD Biosciences, CA).

2.4. Analysis of Apoptotic Parameters. Apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and flow cytometry, as described previously [22].

2.5. Plasmid and Transfection. Microtubule-associated protein 1 light chain 3 beta (LC3B) fused to green fluorescent protein (GFP) (kind gift of Dr. Tamotsu Yoshimori, National Institute of Genetics, Mishima, Japan) was transfected using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA) as recommended by the vendor. After 24 h of transfection, cells were exposed to 50 μM DHA for 2 h. The transfected cells were then observed under a fluorescence microscope (Olympus iX70, Japan), and fluorescence imaging was conducted to detect the punctate pattern of GFP-LC3B.

2.6. Western Blot Analysis. Cells were harvested, washed once with ice-cold phosphate buffered saline, and subjected to Western blot analysis, as described previously [23]. The antibodies were as follows: Akt (#9272), phospho-Akt (S473, #4060), mTOR (#2972), phospho-mTOR (S2448, #2971), and LC3B (#3868) were obtained from Cell Signaling Technology; phospho-Akt (T308, sc-16646), poly (ADP-ribose) polymerase (PARP, H-250, sc-8007), and actin (I-19, sc-1616) were obtained from Santa Cruz Biotechnology. Goat anti-rabbit (401315) and anti-mouse (401215) secondary antibodies were purchased from Calbiochem.

2.7. Statistical Analysis. Results are expressed as means ± SD. Significance was assessed by Student's t test. $P < 0.05$ was considered statistically significant ($^*P < 0.05$).

3. Results and Discussion

3.1. DHA Exerts Cytotoxic Effects on Prostate Cancer Cell Lines with Altered p53. Since the ω3-PUFA DHA kills cancer cells with wild-type 53 [22], two commonly used human

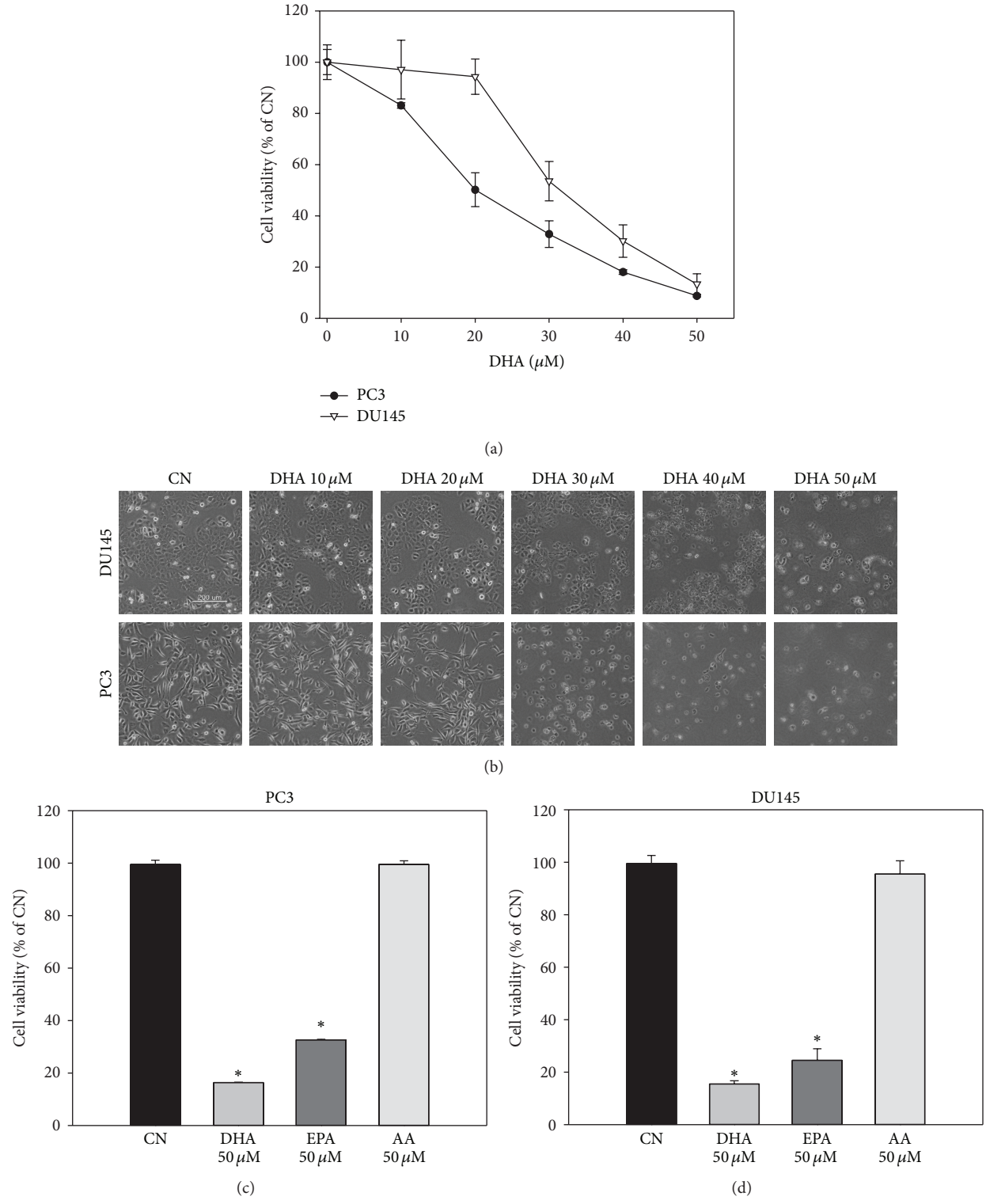
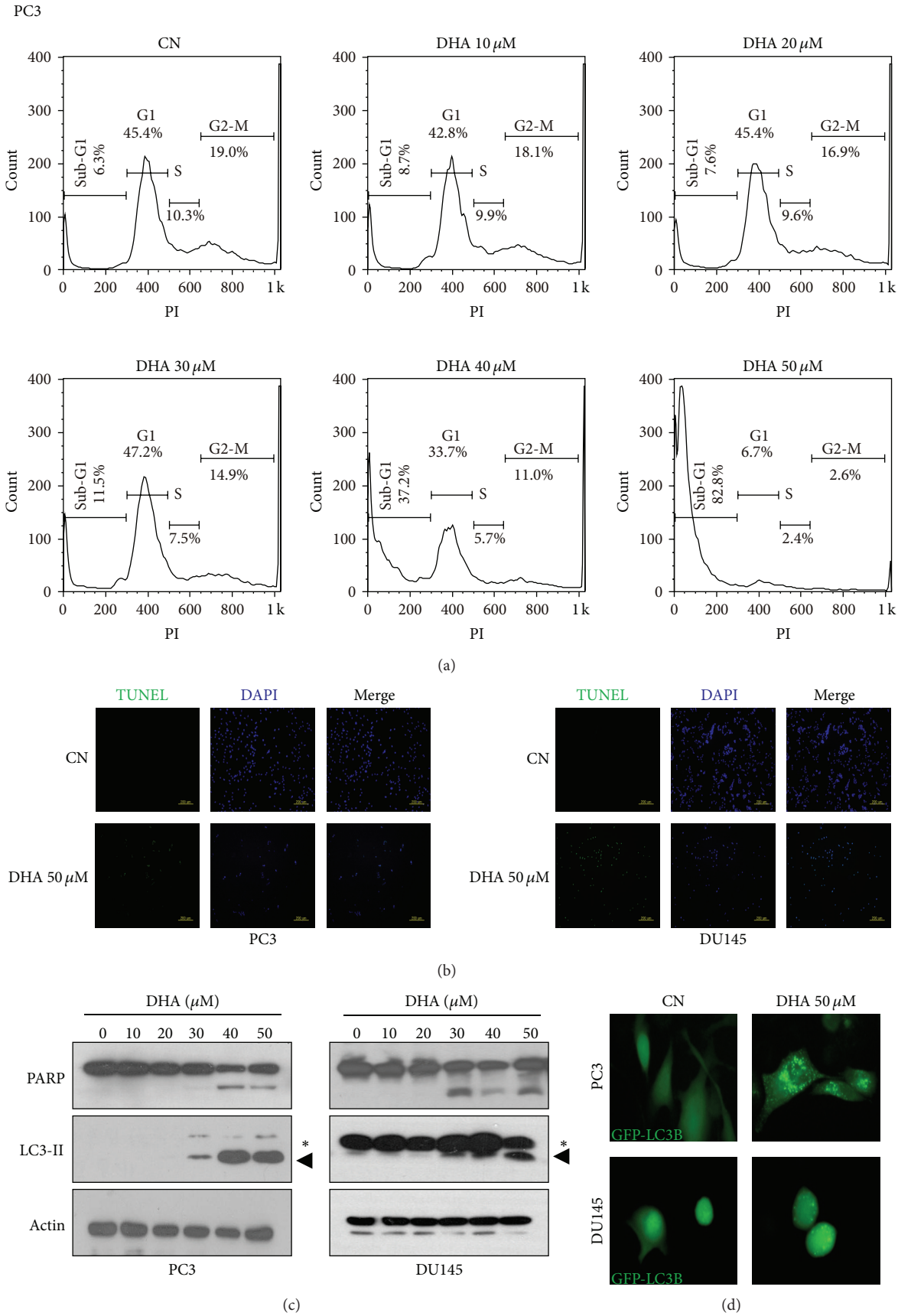


Figure 1: DHA induces cytotoxicity in PC3 and DU145 prostate cancer cells. (a) Reduced cell viability in response to DHA. PC3 and DU145 cells were exposed to 0–50 μM DHA for 24 h and cell viability was measured. (b) Representative images of PC3 (bottom) and DU145 (top) cells exposed to DHA for 24 h (scale bar, 200 μm). (c), (d) Different effects of ω3- and ω6-PUFAs on the viability of PC3 and DU145 cells. PC3 cells (c) and DU145 cells (d) were exposed to 50 μM DHA, EPA, and AA for 24 h and cell viability was measured by MTT. Data are displayed as means ± SD ($n > 5$; $^{*}P < 0.05$).



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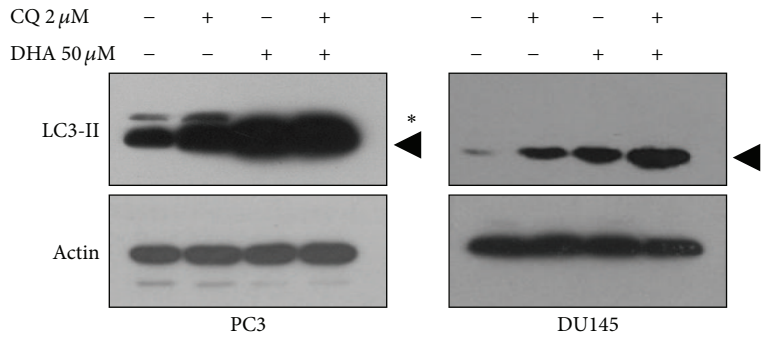


Figure 2: DHA induces apoptosis and autophagic activation in PC3 and DU145 prostate cancer cells. (a) Cell cycle analysis of PC3 cells exposed to DHA. PC3 cells were incubated with 0–50 μM DHA for 24 h and then subjected to flow cytometry after staining with PI. (b) Increased number of TUNEL-positive cells in DHA-treated cells. PC3 (left) and DU145 (right) cells were incubated with 50 μM DHA for 24 h before staining with TUNEL reagents (scale bar, 200 μm). (c) Levels of cleaved PARP and expression levels of LC3-II in response to DHA. PC3 (left) and DU145 (right) cells were incubated with 0–50 μM DHA for 24 h, and PARP cleavage and the expression levels of LC3-II were monitored by Western blot analysis. (d) Representative images of PC3 (top) and DU145 (bottom) cells transiently expressing GFP-LC3B upon exposure to 50 μM DHA for 2 h (magnification, 600x). (e) Effect of DHA on autophagic flux. PC3 (left) and DU145 (right) cells were incubated with 50 μM DHA and/or 2 μM CQ for 24 h and were then subjected to Western blotting for the detection of LC3-II. Black triangle indicates the lipidated form LC3B (LC3-II), and asterisk indicates nonlipidated form of LC3B (LC3-I).

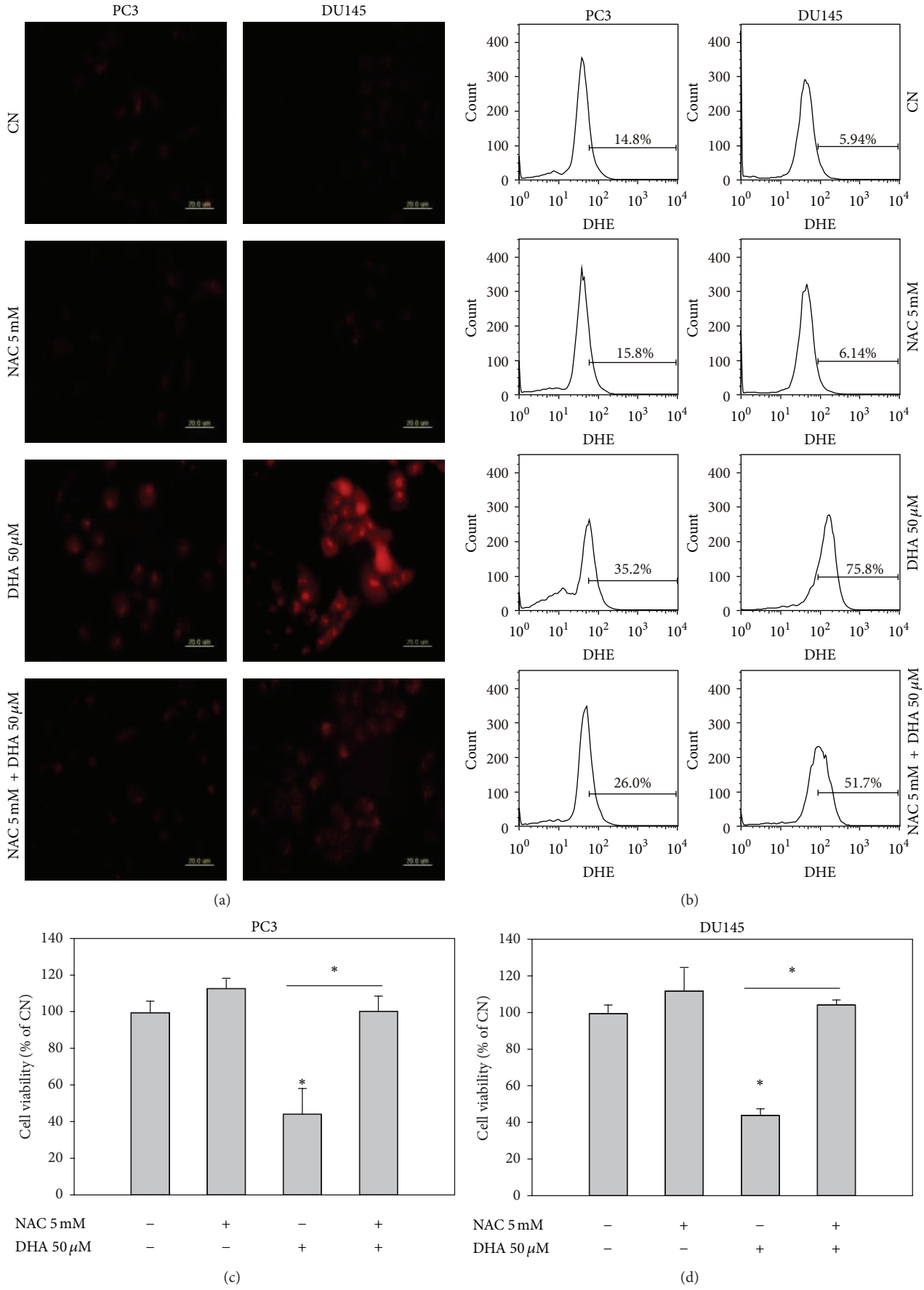
prostate cancer cell lines with altered p53 status, PC3 (p53-null) and DU145 (P223L/V274F) [24], were used to determine whether the anticancer effect of DHA is dependent on functional p53. DHA decreased PC3 and DU145 cell viability in a concentration-dependent manner, and the DHA concentration of 30 μM suppressed viability of DU145 and PC3 cells by about 50% and 70%, respectively, in the MTT assay (Figure 1(a)), suggesting that DHA is cytotoxic to cancer cells with mutant p53 and that p53 function is not essential to DHA anticancer activity. This observation is consistent with a previous study by Kato et al. [25] showing that the inhibitory effect of DHA on colon cancer growth in vitro and in vivo is not extremely dependent on functional p53.

Morphological analysis of PC3 and DU145 cells demonstrated that DHA induces extensive cell death as evidenced by cell rounding, detachment, and shrinkage (Figure 1(b)). Further, at the same concentrations, another ω3-PUFA, EPA, had a lesser cytotoxic effect on PC3 cells and DU145 cells than DHA, while the ω6-PUFA AA had no effect or decreased cell growth only slightly (Figures 1(c) and 1(d)). These observations indicate that DHA may exert cytotoxicity mainly by induction of cell death, and that the effects observed in mutant p53 prostate cancer cells might be restricted to ω3-PUFAs.

3.2. DHA Simultaneously Induces Apoptosis and Autophagy in Prostate Cancer Cells with Altered p53. To determine whether, besides cell death, growth arrest may mediate the effect of DHA in prostate cancer cells with mutant p53, cell cycle analysis was performed using PC3 cells exposed to the same concentrations of DHA as those used in the cell viability assays (Figure 2(a)). DHA did not cause G1, S, or G2-M phase cell cycle arrest; instead, it remarkably increased the number of cells with Sub-G1 DNA content, which represents hypodiploid nuclei, a typical characteristic of apoptotic cells [26]. These results provide evidence that DHA reduces the viability of p53-mutant prostate cancer cells by inducing cell death and indicate that apoptosis is involved in that cell death process. Next, we performed experiments to detect TUNEL-positive cells (DNA nicks) and PARP cleavage, two commonly used markers of apoptosis [26], in DHA-treated cells. Results showed marked increases in the number of TUNEL-positive cells (Figure 2(b)) and in the levels of cleaved PARP (Figure 2(c)) in both PC3 and DU145 cells exposed to DHA, confirming that DHA induces apoptosis in prostate cancer cells with altered p53 status.

These findings are consistent with previous studies demonstrating that apoptosis is a common response to DHA in prostate and other tumor cell lines [8, 14, 27–29]. The apoptosis machinery consists of the intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) pathways [26], and DHA has been shown to induce apoptosis by both pathways through various cell surface receptors [30–32] and intracellular signaling molecules [33], including the well-documented apoptotic inducer p53 [34]; however, since p53 is mutated in PC3 and DU145 cells, DHA-induced apoptosis in these cells must occur through a mechanism other than p53 activation.

As apoptosis and autophagy are highly interconnected [26] and because the ability of DHA to induce apoptosis is related to its ability to stimulate autophagic activation in tumor cells with wild-type p53 [22], we investigated whether autophagy may also be stimulated by DHA in cell models carrying altered p53. To this end, we first examined the level of lipidated form LC3B (LC3-II), a marker of autophagic membranes [35, 36], in PC3 and DU145 cells exposed to DHA. Western blot analysis showed that DHA increased the expression levels of LC3-II in both cell lines (Figure 2(c)), indicating that DHA increases the amount of autophagic



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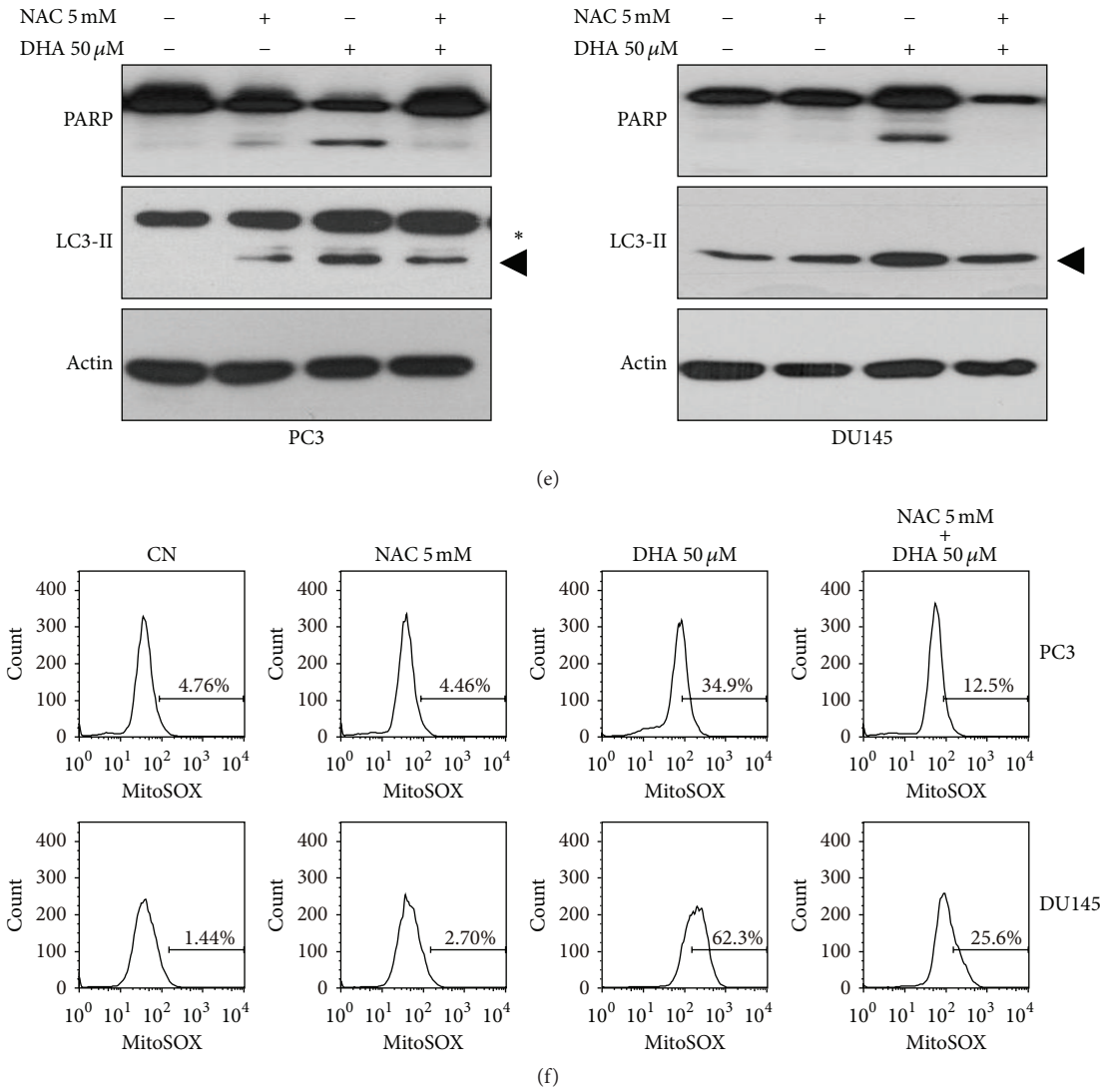

Figure 3: Mitochondrial ROS overproduction mediates DHA-induced apoptosis and autophagy. (a), (b) Increased levels of intracellular ROS induced by DHA in PC3 and DU145 cells. DHE-loaded PC3 and DU145 cells were examined for intracellular ROS accumulation by microscopy (a) and flow cytometry (b) after incubating with 50 μM DHA in the absence or presence of 5 mM NAC for 2 h (scale bar, 200 μm). (c), (d) Effect of the ROS scavenger NAC on the reduction in cell viability induced by DHA. PC3 (c) and DU145 (d) cells were exposed to 50 μM DHA and/or 5 mM NAC for 12 h and cell viability was measured by MTT. Data are displayed as means ± SD. ($n > 5$; $^*P < 0.05$). (e) Effect of NAC on the levels of cleaved PARP and on the expression levels of LC3-II in DHA-treated cells. PC3 (left) and DU145 (right) cells were exposed to 50 μM DHA and/or 5 mM NAC for 12 h, and PARP cleavage and the expression levels of LC3-II were examined by Western blot analysis. Black triangle indicates the lipidated form LC3B (LC3-II), and asterisk indicates nonlipidated form of LC3B (LC3-I). (f) Increased levels of mitochondrial ROS induced by DHA. MitoSOX-loaded PC3 (top) and DU145 (bottom) cells were examined for mitochondrial ROS levels by flow cytometry after incubating with 50 μM DHA in the absence or presence of 5 mM NAC for 2 h.

membranes. This observation was confirmed in cells transfected with GFP-LC3B, which showed a higher number of GFP-LC3B puncta than control cells (Figure 2(d)) upon exposure to DHA. Moreover, autophagic flux assays in cells pretreated with CQ, an inhibitor of lysosomal acidification [37], revealed that DHA further increased the expression levels of LC3-II (Figure 2(e)). These observations clearly demonstrate that DHA also stimulates autophagy in prostate cancer cells with mutant p53 and indicate that functional p53 is not essential to DHA-induced autophagy.

Our results so far suggest that, in addition to apoptosis, autophagy is induced by DHA, and that apoptosis and autophagy may act together to mediate DHA cytotoxicity in prostate cancer cells with altered p53. As an evolutionarily conserved process, basal levels of autophagy maintain cellular homeostasis, while overactivated autophagy causes either cell damage by degrading essential cellular components or cell survival by conferring resistance to stress [35, 37]. As previous studies conducted by us and others show that the autophagic activation induced by DHA and its derivatives contributes

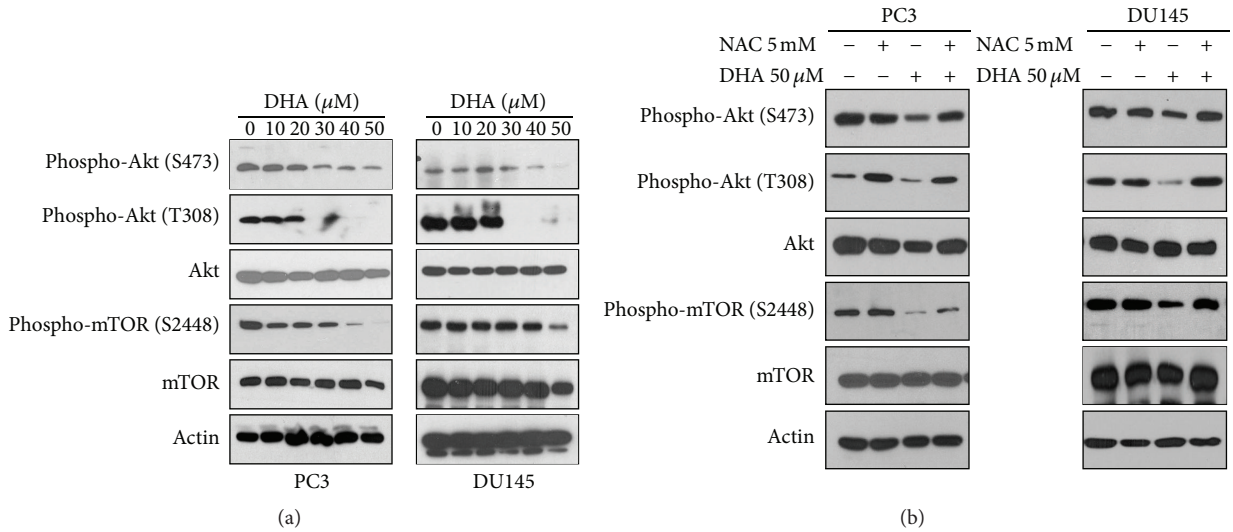

Figure 4: Inhibition of Akt-mTOR signaling contributes to the ROS-mediated apoptosis and autophagy. (a) Inhibition of Akt-mTOR signaling induced by DHA. PC3 (left) and DU145 (right) cells were incubated with 0–50 μM DHA for 24 h, and then the expression levels of Akt-mTOR pathway molecules were detected by Western blot analysis. (b) Effect of NAC on the expression levels of Akt-mTOR pathway molecules in DHA-treated cells. PC3 (left) and DU145 (right) cells were exposed to 50 μM DHA and/or 5 mM NAC for 12 h, and the expression levels of Akt-mTOR pathway molecules were examined by Western blot analysis.

to the death of cancer cells with wild-type p53 [21, 22], it seems reasonable to speculate that the autophagy induced by DHA might be prodeath in cells expressing mutant p53 as well. Investigation is currently underway to address this issue.

3.3. DHA-Induced Apoptosis and Autophagy Are Mediated by Mitochondrial ROS. Multiple lines of evidence indicate that ROS accumulation induced by DHA is responsible for the proapoptotic effect of DHA in tumor cells [33], and ROS have also been implicated in autophagic activation [19, 20]. This knowledge led us to ask whether the simultaneous induction of apoptosis and autophagy by DHA in our cell systems was mediated by ROS. PC3 and DU145 cells were exposed to DHA and the level of intracellular ROS was monitored using the oxidation-sensitive fluorescent probe DHE. DHA resulted in a substantial increase in intracellular ROS accumulation, whereas the antioxidant NAC effectively inhibited that effect in both cell lines, as shown by fluorescence microscopy and flow cytometry (Figures 3(a) and 3(b)). More importantly, NAC pretreatment completely blocked the DHA-induced reduction in cell viability (Figures 3(c) and 3(d)) and reduced the elevated PARP cleavage and LC3-II levels caused by DHA (Figure 3(e)). On the basis of these findings, we conclude that ROS are involved in DHA-induced cytotoxicity in PC3 and DU145 prostate cancer cells and that DHA induces autophagy and apoptosis by triggering intracellular ROS accumulation in these cells.

Moreover, since the major source of intracellular ROS is mitochondria [38], we further examined whether the observed DHA-induced intracellular ROS accumulation originated from mitochondria. As shown in Figure 3(f), in both PC3 and DU145 cells, DHA dramatically increased the signal of the mitochondrial ROS-specific dye MitoSOX, whereas NAC repressed that effect, supporting the mitochondrial origin of DHA-induced ROS in our experimental conditions. Altogether, these data indicate that DHA induces autophagy and apoptosis by triggering mitochondrial ROS overproduction. Although it is not clear how DHA induces mitochondrial ROS in PC3 and DU145 cells, DHA supplementation has been shown to result in its extensive incorporation into mitochondrial membrane phospholipids and to enhance mitochondrial lipid oxidation and ROS generation, leading to mitochondrial dysfunction [39, 40]. It is therefore highly likely that the mitochondrial ROS overproduction observed in PC3 and DU145 cells may result from the direct accumulation of DHA in cell mitochondrial membranes; further study is required to test this hypothesis.

Together, these results are in line with those of others [33, 39, 40], supporting that ROS induction contributes to the antitumor activity of ω3-PUFAs; however, it is noteworthy that the effect of ω3-PUFAs on ROS has been shown to be inconsistent. Schmidt et al. [41] recently reported that supplements containing EPA and DHA enhance the transcriptional levels of genes related to antioxidative enzymes in both normal and dyslipidemic subjects, indicating an inhibitory effect of ω3-PUFAs on oxidative stress. Further, human aortic endothelial cells exposed to EPA and DHA also show reduced ROS production [42]. The inconsistency between augmentation and reduction in oxidative stress caused by ω3-PUFAs is not fully understood, but it might be associated with different cellular milieu in which they react with oxidants [43].

3.4. ROS-Mediated Akt-mTOR Signaling Inactivation Participates in the Autophagy and Apoptosis Induced by DHA. The finding that ROS regulates the simultaneous induction of autophagy and apoptosis by DHA prompted us to investigate the molecular mechanism underlying ROS-induced cytotoxicity. The Akt-mTOR pathway plays a crucial role in prostate cancer development [44], and inhibition of this signaling

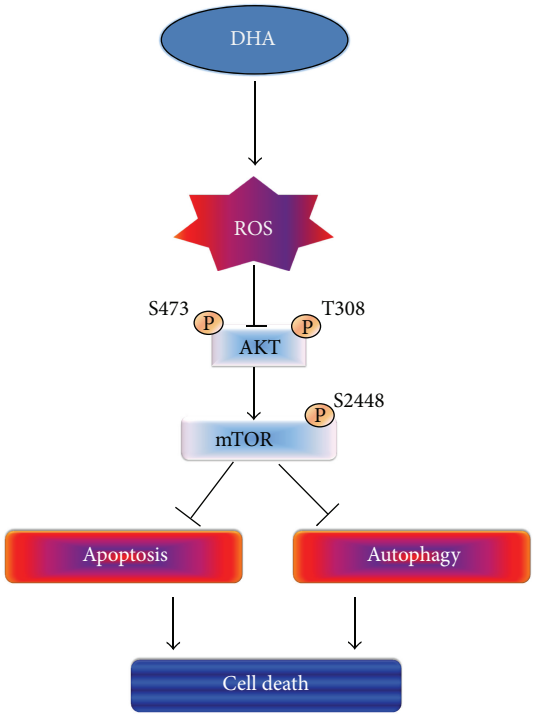

Figure 5: Proposed mechanism of DHA-induced apoptosis and autophagy in prostate cancer cells with altered p53 status.

by ROS is associated with both autophagic activation and apoptosis in cancer cells [20]. Therefore, we hypothesized that Akt-mTOR signaling inactivation might be the mechanism by which DHA induces ROS-mediated autophagy and apoptosis. When PC3 and DU145 cells were exposed to increasing concentrations of DHA, the levels of phosphorylated Akt and mTOR decreased in a concentration-dependent manner whereas the levels of total Akt and mTOR remained unchanged (Figure 4(a)), implying Akt-mTOR signaling inactivation. Next, to obtain direct evidence for the interconnection between Akt-mTOR signaling inhibition and ROS accumulation, PC3 and DU145 cells were preincubated with or without NAC and then exposed to DHA. Western blot analysis revealed that the levels of phosphorylated Akt and mTOR were restored by NAC (Figure 4(b)). These data indicate that the ROS-mediated Akt-mTOR signaling inactivation may be responsible for the autophagy and apoptosis induced by DHA in prostate cancer cells expressing altered p53.

4. Conclusions

In summary, the present study shows that the ω3-PUFA DHA simultaneously induces autophagy and apoptosis in p53-mutant PC3 and DU145 cells by triggering mitochondrial ROS generation and that inhibition of Akt-mTOR signaling is involved in this process (Figure 5). These findings suggest that DHA may be beneficial to patients with p53-mutant prostate cancer and provide a strong rationale for the use of DHA in the treatment of prostate cancer.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contribution

Soyeon Shin and Kaipeng Jing contributed equally to this work.

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Review Article

Mechanisms of Omega-3 Polyunsaturated Fatty Acids in Prostate Cancer Prevention

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This review focuses on several key areas where progress has been made recently to highlight the role of omega-3 polyunsaturated fatty acid in prostate cancer prevention.

1. Introduction

The health benefits of omega-3 polyunsaturated fatty acids (n-3 PUFA), mainly eicosapentaenoic acid (EPA 20:5) and docosahexaenoic acid (DHA, 22:6), have been long known. Epidemiologic studies dating back to the 1970s were among the first to suggest that dietary PUFA may be beneficial in preventing disease [1, 2]. Still today, studies continue to demonstrate the health benefits of n-3 PUFA; however, the mechanisms of action of n-3 PUFA are still not fully understood. Many new discoveries have advanced our understanding about the activities of n-3 PUFA against human disease. For example, DHA-receptor GPR120 has been demonstrated to play a role in sensing and controlling obesity and metabolic syndrome [3]; the recently identified omega-3 mediators, resolvins, and protectins have been demonstrated to have anti-inflammatory and proresolving activities [4]. The purpose of this review is to highlight the recent advances in our understanding of the mechanisms by which n-3 PUFA modulate prostate cancer development.

2. Fatty Acids

There are two major classes of PUFA: n-6 and n-3. Unlike saturated and monounsaturated fatty acid, PUFA cannot be synthesized *de novo* by mammals because they lack the required enzymes and, therefore, PUFA must be obtained from the diet. The n-6 and n-3 PUFA also cannot be interconverted in mammals, but within each series, their metabolism can produce various lipids that differ in chain length and number of double bonds. Linoleic acid (LA, 18:2 n-6) is an n-6 PUFA found in high concentration in grains as well as many seeds and meats. LA serves as a substrate to be converted into a longer fatty acid, arachidonic acid (AA, 20:4 n-6), via a series of oxidative desaturation and elongation reactions. Of the n-3 fatty acids, alpha linolenic acid (ALA, 18:3 n-3) is found at moderate levels in plants, seeds, leafy vegetables, legumes, and nuts. ALA is not metabolized efficiently to longer-chain n-3 PUFA, such as EPA and DHA.

Although they belong to two distinct families, n-3 and n-6 PUFA are metabolized by some of the same enzymes, specifically, delta-5-desaturase and delta-6-desaturase. Excess in one family of fatty acid can interfere with the metabolism of the other and alter their overall biological effects [5]. During n-6 PUFA conversion, delta-6-desaturase, or fatty acid desaturase 2 (FADS2), converts LA to gamma-linolenic acid (GLA, 18:3 n-6). This enzyme represents a rate-limiting step in the synthesis of AA from LA [6]. GLA is elongated to dihomo-gamma-linolenic acid (DGLA, 20:3 n-6) through a chain reaction of four enzymes: a condensation reaction of the fatty acyl chain with malonyl-CoA, catalyzed by an enzyme encoded by the ELOVL5 gene (elongation of very long-chain

fatty acids, family member 5); a reduction reaction mediated by 3-ketoacyl-CoA reductase (KAR); a dehydration reaction catalyzed by 3-hydroxyacyl-CoA dehydratase (HACD); and a second reduction reaction catalyzed by trans-2,3-enoyl-CoA reductase (TECR). Finally, DGLA is converted to AA by delta-5 desaturase, or fatty acid desaturase 1 (FADS1) [6, 7]. Interestingly, malonyl-CoA, which is necessary for fatty acid elongation, is derived from the rate-limiting enzyme of the *de novo* fatty acid synthesis pathway, acetyl-CoA carboxylase. Fatty acid synthesis is well described as an overactive pathway in many cancers [8–11], and its upregulation may also contribute to the elongation of PUFA.

In contrast to AA, the efficiency of ALA conversion to DHA appears to be very low, below 5% in humans. Most ingested ALA is subject to beta-oxidation to provide energy, and only a small fraction is converted to EPA [12, 13]. It was estimated that as low as 0.2% of ALA is converted to EPA, 64% of EPA to docosapentaenoic acid (DPA, 22:5 n-3), and 37% of DPA to DHA [14]. Thus, the overall amount of DPA and DHA converted from ALA is about 0.13% and 0.05% of the starting ALA, respectively. These findings suggest that any contributions from the fatty acid synthesis pathway toward PUFA metabolism most likely favor n-6, rather than n-3, PUFA elongation. It is also very likely that synthesis of the longer n-3 fatty acids from ALA within the body is competitively hindered by the n-6 analogues. It has been reported that the n-3 conversion efficiency is greater in women, possibly because of the importance of meeting the DHA demands of the fetus and neonate [14].

3. PUFA and Cancer

Total fat intake and the ratio of n-6 to n-3 PUFA in the Western diet have increased significantly since the Industrial Revolution [15, 16]. Increased fat consumption has been associated with the development of specific types of cancer such as breast, colon, and pancreatic and prostate cancers, with the notable exception of n-3 PUFA, which show protective effects against colon, breast, and prostate cancers in a number of experimental systems [17–23]. Epidemiological studies about the association of dietary fat and cancer suggests a protective effect of n-3 PUFA and a promoting effect of n-6 PUFA on cancer. Most clinical data regarding the effects of dietary fat on cancers are observational [24], and the results of such studies are mixed, as many fail to demonstrate a significant association between n-3 PUFA and reduced prostate cancer risk or tumor growth [20, 25–27].

The Western diet contains disproportionally high amounts of n-6 PUFA and low amounts of n-3 PUFA, denoted as a high n-6 to n-3 PUFA ratio. Most data regarding the effects of high dietary n-6 PUFA are positively associated with prostate cancer incidence [28–30]. In a study of Jamaican men undergoing prostate biopsy for elevated PSA levels, a positive correlation was observed between n-6 fatty acid LA and Gleason score and n-6 (LA) to n-3 (DHA) ratio in erythrocyte membranes and prostate tumor volume [31]. By comparing PUFA content from malignant and benign prostatic tissues from the same prostate specimens, a Swedish research group found that n-6 PUFA and n-6 PUFA precursors were significantly higher in malignant tissues. This finding further demonstrates that n-6 dietary fat is associated with prostate carcinogenesis [28]. In race-specific analyses based on a case-control study comprising 79 prostate cancer cases and 187 controls, Williams and colleagues found that a high ratio of n-6 to n-3 fatty acids may increase the overall risk of prostate cancer among white men and possibly increase the risk of high-grade prostate cancer among all men [29].

At the same time, epidemiological literature on the association of n-3 PUFA and cancer, including correlational studies and migrational studies, suggest a protective role played by n-3 PUFA. In a recent population-based prospective cohort study of 90,296 Japanese subjects, Sawada et al. reported that consumption of n-3-rich fish or n-3 PUFA, particularly EPA, DPA, and DHA, appears to protect against the development of hepatocellular carcinoma (HCC) [32]. In another population-based prospective study in Japan, there was an inverse relationship between marine n-3 PUFA intake and the risk of colorectal cancer, but this association was only statistically significant in the proximal site of the large bowel [33]. Chavarro et al. performed a nested case-control study by analyzing blood samples of 14,916 healthy men and concluded that higher blood levels of long-chain n-3 fatty acids were associated with a reduced risk of prostate cancer [34]. Szymanski et al. conducted a meta-analysis of fish intake and prostate cancer by focusing on the incidence of prostate cancer and prostate cancer-specific mortality. Their results did not establish a protective association of fish consumption with prostate cancer incidence but showed a significant 63% reduction in prostate cancer-specific mortality [35].

The results of correlational studies are mixed, some of them failing to demonstrate a statistically significant effect. Several confounding factors could account for the inconsistent results on the association between n-3 PUFA and prostate cancer. First, population-based studies mainly rely on data from self-reported dietary fatty acid intake or from estimates based on national consumption, and these assessments correlate poorly with direct measurements of fatty acids in patient samples. In addition, the actual intake in n-3 PUFA may be too low for a protective effect in some cases. Second, the ratio of n-6 to n-3 fatty acids may be more important than the absolute amount of n-3 PUFA, as suggested by animal and human studies [16, 36]. Using a prostate-specific Pten knockout mouse prostate cancer model, we showed that a ratio of n-6 to n-3 below 5 was effective in slowing cancer progression [3]. Brown et al. reported that AA might potentiate the risk of metastatic prostate cancer cell migration and seeding at the secondary site *in vivo*, and lowering the n-6/n-3 ratio in diet by uptake of n-3 PUFA might reduce this risk [37].

4. Mechanisms of Action

4.1. Integration of PUFAs into Plasma Membrane Glycerophospholipids. Although fatty acids are consumed at high levels in a typical Western diet, tumor cells display a strong

dependence on *de novo* fatty acid synthesis [9, 10]. The increased proliferation and metabolism of cancer cells could be the trigger for the abnormal requirement for fatty acid compared to normal cells. Most newly synthesized fatty acids are used to support membrane biogenesis in the form of glycerophospholipids, a class of lipids that are a major component of all cell membranes. Glycerophospholipids, including phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), contain a diglyceride, a phosphate group, and a simple organic molecule, such as choline or serine.

Dietary PUFA can influence the fatty acid composition of glycerophospholipids in cell membranes. In mammals, the *sn*-1 position on the glycerol backbone of glycerophospholipids is usually linked to a saturated fatty acid such as stearic acid (SA, 18:0), and the *sn*-2 position is linked to an n-6 PUFA, such as AA. Feeding cells in culture, or animals, with n-3 PUFA can replace n-6 with n-3 fatty acid at the *sn*-2 position of glycerophospholipids; this is considered a diet-induced *sn*-2 fatty acid moiety change [17, 38, 39]. We have analyzed the incorporation efficiency of PUFAs into glycerophospholipids in prostate cancer cells. Approximately 25% of input albumin-conjugated fatty acids were incorporated into cells within 48 hours. The majority of these newly integrated PUFAs were in the form of PC and PE [40]. These data clearly suggest that the fatty acid at the *sn*-2 position of glycerophospholipids is influenced by cellular PUFA uptake.

n-3 PUFA influences cell membrane conformation and signaling dynamics. The greater density of n-3 PUFA, compared to n-6 PUFA, dictates their aggregation near the lipid-water interface. This characteristic can significantly affect plasma membrane properties, including membrane fluidity, phase behavior, and permeability [41]. These membrane perturbations can bring about changes associated with receptor activation, such as diffusional coupling of various peripheral proteins required in G protein-coupled receptor signaling (GPCR) [42, 43]. Additionally, because of its high level of unsaturation, DHA has very poor affinity for cholesterol, which is enriched in lipid rafts of the cell membrane. Lipid rafts are important membrane domains for cell signaling as many receptors and proteins are enriched in this domain, such as epidermal growth factor receptor (EGFR) [44]. Hence, incorporation of n-3 PUFA into membrane lipids can disturb the formation of lipid rafts and suppress raft-associated cell signal transduction [13, 44].

The serine/threonine protein kinase AKT (protein kinase B) is activated in many solid tumors and hematological malignancies. AKT acts downstream of phosphatidylinositol 3-kinase (PI3K) signaling and is a key regulator of multiple survival pathways. AKT is known to phosphorylate and inactivate the proapoptotic Bcl-2 family member BAD as one of its prosurvival tactics [45]. Phosphatidylinositol (PI) is a negatively charged constituent of lipid membranes. Specific kinases phosphorylate the hydroxyl groups on positions 3′, 4′, or 5′ of the inositol ring, and PI3K primarily generates PI-3,4,5-trisphosphate (PIP_3) from PI-4,5-bisphosphate ($PI(4,5)P_2$). PIP_3 acts as a second messenger to activate pleckstrin homology (PH) domain-containing proteins, including AKT. Conversely, PIP_3 is hydrolyzed to $PI(4,5)P_2$ by PTEN, opposing the action of PI3K [46, 47]. AKT can also be phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDPK1), a PH domain-containing kinase downstream of PI3K (for review, see Franke, 2008) [48]. Hence, intracellular PIP_3 plays a pivotal role in this PI3K/PIP_3/AkT cascade pathway.

Using prostate-specific Pten knockout mice, an immune-competent, orthotopic prostate cancer model, and diets with defined PUFA levels, we found that n-3 fatty acid reduced prostate tumor growth, slowed histopathological progression, and increased survival, whereas n-6 fatty acid had opposite effects. Introducing an n-3 desaturase, which converts n-6 to n-3 fatty acid, into the Pten knockout mice fed an n-6 diet reduced tumor growth similarly to mice fed the n-3 diet. Tumors from mice on the n-3 diet had lower proportions of phosphorylated BAD and higher apoptotic indexes compared with tumors from mice on the n-6 diet. These data suggest that n-3 PUFA can promote BAD-dependent apoptosis to modulate prostate cancer development [17]. We also found that PUFAs modify glycerophospholipid content. DHA can replace the fatty acid at the *sn*-2 position of the glycerol backbone, thereby changing the species of phospholipid. DHA also inhibited AKT^{T308} but not AKT^{S473} phosphorylation, altered PIP_3 and phospho-AKT^{S473} protein localization, decreased $pPDPK1^{S241}$-AKT and AKT-BAD interaction, and suppressed prostate tumor growth. Knockdown of BAD eliminated n-3 PUFA-induced cell death, and reintroduction of BAD restored the sensitivity to n-3 fatty acids *in vitro*. Knockout of BAD diminished the suppressive effect of n-3 PUFA on prostate tumor growth *in vivo*. These data suggest that modulation of prostate cancer development by PUFA is mediated in part through the PI3K/AKT survival pathway [17, 40].

Hu et al. reported that n-3 PUFA-induced apoptosis in human prostate cancer cells occurs through upregulation of syndecan-1 (SDC-1) expression followed by concomitant suppression of PDPK1/AKT/BAD phosphorylation [49]. n-3 fatty acids may also decrease cell proliferation and induce apoptotic cell death in human cancer cells by decreasing signal transduction through the AKT/NFκB cell survival pathway and by modulating the PI3K/AKT/p38 MAPK pathway [50, 51].

4.2. PUFA Mediator. A common fate of unsaturated lipids released from the membrane is oxidation. AA is the precursor of highly bioactive lipid mediators metabolized by a number of enzymes belonging to the cyclooxygenase (COX) and lipoxygenase (LOX) families, as well as cytochrome P450. COXs have two well-characterized isoforms, COX1 and COX2. COX1 is a constitutively expressed gene in most tissues, whereas COX2 is an immediate-early response gene, which is strongly induced in many human malignancies [52]. Signal transduction of n-6 PUFA-derived lipids and the effect of these lipid mediators on the organism have been well characterized. For example, AA-derived lipid mediators are associated with a variety of activities, including inflammation and cancer. Evidence from human studies also supports the important role of COXs and LOXs in PUFA metabolism and

cancer [53–56]. Because of its high expression in inflammation and cancer, COX2 has been the subject of intense study and proposed as a target for cancer therapy [57–59].

In contrast to n-6 PUFA, the metabolism of n-3 PUFA is not well understood. Interest in n-3 PUFA-derived lipid mediators began with observations of Greenland Eskimos whose diet is rich in marine-derived fish and showed lower mortality from coronary heart disease and lower prevalence of inflammation-related diseases, such as psoriasis, inflammatory bowel disease, asthma, rheumatoid arthritis, and other autoimmune diseases [60, 61]. Serhan et al. reported that inflammatory exudates in the murine air pouch from mice treated with n-3 PUFA and aspirin contained a series of bioactive compounds. Using an unbiased lipidomics approach, they identified and named the EPA-derived E-resolvins (RvE1 and RvE2), DHA-derived D-resolvins (RvD1 and RvD2), and (neuro-)protectin (PD1) [62].

RvE1 and RvE2 are protective in a wide variety of disease models, mainly through their anti-inflammatory activities. RvE1 can resolve inflammation caused by bacterial infection of periodontal disease in a rabbit model [63], prevent neovascularization after oxygen-induced retinopathy [64], and suppress neutrophil infiltration in an acute peritonitis model [65]. RvD1, RvD2, and PD1 also have protective activities in a variety of animal models, including models of lung injury, insulin resistance, peritonitis, wound healing, and atherosclerosis [66]. RvD1 was shown to reduce leukocyte infiltration in murine inflammatory exudates [67] and RvD2 was shown to reverse inflammatory pain in mice [68]. PD1 has been reported to regulate amyloid beta secretion and thereby improve neuronal survival in a mouse model of Alzheimer's disease [69]. Although ample data indicate that these n-3 PUFA-derived mediators can resolve inflammation, little is known about their role in inflammation-related cancers, such as prostate and colon cancers.

Due to the existence of multiple oxygenases, the role of each enzyme in the development of prostate cancer has not been studied systematically in a single system or animal model. Furthermore, studies performed in animals rarely take diet into account. To systematically assess the interaction between oxygenases and dietary PUFA in a single animal model of prostate cancer, we knocked out Cox1, Cox2, Lox5, Lox12, or Lox15 in prostate-specific Pten null mice. Our preliminary results indicate that tumor growth was significantly increased in $Cox1^{-/-}$ Pten null mice on n-3 diet compared to Cox1-wild-type Pten null littermates. This result suggests that Cox1 is required for the protective effects of n-3 PUFA. Interestingly, tumor growth was decreased in n-6 PUFA fed $Cox1^{-/-}$ Pten-null mice compared to n-6 fed Cox1-wildtype Pten-null mice, suggesting that n-6 metabolites of Cox1 promote tumor growth. Loss of Cox2 reduced prostate tumor growth on both n-3 and n-6 diets, suggesting that the suppressive effect of n-3 PUFA is independent of Cox2 metabolism. Loss of Lox5 reduced prostate tumor growth on n-6 diet but had no effect on n-3 diet; loss of Lox12 or Lox15 did not affect prostate tumor growth on either diet. These results suggest that the promotion of prostate tumor growth by n-6 diet is dependent on Lox-5 metabolism and both Lox12 and Lox15 metabolites are not critical for prostate cancer growth in this model (Chen et al., unpublished).

4.3. Fatty Acids Receptors. Lipids are ligands for cell-surface G protein-coupled receptors (GPCRs), toll-like receptors (TLRs), and peroxisome proliferator-activated receptors (PPARs). G protein-coupled receptors (GPCRs) are important signaling molecules for many aspects of cellular function. They are members of a large family that share common structural motifs, such as seven transmembrane helices and the ability to activate heterotrimeric G proteins. Recently, several groups reported that unbound free fatty acids can activate GPCRs, including GPR40, GPR41, GPR43, GPR84, and GPR120 [3]. Short-chain fatty acids are specific ligands for GPR41 and GPR43, medium-chain fatty acids for GPR84, and long-chain fatty acids for GPR40 and GPR120 [70–73]. Activation of GPR84 receptor by medium-chain fatty acids triggered the production of the proinflammatory cytokines from leukocytes and macrophages. The function of GPR84 may be associated with chronic low-grade inflammation-associated disease [74].

GPR40 and GPR120 have been reported to be activated by long-chain fatty acids such as DHA, EPA, and AA [73, 75]. As a G protein-coupled receptor, GPR40 can activate the phospholipase C and phosphatidylinositol signaling pathways [76]. Although GPR40 is preferentially expressed in pancreatic β-cells and is known to mediate insulin secretion [77], several groups showed that it is expressed in the brain where it mediates the antinociceptive activity of DHA [78, 79]. Recently, Oh and others reported that GPR120 functions as an n-3 PUFA receptor *in vitro* and *in vivo* [3] and suggested that diminished activation of GPR120 can be an important contributor to obesity, insulin resistance, and tissue inflammation [80, 81]. GPR120 is highly expressed in adipose tissue, proinflammatory bone marrow-derived $CD11C^{+}$ macrophages (BMDCs), mature adipocytes, and monocytic RAW 264.7 macrophage cells. DHA strongly inhibited LPS-induced phosphorylation of JNK and IKKβ, IκB degradation, cytokine secretion- and inflammatory gene expression level in GPR120-positive cells. These effects of DHA were completely prevented by GPR120 knockdown, demonstrating that these anti-inflammatory effects were specifically exerted through GPR120. An n-3 PUFA diet containing 27% fish oil led to improved insulin sensitivity with increased glucose infusion rates, enhanced muscle insulin sensitivity, and greater hepatic insulin sensitivity. The n-3 PUFA diet had no effect in the GPR120 knockout (KO) mice. On chow diets, the GPR120 KO mice showed moderate insulin resistance with no changes in food intake or body weight. On high-fat diet (HFD), the GPR120 KO mice gained more weight than wild-type controls [3]. In humans, GPR120 expression in adipose tissue is significantly higher in obese individuals than in lean controls [80]. Ichimura and colleagues compared sequences of GPR120 exons in obese populations and discovered a deleterious nonsynonymous mutation (R270H) [80]. Their population study showed that the $GPR120^{R270H}$ variant correlated with obesity. Further investigation *in vitro* demonstrated that the $GPR120^{R270H}$

variant was unable to respond to long-chain fatty acid stimulation. This inactive mutant of GPR120 may contribute to its significant association with obesity [80].

Toll-like receptors (TLRs) are transmembrane glycoprotein receptors that are important regulators of the innate immune system. TLRs are considered a link between innate (nonspecific) and adaptive (specific) immunity and contribute to the immune system's capacity to efficiently combat pathogens [82]. TLR expression is increased in tumors, including breast, colorectal, melanoma, lung, prostate, pancreatic, and liver cancer [83]. Activation of TLRs triggers a signaling cascade producing inflammatory cytokines that recruit components of the adaptive immune system to kill the pathogen [84]. Among the family of TLRs, TLR4 and TLR9 have been reported to be associated with prostate cancer [85–87]. Panigrahy et al. reported that saturated fatty acids activated, and DHA inhibited, TLR2- and TLR4-mediated proinflammatory activity in a cell culture system [88]. Saturated fatty acids may stimulate the TLR4 signaling pathway to trigger the production of proinflammatory mediators, which may contribute to neuronal death [89].

PPARs (PPARα, PPARβ/δ, and PPARγ) are a superfamily of ligand-activated transcription factors and nuclear hormone receptors. The syndecan family of cell surface proteoglycans share a structure of small, conserved cytoplasmic and transmembrane domains and larger, distinct ectodomain. They are implicated in a variety of physiologic and pathologic processes such as nutrient metabolism, energy homeostasis, inflammation, and cancer. Growing evidence has demonstrated that PPARγ serves as a tumor suppressor in cancer (see review of Robbins and Nie, 2012) [90]. n-3 PUFA can induce apoptosis in human prostate cancer cells by activating the nuclear receptor PPARγ and upregulating the PPARγ target gene, syndecan-1 (SDC-1) [49, 91]. It has been suggested that n-3 PUFA induces cell apoptosis in prostate cancer via a mechanism of LOX15-mediated SDC-1-dependent suppression of PDPK1/AKT/BAD phosphorylation [49, 91]. SDC-1 upregulation by DHA has also been demonstrated in human breast cancer cells [19, 92–94] and in n-3 PUFA-enriched mammary glands and liver of fat-1 mice [95].

SDC-1 plays several important cellular functions. It regulates many steps of leukocyte recruitment in noninfectious inflammatory diseases, attenuates inflammation by modulating heparin sulfate-binding proinflammatory factors, and plays a key role in the normal remodeling of injured cardiac tissues [96]. Loss of cell surface SDC-1, seen in many carcinomas such as skin cancer and colorectal adenocarcinomas, favors acquisition of the metastatic phenotype in cancer cells [97]. There is very limited information about SDC-1 expression in prostate cancer. Some studies reported an inverse relationship between SDC-1 and Gleason score [98, 99], but a tissue microarray analysis in another study showed an increase in SDC-1 with tumor progression [100]. Our own studies have shown reduced expression of SDC-1 in prostate cancer cell lines compared to normal prostate epithelial cells and lower expression in androgen-dependent LNCaP cells compared to androgen-independent PC3 and DU145 cells [49]. In a mouse prostate cancer model, the reduction in tumor growth as a result of dietary n-3 PUFA is accompanied by an increase in the expression of secreted SDC-1 [49, 101].

Several other receptors have been suggested as targets for n-3 PUFA action. Turk et al. reported that DHA can induce the alteration in both the lateral and subcellular localization of EGFR and suppress EGFR signaling, which suggests implications for the molecular basis of cancer prevention by DHA [44, 102]. It is also reported that DHA can increase CD95 (Fas ligand death receptor) cell surface expression and may mediate CD95-induced apoptosis [103]. For more information about PUFA receptor interaction, please refer to a review by Lee et al. [104].

4.4. Other Mechanisms. Nuclear factor erythroid-2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor. Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) under basal conditions. When the cell is challenged by oxidative stress, Nrf2 is released from Keap1 inhibition, translocates to the nucleus, forms a complex with other factors, and activates transcription of genes containing an antioxidant response element (ARE) in their promoter region. Nrf2 has been reported to play an important role in lung injury reversal, human endothelial cell survival, neuroinflammation, hyperoxia, lung damage from cigarette smoking, and impaired function of macrophages [105]. Other studies suggest that Nrf2 suppresses inflammation by inhibiting NFκB activation through regulation of redox balance, calcium signaling, and PPARs [106]. Various human cancers, such as lung cancer, frequently exhibit increased levels of Nrf2 [107]. Downregulation of nuclear Nrf2 gene expression by RNAi-mediated silencing in nonsmall cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy [108]. Cancer cells are suspected to hijack the Keap1-Nrf2 system as a means to acquire malignant properties. Indeed, the prognosis of patients carrying Nrf2-positve cancers is poor [105]. Oxidized n-3 fatty acids can react directly with the negative regulator of Nrf2, Keap1, by dissociating them and inducing Nrf2-directed gene expression [109]. For example, n-3 PUFA mediators can activate Nrf2 in vascular endothelial cells to prevent oxidative stress-induced cytotoxicity [110]. DHA and EPA can induce Nrf2 expression and suppress lipopolysaccharide-(LPS-) induced inflammation [111]. Evidence of Nrf2-mediated response modulated by n-3 PUFA in prostate cancer came from a randomized clinical trial. Eighty-four men with low-risk prostate cancer were stratified based on self-reported dietary consumption of fish oil. Exploratory pathway analyses of rank-ordered genes revealed the modulation of Nrf2 or Nrf2-mediated oxidative response after 3 months of fish oil supplementation ($P = 0.01$) [112].

Calcium (Ca^{2+}) signaling is a ubiquitous mechanism in the control of cell function. The transient receptor potential channels (TRP channels) are 6 transmembrane-spanning proteins with both amino and carboxyl tails located on the intracellular side of the membrane. Ca^{2+} flux through TRP channels located in the plasma membrane and in the membranes of excitable intracellular organelles can promote changes in intracellular free Ca^{2+} concentrations and the

Table 1: Mechanism of n-3 PUFA action.

Target	Mechanism of action	References
Membrane PIPs	Suppress PI3K/AKT/BAD survival pathway	[17, 40]
SDC-1	Suppress PI3K/AKT/BAD and AKT/NFκB survival pathway	[49]
Unknown	Suppress AKT/NFκB or PI3K/AKT/p38 MAPK pathway	[50, 51]
GPR40	Mediate insulin secretion, cell proliferation	[75–77]
GPR120	Anti-inflammation	[3, 80]
TLRs	Suppress the production of proinflammatory mediators	[88, 89]
PPARg	Induce cell apoptosis	[90]
Nrf2	Suppress inflammation, reduce oxidative stress	[106, 109, 110]
Calcium signaling	Inhibit cell proliferation	[114]
COXs	Promote inflammation	[52]
LOXs	Promote inflammation	[53]
E-resolvins	Resolve inflammation	[62]
D-resolvins	Resolve inflammation	[62]

membrane potential, which can modulate the driving force for other ions and Ca^{2+} itself [113]. Evidence suggests that TRP channel function can be modulated both directly and indirectly by n-3 fatty acids [114]. It was demonstrated that DHA and EPA at physiological concentrations have the ability to evoke small currents, which seems to be dependent on the previous sensitization of the channel by protein kinase C (PKC). Whether these effects are due to the action of these PUFAs on the agonist binding site or are due to conformational changes caused by TRP protein interactions with the lipid bilayer requires further investigation [115]. Recent findings also indicate that TRP channel function can be modulated by D and E resolvins. Resolvin binding on GPCRs seems to be part of the mechanism underlying the resolvin-mediated regulation of TRP channel function [116]. DHA significantly reduces oxidative stress-induced endothelial cell Ca^{2+} influx. This effect might be associated, at least in part, with altered lipid composition in membrane caveolar rafts [117]. Ca^{2+} has been shown to be essential for increased cell proliferation in prostate cells [118]. Sun et al. observed significantly higher Ca^{2+} influx in prostate cancer cells. They reported that high ratio of Ca^{2+}/Mg^{2+} facilitated Ca^{2+} influx and led to a significant increase in cell proliferation of prostate cancer [119]. Thus, one could speculate that n-3 PUFA might indirectly modulate prostate cancer growth by directly modulating Ca^{2+} influx.

5. Conclusions

Cancer incidence and mortality are high in the Western world and a high n-6 to n-3 PUFA ratio in the Western diet may be a contributing factor. There is much evidence to suggest that n-3 PUFA has antiproliferative effects in cancer cell lines, animal models, and humans. Direct effects on cancer cells and indirect effects on the host immune system (anti-inflammation) likely contribute to the inhibitory effect of n-3 fatty acids on tumor growth; however, further investigation is warranted. n-3 PUFA may also regulate other complex metabolic processes, including β-oxidation, lipid release from glycerophospholipids, cellular signaling of membrane bound proteins, eicosanoid synthesis, and direct activation of nuclear receptors and gene transcription, all of which may influence the development and progression of prostate cancer. Overall, there seems to be an exceptionally broad potential for the mechanisms mediating cancer prevention by n-3 PUFA (summarized in Table 1). We expect that new research in lipidomics and metabolomics will provide new techniques and approaches to answering the many questions that remain regarding the mechanisms underlying the health benefits of n-3 PUFA.

Abbreviations

PUFA: Polyunsaturated fatty acid
EPA: Eicosapentaenoic acid (20:5, n-3)
DHA: Docosahexaenoic acid (22:6, n-3)
LA: Linoleic acid (18:2, n-6)
AA: Arachidonic acid (20:4, n-6)
ALA: Alpha linolenic acid (18:3, n-3)
FADS: Fatty acid desaturase
GLA: Gamma-linolenic acid (18:3, n-6)
DGLA: Dihomo-gamma-linolenic acid (20:3, n-6)
KAR: 3-Ketoacyl-CoA reductase
HACD: 3-Hydroxyacyl-CoA dehydratase
TECR: Trans-2,3-enoyl-CoA reductase
DPA: Docosapentaenoic acid (22:5, n-3)
PC: Phosphatidylcholine
PS: Phosphatidylserine
PE: Phosphatidylethanolamine
PI: Phosphatidylinositol
SA: Stearic acid (18:0)
GPCR: G Protein-coupled receptor
EGFR: Epidermal growth factor receptor
AKT: Serine/threonine protein kinase (protein kinase B)
PI3K: Phosphatidylinositol-3-kinase
PIP_3: PI-3,4,5-trisphosphate

PH: Pleckstrin homology
PDPK1: Phosphoinositide-dependent kinase-1
SDC-1: Syndecan-1
COX: Cyclooxygenase
LOX: Lipoxygenase
RvE1: E-Resolvin1
RvD1: D-Resolvin1
TLR: Toll-like receptor
PPAR: Peroxisome proliferator-activated receptor
MCFA: Medium-chain fatty acid
BMDC: Bone marrow-derived $CD11C^+$ macrophage
KO: Knockout
HFD: High-fat diet
Nrf2: Nuclear factor erythroid-2-related factor 2
Keap1: Kelch-like ECH-associated protein 1
ARE: Antioxidant response element
LPS: Lipopolysaccharide
PKC: Protein kinase C.

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Review Article

Dietary ω-3 Polyunsaturated Fatty Acid DHA: A Potential Adjuvant in the Treatment of Cancer

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ω-3 Polyunsaturated fatty acids (PUFAs), mainly present in fish oil, are part of the human diet. Among PUFAs, docosahexaenoic acid (DHA) has received particular attention for its anti-inflammatory, antiproliferative, proapoptotic, antiangiogenetic, anti-invasion, and antimetastatic properties. These data suggest that DHA can exert antitumor activity potentially representing an effective adjuvant in cancer chemotherapy. This review is focused on current knowledge supporting the potential use of DHA for the enhancement of the efficacy of anticancer treatments in relation to its ability to enhance the uptake of anticancer drugs, regulate the oxidative status of tumor cells, and inhibit tumor cell invasion and metastasis.

1. Introduction

Dietary fish oil (FO) has been shown to have beneficial effects on some chronic degenerative diseases such as cardiovascular disease [1, 2], rheumatoid arthritis [3], diabetes [4], other autoimmune diseases [5, 6], and cancer [7, 8]. The beneficial effects of FO seem to be due to its high content of the ω-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). 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). Both agents are essential fatty acids (FAs) that cannot be synthesized by mammals and thus must be obtained from dietary sources.

DHA and EPA, as well as the other FAs, once ingested, can be uptaken and undergo to both cell passive translocation and carrier-mediated transmembrane translocation that involves various membrane-associated proteins [9]. Upon incorporation in cell membranes, PUFAs can be found either as constituents of membrane phospholipids (i.e., esterified FAs) or free molecules (i.e., free FA-FFA). In both forms, PUFAs give a substantial contribution to the physical properties of biological membranes, including membrane organization, ion permeability, elasticity, and eicosanoid formation [10, 11]. Taking into account these considerations, dietary DHA and EPA were established as significant nutrients involved in metabolic regulation. Moreover, some researches have established the capability of EPA and in particular of DHA to influence cancer proliferation [12], apoptosis [12, 13] and differentiation [12], as well as, to inhibit angiogenesis [14], tumor cell invasion [15] and metastasis [16]. These data suggest that DHA can both exert antitumor activity potentially representing an effective adjuvant in cancer chemotherapy and ameliorate some of the secondary complications associated with cancer, like cachexia [17, 18].

Despite progress made in recent years in cancer chemotherapy, advanced solid tumors, including advanced carcinomas, sarcomas, melanoma, and glioblastomas, still pose major difficulties in their treatment, and the traditional therapeutic modalities alone have not provided satisfactory long-term clinical results [19, 20]. Indeed, after one or several distinct lines of chemotherapy, in most cases only partial responses are obtained, meaning that after an initial pyrrhic success, tumors will resume growth, select therapy-resistant variants, and seal the patient's fate [21]. Even in those cases in which the tumor has apparently been removed completely (complete remission), micrometastases of dormant tumor cells (or cancer stem cells) often lead to relapse and to final

therapeutic failure. Therefore, given the complexity of escape and survival to cancer development, most oncologists have reached the idea that no single therapy is sufficient to treat cancer [22, 23].

Evidence exists on the efficacy of DHA as anticancer adjuvant, with particular emphasis to its capability both to enhance the uptake of anticancer drugs, especially in cells otherwise resistant to these drugs, and to increase the prooxidant and proapoptotic efficacy of some chemotherapies [17]. This review focuses on the investigations on the potential use of DHA as adjuvant to improve the efficacy of anticancer treatment, acting at multiple levels such as the regulation of the oxidative status of tumor cells and the inhibition of tumor cell invasion and metastasis.

2. DHA as Adjuvant to Improve Tumor Cell Cytotoxicity Induced by Lipid Peroxidation and Oxidative Stress

2.1. Redox Status Management in Tumor Cells. As a consequence of aerobic metabolism, aerobic organisms produce a wide range of oxygen radicals and other reactive oxygen species (ROS), including free radicals (e.g., $O_2^{\bullet -}$ and hydroxyl radical $OH^{\bullet -}$) and nonradical compounds (e.g., H_2O_2). ROS and reactive nitrogen species (RNS) are constantly generated inside cells by dedicated enzyme complexes (like NADPH oxidase and nitric oxide synthases) or as by-products of oxidation-reduction reactions, including those arising from mitochondrial respiration [24]. While some of these intermediates are useful against pathogens in the context of innate immunity, most are harmful to cells because they irreversibly damage proteins, lipids and nucleic acids and positively contribute, in different ways, to carcinogenesis and to malignant progression of tumor cells.

When normal cells become cancerous, they exhibit elevated levels of endogenous ROS principally due to the accelerated metabolism needed to maintain the high proliferation rate typical of cancer cells [25]. Moreover, several studies indicate that high levels of mitochondrial ROS generation are essential for cellular proliferation and tumorigenesis. ROS can affect target gene expression through phosphorylation, activation and oxidation of transcription factors such as APEX1, NF-kB, p53 and HIF-1α [26–28]. Moreover, ROS can oxidize and inhibit signaling molecules such as p38 mitogen-activated protein kinase (MAPK) phosphatase, resulting in enhanced proliferation and survival of cancer cells [29].

High ROS levels imply that tumor cells also need to defend themselves from oxidative damage in order to survive and successfully spread. For example, the transforming activity of some oncogenes has been linked to their capacity to maintain elevated intracellular levels of reduced glutathione (GSH) (the principal redox buffer) [30] and high levels of antioxidant enzymes like superoxide dismutase 1 (SOD1) [31, 32]. Moreover, the "Warburg effect" adopted by cancer cells leads to activation of glucose metabolism and inhibition of mitochondrial respiration (i.e., cell catabolic processes that provides the highest quantity of reactive species), constituting thus a mechanism of protection that rescues tumor cells from oxidative stress and allows them to continue to proliferate [33]. On the other hand, ROS have been proposed as common mediators of apoptosis. Indeed, the vast majority of cytotoxic anticancer agents (including ionizing radiation, most chemotherapeutic agents, and some targeted therapies) work through the generation of ROS either directly or indirectly [34]. In fact, the accumulation of intracellular ROS causes the disruption of the mitochondrial membrane potential, the release of cytochrome c with consequent activation of the caspase cascade and ultimately cancer cell's demise through tumor cell death for apoptosis [35]. However, although various anticancer drugs initially induce ROS production, in most cancer cells the prolonged treatment with these drugs reduces the levels of ROS, resulting in drug-resistance. For example, it has been observed that cisplatin or chlorambucil initially induces ROS production in ovarian carcinoma A2780 cells, whereas prolonged drug treatments reduce ROS levels making those cells resistant to chemotherapy [36, 37]. Thus, a decrease of ROS level in prolonged drug-treated cells is not a secondary cellular outcome, but a primary mechanism of drug-resistance. Taking into account these considerations, "drug-combination" therapies might represent a good strategy to increase the efficacy of conventional anticancer treatments by acting as follows: (i) maintaining higher ROS levels in cancer cells, thereby precluding drug resistance; (ii) reducing cancer endogenous antioxidant defenses; and (iii) increasing drug uptake and thus apoptosis.

2.2. DHA as Adjuvant in the Modulation of the Redox Status in Tumor Cells. As mentioned above, in a variety of cancer types different anticancer chemotherapeutic agents have been shown to be more therapeutically effective when marine n-3 long-chain PUFAs were added to the diet [38]. The specific mechanisms behind these effects have not fully elucidated yet, but many scientific researches suggest that n-3 PUFAs may act at several levels.

One of the main characteristics of PUFAs is the fact that they are highly susceptible to oxidation. Indeed, methylene group, located between two double bonds (–CH=CH–CH_2–CH=CH–), is particularly vulnerable to radical attack by reactive species which entails the abstraction of hydrogen [39]. Among PUFAs, DHA having five of these methylene groups, is more susceptible to nonenzymatic lipid peroxidation [40]. DHA nonenzymatic oxidation is initiated after free radical attack (e.g., $^{-}O_2$, H_2O_2), resulting in an unstable DHA-radical state that quickly undergoes isomerization and rearrangement of double bonds. These changes lead to the formation of conjugated dienes, which successively, after a further oxidation, are converted into lipid hydroperoxides. At this step, lipid hydroperoxides can follow two different ways: one leads to disintegration of the carbon chain and makes alkoxy radicals such as aldehydes (e.g., malondialdehyde, MDA) and alkanes and/or alkenes as byproducts (e.g., pentane); the other keeps the carbon chain intact, with the formation of peroxy radicals like isoprostane, isofuran and mono- or dihydroxy FAs [41]. The lipid peroxidation products, such as MDA, are highly toxic molecules for the cell, that are able to interact with nucleic acid bases to form several different adducts [42]. Moreover, the nonenzymatic

lipid peroxidation into membranes triggers a further increase of formation of radical species in the cell. Therefore, as described above, considering that cancer cells contain a higher rate of reactive species compared to normal cells, the presence of DHA can lead to a high level of nonenzymatic lipid peroxidation into membranes, that keeps consistently high levels of ROS in the cells. Hossain et al. showed that DHA dose-dependently stimulated reactive species production and hence membrane lipid peroxidation, in HT-29 and Caco-2 colon carcinoma cells [43]. Moreover, other works show that this effect was enhanced when DHA was given in combination with some chemotherapeutic agents. In a report by Guffy et al., it has been shown that *in vitro* administration of DHA improved adryamicin uptake, cytotoxicity towards L1210 murine leukemia cells and increased tumor cell lipid peroxidation and oxidative damage [44]. Other studies have highlighted similar activities of DHA in combination with vincristine (VCT) chemotherapeutic agent. Indeed, DHA was able to increase VCT influx and cytotoxicity against both the KB-3-1 human cervical carcinoma cell line and the KB-ChR-8-5 VCT resistant cell variants. Similar results were observed in the NCG human neuroblastoma cell line and in NCG/VCR1 vincristine-resistant cells, where DHA enhanced the vincristine sensibility, by the enhancement of drug uptake and lipid peroxidation [45, 46]. These observations suggest that DHA may be capable of increasing the uptake of anticancer drugs in both resistant and sensible cells and that the incorporated DHA-mediated lipid peroxidation may lead to a greater cytotoxic effect compared to chemotherapy alone. However, the increased drug uptake by DHA was not seen in all tumor cell models. In fact, although it has been demonstrated that DHA is able to increase doxorubicin uptake in both P388 and P388/DOX (doxorubicin resistant) mouse leukemia cells [47] and in MDA-MB-231 and MCF-7dox (doxorubicin-resistant cells) human breast carcinoma cell lines, the same action has not been observed in parental MCF-7 human breast carcinoma cells [48]. This discrepancy might be due to the fact that different cell lines might have different cellular characteristics, such as membrane formation and configuration. However, even if DHA does not act in some cells as a drug uptake inducer, it can make cells more vulnerable to oxidative damage induced by exogenous agents. Thus, in some cases, DHA can make possible to overcome the maximum threshold of ROS tolerability by cancer cells, bypassing thus the oxidative stress resistance [49].

As above mentioned, cancer cells are capable of increasing their resistance to oxidative stress by increasing their endogenous antioxidant defenses. On this point, in our laboratory we have highlighted the ability of DHA to induce active GSH extrusion in the PaCa-44 pancreatic cell line [50]. We observed that intracellular GSH was dramatically reduced (more than 60%) by an active extrusion process after 6 h of cell treatment with DHA and that, in the presence of two specific inhibitors of carried-mediated GSH extrusion (such as cystathionine and methionine), DHA-induced GSH-extrusion process was reversed. Moreover, Ding and Lind showed that DHA treatment induced a 50% reduction of glutathione peroxidase-4 (GPx-4) protein expression and cytotoxicity in human ovarian cancer cell lines. Moreover, DHA-mediated cytotoxic effect was reversed by pretreatment with vitamin E, suggesting that GPx-4 downregulation was due to oxidative stress [51]. Similarly, downregulation of SOD1 has been found in DHL-4 lymphoid cell line treated with DHA [31]. In the manuscript by Vibet et al., DHA-doxorubicin co-treatment caused an increase of ROS levels and a concomitant decrease of cytosolic GPx1 activity. This effect was detected both in MDA-MB-231 breast cancer cell line *in vitro* and in rat mammary tumors *in vivo* [52]. Furthermore, among the endogenous defenses to ROS, the Warburg effect (above mentioned) is another metabolic pathway adopted by cancer cells. In a recent paper, we have demonstrated that although the metabolism of human PaCa-44 pancreatic cancer cell lines mainly leans on glycolytic pathways (thus implementing the Warburg effect), after DHA treatment there was an overexpression of Kreb's cycle enzymes, indicating that cancer cell metabolism was switched in the Kreb's cycle activation [53].

As commented above, it has become clear the role played by the apoptotic alterations occurring during the development of neoplastic diseases. Thus, researches for possible therapeutic strategies involving the modulation of the apoptotic pathways have attracted considerable interest in the past few years. Oxidative stress has a fundamental role in apoptosis induction and many chemotherapeutic agents work generating directly or indirectly ROS, which lead to the key step in blocking cell cycle and apoptosis induction. Apoptosis occurs mostly through two mechanisms: the intrinsic and the extrinsic pathways. The first is caused by the disruption of mitochondrial membranes, the release into the cytoplasmic compartment of the mitochondrial cytochrome c, which in turn binds to the cytoplasmic apoptotic protease-activating factor (APAF) complex, triggering at first the activation of the initiator caspase-9 and then the executor caspases-3, -6, -7. The second pathway is initiated by the activation of death receptors by ligands like FasL, followed by the assembly of DISC (Death Inducing Signaling Complex), which, hydrolyzing the procaspase-8 or -10, causes caspase-8 or -10 activation. Then, caspase-8 or -10 activation results in the activation of executor caspases, which will be the real effectors of apoptosis [54]. Several evidence in the literature suggest the proapoptotic role of DHA, either alone or in combination of anticancer chemotherapies [17]. The apoptotic effect of DHA appears to take place through both the intrinsic and extrinsic pathways [13]. This role is further emphasized by the fact that DHA seems to be a potent inducer of apoptosis only for cancer cells and not for normal cells. For instance, it acts as a proapoptotic factor in colon cancer cells, whereas no significant proapoptotic effect was observed in the NCM460 normal human colon mucosal epithelial cell line [55], as well as cytotoxic effects were not observed in normal skin fibroblasts, microvascular endothelial cells and peripheral blood mononuclear cells derived from healthy donors [56]. These observations can be explained by the fact that normal cells might produce enhanced amounts of cytoprotective molecules such as lipoxins, resolvins and protectins in contrast to tumor cells which produce cytotoxic lipid hydroperoxides and other peroxides

(as mentioned above) [57]. It has been demonstrated that the proapoptotic action of DHA is carried out by different ways, including the induction of lipid peroxidation and oxidative stress. DHA can be rapidly incorporated in mitochondrial membranes, altering their permeability and decreasing the mitochondrial membrane potential (MMP) [58, 59]. It has been also reported that DHA is mostly present in the mitochondrion in association with cardiolipins [60]. Cardiolipin-DHA molecules are under attack of radical species (highly presents in cancer cells) with the consequent decrease of their binding affinity for cytochrome c. Enhancement of their release as well as of other proapoptotic factors (e.g., the apoptosis-inducing factor, Smac/Diablo, Omi/HtrA2, and endonuclease G) from mitochondria to cytosol, leads to the induction of the activation of intrinsic apoptosis [61]. Sturlan et al. showed that DHA enhanced arsenic-trioxide-induced apoptosis in the arsenic-trioxide resistant HL-60 (myeloid leukemia), SH-1 (hairy cell-leukemia), and Daudi (Burkitt-lymphoma) cell lines and this effect was due to an increase of lipid peroxidation and a reduction of the mitochondrial membrane potential. Moreover, the authors showed that these effects were reversed by the addition of vitamin E [62]. Similar effects have been found in DHA-combined treatment with 5-fluorouracil (5-FU), oxaliplatin (OX) and irinotecan (IRI) in HT-29 human colorectal adenocarcinoma cells. The anticancer action of DHA, observed in presence of low doses of chemotherapeutic drugs (1 μM 5-FU, 1 μM OX and 10 μM IRI), was carried out first by loss of mitochondrial membrane potential and then by caspase-9 activation [63].

3. DHA as Adjuvant to Improve the Antimetastatic Efficacy of Anticancer Therapies

Tumor metastasis is the primary cause of mortality in most cancer patients and thus the most life-threatening aspect of different types of tumors. Conventional chemotherapeutics exert cytotoxic activity against tumor cells affecting thus tumor growth. Therefore, chemotherapeutic effects on survival of cancer patients are generally interpreted as the consequence of their control on tumor cell growth, which in turn decreases tumor systemic spread. However, the initial chemotherapeutic efficacy is often hampered by the development by tumor cells of mechanisms of escape from the chemotherapeutic control, allowing residual cancer (stem) cells to growth, invade and metastasize. Therefore, the investigation on possible adjuvants in anticancer treatment affecting tumor cell invasion and metastasization is crucial to improve long-term therapeutic success of conventional anticancer agents, namely to decrease the mortality rate for cancer disease.

There is evidence that the intake of ω-3 PUFAs and in particular DHA inhibits not only the initiation of many kinds of tumors but also their progression, in that it inhibits metastases of murine and human tumors *in vivo* [64–67]. Indeed, marine fatty acid (ω-3 PUFA DHA and EPA) intake is associated with reduced all-case mortality in breast cancer patients [68]. In addition, there is some evidence that DHA increase the antimetastatic effect of anticancer drugs or other chemical compounds [16, 69, 70].

Although the metastatic process is very complex and its molecular knowledge is still very limited, metastasization can be described as a sequence of phases in which cancer cells leave the original tumor site and migrate to other parts of the body via blood and lymphatic vessels [71]. The initial phase of the metastatic process results in the invasion by tumor cells of the surrounding primary tissue and the basement membrane. The invasion phase includes the following steps: (i) the detachment of tumor cells from surrounding primary cancer cells (i.e., loss of cell-to-cell-adhesion through downregulation of E-cadherin expression), (ii) the increased cell capacity to interact with extracellular matrix (ECM) proteins through adhesion molecules (e.g., integrins, cadherins, CD44), (iii) the degradation and remodeling of ECM by the secretion of hydrolytic enzymes (e.g., matrix metalloproteases-MMPs), and (iv) the migration through the degraded ECM (via mitogen-activated protein kinases-MAPKs, complex cascade of cytoskeleton rearrangement, cytokines and chemokines) towards blood and lymphatic vessels [71–73]. The second critical phase of the metastatic process includes the intravasation of tumor cells into blood or lymphatics, tumor cell survival and transport in the blood stream or the lymphatic system, followed by the arrest and extravasation at a distal site. Invasion, intravasation and extravasation underlie the tumor cell dissemination process that is often indicated in carcinomas as epithelial to mesenchymal transition (EMT) [73, 74]. Of note, EMT also occurs during normal embryonic development as precursor cells migrate along directions dictated by morphogenetic gradients [75]. Thus, EMT confers embryonic stem cell-like properties to tumor cells. Finally, the full accomplishment of the metastatic program requires that cells at a distant site survive and grow into secondary tumor masses. For clonal outgrowth at metastatic sites, as for final developmental cellular differentiation, EMT reversion such as mesenchymal to epithelial transition (MET) is required [76, 77]. This metastatic final step involves the capacity of tumor cells to proliferate, stimulate angiogenesis, and crosstalk with the component of the new microenvironment including parenchymal, stromal, and inflammatory cells [78]. The microenvironment surrounding both the primary tumor and metastases is regarded to be a prominent regulator of the metastatic potential [78]. In fact, stromal interactions contribute to invasion at the primary tumor site, where newly formed leaky blood vessels facilitate cancer cell intravasation; growth factors and cytokines produced by stromal cells (like tumor-associate macrophages) stimulate in trans-EMT [79, 80]. On the other hand, tumor cell-microenvironment interaction also involves the secondary tumor site, with promotion of neoangiogenesis, cell proliferation and crosstalk with protumorigenic inflammatory immune cells and molecules [79–81].

In this second part of the review, we first report some key evidence supporting the inhibitory effect of DHA on tumor metastasis and then we focus our attention on the influence of DHA on the initial phase of the metastatic process, namely the invasion phase. We also illustrate data supporting the idea that DHA might be combined with anticancer therapeutic

strategies as adjuvant to improve their efficacy against tumor metastasization, thereby cancer patient survival extension.

3.1. DHA and Tumor Metastasis. As early as 1994, Rose et al. started a certain number of studies on the effects of dietary fish oil ω-3 PUFAs, including DHA, on the growth and metastases of MDA-MB-435 human breast cancer cells in female nude mice [82]. Animals were fed with three isocaloric diets containing 23% total fat but different proportion of corn oil (rich in linoleic acid-LA) and menhaden oil (rich in EPA and DHA) [65, 82]. The authors reported that, in contrast to mice fed with diets rich in LA, mice receiving diets supplemented with EPA or DHA showed a significant suppression of both primary tumor growth rate and lung metastasis occurrence and severity, suggesting a role for EPA and DHA in the inhibition not only of tumor growth but also of metastasization of human breast cancer cells [82, 83]. Successively, the same authors investigated tumor responses to EPA or DHA administered immediately after surgical excision of the primary tumor ("postoperative adjuvant" activity). They found that DHA, but not EPA, significantly reduced lung involvement following the postexcision [84]. Therefore, based on these results, the authors suggested that PUFAs and in particular DHA may have a place in nutritional therapy of breast cancer as part of both neoadjuvant as well as postoperative adjuvant antimetastatic regimen. Similar results were obtained by Kinoshita et al., using purified DHA in a murine mammary metastatic tumor model [85]. Indeed, the authors observed that DHA suppressed not only the growth but also metastases of the MM48 murine mammary tumor transplanted into C3H/He mice. Of note, more recently, using a mouse model of MDA-MB-231 human breast cancer cell metastasis to bone, Mandal et al. showed that dietary fish oil DHA and EPA prevented the formation of osteolytic lesions in bone, suggesting a novel health effect of DHA or EPA on breast cancer cell metastasis to bone [86].

The antimetastatic property of DHA was also investigated using different experimental models of colon cancer metastases. It has been shown that dietary marine oil (ω-3 PUFA EPA and DHA) inhibited the growth as well as the pulmonary colonization of a transplantable colon tumor (CT-26) implanted at the descending colon of male Balb/c mice [64]. Then, Iigo et al. and Suzuki et al., using the same subcutaneous implanted highly metastatic colon carcinoma 26 (Co 26Lu) model, found that a DHA-rich diet, when administered together with tumor cells, dramatically suppressed lung metastases (58% fewer colonies than control). Moreover, they found that *in vivo* DHA-treated tumor cells maintained their low potential for lung colony formation when transferred to new hosts, proposing that the effect of DHA was exerted directly on the metastatic ability of the tumor cell and not on the microenvironment [87, 88]. One further study, was performed on a model of colorectal metastasis in male rats (WAG/Rij) fed with a diet containing an EPA/DHA mixture (1.96% fish oil; EPA : DHA ratio 3 : 2) three days before and 28 days after splenic injection of CC531 cells (a moderately differentiated colon adenocarcinoma). A 70% reduction in incidence and 50% reduction in liver metastasis size as compared to control rats fed with 15% coconut oil was found [89]. Finally, Ichihara et al. reported the high therapeutic effects of intravenous injection of hybrid liposomes DMPC/DHA (composed of 50 mol% L-α-dimyristoylphosphatidylcholine-DMPC and 50 mol% DHA) on the hepatic metastasis mouse model of HCT116 human colon carcinoma cells. This effect was also associated with a prolonged murine survival [90].

Gleissman et al. showed that DHA, given daily by gavage in atymic rats, delayed the progression of established aggressive human neuroblastoma xenografts [67].

Finally, the antimetastatic activity of DHA was also observed by Yam et al. in the well-characterized model of Lewis Lung Carcinoma (3LL) metastases in C57BL/6J mice [66]. Interestingly, in the same experimental model, these authors also investigated the antimetastatic property of dietary fish oil (DHA and EPA) administered in combination with vitamins E and C and cisplatin [69]. Indeed, C57BL/6J mice bearing Lewis lung carcinoma (3LL) were fed ad libitum with one of the three isocaloric diets containing 5% soybean oil supplemented with 40 mg/kg α-tocopherol acetate (SO diet), or 4% fish oil plus 1% corn oil and basal amounts of vitamin E (FO diet) or FO diet supplemented with vitamins E and C (FO + E + C diet). These diets were tested in combination with the conventional cytotoxic agent cisplatin in a series of regimens and tumor growth and lung metastasis were monitored. Both the FO dietary groups showed significantly lower tumor development than the SO group in all examined parameters, indicating that ω-3 PUFAs exert anticancer activity. However, the FO diet, in comparison with the FO + E + C diet induced a significantly slower rate of tumor growth as well as lower metastatic load, as reflected in lung weight. The authors proposed that the decreased anticancer activity of FO by the addition of vitamins E and C could be explained by the decrease of oxidized ω-3 PUFAs, that, accumulated in the membranes and the cytosol of tumor cells, reduced their vitality and eventually lead to their death. Cisplatin treatment with the SO diet had no apparent therapeutic effect, while cisplatin combined with the FO diets significantly reduced the metastatic load. Therefore, these results suggest an adjuvant function of DHA and EPA in chemotherapy on spontaneous metastatic dissemination [69].

Despite evidence from preclinical studies for antimetastatic activity of DHA, to the best of our knowledge no published studies have yet investigated the effect of DHA in patients with metastatic tumors. We identified only one published human study on the antimetastatic effect of combining DHA with a conventional chemotherapeutic regimen. On the basis of the ability of DHA to increase the efficacy of anticancer agents by induction of oxidative stress, a phase II study evaluated the addition of 1.8 g DHA daily to an anthracycline-based chemotherapy regimen for 25 patients with metastatic breast cancer. Patients were dichotomised into two groups based on high or low DHA incorporation into plasma phospholipids. The high DHA-incorporation group had a significantly longer time to disease progression (median 8.7 months versus 3.5 months) and overall survival (median 34 months versus 18 months). Although the small number of patients involved in the study does not allow a definitive

conclusion on the efficacy of this combined treatment, the data indicate that adjuvant treatment with DHA may improve the outcome of chemotherapy, in terms of response rate, time to progression, and overall breast cancer survival [70].

The molecular mechanisms by which DHA, alone or in combination with other agents, may affect the metastatic potential of tumors remain unclear. However, several molecular mechanisms have been proposed. In most studies abovementioned, the investigations were focused on changes in the chemical content of tumor FAs and alterations of tumor membrane characteristics induced by the uptake of DHA associated to the displacement of arachidonic acid (AA) in phospholipid membranes of tumor cells [65, 66, 82, 85, 87, 91]. Incorporation of DHA in the tumor cell membrane results to some extent in a change in its lipid composition, which might make plasma membrane considerably less fluid and less deformable [92]. Thus, it has been proposed that the antimetastatic activity of DHA may be related to pronounced changes in the FA composition of tumor cells, which impair tumor cell membrane and decrease the ability to metastasize [82, 87]. Moreover, it has been observed that the increased representation of DHA in tumor phospholipids, associated with a statistically significant reduction in AA concentrations, suppresses AA-derived eicosanoid (PGs) biosynthesis thus decreasing prostaglandin (PG) E2 concentration [65, 66, 85]. This point may be crucial, since PGE2 production results in suppression of immune responses to cancer cells and in promotion of inflammation, as well as, enhancement of cell proliferation, neo-angiogenesis and invasion. Furthermore, Suzuki et al., in the metastatic colon carcinoma model, found that dietary DHA caused a decrease in metalloprotease-9 (MMP-9), which was well correlated with AA content in tumor tissues ($r = 0.900$, $P < 0.001$), suggesting that inhibition of metastasis by DHA might be due to depressed type-IV collagenase activity [88]. This is consistent with data in the literature reporting the influence of DHA on the one-carbon cycle, thereby contributing to increased homocysteine and oxidative stress leading to decrease gene expression of MMPs and to increase that of specific endogenous inhibitors of MMPs such as tissue inhibitors of metalloproteinases (TIMPs) [93]. Finally, Mandal et al., using the mouse model of human breast cancer cell metastasis to bone, found that fish oil supplemented with DHA and EPA significantly inhibited mRNA and protein levels of the cell-surface CD44 adhesion molecule (involved in cell-cell interactions, cell adhesion and migration) in the aggressive MDA-MB-231 tumors, thus identifying a novel DHA function in tumor cells that is the targeting of the cell-intrinsic pro-metastatic CD44 molecule expression [86]. This is a very interesting data, since the acquisition of increased expression of CD44 by noninvasive breast cancer cells correlates with the induction of EMT necessary for metastatic potential [94]. However, several other targets of DHA including cyclooxygenase-2 (COX-2), nuclear factor kappa-light-chain enhancer of activated B cells (NFkB), peroxisome proliferator-activated receptor-γ (PPAR-γ), mitogen-activated protein kinases (MAPK), Akt (also known as Protein Kinase B), and B-cell lymphoma/Bcl-2-associated X protein (BCL-2/BAX) play an important role in the suppression of metastases and excellent reviews are already available on their implication in DHA influence on tumor cell proliferation [8], angiogenesis [14] and immune system response [95], all critical events in the metastatic process. Therefore, in the next chapter we focus our attention on the influence of DHA on tumor cell invasion, the first phase of the metastatic process, illustrating studies that investigated the effect DHA on the invasion capability of murine and human cancer cells *in vitro*.

3.2. DHA and Tumor Cell Invasion. An early study on the effect of DHA on tumor cell invasion is dated 1993, when Connolly and Rose, using an *in vitro* invasion assay, examined the effect of LA, EPA, and DHA on the invasive capacity of the aggressive MDA-MB-435 human breast cancer cell line. They reported that although all these agents did not affect the migration of tumor cells through gelatin, EPA and DHA (at concentration of 0.25 and 0.5 μg/mL, that did not inhibit cell growth), but not LA, significantly inhibited the invasion of tumor cells through Matrigel [96]. Recently, Altenburg and Siddiqui found that treatment of the aggressive MDBA-MB-231 breast cancer cells with ω-3 PUFAs resulted in reduced surface but not overall C-X-C chemokine receptor type 4 (CXCR4) expression and subsequently in reduced CXCR4-mediated cell migration. The authors also suggest that the possible mechanism behind the reduced CXCR4 activity may be the disruption of the lipid raft domains by PUFAs, which results in a partial displacement of CXCR4 [97]. According to the anti-invasion activity of DHA in aggressive breast cancer cell lines, Blanckaert et al. showed that the invasive phenotype of the MDA-MB-231 human breast carcinoma cell line was markedly decreased following cell incubation with 100 μM of DHA for 24 h, whereas they could not observe any effect when cells were treated with 20 μM of DHA whatever the incubation time, suggesting that high doses of DHA are required for this activity [98]. Other authors investigated the anti-invasion effect of combining DHA with another nutritional compound such as genistein [16]. Genistein, an isoflavonoid isolated from soybean, has been shown to possess anticancer activities and is a potent inhibitor for a number of tyrosine kinases. Horia and Watkins tested the combination of genistein and DHA for the synergistic inhibition of cell invasiveness, suppression of PGE2 production and COX-2 expression in MDA-MB-231 cancer cells. Their data demonstrated an additive effect of DHA and genistein in suppressing cell invasiveness and the endogenous production of PGE2. Furthermore, the combination of DHA and genistein did not enhance the suppression of COX-2 gene expression but appeared to work through peroxisome proliferator-activated receptor-c/pregnane X receptor-α- (PPARc-PXRα-) mediated pathways for reduced PGE2 production [16], thus suggesting alternative molecular targets for DHA to PGE2 inhibition.

The anti-invasive activity of DHA was also investigated using other types of experimental tumor models. McCabe et al. demonstrated that DHA (10 μM for 24 h, which had no effects on cell proliferation) significantly inhibited (48.48%) the invasion of caki-1 renal cell carcinoma cell line through Matrigel. They also reported that this effect was associated to increased tumor cell levels (17.42%) of TIMP-1, and that similar increased levels were found when PGE2 production

was inhibited, suggesting that the reduction of the invasive profile is regulated by tumor PGE2 production levels [99]. In the 70W human melanoma cell line (that metatstazise to the brain in nude mice), Denkins et al. demonstrated that DHA (50 μM for 24 h) decreased Matrigel invasion and that this effect was associated to the inhibition of the COX-2 expression, which in turn downregulated PGE2 production [100]. Moreover, Xia et al. examined the effect of alteration in the n-6/n-3 fatty acid ratio on the invasive potential of human lung cancer A549 cells. These cells had a marked reduction of the n-6/n-3 fatty acid ratio, because they were transfected with the Caenorhabditis elegans fat-1 gene, which encods an n-3 desaturase that converts n-6 to n-3 fatty acids. Cell adhesion assay showed a significant delayed adhesion and retarded colonization. Matrigel assay indicated a 2-fold reduction of cell invasion in the fat-1 transgenic cells when compared with the control cells. Microarray and quantitative polymerase chain reaction revealed a downregulation of several adhesion/invasion-related genes (MMP-1, integrin-α2 and nm23-H4) in the fat-1 transgenic cells, suggesting that the reduced invasion and colonization potential of human lung cancer cells induced by decreased n-6/n-3 fatty acid ratio were probably due to downregulation of cell adhesion/invasion-related molecules [101]. Furthermore, in U87 malignant glioma cells, Mita et al. showed that DHA inhibited and AA stimulated tumor cell migration. The authors also showed that the antimigratory effect by DHA was dependent on its binding with the brain fatty acid-binding protein (FABP7) which move to the nucleus and cooperates with DHA for the activation of the PPARγ transcription factor, thereby downregulating the COX-2-PGE2 pro-migratory pathway. The authors thus proposed FABP7 and its fatty acid ligands as key therapeutic targets for controlling the dissemination of malignant glioma cells within the brain [102]. Very recently, Sun et al. also found that DHA (100 μM for 24 h) inhibited migration as well as invasion of the Bel-7402 human hepatocellular carcinoma cell line and that those inhibitions paralleled MMP-9 decrease [103]. Finally, in our laboratory we recently showed that, in contrast to AA, DHA (25, 50, and 100 μM for 24 h, that did not affect cell proliferation) inhibited in a dose-dependent manner the invasion of RT112 urinary bladder and PT45 pancreatic carcinoma cell lines through Matrigel. Moreover, we showed that, in contrast to AA, the inhibition of cancer cell invasion paralleled DHA-induced downmodulation of the tumor-expressed chymotrypsin-like serine protease granzyme B (GrB) [15]. Although GrB was originally known as a cyotoxic molecule of cytoplasmic granules of cytotoxic lymphocytes [104], it was recently also characterized for its extracellular functions [105], such as invasion promotion of cancer cells [106]. The inhibitory effect of DHA on GrB expression is consistent with results from Kun et al., showing that dietary ω-3 PUFAs inhibited the expression of GrB in a rat model of small bowel transplant chronic rejection [107]. We also demonstrated that GrB was expressed in cancer cells *in vitro* and *in vivo*. GrB was capable of degrading ECM components and promoting invasion of bladder and pancreatic cancer cells *in vitro*. Moreover, GrB tumor expression was significantly associated with tumor EMT *in vivo*, as well as, with the pathological tumor spreading [15, 107]. Taking into account these results, we proposed a possible causative role of GrB in the inhibition of bladder and pancreatic cancer cell invasion by DHA [15].

4. Conclusions

In conclusion, taking into consideration the data in the literature, it appears that DHA has the ability to inhibit metastasis in preclinical *in vivo* tumor models as well as invasion and migration in *in vitro* tumor cells. It also appears that a "combination therapy" of DHA and antitumor drugs may increase the cytotoxic efficacy of drug treatment alone, since it should allow cancer cells to maintain higher levels of ROS (thereby precluding drug resistance), reduce endogenous antioxidant tumor cell defenses, and increase drug uptake. Despite these encouraging results, there is still a need to verify whether DHA supplementation can improve the antimetastatic efficacy of chemotherapeutic and radiotherapeutic anticancer regimens in humans.

Conflict of Interests

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

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Review Article

Oxidation of Marine Omega-3 Supplements and Human Health

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Marine omega-3 rich oils are used by more than a third of American adults for a wide range of purported benefits including prevention of cardiovascular disease. These oils are highly prone to oxidation to lipid peroxides and other secondary oxidation products. Oxidized oils may have altered biological activity making them ineffective or harmful, though there is also evidence that some beneficial effects of marine oils could be mediated through lipid peroxides. To date, human clinical trials have not reported the oxidative status of the trial oil. This makes it impossible to understand the importance of oxidation to efficacy or harm. However, animal studies show that oxidized lipid products can cause harm. Oxidation of trial oils may be responsible for the conflicting omega-3 trial literature, including the prevention of cardiovascular disease. The oxidative state of an oil can be simply determined by the peroxide value and anisidine value assays. We recommend that all clinical trials investigating omega-3 harms or benefits report the results of these assays; this will enable better understanding of the benefits and harms of omega-3 and the clinical importance of oxidized supplements.

1. Introduction

Marine omega-3 rich oils (marine oils) are the most popular supplements in the United States; after a rapid rise in popularity, they are now used by more than a third of American adults [1, 2]. Marine oils (derived from fish, krill, shellfish, calamari, or algae) differ from terrestrial plant sources of omega-3 fatty acids such as flaxseed as they contain the long chain polyunsaturated fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). They show promise particularly in the prevention of cardiovascular disease [3], the treatment of inflammatory disease [4], improving early life neurodevelopment, preventing cognitive decline [5], and potential benefits to metabolism [6]. However, it is important to understand that unlike most pharmacological and neutraceutical interventions, these oils are highly susceptible to oxidation. There has been concern about the safety of oxidized fish oil since the 1950s [7], and although there is evidence that over-the-counter supplements are frequently oxidized, this has had no impact on the requirements for storage and labelling or on the design of human clinical trials. No human efficacy trials have reported the oxidative state of the trial oil which would question the validity of the results and conclusions of these trials. It is currently unclear to what degree the oxidation of fish oil influences its efficacy or harm in humans. This commentary discusses these issues and outlines the implications for interpreting the literature and improving clinical trial design.

2. How Stable Are Marine Omega-3 Supplements?

n-3 LC-PUFAs are chemically unstable, so that marine oils rapidly oxidize during storage to a complex chemical soup of lipid peroxides, secondary oxidation products, and diminishing concentrations of unoxidized fatty acids. As a result, the composition of a fish oil supplement cannot be simply inferred from the labelled EPA and DHA concentrations.

n-3 LC-PUFAs are highly prone to oxidation due to their large number of double bonds and their position within the fatty acid chain [8, 9]. This makes them prone to oxidation because bisallylic carbons, those between two double-bonded carbon atoms, have a low activation energy for hydrogen loss and free radical formation [8]. n-3 LC-PUFAs have

more of these vulnerable bisallylic carbons (EPA : 4, DHA : 5) than the short n-3 PUFA (α-linolenic acid : 2) or n-6 PUFAS (arachidonic acid : 3) while the monounsaturated fatty acids and saturated fatty acids have none. In the presence of various initiators, a lipid radical is formed starting an expansive chain reaction which creates lipid peroxides and more radicals from unoxidized PUFAs. A complex array of different peroxide molecules arises depending on the position of the oxidized carbon, and after undergoing cis-trans isomerisation and a shift of double bonds, conjugated dienes and trienes are produced which have different polarity and shape to the original fatty acid [8]. A potentially important class of n-3 peroxidation products, the prostaglandin-like F3-isoprostanes and F4-neuroprostanes are formed from EPA and DHA, respectively [10]. Thus, the primary oxidation products of n-3 LC-PUFAs are chemically different from unoxidized n-3 LC-PUFAs and may have different biological properties.

Lipid peroxides are unstable and further degrade to form secondary oxidation products including aldehydes such as 4-hydroxyhexenal (HHE) and malondialdehyde (MDA) [11]. As the oil oxidizes over time, there is an initial exponential increase in the concentration of lipid peroxides. These later degrade and the concentration of potentially harmful secondary oxidation products increases as the lipid peroxides decrease.

The rate of lipid peroxidation is influenced by light, heat, and oxygen concentration even at normal room conditions. Moreover, even oil stored in the dark at 4°C may oxidize unacceptably within a month of storage [12]. Added antioxidants reduce but do not prevent oxidation [13]. The tendency of n-3 LC-PUFAs to oxidize under light is also influenced by the presence of impurities such as protein or heavy metals and its conjugate; phospholipids are more prone to oxidation than triglycerides [8]. Because peroxidation is an accelerating chain reaction, small concentrations of peroxides in the source oil, or exposure to oxidising conditions during processing could have a large effect on the rate of oxidation. In addition, deodourisation to remove fishy odour often involves high temperature which may accelerate secondary oxidation. Significant peroxidation is highly likely to occur in over-the-counter supplements which are commonly kept at room temperature both in retail shops and in the home.

Oil in an omega-3 supplement may differ substantially from the oil in fresh fish depending on its age, heat and light exposure. As a result, these supplements should be viewed as a complex mix of EPA, DHA, other fatty acids, additives, and an unspecified concentration of potentially toxic lipid peroxides and secondary oxidation products.

3. Can Oxidation Be Easily Quantified and Reported?

Measurement of specific lipid peroxide species and secondary oxidation products requires gas-chromatography mass-spectrometry [14–17] or other chromatographic techniques [9] which are expensive and require significant technical expertise. However, the oxidative status of supplemental oils can be easily estimated using the peroxide value (PV) and anisidine value (AV) assays. While these are nonspecific, they are repeatable, simple, and cost effective, and guidelines exist for recommended maximum levels in marine omega-3 supplements. The peroxide value (PV) is a simple titration enabling quantification of the concentration of peroxide groups in oil [18] while the anisidine value (AV) is a colorimetric test which enables estimation of the concentration of secondary oxidation products. Both measurements are required to estimate total oxidation (TOTOX = $2 \times \mathrm{PV} + \mathrm{AV}$).

A number of organisations have endorsed maximum recommended levels of oxidation in supplements [19–22]; though due to the paucity of human evidence, these are based on palatability and not the effect on human health [20]. Clearly, this implies a need for evidence-based guidelines. The simplest way to improve the knowledge base would be for clinical trials to report the oxidative status of their trial oils, so that benefits and harms could be associated with the oxidative state.

4. Are Over-the-Counter Marine Omega-3 Supplements Significantly Oxidized?

The oxidative states of retail oils are not routinely labelled and it is surprising that there has not been more formal evaluation of the oxidative stability of marketed omega-3 supplements. When over-the-counter supplements have been investigated, the frequency of excess oxidation [19–22] was highly variable but not uncommon, affecting between 11%–62% of products [23–27]. Thus, consuming purchased supplements entails risk of exposure to unacceptably oxidized oil, and it is likely that the omega-3 supplements used in many clinical trials have also been significantly oxidized. Understanding the effects of oxidized omega-3 LC-PUFAs on health is thus important both for the vast number of supplement consumers and for scientists and clinicians interpreting the medical literature.

5. Are Oxidized Omega-3 Oils Efficacious?

To our knowledge, no clinical trial investigating the efficacy of omega-3 in humans has reported the oxidative state of the trial oil or compared oxidized and nonoxidized oils. The relative efficacy of highly oxidized and nonoxidized oil cannot be inferred. However, it is likely that there is a difference.

The mechanisms of action of omega-3 are not fully understood, but there are multiple interacting mechanisms including acting as a ligand for intracellular and extracellular receptors, competition for metabolism by enzymes, structural roles in cell membrane, and stearic interference with ion channels. For illustration, triglyceride lowering is mediated by interaction with sterol receptor binding protein 1-c (SREBP1-c) and the peroxisome proliferator activated receptor alpha (PPAR-α) [28]. Anti-inflammatory, hypotensive, and antiplatelet effects may be mediated by competition with arachidonic acid for synthesis of eicosanoids by the enzyme cyclooxygenase [3]. Antiarrhythmic effects are in part due to stearic interference with ion channels [29]. Insulin sensitisation is partly mediated by interaction with PPAR-γ an intracellular transcription factor [6] and binding to the recently discovered G-protein linked receptor GPR120 on

the cell surface [30]. Omega-3 fatty acids may also have anti-oxidant effects [31] and influence cell membrane fluidity [32].

As lipid peroxides have different shape, polarity, and reactivity to their parent fatty acid, it is likely that they will be ineffective through some if not all of these mechanisms. Because these mechanisms are diverse, the effect of oxidized supplements may be divergent, some beneficial effects may be lost but not others; lipid peroxides may even have their own unique functions.

Surprisingly, there are no specific clinical trials investigating the effects of oxidation on the efficacy of marine n-3. However, in a clinical trial of fish oil supplementation with and without the anti-oxidant vitamin E, triglycerides decreased significantly more in the vitamin E group [33]. Increased efficacy with vitamin E is most likely due to prevention of oxidation of the oil either prior to consumption or *in vivo*. Interestingly, in a study of liver tissue in culture, oxidized EPA inhibited the inflammatory NF-κB pathway [34]. This may be mediated by n-3 derived isoprostanes, as these peroxides have been shown to be biologically active, inhibiting macrophage NF-κB activation in tissue culture [35]; and affecting vascular and platelet function [10]. It is not yet clear whether these effects are important *in vivo*; however, they provide evidence for a divergence of effects when n-3 LC-PUFAs are oxidized. Clearly, the effect of oxidation on efficacy of omega-3 requires more investigation; at minimum, the oxidative state of supplements used in clinical trials must be reported. Further, detailed studies are also required to establish both the bioavailability of individual oxidized lipid species and to provide greater insights into their biological functioning.

6. Are Oxidized Omega-3 Supplements Harmful?

There are insufficient interventional human studies that examine potential biological functions of oxidized marine n-3; however, there is evidence that lipid peroxidation is involved in human disease. In addition, animal studies show that oxidized lipids may cause organ damage, inflammation, carcinogenesis, and advanced atherosclerosis. These deleterious effects cannot be ignored, particularly when marine n-3 is taken during vulnerable stages of life such as pregnancy, early childhood, and old age and for long periods of time.

Lipid peroxides are absorbed through the gut and incorporated into chylomicrons [36], LDL [37], and VLDL [38]. Their active transport in LDL particles and particularly subsequent oxidation of LDL may be important in atherogenesis [11, 39]. Lipid peroxides also partially decompose to secondary oxidation products in the gut which are absorbed [40].

Lipid peroxides hasten oxidation of other fatty acids to create further lipid peroxides in an expansive chain reaction. We speculate that ingested omega-3 peroxides could lead to lipid membrane peroxidation, cell damage, and oxidative stress, which are known to be mechanisms of disease. Endogenous membrane lipid peroxidation results in altered membrane fluidity, transport, and cell signalling [8] which also may be an important disease mechanism. For example, acute severe lipid and protein peroxidation has been shown to be the cause of death when, despite appropriate treatment, people die from organophosphate poisoning [41]. Chronic lipid peroxidation may be a mechanism in carcinogenesis [42] and in the pathogenesis of Alzheimer's disease where the secondary oxidation product 4-hydroxynonenal (HNE) appears to have a role in both the formation of neurofibrillary tangles and neurotoxicity [43]. Oxidative stress further activates the NF-κB pathway and increases production of proinflammatory cytokines [44]. Chronic low grade inflammation is involved in degenerative disease including atherogenesis [45] and the generation of insulin resistance in the metabolic syndrome [46].

Animal studies provide clear evidence that oxidized lipids are harmful, though typically using higher doses of oil than humans consume or administering oxidation products in nonphysiological ways [11]. Chronic feeding of oxidized PUFAs to rats led to growth retardation, intestinal irritation, liver and kidney enlargement, haemolytic anaemia, decreased vitamin E, increased lipid peroxides and inflammatory changes in the liver, cardiomyopathy, and potentially malignant colon cell proliferation [11]. A major secondary oxidation product of omega-3 oils is the aldehyde HHE. HHE when injected into the peritoneum causes necrotising peritonitis and when injected intravenously causes liver damage. It is chemically similar to the better studied omega-6 oxidation product HNE which is known to be highly toxic and causes DNA damage [11, 42].

There is increasing evidence that *in vivo* oxidation of LDL has a role in atherogenesis [47]. Unmodified LDL cannot induce foam cell formation; however, after oxidative modification it can be recognised by the scavenger receptor of macrophages and is rapidly absorbed [9, 48]. Given that ingested peroxides are transported in LDL [37], it is possible that they could have a role in enhancing LDL oxidation and atherogenesis. This is supported by a study in rabbits where addition of fish oil to a high cholesterol diet led to rapid atherosclerosis [49]. We speculate that if this is due to oxidation of LDL, ingested oxidized marine n-3 could be atherogenic in humans. This could contribute to the disappointing results in primary and secondary cardiovascular prevention trials [50] and requires further investigation.

Consuming marine oil leads to increased plasma [33] and urinary [51] MDA in humans and mice, due to both absorption of peroxidized oil and *in vivo* oxidation with subsequent degradation of peroxides [51]. This is only partially reduced by addition of antioxidants [51–53]. MDA induces transition, transversion, and frame shift DNA mutations [54]. It has been shown to cause thyroid tumours when fed to rats and skin cancer with topical application [55]. The little evidence in humans is unclear; however, women with breast cancer have higher concentrations of MDA-DNA adducts in their normal breast tissue than controls, consistent with MDA exposure increasing risk [56].

One human-randomized placebo-controlled trial has examined the effects of oxidized versus nonoxidized oil over 7 weeks [57]. No difference was found in markers of *in vivo* lipid peroxidation (urinary 8-isoFGF2α, plasma HHE and HNE), markers of antioxidant activity, C-reactive protein, or liver

function tests. This suggests that oxidized marine n-3 may not be associated with acute oxidative toxicity. However, this is not reassuring as the study was short and did not assess important pathological markers associated with atherosclerosis such as oxidized LDL or carotid artery intimal thickness. Further, there was no assessment of specific inflammatory markers such as prostaglandins and cytokines or of markers of DNA damage. Thus, the risks of atherosclerosis, DNA damage, malignancy, and inflammation especially at tissue level remain open. If low grade, chronic peroxide, aldehyde, or MDA exposure is important in disease it may require long periods of followup to identify an effect. Some pathological effects such as tissue level inflammation may be difficult to detect without invasive methods such as muscle, liver, or adipose tissue biopsy.

In summary, given the paucity of specific evidence, it is currently impossible to know whether marine oils, some of the world's most popular supplements, are safe after oxidation. The effects of oxidation on the biological effects of these oils may be complex, there could be both beneficial [10, 34, 35] and harmful effects. Thus, long-term safety studies of marine oil are required, looking at appropriate disease outcomes and surrogates and relating these to the oxidative state.

7. Why Is the Omega-3 Supplementation Literature Conflicting?

The omega-3 supplementation literature is highly conflicting, especially in the area most heavily researched, the effect on cardiovascular disease. Oxidation may be a major cause of these conflicting results; however, it has never been reported in these trials.

Epidemiological studies link higher dietary [58–64] or plasma n-3 LC-PUFAs [63, 65–67] to lower risk of diabetes and cardiovascular disease. Furthermore, supplementation with encapsulated fish oil or fortified foods improves a wide range of cardiovascular risk factors including lipid profile [68–73], blood pressure [69, 74, 75], heart rate [76] and variability [77, 78], platelet aggregation [79, 80], endothelial function [81], and atherosclerotic plaque stability [82]. After myocardial infarction, fish oil reduces sudden cardiovascular death probably due to an antiarrhythmic effect [29, 83–85]. Systematic reviews of minor outcomes such as blood pressure [86, 87] and plasma triglycerides [88] are overall positive; however, individual studies are mixed. Moreover, despite the abundant evidence for improvement of cardiovascular risk factors, the results of primary and secondary prevention trials have been conflicting [89–91], and a recent systematic review found no overall effect of marine oil supplementation on the risk of all-cause mortality, cardiac death, sudden death, myocardial infarction, or stroke [92]. In explaining the conflicting effects of marine oil on health, authors have overlooked oxidized supplement as an explanation. Alternative explanations include a true lack of efficacy, obscuration of benefit by other cotreatments that improve cardiovascular risk such as statins, aspirin and beta-blockers [50], high background fish intake in some populations [93], and underpowered studies. However, it must be recognised that the oxidative status of the trial oil could also explain these disappointing results. If oxidized oils are less efficacious, or if they cause harm, for example, by advancing atherosclerosis then provided some studies used oxidized supplements, these results would be expected. We are currently in danger of concluding that marine n-3 supplements are ineffective in the prevention of cardiovascular disease, before they have been adequately investigated.

8. What Are the Implications for Interpretation of the Literature and Future Clinical Trials?

To assess the degree to which the importance of oxidation of marine oil is understood, we identified all human clinical trials published in 2012 using Pubmed. Of 107 reports, only one study investigating short-term harm reported the oxidative state of the trial oil (previously described) [57]. This strongly suggests that the instability of marine oil is generally unrecognized or not considered important.

It is currently impossible to determine how oxidation affects the efficacy or potential harms of marine oil. This makes interpretation of the clinical trial literature problematic. If the oxidative state of marine oils may affect efficacy or harm, then physicians should recommend, and consumers select, a supplement with the same oxidative state as the oils used in clinical trials that have shown benefit and safety. This is currently impossible because although over-the-counter-supplements are frequently oxidized [23–27]; the oxidative state of trial oils and retail supplements remain unreported.

That marine oils have beneficial effects on many indices such as plasma triglycerides, blood pressure, inflammation, and insulin sensitivity (in rodents) is not in question. The purpose of this commentary is to highlight the limited knowledge about the importance of oxidation to these effects. For example, some *in vitro* and animal studies have stored oil under conditions likely to prevent oxidation such as under nitrogen or at very low temperature [94–97]. This confirms for example, that unoxidized marine oil prevents insulin resistance in the rat [95]. However, whether oxidized oil has the same effect is unknown. In contrast, emerging evidence has shown that some *in vitro* anti-inflammatory effects are solely mediated by oxidized oil, but the clinical relevance of this is unclear.

Future safety and efficacy trials, particularly in humans, should report the oxidative state of the marine oil. This could most easily be done by reporting the peroxide, anisidine, and TOTOX values. Even established benefits of marine oil need to be reinvestigated with provision of this information. In parallel, there should be a move to labelling marine oil supplements with these same oxidative indices and a production and storage chain that minimizes oxidation prior to purchase. Only then can we generalise efficacy and safety trial data to the available omega-3 supplements and provide informed recommendations to patients and consumers.

Conflict of Interests

The authors have no conflict of interests to declare.

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Review Article

Experimental Evidence of ω-3 Polyunsaturated Fatty Acid Modulation of Inflammatory Cytokines and Bioactive Lipid Mediators: Their Potential Role in Inflammatory, Neurodegenerative, and Neoplastic Diseases

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A large body of evidence has emerged over the past years to show the critical role played by inflammation in the pathogenesis of several diseases including some cardiovascular, neoplastic, and neurodegenerative diseases, previously not considered inflammation-related. The anti-inflammatory action of ω-3 polyunsaturated fatty acids (PUFAs), as well as their potential healthy effects against the development and progression of the same diseases, has been widely studied by our and others' laboratories. As a result, a rethinking is taking place on the possible mechanisms underlying the beneficial effects of ω-3 PUFAs against these disorders, and, in particular, on the influence that they may exert on the molecular pathways involved in inflammatory process, including the production of inflammatory cytokines and lipid mediators active in the resolving phase of inflammation. In the present review we will summarize and discuss the current knowledge regarding the modulating effects of ω-3 PUFAs on the production of inflammatory cytokines and proresolving or protective lipid mediators in the context of inflammatory, metabolic, neurodegenerative, and neoplastic diseases.

1. Introduction

A number of studies has supported the hypothesis that a wide range of chronic diseases, including cardiovascular [1, 2], neoplastic [3, 4], and neurodegenerative diseases [5, 6], may be beneficially influenced by the increased intake of dietary ω-3 polyunsaturated fatty acids (PUFAs). Many molecular mechanisms have been invoked to explain such a pleiotropic effect of these fatty acids (FAs) [3, 7]. Meanwhile, plenty of experimental preclinical data has suggested the potential healthy effects of these compounds also on diseases with a clear inflammatory/immune pathogenesis, including asthma, rheumatoid arthritis, or inflammatory bowel diseases [8, 9].

It should be noted that conflicting results have been obtained so far by the clinical trials conducted in this research field [2, 8–12]. However, on the whole, the human intervention studies [9, 10, 12] are still too scarce in number and often show severe pitfalls. The ω-3 PUFA potential of modulating the molecular pathways involved in the inflammatory processes has been intensely investigated in experimental preclinical studies, and a substantial body of evidence has accumulated over the years to clarify that an inflammatory component may play an essential role in the pathogenesis of diseases that before had not been considered inflammation-related, such as some cardiovascular, neoplastic, and neurodegenerative diseases, and particularly metabolic disease, inflammation-associated cancers, Alzheimer's disease (AD), cognitive decline of aging, and so forth [13–16]. The altered production of inflammatory cytokines and bioactive lipid mediators with proresolving or protecting activities, either by

inflammatory cells or by cells residing in the affected tissues, has attracted considerable attention as possible critical steps in the pathogenesis of these diseases.

On this basis, in the present review we will analyze the published evidence that has so far contributed to show the relationships existing between the potential beneficial effects of ω-3 PUFAs against several inflammatory diseases, as well as against pathologies having an inflammatory component, and the modulatory effects that these FAs exert on the amount and quality of inflammatory cytokines and bioactive lipid mediators produced during the development and progression of these diseases. In the first part we will treat in general the anti-inflammatory effects of these FAs, with particular attention to important inflammatory and dysmetabolic conditions affecting different tissues and organs, trying to elucidate the underlying mechanisms and reviewing the modulating effects that these FAs may have on cytokine and lipid mediators productions in these disorders. Afterwards, we will examine in detail the relationships between the anti-inflammatory potential of these FAs and their beneficial effects against the development and progression of neurodegenerative diseases and cancer.

2. ω-3 PUFA Chemistry and Biochemistry

There is wide consensus that among the ω-3 PUFAs the most powerful are the ω-3 long-chain-PUFAs (ω-3 LCPUFAs) docosahexaenoic (DHA, 22:6ω-3) and eicosapentaenoic acid (EPA, 20:5ω-3) [17]. We can produce them endogenously metabolically converting α-linolenic acid (ALA, 18:3ω-3), an essential PUFA largely available in vegetables, through sequential desaturations and elongations followed by peroxisomal β-oxidation [18] (Figure 1). However, the efficiency of these conversions is quite low in the presence of high levels of linoleic acid (LA, 18:2ω-6), as those present in many Western diets [19]. Instead, we can directly obtain EPA and DHA by components of our diet that contain high levels of these FAs, such as fish and seafood.

Irrespective of their dietary origin, ω-3 PUFAs, as well as ω-6 PUFAs can be converted in our cells and tissues into potent lipid mediators playing an important role in the regulation of inflammation. For many years the predominant focus in the understanding of lipid mediators was on the ω-6 arachidonic acid (AA) and its derived compounds such as prostaglandins (PG) and leukotrienes (LT), which were initially described early in the 20th century. Generally, these ω-6 PUFA-derived lipid mediators promote inflammation whereas a wide variety of experimental studies has assigned ω-3 PUFAs as anti-inflammatory. This was traditionally attributed to their ability to inhibit the formation of ω-6 PUFA-derived proinflammatory PG and LT. However, newly discovered hydroxylated lipid metabolites derived from EPA and DHA, such as resolvins and protectins (Figure 2), are potent anti-inflammatory lipid mediators derived directly from ω-3 PUFAs with distinct pathways of action [20–23].

Most of preclinical *in vitro* and *in vivo* studies have been conducted treating cells and animals with fish or algal oils containing EPA and DHA, or with purified EPA or DHA. Moreover, a recently developed transgenic mouse model, the *fat-1* mouse [24], that has endogenously increased ω-3 PUFAs has been used in a large number of studies. This transgenic mouse expresses a *Caenorhabditis elegans* desaturase, leading to the formation of endogenously high levels of ω-3 PUFAs from ω-6 PUFAs. This model has been extremely useful to establish the effect of increased ω-3 PUFAs on several chronic pathologies, since it offers several advantages over conventional dietary approaches using ω-3 PUFAs, as it eliminates confounding factors of diet (content of, e.g., antioxidants) that could have significant effects themselves on animal models of inflammatory disease.

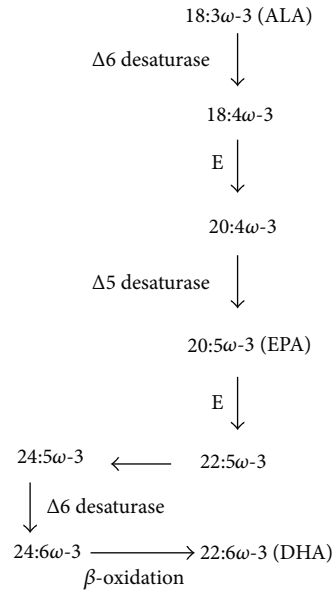

Figure 1: Endogenous synthesis of long-chain-PUFAs. ALA: α-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; E: elongase. 24:5ω-3 is converted to DHA by β-oxidation in peroxisomes.

3. Effects of ω-3 PUFAs in Disorders of GI-Tract and in Metabolic Diseases: Involvement of Cytokines and Lipid Mediators

3.1. Effect of ω-3 PUFAs in the Colon. Using this transgenic mouse model in experimental dextran sodium sulfate (DSS) colitis demonstrated protection from colitis in the *fat-1* mice with increased ω-3 PUFA status. This was accompanied by decreased nuclear factor-κB (NF-κB) activity and decreased expression of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (NOS), and interleukin-1β (IL-1β). Analyzing hydroxylated lipid mediators derived from ω-3 PUFAs in this context demonstrated significant formation of anti-inflammatory resolvins [25]. Another study with DSS colitis in *fat-1* mice established decreased cyclooxygenase-2 (COX-2) expression and decreased PGE_2 levels in the *fat-1* mice and confirmed the suppression of the cytokine response in these mice with a lower expression of IL-18, IL-1α, IL-1β, IL-6, monocyte chemotactic protein-1,2,3 (MCP-1,2,3), matrix metalloproteinase-9 (MMP-9), and TNF-α [26]. However,

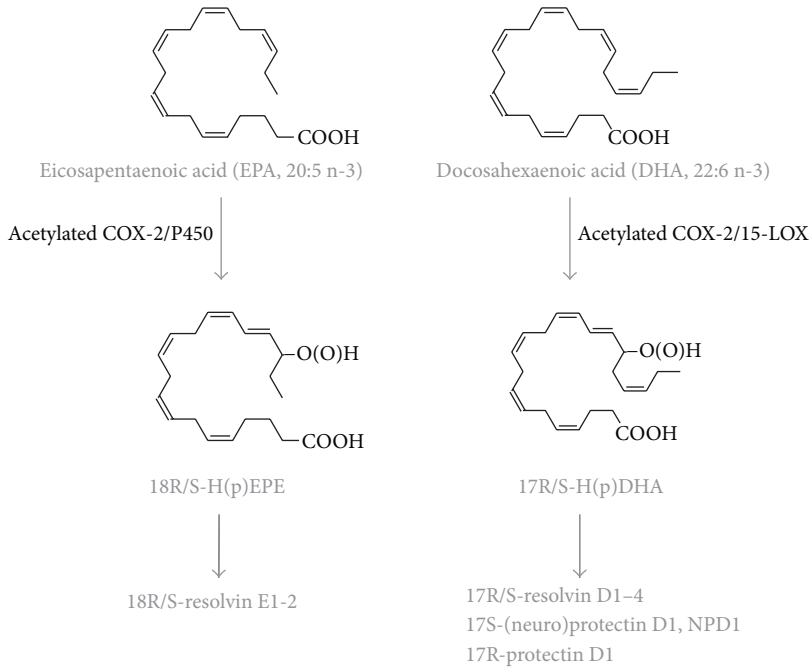

Figure 2: Production of anti-inflammatory lipid mediators from EPA and DHA. E-series resolvins are formed via a common precursor 18R/S-H(p)EPE, while D-series resolvins are synthesized via the common precursor 17R/S-H(p)DHA. Note that anti-inflammatory activity has been described for several of the R, as well as the S stereoisomers.

when we compare these findings obtained in animals to studies in humans, the picture becomes less clear. While one study has shown beneficial effects of ω-3 PUFAs on human Crohn's disease [27], the data so far are not conclusive, as another trial in patients with Crohn's disease in remission could not find a benefit of supplementation with ω-3 PUFAs [28]. Furthermore, a Cochrane Database systematic review could not establish a benefit for ω-3 PUFA treatment for the maintenance of remission in ulcerative colitis [29].

3.2. Effect of ω-3 PUFAs on the Liver. In *fat-1* mice with a balanced ω-6/ω-3 PUFA tissue content, the increased ω-3 PUFA content decreased the inflammatory response in the macrophage-dependent D-Gal/LPS acute hepatitis model. This was associated with decreased plasma TNF-α levels and reduced expression of IL-1β, interferon-γ (IFN-γ) and IL-6 in *fat-1* mice, leading to a decreased rate of apoptosis in livers from *fat-1* animals [30]. This could be due to the formation of ω-3 PUFA-derived lipid mediators, as lipidomics analyses of liver tissue revealed significantly increased levels of the ω-3 PUFA-derived 18-hydroxyeicosapentaenoic acid (18-HEPE) and 17-hydroxydocosahexaenoic acid (17-HDHA) in the livers of *fat-1* animals with chemically induced liver tumors [31]. In another study, DHA supplementation led to increased formation of DHA-derived lipid mediators such as 17-HDHA and protectin D1, which were able to protect the liver from CCL4-induced inflammatory damage [32]. The study also showed that 17-HDHA can suppress TNF-α secretion from cultured murine macrophages. This was confirmed in other *in vitro* experiments showing that 17-HDHA—and EPA-derived 18-HEPE—could effectively suppress LPS-triggered TNF-α formation in a murine macrophage cell line [31].

3.3. Metabolic Disease and ω-3 PUFAs. Other evidence demonstrates that ω-3 PUFAs may also play an important role in the alleviation of metabolic disturbances in the liver. A study using a high-fat diet in *fat-1* and wild-type mice demonstrated very mild steatosis in *fat-1* mice as compared to a moderate-to-severe steatosis in wild-type animals with normal transaminase levels in *fat-1* mice as compared to elevated values in high-fat diet-fed wild-type mice. This study also demonstrated the well-established lipidologic effects of ω-3 FAs with significantly decreased serum triglycerides, very low-density lipoprotein cholesterol, and chylomicrons on *fat-1* mice [33]. These findings are in accordance with other data using dietary supplementation of ω-3 PUFAs in which these FAs were shown to alleviate hepatic steatosis and to up regulate PPARγ, glucose transporter-2 (GLUT-2) and GLUT-4, and insulin receptor signaling (IRS-1/IRS-2) genes as well as the anti-inflammatory and insulin-sensitizing adiponectin, while concomitantly inducing AMP-activated protein kinase (AMPK) phosphorylation. Omega-3-PUFA suppressed the formation of ω-6-PUFA-derived eicosanoids and triggered formation of ω-3-PUFA-derived lipid mediators [34]. Interestingly, the administration of ω-3 PUFA-rich parenteral nutrition has been established as beneficial in pediatric patients with parenteral nutrition-associated liver disease. In these children administration of a fish-oil-based parenteral nutrition led to a significant decrease of the serum bilirubin as well as of triglycerides, and total and LDL-cholesterol [35]. These metabolic effects of ω-3 PUFAs leading to the alleviation of liver steatosis, as well as lipid and glucose status are therefore associated with the clinically proven—and widely used—beneficially effect of ω-3 PUFAs in hypertriglyceridemia. Hypertriglyceridemia is one of the indications for

which high-dose ω-3 PUFA preparations are approved by the Food and Drug Administration (FDA) and the European regulatory authorities, and many studies consistently show this effect, with doses of the ω-3 PUFA preparations of up to 12 g/d [36–38]. Furthermore, as high triglyceride levels predispose to the development of pancreatitis [39, 40], it is noteworthy that experimental data suggest an anti-inflammatory effect of ω-3 PUFAs also in the context of pancreatitis. In acute pancreatitis, *fat-1* mice showed a decreased systemic inflammatory response as measured by plasma IL-6 levels and neutrophil infiltration in the lung, as well as a trend towards decreased pancreatic necrosis. Possibly of importance for the prevention of long-term complications of chronic pancreatitis, such as chronic pain and exocrine and endocrine pancreatic insufficiency, chronic pancreatitis in *fat-1* was associated with decreased pancreatic fibrosis [41]. Among others, these animal data and some human studies [42, 43] therefore suggest a beneficial potential for ω-3 PUFA supplementation in pancreatitis.

3.4. Lipidomics in Metabolic Liver Disease. Recent studies have analyzed lipidomic aspects in the context of nonalcoholic steatohepatitis (NASH) pathology [44, 45], demonstrating an increase in the ratio of ω-6 to ω-3 PUFAs in NASH liver tissue as well as in the plasma levels of the AA metabolites 5-HETE, 8-HETE, and 15-HETE in the progression from normal to NASH. These data indicate that the liver is critical not only for lipoprotein and triglyceride metabolism, but also for the conversion of essential PUFAs to bioactive lipid mediators, due to the expression of COX, lipoxygenase (LOX), and cytochrome P450 enzymes. The ω-6/ω-3 PUFA ratio in liver tissue therefore probably determines the lipid mediator profile generated and could thus be an important factor in the development of metabolic (liver) disease. Mass spectrometry techniques allowing for a comprehensive and concomitant assessment of a multitude of ω-3 and ω-6 PUFAs and their derived lipid metabolites in human blood and tissue samples [46] will thus have an important role now for the evaluation of lipid metabolite formation and of their role in different physiological and pathophysiological contexts. Measurement of ω-3 derived lipid metabolites and mediators such as 18-HEPE, 17-HDHA, and their resolvins in blood might thus be one of the most important tools to better understand the biological impact of ω-3 PUFAs on the human body (Figure 2).

4. Beneficial Effects of ω-3 PUFAs on Neurodegenerative Diseases: Involvement of Cytokines and Lipid Mediators

Among ω-3 PUFAs, DHA is specifically enriched in the brain and is essential for normal neurological function [6]. In the mammalian brain, lipids make up 10% of the fresh weight and 50% of the dry weight, and DHA and AA (20:4ω-6) are the major PUFAs found in the neuronal membrane [47]. Most DHA and AA accumulation in the brain takes place during brain development in the perinatal period from the beginning of the third trimester of gestation to 2 years after birth in humans and from prenatal day 7 to postnatal day 21 in rats [48–50]. However, hippocampal DHA levels decrease with age in rats [51, 52] and are reduced in the neurodegenerative Alzheimer's disease (AD) [22, 53, 54]. Deficiency of hippocampal DHA is associated with reduced learning and memory ability in rats [55] and with cognitive decline in AD patients [56].

Although the liver is the major site of DHA biosynthesis [57], DHA can be synthesized locally in the developing brain [58, 59]. In contrast, the human adult can convert ^{13}C-labeled ALA to EPA and, to a lesser extent, docosapentaenoic acid (22:5ω-3), but very little is converted to DHA [60]. In addition, human blood levels of EPA, but not DHA, are increased in healthy humans supplemented with ALA (2–40 g/day for 3–26 weeks) or EPA (1–4 g/day for 4–12 weeks) [61]. The 15-lipoxygenase-1 expression and NPD1 levels are reduced in the hippocampus of AD patients [22]. These findings indicate that the developing brain has the ability to synthesize and take up DHA, while, in the adult, DHA synthesis is low, and, in the patient of AD, NPD1 biosynthesis is reduced.

4.1. Specific Effect of ω-3 PUFAs on Neuron Protection. Evidence is accumulating that neuron protection is provided by ω-3 fatty acids, but not other fatty acids. DHA or NPD1, but not AA, attenuates TNF-α- and H_2O_2 -induced apoptosis of human retina pigment epithelial ARPE-19 cells [62]. DHA, but not 22:5ω-6, AA, or oleic acid (OA, 18:1ω-9), protected mouse neuroblastoma neuro 2A cells against apoptosis induced by 2-day serum starvation [63, 64], and DHA, but not AA or OA, prevented oxidative stress-induced apoptosis of neuro 2A cells [65]. DHA, but not AA, OA, or palmitic acid (16 : 0), prevented apoptosis of rat retina photoreceptor cells during development or induced by oxidative stress [66, 67]. ALA, but not 16 : 0, provided protection against ischemia-induced hippocampal cell death and prevented kainic acid-induced seizures in rats [68]. These findings indicate a specific effect of ω-3 PUFAs in neuron protection.

4.2. ω-3 PUFAs Reverse Age-Related Inflammation Changes. Levels of mRNAs coding for major histocompatibility complex molecule II and CD40, markers of microglial cell activation indicating neuronal inflammation, and protein levels of IFN-γ and IL-1β were increased in the hippocampus in aged rats (22-month-old) compared to young rats (4-month-old), and these effects on aged rats were overcome by supplementation with EPA (125 mg/kg/day for 4 weeks) [69].

4.3. ω-3 PUFAs in AD Prevention. AD is a progressive neurodegenerative disease characterized by dementia with impaired cognitive performance and accounts for 70% of dementia patients. The main pathology of AD is related to extracellular deposits of diffusible assemblies (oligomers) of amyloid β peptide (Aβ), fibrillar aggregated of Aβ as plaques, and of intracellular phosphorylated tau protein as tangles, which cause neuronal death [70–72]. In AD patients, the hippocampus is one of the first brain regions to suffer damage [73, 74]. In these patients, DHA and Neuroprotectin D1 (NPD1) levels are reduced in the hippocampus but are unchanged in the frontal cortex, thalamus, or occipital lobes [22, 49]. Treatment of human SH-S5Y5 neuronal cells with

DHA inhibits the formation of Aβ fibrills and oligomers and their cytotoxicity [75]. Moreover, in studies on a primary coculture of human neurons and glia supplemented with DHA, NPD1 biosynthesis is increased, Aβ production is reduced, antiapoptotic gene expressions, Bcl-2 and Bfl-1, are upregulated, and cell survival is increased [22].

In addition, NPD1 downregulates Aβ-induced expression of proinflammatory elements including COX-2, tumor necrosis factor alpha (TNF-α), and a TNF-α-inducible proinflammatory element B-94 to promote cell survival in the human neuronal-glial cells [76]. Moreover, DHA enhances NPD1 synthesis and NPD1 reduces IL-1β-stimulated COX-2 expression, and DHA/NPD1 up regulates anti-apoptotic Bcl-2 and Bfl-1 proteins and down-regulate proapoptotic BID, BAX, BAD, and caspase-3 proteins in human ARPE-19 cells [62, 77]. These studies suggest that DHA increases NPD1 biosynthesis, reduces Aβ production, and inhibits inflammatory cytokine secretion in neuron cells.

In studies of ω-3 PUFA supplementation in an AD animal model, Aβ plaques in the hippocampus were reduced in aged (22.5-month-old) AD mice fed with a DHA-enriched diet (0.6% w/w in chow diet) for about 103 days [78], DHA levels were increased, soluble Aβ levels reduced, and levels of phosphorylated tau protein decreased in the brain in adult (3-month-old) AD mice fed with a DHA-enriched diet (1.3% w/w in control diet) for 3–9 months [79] and reactive oxygen species levels and the number of apoptotic neurons in the hippocampus were decreased, hippocampal DHA levels increased, and radial-maze learning memory performance improved in Aβ-infused adult rats supplemented with DHA or 20:5ω-3 (300 mg/kg/day for 12 weeks) [80–82]. Phosphorylation of the antiapoptotic protein BAD was increased and levels of oxidized proteins decreased in the cortex and water-maze learning memory performance improved in aged (17-month-old) AD mice fed with an ω-3 fatty acid-deficient diet when the diet was supplemented for 103 days with DHA (0.6% w/w in the ω-3 deficient diet) [83]. These findings indicate that reduced brain DHA levels are restored, NPD1 biosynthesis is increased, Aβ production is reduced, antiapoptosis proteins are increased, and learning memory improved, by ω-3 PUFAs consumption.

In humans, DHA levels do not change in the healthy hippocampus between the ages of 33 to 90 years [53], but, in AD patients, they are reduced by 53% to 8% of total fatty acids in the PE fraction compared to 17% in healthy age-matched controls [53], and, in patients with mild AD, fish oil supplementation (600 mg of EPA + 1700 mg of DHA/day for 6 months) increased plasma DHA and EPA levels, decreased IL-1β, IL-6, granulocyte colony-stimulating factor and prostaglandin $F_{2\alpha}$ after the stimulation of peripheral blood mononuclear cells with lipopolysaccharide, regulated inflammatory gene expression, and delayed cognitive decline [84–87]. However, in the same study, IL-6, TNF-α, Aβ, tau protein, and phosphorylated tau protein in cerebrospinal fluid were not changed in AD patients with fish oil supplementation [88]. In addition, serum DHA levels in AD patients gradually decrease with the severity of clinical dementia compared to healthy age-matched controls [18], while AD risk is reduced, and cognitive decline is delayed by higher DHA levels in blood [89, 90] or ω-3 PUFA consumption [91, 92]. These studies indicate that ω-3 PUFA consumption reduces AD pathology, inhibits inflammatory cytokine secretion, upregulates anti-apoptotic proteins, and downregulate pro-apoptotic protein expression to protect neuron, reverse age-related inflammatory changes, and decrease cognitive decline for AD prevention summarized in Figure 3.

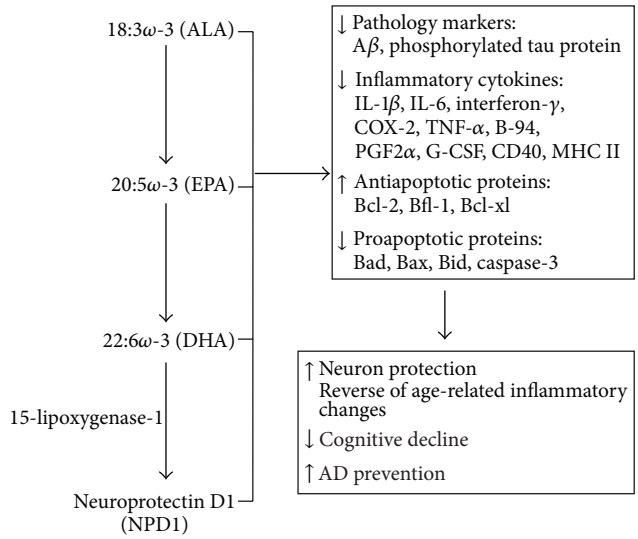

Figure 3: Preventive effect of ω-3 PUFAs on the neurodegenerative Alzheimer's disease. ALA: α-linolenic acid; DHA docosahexaenoic acid; EPA: eicosapentaenoic acid; DHA: is converted to NPD1 by 15-lipoxygenase-1 in neuronal cells. ω-3 PUFAs reduce AD pathology, inhibit inflammatory cytokines secretion, upregulate antiapoptotic proteins, and downregulate proapoptotic proteins expression to protect neuron, reverse age-related changes, and decrease cognitive decline for AD prevention.

5. Beneficial Effects of ω-3 PUFAs in Cancer: Involvement of Cytokines and Lipid Mediators

5.1. Inflammatory Cytokines on the Pathogenesis of Cancer: Modulation by ω-3 PUFAs. An impressive body of evidence supports the existence of a relationship between chronic inflammation and the development of some kinds of cancer. Among them cervix, colon, bladder, gastric, liver, esophageal, ovarian, and prostate cancer are included [93–95]. All these cancers have been called "inflammation-related" tumors, since they share the feature to unfold slowly on a background of chronic injury and inflammation. In turn, the definition "cancer-related inflammation" has been also widely used to indicate the link that many epidemiological, experimental and clinical studies have established between persistent inflammatory conditions and the upsurge of cancer in the inflamed tissues of these patients. Altogether, it has been deduced that at least 25% of tumors have a causal relationship with a preexisting inflammation affecting the tissue where the cancer arises [94, 96]. Moreover, in many tumors, such as breast cancer, for which a clear inflammatory pathogenesis has not been directly established, the typical histological and

molecular features of a "cancer-related" inflammation are still evident [96].

Beside the observations that the "inflammation-related" tumors are rich in inflammatory cells and often surrounded by inflamed tissues, the hypothesis of their inflammatory pathogenesis is also supported by the observation that the incidence of these cancer may be reduced by the administration of anti-inflammatory drugs [97]. Many important mechanistic pathways underlying inflammation associated to cancer have been so far identified, even though the molecular links interconnecting inflammation and cancer are not completely understood [98]. A series of molecular mediators able to trigger inflammation and simultaneously induce oncogenic pathways has been uncovered, including some bioactive eicosanoids originated by the metabolic conversion of AA, as well as reactive oxygen or nitrogen species (ROS and RNS) and cytokines, which are produced by both the inflammatory cells present in the microenvironment surrounding the "inflammation-related" tumors, and by the tumor cells themselves [96, 98, 99].

5.2. Macrophages and Tumorigenesis. A central pathogenetic role has been attributed also to the polarization of the tumor environment infiltrating macrophages towards the M1 phenotype in preneoplastic environment, then substituted by M2 mononuclear cells in advanced cancer lesions [100]. M1 are high producers of proinflammatory cytokines TNF-α, IL-1 and IL-12 and secrete the immunosuppressive IL-10 and transforming growth factor β (TGF-β) at low levels. The high production of pro-inflammatory TNF-α, IL-1 and IL-12 is driven by the activation of the transcription factor NF-κB in these macrophages. On the other hand, M2 macrophages show scarce NF-κB activity that leads to low production of TNF-α, IL-1, and IL-12 and high production of immunosuppressive IL-10 and TGF-β [100, 101].

Among the cytokines that have been recently recognized as important pathogenetic factors in the induction of the growth, invasion, and metastasis of "inflammation-related cancers" the most prevalent and studied in tumors microenvironment are TNF-α, IL-6, and IL-1. The detailed description of their pathogenetic role in cancer is beyond the scope of these review, and a recent exhaustive review has been recently published on the subject [96]. The role exerted by the inflammatory cytokines in the development and progression of "inflammation-related" tumors has been further clarified as the resolving phase of acute inflammation and the molecular factors involved in it are being better comprised. It is becoming increasingly clear that the derangement of the processes involved in the resolution of acute inflammation facilitates the onset of chronic inflammation conditions and related cancer [94]. Interestingly, it has been observed that several bioactive lipid mediators endogenously formed and exhibiting a strong proresolving activity play protective roles against tumorigenesis. One hypothesis that has been put forward to explain their effect is that they may inhibit the production of cytokines involved in tumorigenesis [94].

5.3. ω-3 PUFAs and Tumorigenesis. As described above, ω-3 PUFAs are known for having strong anti-inflammatory activity. To explain their potent anti-inflammatory action, it has been especially invoked their inhibitory effect on the production of inflammatory eicosanoids derived from AA metabolism, as well as their ability to modulate the production of cytokines and ROS [102]. Recently, their ability to be metabolized to proresolving molecules (i.e., resolvins) and to influence the prevalence of the M1/M2 mononuclear cell phenotype in tissues [20, 103, 104] has been also invoked. We have just considered that all these molecules and factors influenced by ω-3 PUFAs have been also recognized as the main mediators involved in the development and progression of "inflammation-related" tumors (see above). On this basis, and in relation to the antineoplastic role of these FAs, supported by over two decades of extensive investigation [105], it is becoming clear that a pathway through which they could exert their anti-cancer action against "inflammation-related tumors" could be the modulation of the activity of inflammatory cells and factors found in the context of these tumors. In this section of the review we focus on the recent research showing a clear relationship between the anti-inflammatory and antineoplastic effects of ω-3 PUFAs. We pay particular attention to those findings that support the ω-3 PUFA modulating effect on cytokine production in inflamed tissues as a crucial mechanism underlying the antineoplastic effect of these dietary compounds. So far, actually, this issue has been barely studied in a direct way, and only a few published reports are available at the moment, even though it should be underlined that the role of ω-3 PUFAs, and especially for EPA, as anticachectic agents reducing the cytokines production in patients bearing advanced tumors is already well recognized [106–108]. For instance, one interesting and recent work by Lou et al. [109] demonstrated that a ω-3 PUFA-rich diet had beneficial effect against UVB-induced carcinogenesis. Besides reducing incidence and size of different skin tumors and increasing the latency for their development, ω-3 PUFA treatment markedly decreased the levels of several inflammatory cytokines in UVB-treated epidermis, including lipopolysaccharide-induced CXC chemokine (LIX), soluble tumor necrosis factor-alpha receptor 1 (sTNF R1), and macrophage inflammatory protein-1c (MIP-1c). Many other cytokines, already scarcely present in control rat epidermis, were not any more observable in the epidermis of ω-3 PUFA-treated rats. In agreement, ω-3 PUFAs were found to inhibit *in vitro* the expression of IL-1β and IL-6 cytokines in AR42J pancreas acinar tumor cells stimulated by the pancreatitis-inducer cerulean [110]. Moreover, recently Rosa et al. [111] found that in rats treated with fish oil (FO) (Galena FO, 4% w/w) and subjected to 1,2-dimethylhydrazine (DHM)-induced colon carcinogenesis a lower incidence of aberrant crypt foci (ACF) was observed as compared to soybean-oil-treated rats (controls), confirming a wide series of previous observation [112]. Interestingly, the authors suggested the existence of a relationship between the anticancer effect of ω-3 PUFAs and their anti-inflammatory effect, since they observed an increased expression of the immunosuppressive factor TGF-β and a decreased expression of the pro-inflammatory and pro-angiogenic chemokine IL-8 in the colonic mucosa of DHM-treated rats fed with a FO-enriched diet. This result is in agreement with previous findings

indicating that ω-3 PUFAs inhibit neo-angiogenesis in animal models of inflamed mucosa and colon cancer [113, 114], and with the observed ω-3 PUFA-induced inhibition of IL-8 production in IL-1β-stimulated endothelial cells (HUVEC) [113] or in UVB- or TNF-α-stimulated HaCaT keratinocytes [115]. Moreover, as it is known that both IL-8 and TGF-β are among the cytokines secreted at high levels and specifically by the M2 monocytic phenotype [116], this finding suggests that ω-3 PUFAs may exert direct influence on the polarization of M1/M2 monocytic cells in the context of tumor microenvironment. In agreement, Chiu et al. [117] recently found that 17-HDHA, a DHA-derived resolvin, increases the macrophage polarization towards the M2 phenotype *in vitro*. However, these ω-3 PUFA-induced alterations may be tissue specific, since a dietary treatment with ω-3 PUFAs of transgenic LDLr−/− or apoE−/−mice, an atherosclerosis animal model, did not alter the M1/M2 proportion in blood, whereas it induced M1 polarization in spleen [104]. The monocyte alternating between the two phenotypes M1 and M2 in tumor microenvironment is currently a field of great interest [100], but we should underline that its relationship with the development and progression of "inflammation-related cancers" are presently far from being completely understood. In particular, the hypothesis of Bögels et al. [116] that the polarization of M1/M2 monocytes in the context of tumor microenvironment may be greatly influenced by the specific microenvironments of different tumor types should be noted. These authors found that monocytes grown *in vitro* in the presence of breast cancer cell supernatant showed an increased expression of IL-8 and other cytokines (IL-10, and the chemokines CCL17 and CCL22), all associated with the alternatively M2 phenotype [116]. By contrast, colon cancer cell supernatants induced monocytes to produce more pro-inflammatory cytokines (i.e., IL-12 and TNF-α) and ROS [116].

It has been widely reported that ω-3 PUFAs exert inhibitory action towards NF-κB activation [3]. This effect has been largely invoked to explain the anti-inflammatory and antineoplastic effects of these FAs, since NF-κB is the main transcription factor involved in the upregulation of inflammatory cytokines, and other inflammation related genes (such as COX-2 or genes codifying adhesion molecules), as well as cell growth-related genes [118]. Recently, a dynamic model of gradual NF-κB inhibition associated with M2 polarization in monocytes associated with tumor microenvironment was proposed [100]. According to this model, the full activation of NF-κB in inflammatory leukocytes resident in preneoplastic sites may exacerbate local M1 inflammation (with high levels of TNF-α, high IL-1, and IL-12 and low levels of IL-10 and TGFβ) and favour tumorigenesis [119]. On the other hand, tumor growth may be associated with the progressive inhibition of NF-κB and in the progressive development of M2 inflammation, characterized by low levels of TNF-α, IL-1, and IL-12, and high levels of IL-10 and TGF-β [120]. On these bases, it can be hypothesized that the preventive or therapeutic efficacy of NF-κB suppressor agents like ω-3 PUFAs against cancer could be subject to tumor stage and polarization of infiltrating leukocytes. This means that they could exert a beneficial major role preventing the development of cancer, but scarcely or negatively affect the progression of established and advanced cancer lesions.

A lower activity of NF-κB was recently observed in colon tumors induced in *fat-1* mice by using the DSS/azoxymethane (DSS/AOM) colon tumorigenesis protocol. These transgenic mice, showing an endogenous tissue enrichment with ω-3 PUFAs, had a lower tumor load, and also higher expression of TGF-β in the colon [121]. Also in another study performed using *fat-1* mice and the same model for tumorigenesis a decreased incidence of colonic adenocarcinomas was shown, as well as increased apoptosis, decreased CD3(+), CD4(+) T helper cells, and ω-6 PUFA-derived eicosanoids in colon cancer tissue [122]. Interestingly, the authors found also decreased macrophage cell numbers per colon, which is in keeping with the decreased number of monocytes of both M1 e M2 phenotype infiltrated in the adipose tissue of animal treated with a diet at high level of FO [103]. Subsequently, several other studies demonstrated decreased liver tumorigenesis in *fat-1* mice. In a transgenic hepatoma model containing double mutations in c-myc and TGF-α a constitutive increase in ω-3 PUFA tissue content led to protection from liver tumor development that was associated with a decrease in NF-κB levels [123]. In the diethylnitrosamine (DEN) model of chemically induced liver carcinogenesis a tumor suppression effect of increased ω-3 PUFA tissue status was demonstrated in the *fat-1* mice as well, with lowered plasma TNF-α levels and decreased liver COX-2 expression [31]. Consistent with the view that COX-2 represents an inflammation-associated factor involved in the pathogenesis of "inflammation-related cancers" [124], plenty of work has considered the ω-3-PUFA-induced reduction of COX-2 expression as essential to explain the antineoplastic effect of these FAs on tumors that are pathogenetically related to chronic inflammation [114, 125, 126].

It has also been suggested that inflammatory components of the tumor microenvironment may impact on mechanisms essential for the generation of metastatic variants [127]. The production of inflammatory cytokines such as TNF-α and IL-1 by tumor-associated macrophages can act as potent stimulator of metastasis [96, 128]. Kim et al. [129] recently reported that ω-3 PUFAs, besides being powerful inhibitors of the production of these cytokines by macrophages, could exert antimetastatic effects by suppressing the invasive and metastatic response of cancer cells to these cytokines [129]. In particular, they observed that EPA inhibited the TNF-α-induced expression of a matrix metalloproteinase (MMP-9) specifically associated to tumor cell invasion and metastases [130, 131] in HaCaT keratinocytes.

It was recently reported that ω-3 PUFAs inhibit EGFR signalling in breast cancer cells [132, 133]. This receptor has been considered the "central hub" capable of integrating and transducing a variety of signals that can have an impact on cancer progression, including inflammatory signals from cytokines and other inflammatory mediators, and accumulating observations support the involvement of EGFR signalling dysregulation in hepatocarcinogenesis [95]. Similarly, it was observed [134] that ω-3 PUFAs inhibit ovarian cancer cell growth by specifically inducing TGF-β1 expression and modulating molecular pathways and get involved in cancer cell

growth. Since it is known that this cytokine may exert both pro- and anti-inflammatory actions, depending on the local tissue environment [135], it would be interesting to investigate if the effect of ω-3 PUFAs on the expression of this cytokine may be related to an anti-inflammatory action in the contest of the ovarian cancer microenvironment.

Certainly, the inflammatory hypothesis does not exclude that other routes may exist through which ω-3 PUFAs may exert their anticancer action. It has been shown that these fatty acids may exert antineoplastic effects also in models of cancers not showing a major inflammatory pathogenesis (i.e., leukemia or breast cancer) [4, 136]. Moreover, most of the mechanisms that have been suggested to explain their antineoplastic effects have been identified using cancer cells cultured *in vitro* [137], that is, using artificial conditions of cell growth that exclude the presence of the inflammatory microenvironment surrounding the tumors *in vivo*. A number of the identified mechanisms through which ω-3 PUFAs may act are common to cancer cells originating from different tissues, whereas others can be cell specific [114, 125, 126, 138, 139]. For instance, we found that ω-3 PUFAs exerted antineoplastic effects (i.e., proapoptotic and pro-differentiating effects) on colon cancer and melanoma cells by inducing opposite effects on the intracellular location of β-catenin [126, 140].

On the whole, however, unifying routes of action have been identified to explain the beneficial effects of these FAs [3], and it has now become clear that, once they are incorporated in our cell membranes, they can chemically and physically alter that microenvironment and, thus, influence the activities of many membrane-bound proteins with carrier, channel, enzyme, or signaling functions [141]. Moreover, as discussed above, they can also be metabolized in the cells and converted to powerful bioactive products able to modulate many cellular process and functions [20]. It is worth stressing that all effects may be relevant on different levels, either if ω-3 PUFAs are incorporated by the tumor cells themselves, or also by incorporation into inflammatory/immune cells surrounding the tumors, and—influencing the process of tumorigenesis—by affecting the normal cells from which tumor cells may originate.

5.4. Antitumor Evidence in Humans. There is currently some direct evidence for an anti-tumor effect of ω-3 PUFAs on human colon and liver. Regarding an antiproliferative action of ω-3 PUFAs in the colon, over 20 years ago a pioneer study was conducted by Anti et al. [142], demonstrating that the abnormal proliferation observed in the colonic mucosa of patients at high risk of colon cancer for sporadic polyposis was suppressed by a combined supplementation with EPA and DHA. This study was confirmed later by Huang et al. [143] who observed that the abnormal proliferation of the colonic epithelium adjacent to surgically resected cancer in patients at high risk of developing a second tumor was reduced by supplementing a fish oil concentrate. In agreement, in a more recent trial in patients with familial adenomatous polyposis the treatment with EPA for 6 months was associated with a reduction in polyp number, a decrease in the sum of polyp diameters and stabilization of polyp burden [144]. ω-3 PUFAs might thus be a safe chemopreventive agent in this context. Furthermore, a recent Japanese epidemiological study found that the consumption of ω-3 PUFA-rich fish and ω-3 PUFAs were associated with decreased development of hepatocellular carcinomas [145].

6. Conclusions

Taken together, a large amount of experimental evidence supports the anti-inflammatory and anti-tumor role of ω-3 PUFAs, and numerous mechanisms for these effects have been elucidated *in vitro* and *in vivo*. These studies have led to a better understanding of the influence exerted by ω-3 PUFAs and their metabolic derivatives on inflammation/resolution, as well as on the molecular pathways underlying the production and action of inflammatory cytokines in the context of inflammatory, metabolic, and neurodegenerative diseases and inflammation-related cancers. However, so far the clinical application of this knowledge has been lagging behind, which is due, at least in part, to inconclusive or contradicting results in a wide variety of clinical studies using ω-3 PUFAs.

The purpose of this review is therefore to refocus on what is known and what is established. For the clinics this is, beyond doubt, the triglyceride-lowering effect of the ω-3 PUFAs.

However, it is our belief, that future human studies will also have to take a better look at the anti-inflammatory, anti-degenerative, and antiproliferative effects of ω-3 PUFAs and their lipid mediators. It is conceivable that the heightened public interest in ω-3 PUFA (fish oil) fatty acids in recent decades has already led to an increase in the ω-3 PUFA uptake in large parts of western populations, leading to a shifting baseline in intervention studies using ω-3 PUFA supplementation and thereby confounding a potential outcome advantage of ω-3 fatty acid supplementation. This could be addressed by more widespread measurements of ω-3 and ω-6 fatty acid levels in the context of these studies—a measurement that has been lacking from most ω-3 PUFA intervention studies performed so far.

Much remains to be done, and given the favorable safety profile of long-term ω-3 PUFA supplementation we propose that testing long-term preventive effects of ω-3 PUFAs in the context of cancer and neurodegenerative disease, as well as in the metabolic syndrome, should become the most important field of ω-3 PUFA research in the coming years.

Authors' Contribution

G. Calviello, H.-M. Su, and K. H. Weylandt contributed equally to this work.

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Review Article

Cardioprotective Effects of ω-3 PUFAs in Chronic Kidney Disease

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The prevalence rate of chronic kidney disease (CKD) is increasing worldwide, and cardiovascular disease (CVD) is a main cause of death in patients with CKD. The high incidence of CVD in CKD patients is related to chronic inflammation, dyslipidemia, malnutrition, atherosclerosis, and vascular calcification. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been shown to reduce the risk of CVD. In this paper, we review the beneficial effects of ω-3 PUFAs on CVD and the possible cardioprotective mechanisms of ω-3 PUFAs in CKD patients by determining the effect of ω-3 PUFAs in the general population. ω-3 PUFAs have several cardioprotective benefits, such as reducing inflammation, decreasing oxidative stress, inhibiting platelet activity, exerting antiarrhythmic effects, and improving triglyceride levels, in the general population and patients with CKD. Modifications of erythrocyte membrane fatty acid content, including an increased ω-3 index and decreased oleic acid, after ω-3 PUFAs supplementation are important changes related to CVD risk reduction in the general population and patients with CKD. Further basic and clinical studies are essential to confirm the effects of ω-3 PUFAs on vitamin D activation, vascular calcification prevention, cardiovascular events, and mortality in CKD patients.

1. Introduction

Chronic kidney disease (CKD) is a public health problem, and the prevalence rate is increasing worldwide. The increasing prevalence rate of CKD is related to increased average life expectancy, the elderly population, obesity, diabetes, and hypertension, which are risk factors of CKD. Cardiovascular disease (CVD) is a main cause of death, the primary comorbid disease, and a frequent cause of hospitalization in patients with CKD [1, 2]. The high incidence of CVD in CKD patients is related to chronic inflammation, dyslipidemia, malnutrition, atherosclerosis, and vascular calcification [3–7]. Vascular calcification (VC) has been shown to be an independent predictor of cardiovascular mortality in CKD patients maintained on dialysis therapy. Therefore, preventive strategies for CVD are essential, especially in CKD patients.

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) supplementation has been linked to reducing the risk of CVD [8]. Dyerberg et al. found that Greenland Inuit who had a diet including a high content of ω-3 PUFAs had low mortality from coronary heart disease [9]. Subsequent studies found that ω-3 PUFAs intake is associated with a reduced risk of CVD [10]. This cardioprotective effect of ω-3 PUFAs is explained by its ability to suppress inflammation, inhibit platelet activation/adhesion, and reduce thrombosis [11]. The main effect of ω-3 PUFAs is reducing triglyceride levels in patients with hypertriglyceridemia, which is associated with CVD [12]. In addition, ω-3 PUFAs reduced oxidative stress and had the possibility to inhibit vascular calcification in human studies and a rat model [13–15]. Furthermore, ω-3 PUFAs may be involved in CVD by modulating cell membrane receptors and affecting signal transduction and eicosanoid metabolism [16–18]. Therefore, ω-3 PUFAs, which have several benefits in CVD, may be helpful to reduce CVD in CKD patients, who have a high prevalence rate of CVD.

In this paper, we review the beneficial effects of ω-3 PUFAs on CVD and possible cardioprotective mechanisms of ω-3 PUFAs in CKD patients by evaluating the effect of ω-3 PUFAs in the general population. We also suggest several investigations to prove the cardioprotective effect of ω-3 PUFAs based on small challenging studies.

2. ω-3 PUFAs, ω-6 PUFAs, and the ω-3 Index

ω-3 PUFAs are commonly found in marine and some plant oils, such as fish oils, algal oil, squid oil, echium oil, and flaxseed oil. They have several double bonds (C=C) beginning after the third carbon atom from the end of the carbon chain. They are considered as essential fatty acids, which cannot be synthesized by mammalian cells de novo but are vital for normal metabolism. ω-3 PUFAs include α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) (Figure 1). ALA is a short-chain ω-3 PUFAs and can be converted into EPA and DHA, which are long-chain ω-3 PUFAs, by desaturase and elongase [19]. To support optimal EPA and DHA levels, these conversions are a rate-limited step and depend on the amount of dietary linoleic acid (LA) and ALA [19, 20].

Omega 6 PUFAs (ω-6 PUFAs) are another family of unsaturated fatty acids that have a final carbon–carbon double bonds in the sixth bond counting from the methyl end. The major source of ω-6 PUFAs is animal meat. ω-6 PUFAs can be converted into arachidonic acid (AA) [21], and AA can be metabolized into ω-6 eicosanoid products, such as prostaglandins, leukotrienes, and thromboxanes, by cyclooxygenase, lipoxygenase, or cytochrome P450 AA monooxygenase. Excessive ω-6 PUFAs promote prothrombotic, proinflammatory, and atherosclerotic processes. Competitive interactions with ω-3 PUFAs affect the relative storage, mobilization, conversion, and action of the ω-3 and ω-6 eicosanoid precursors.

Harris and Von Schacky announced the omega-3 index (ω-3 index), a new risk factor for death caused by coronary artery disease (CAD) [22]. This index is defined as the percentage of EPA + DHA content in red blood cells (RBCs) membranes. The rationale for this index is that the FA content of erythrocyte membranes has been shown to highly correlate with the FA content of the myocardium [23]. The erythrocyte FA composition is less variable than plasma. Therefore, the cardiac FA content can be determined by estimating the erythrocyte membrane FA content. The modification of the FA content can either reduce or increase the risks of cardiovascular events. It is necessary to measure the erythrocyte membrane FA content, including the ω-3 index, which can be affected by dietary habits, especially in clinical trials for evaluating the effect of ω-3 PUFAs.

3. Anti-Inflammatory and Antithrombotic Effects of ω-3 PUFAs

Inflammation results from immunological processes in response to injury, invading pathogens, allergens, and toxins and leads to the repair of damaged tissue. Chronic and persistent inflammation plays a central role in the development and progression of CAD. The inflammatory response is regulated by a complex network of mediators, including lipid mediators (eicosanoids, docosanoids, and platelet-activating factors), cytokines, and chemokines [24]. These mediators promote platelet aggregation and have proinflammatory effects.

ω-6 PUFAs are converted to AA-derived eicosanoids, which have prothrombotic, proinflammatory, and proarteriosclerotic effects. In contrast, ω-3 PUFAs compete with the AA cascade, and ω-3 PUFAs consumption increases EPA, ω-3 PUFAs in the cell membrane. EPA is a competitive inhibitor of cyclooxygenase. EPA reduces the production of the 2-series prostaglandins, thromboxanes, and prostacyclins and the 4-series leukotrienes and produces the 3- and 5-series prostanoids, which are less biologically active. DHA can inhibit AA metabolism and platelet aggregation by reducing the affinity of the platelet TXA2/PGH2 receptor. These actions of ω-3 PUFAs may contribute to benefits regarding CVD. ω-3 PUFAs also decrease the risk of thrombosis by inhibiting platelet aggregation [25]. The effects of ω-3 PUFAs on platelet function and thrombosis are still controversial. Some studies have reported that there is no significant association between ω-3 PUFAs supplementation and coagulation factors [26]. Therefore, further investigations on ω-3 PUFAs are necessary to clarify its antithrombotic effect.

4. Anti-Inflammatory and Antithrombotic Effects of ω-3 PUFAs in CKD

CKD is a microinflammatory state, and inflammation is a salient feature in CKD patients on predialysis and dialysis [27]. Previous studies have shown that renal dysfunction is associated with inflammation. In a study using data from NHANES III, C-reactive protein (CRP), serum homocysteine, and plasma fibrinogen levels were elevated in CKD patients [28]. These inflammatory markers may play a role in increasing the cardiovascular risk among patients with CKD. Increased inflammatory mediators have been associated with increased oxidative stress and the accumulation of advanced glycation end (AGE) products. Various studies have reported that omega-3 fatty acid (ω-3 FA) can decrease inflammatory markers [29–31]. ω-3 PUFAs supplementation significantly attenuated oxidative and inflammatory pathways by decreasing NOX-4, gp91phox, p47phox, p22phox, MCP-1, NF kappa-B, and COX-2 expression in a 5/6 nephrectomy rat model [32]. As a result of ω-3 PUFAs actions, renal fibrosis was also decreased [32]. Recent studies have reported that resolvins and protectins, which are a new class of lipid mediators, are associated with the resolution of renal inflammation [33]. ω-3 PUFAs are used as an additional treatment for inflammatory diseases, such as rheumatoid arthritis [34]. However, some studies have reported that ω-3 PUFAs intake does not have anti-inflammatory benefits in CKD patients [35]. Further clinical studies are required to identify the anti-inflammatory effect of ω-3 PUFAs in CKD patients with chronic inflammation.

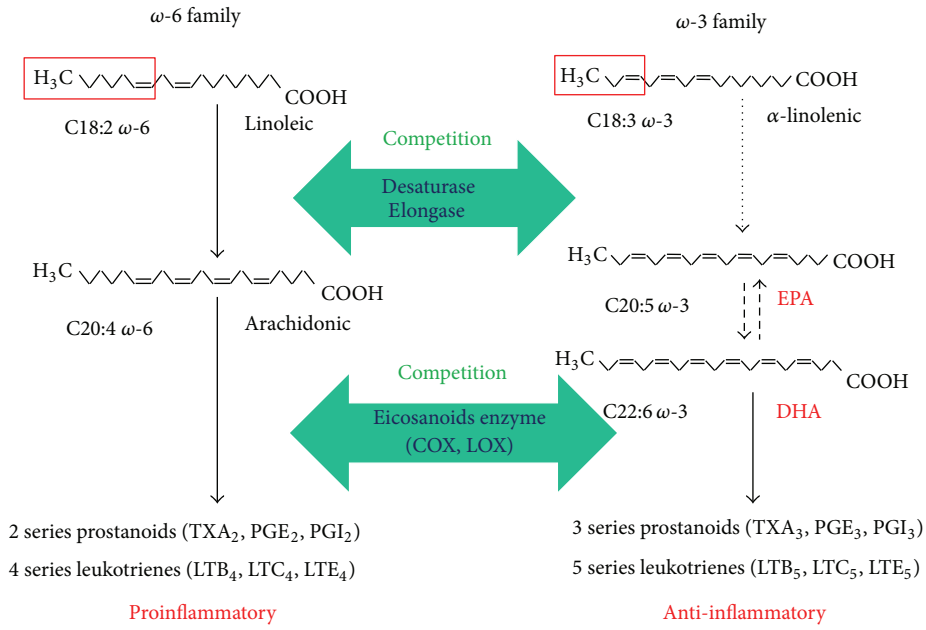

Figure 1: Biochemical pathways of ω-6 and ω-3 fatty acids.

5. Antiarrhythmic Effects of ω-3 PUFAs

ω-3 PUFAs have beneficial effects on cardiovascular health and mortality through reducing arrhythmia in the myocardium. ω-3 PUFAs may contribute to reducing the resting heart rate, promoting a faster return to resting heart rate after exercise, and increasing heart rate variability and left ventricular filling capacity [36–38]. The mechanism of these benefits is derived from preventing calcium overload by maintaining the activity of L-type calcium channels during periods of stress and increasing the activity of cardiac microsomal Ca^{2+}/Mg^{2+} ATPase (adenosine triphosphatase). In addition, ω-3 PUFAs may attenuate delayed depolarization by reducing Na^{+}/Ca^{2+} exchange currents, which may alter membrane electrical activity [39, 40]. Thus, ω-3 PUFAs increase myocardiocyte membrane electrical stability and thereby prevent malignant dysrhythmia [17]. However, further basic studies are necessary to elucidate how ω-3 PUFAs stabilize these channels and are involved in membrane activity. Still, there are few studies on the effect of ω-3 PUFAs on arrhythmia in CKD patients, and further clinical studies are necessary.

6. Effects of ω-3 PUFAs on Erythrocyte Membrane Fatty Acid Content in CKD

Fatty acids are required for membrane synthesis and protein and carbohydrate modification, and the necessity of specific fatty acid compositions is different according to specific medical conditions. In several studies, ω-3 PUFAs supplementation augmented the erythrocyte membrane EPA and DHA content and consequently the ω-3 index, in CVD patients. These modifications of erythrocyte membrane fatty acid content were also shown in CKD patients. ω-3 PUFAs supplementation may be helpful for reducing the risk of CVD with regard to the increased content of ω-3 PUFAs, and consequently the ω-3 index. However, the precise mechanism underlying the cardioprotective effect and how increased amounts of ω-3 PUFAs affect the cellular functions and cell membrane receptors are unknown. Further studies are needed to evaluate the effect of ω-3 PUFAs on the conformational changes of membrane receptors and functional changes of cell membrane receptors.

A higher intake of saturated fatty acids increases the cell membrane content of total saturated fatty acid and is related to an increased incidence of CAD [41, 42]. Therefore, decreasing erythrocyte total saturated fatty acid content may be helpful to reduce the incidence of CAD. The total saturated fatty acid content was decreased by ω-3 PUFAs supplementation in the general population and CKD patients [43–45].

Oleic acid has been shown to stimulate vascular smooth muscle cell migration and proliferation via the direct activation of extracellular signal-regulated kinase *in vitro*. In addition, oleic acid amplifies angiotensin II-induced protein kinase C (PKC) activation and reactive oxygen species generation *in vitro* [46, 47]. Erythrocyte membrane oleic acid levels were significantly higher in patients with acute coronary syndrome compared with a control group [48, 49]. In a previous study, erythrocyte membrane oleic acid levels were also elevated in CKD patients, who are at higher risk for CVD, treated with dialysis [11, 43]. Furthermore, erythrocyte membrane oleic acid levels were associated with the vascular calcification score on plain radiographs, which was related to CVD and CAD in hemodialysis patients [50]. Therefore, based on these results, erythrocyte membrane oleic acid levels may be related to CVD. ω-3 PUFAs supplementation

decreased erythrocyte membrane oleic acid levels in CKD patients treated with dialysis [43, 44]. One study showed that a 30% reduction in arachidonic acid/EPA ratio was associated with a 70% reduction in the risk of myocardial infarction [31]. The AA/EPA ratio can be easily reduced by ω-3 PUFAs because ω-3 PUFAs can decrease erythrocyte membrane content of AA, which is related to inflammatory process. The modification of fatty acid content in erythrocyte membranes by ω-3 PUFAs plays an important role in preventing CVD. Several studies have reported an inverse relationship between renal function and risk of CVD [2, 4]. Further investigations may be needed to evaluate the modification of erythrocyte membrane fatty acid content according to renal function in CKD patients.

7. Effects of ω-3 PUFAs on Lipid Metabolism in CKD

Another main effect of ω-3 PUFAs is regulating lipid homeostasis. ω-3 PUFAs affect lipid metabolism by decreasing the synthesis of very low-density lipoprotein (VLDL), promoting β-oxidation in mitochondria and/or peroxisomes, and reducing remnant lipoprotein levels, such as apo-B degradation [51–53]. ω-3 PUFAs especially decrease triglyceride levels by inhibiting the activities of diacylglycerol acyltransferase and phosphatidic acid phosphohydrolase. Recently, high triglyceride levels and low high-density lipoprotein (HDL) cholesterol were identified as residual risk factors for CVD in patients with strictly controlled low-density lipoprotein (LDL) cholesterol [54]. Therefore, physicians occasionally prescribe ω-3 PUFAs to control hypertriglyceridemia.

The primary finding of dyslipidemia in CKD and dialysis patients is hypertriglyceridemia. Over 40 percent of patients with CKD have triglyceride levels greater than 200 mg/dL. Therefore, ω-3 PUFAs are useful for controlling dyslipidemia and reducing CVD risk in CKD patients. However, the total cholesterol concentration, triglyceride level, and LDL cholesterol are sometimes normal or low in CKD patients with malnutrition [55]. In fact, ω-3 PUFAs supplementation did not affect triglyceride levels in dialysis patients with normal triglyceride levels [43, 44].

8. Vitamin D and ω-3 PUFAs in CKD

Vitamin D deficiency is a common condition that affects one billion people worldwide, and the prevalence rate of this condition is increasing [56]. Vitamin D deficiency leads to many health problems, such as CVD, hypertension, insulin resistance, diabetes mellitus, and cancer progression [57–60]. Although there is evidence of vitamin D deficiency affecting CVD, the mechanisms underlying how vitamin D protects the cardiovascular system are unclear. *In vitro* and clinical studies suggest that vitamin D receptor activation leads to downregulation of the renin-angiotensin system, inflammation inhibition, smooth muscle proliferation suppression, and vascular calcification [61–63]. Vitamin D receptor knockout mice develop hypertension and cardiac hypertrophy [64]. Epidemiologic studies have reported that vitamin D deficiency is associated with cardiovascular events in subjects with renal dysfunction and even in the general population [65, 66]. Vitamin D deficiency is much more common in patients with decreased renal function than in those with normal renal function. Several studies have reported an association between vitamin D deficiency and CVD in CKD patients [67, 68]. Thus, providing proper vitamin D supplementation may contribute to public health benefits similar to ω-3 PUFAs supplementation. The Vitamin D and Omega-3 Trial (VITAL), a randomized, double-blind, placebo-controlled, large-scale intervention trial, is currently ongoing. The VITAL study is evaluating whether vitamin D and ω-3 PUFAs reduce the risk of cancer and major cardiovascular events and is recruiting 20,000 participants who have no previous illness. The results of the VITAL study may define the effect of vitamin D and ω-3 PUFAs in the primary prevention of CVD.

Vitamin D is hydroxylated to 25(OH)D in the liver and is then converted to a potent biological metabolite (1,25(OH)2D) by the enzyme 1α-hydroxylase [69]. The biologically active metabolite 1,25(OH)2D has anti-inflammatory and antiproliferative effects on the endothelial cells of the vascular wall [70]. A recent study showed that 1,25(OH)2D concentrations were significantly increased after 3 and 6 months in a ω-3 PUFAs supplemented group compared to baseline in dialysis patients [44]. Therefore, further studies are needed to confirm the cardioprotective effect of ω-3 PUFAs through activating vitamin D.

9. Vascular Calcification and ω-3 PUFAs in CKD

Vascular calcification is highly prevalent in patients with CKD, and it is an independent predictor of cardiovascular mortality in CKD patients [71]. ω-3 PUFAs have a beneficial effect on the vascular system by reducing pulse wave velocity. The pulse wave velocity is associated with vascular calcification on plain radiographs in subjects on dialysis [72]. Fetuin-A also antagonizes the vascular calcifying effects of bone morphogenetic protein-2 [73]. A recent study showed that fetuin-A levels after ω-3 PUFAs supplementation were significantly increased in dialysis patients. However, whether vascular calcification is inhibited by ω-3 PUFAs is unknown, despite an animal study [15]. Further prospective studies are necessary to evaluate the effects of ω-3 PUFAs on preventing vascular calcification in CKD patients.

10. Effects of ω-3 PUFAs on Cardiovascular Events and Mortality in CKD

Several clinical trials have reported that elevated ω-3 PUFAs levels reduce the risk of CVD. The Diet and Reinfarction Trial (DART) investigated the effect of dietary intervention in patients with recent myocardial infarction [74]. The patients in the fatty fish advice group showed decreased mortality. In the Gruppo Ialiano per la Sperimentazione della Streptochinasi nell'infarto Miocardio Prevenzione (GISSI) trial, the ω-3 PUFAs supplemented group demonstrated a reduction in

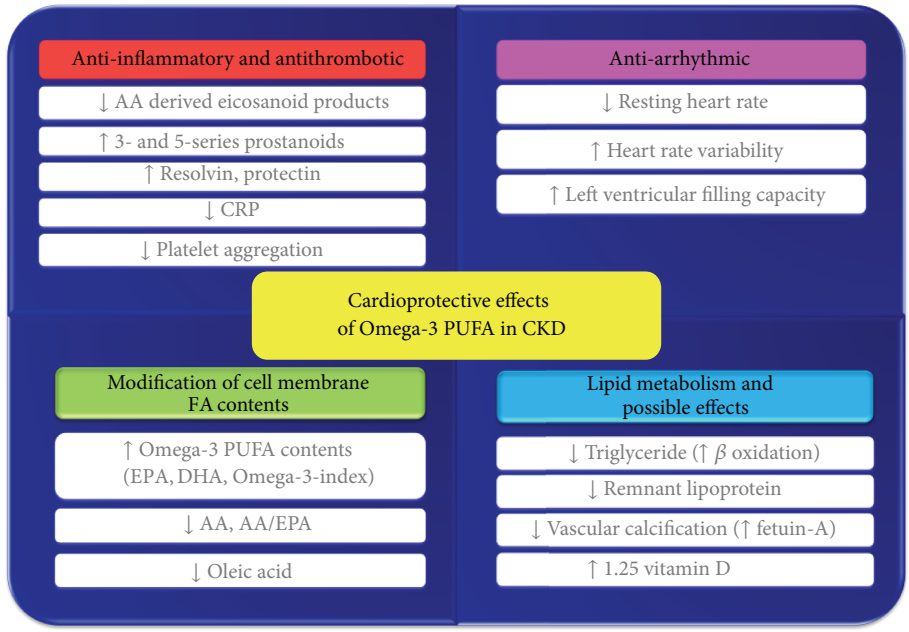

Figure 2: Cardioprotective effects of ω-3 PUFAs in chronic kidney disease (CKD).

cardiovascular death, coronary death, and sudden cardiac death [8]. In 2002, the American Heart Association (AHA) recommended that subjects with heart disease ingest 1 g fish oil daily. The AHA also recommends that CKD patients who have a high risk of CVD consume at least 1 g of ω-3 PUFAs PO daily. However, some studies have reported that ω-3 PUFAs are not significantly associated with a cardioprotective effect. Kromhout et al. demonstrated that patients who had previous myocardial infarction and were undergoing proper medical care did not show a reduced rate of cardiovascular events despite supplementation with low-dose EPA-DHA [75]. Another randomized clinical trial reported that ω-3 PUFAs supplementation was not associated with cardiovascular events [76]. Some studies did not survey actual dietary habits, and some studies did not measure plasma or membrane ω-3 PUFAs levels, including the ω-3 index. These points are very important to interpret the results of clinical trials. In addition, the doses of supplemented ω-3 PUFAs are another important point affecting the results of clinical trials. To our knowledge, there are no large-scale clinical trials with inclusion criteria based on the baseline content of ω-3 PUFAs. Therefore, the effects of ω-3 PUFAs supplementation may be different according to the doses and baseline content of ω-3 PUFAs. In addition, currently, many patients and healthy individuals frequently consume healthy food and pills derived from healthy food, such as ω-3 PUFAs and multivitamins. Patients with CVD are highly informed regarding cardiovascular health and take cardioprotective drugs, such as angiotensin-converting enzyme inhibitors or statins. These environmental factors affect the results of clinical trials on CVD and CVD-related mortality. In CKD patients, more factors are related to CVD and CVD-related mortality. ω-3 PUFAs studies have identified various clinical outcomes that are relevant to patients with renal dysfunction. ω-3 PUFAs supplementation may reduce the cardiovascular risk in subjects with decreased renal function and even in dialysis patients [77]. Another study has shown that ω-3 PUFAs may have beneficial effects against CVD through improving blood pressure and heart rate [78]. Nevertheless, the cardioprotective effect of ω-3 PUFAs is still controversial. Larger controlled clinical trials are needed to establish the cardioprotective effect of ω-3 PUFAs in CKD patients.

11. Conclusions

ω-3 PUFAs have several benefits for minimizing CVD risks by reducing inflammation, decreasing oxidative stress, inhibiting platelet activity, exerting antiarrhythmic effects, and improving triglyceride levels in the general population and patients with CKD (Figure 2). In addition, the modification of erythrocyte membrane fatty acid content by ω-3 PUFAs supplementation is an important process related to CVD risk reduction which may help in the interpretation of clinical trials in general populations and patients with CKD. Increasing erythrocyte membrane content of ω-3 PUFAs, and consequently the ω-3 index, and decreasing total saturated fatty acids, oleic acids, and AA may affect cellular function by changing transmembrane proteins and inflammatory mediators involved with cell signaling systems. The role of ω-3 PUFAs in vitamin D activation, vascular calcification prevention, cardiovascular events, and mortality should be further investigated in CKD patients.

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