

Nanomedicine-based formulations containing ω -3 polyunsaturated fatty acids: potential application in cardiovascular and neoplastic diseases

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Abstract: Omega-3 polyunsaturated fatty acids (ω -3 PUFAs) are dietary factors involved in the prevention of cardiovascular, inflammatory, and neoplastic diseases. A multidisciplinary approach – based on recent findings in nutritional science, lipid biochemistry, biotechnology, and biology of inflammation and cancer – has been recently employed to develop ω -3 PUFA-containing nanoformulations with an aim to protect these fatty acids from degradation, increase their bioavailability and delivery to target tissues, and, thus, enhance their bioactivity. In some cases, these nanoformulations were designed to administer ω -3 PUFAs in combination with other nutraceuticals or conventional/innovative drugs. The aim of this strategy was to increase the activities of the compounds contained in the nanoformulation and to reduce the adverse effects often induced by drugs. We herein analyze the results of papers evaluating the potential use of ω -3 PUFA-containing nanomaterials in fighting cardiovascular diseases and cancer. Future directions in this field of research are also provided.

Keywords: innovative biotechnologies, cardiovascular diseases, nanoparticles, ω -3 PUFAs, cancer

Introduction

There is a large body of evidence supporting a beneficial role of omega-3 polyunsaturated fatty acids (ω -3 PUFAs) against several pathologies, including cardiovascular diseases (CVDs) and cancer.¹⁻³ The results have been obtained by using either the essential fatty acid α -linolenic acid (ALA, 18:3 ω -3), mostly found in vegetables and nuts or, and particularly, its metabolic products, the long-chain (LC)- ω -3 eicosapentaenoic acid (EPA, 20:5 ω -3) and docosahexaenoic acid (DHA, 22:6 ω -3). However, in mammal cells these two highly bioactive compounds are produced endogenously from ALA at very low levels; therefore, it is necessary to increase their main dietary sources (fish and seafood) to reach sufficient amounts in tissues. However, this requires a frequent intake of fish and seafood that appears unsustainable, particularly in the future.⁴ Moreover, wild fish is often contaminated with heavy metals or pesticides,⁵ whereas farmed fish contains much lower levels of LC- ω -3 PUFAs and high levels of antibiotics.⁶ In order to overcome these problems, alternative LC- ω -3 PUFA sources are now being explored, such as microalgae grown in controlled environments,⁷ or genetically modified plants and marine protists.⁸⁻¹¹ An alternative approach may be the new nanotechnology-based strategies that are being developed to effectively deliver purified ω -3 PUFAs to the target tissues. These strategies are aimed to overcome the scarce solubility of these fatty acids, protect them from degradation, make them specifically active to target the

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site of injury, and/or distribute them in strict combination with other bioactive compounds/drugs. The ultimate goal is to enhance their bioavailability, thus reducing the level of intake of these fatty acids or of other co-transported drugs.^{12–14} For the first time, we have comprehensively and critically analyzed in the present review all the reports concerning the nanotechnological ω -3 PUFA-containing formulations hitherto developed, limiting our investigation to the in vitro and in vivo preclinical studies concerning the use of these nanoformulations in cellular and animal models of CVDs and cancers.

Literature search

A systematic literature search of the PubMed database was conducted from July 2017 to July 2018 to identify published peer-reviewed original articles regarding in vitro studies, in vivo animal studies, and human studies on the delivery of ω -3 PUFAs, alone, or in combination with other bioactive compounds, through nanoformulations. The key words used for the search of titles and abstracts were: “omega-3” or “n-3 PUFA” or “docosahexaenoic acid” or “eicosapentaenoic acid” or “ α -linolenic acid” or “fish oil;” and “animal studies,” or “in vitro studies,” or “in vivo studies” or “human studies;” and “nanoparticles” or “nanoformulations” and “encapsulation” and “delivery” and “nanomedicine” and “cancer” or “tumor” and “cardiovascular diseases” or “heart” and “inflammation.” We identified full-text articles written in English. The papers were chosen without restriction of time. We analyzed only the studies evaluating the biological effects of ω -3 PUFA nanoformulations and, in particular, in the cardiovascular and cancer conditions.

ω -3 PUFA-containing nanoformulations for the prevention of CVD and therapy

The prevention of CVDs is considered the main setting for ω -3 PUFA clinical application, and the major processes involved in the pathogenesis of most CVDs, including inflammation, oxidative stress, and abnormal cell proliferation,^{15,16} also represent the main targets of these fatty acids. However, the research investigating potential innovative nanomedicine strategies in CVDs is still very scarce, and currently restricted only to occlusive vasculopathies and atherosclerosis^{17,18} (Table 1; Figure 1). In the first report on this topic, Deshpande et al¹⁷ investigated nanotechnology-based approaches to deliver ω -3 PUFAs in combination with other nutraceuticals/drugs to vascular walls in order to prevent occlusive vasculopathies following vascular injuries.

Table 1 Application of ω -3 PUFA nanoformulations in cardiovascular disease settings

Nanoparticle (NP) type	Chemical form of ω -3 PUFA used	NP size (nm)	EE (%)	Cargo molecule	Zeta potential (mV)	Function of the NP	Experimental model	Mechanisms involved in the NP effects	Reference
ALA-rich* flaxseed oil-based oil-in-water nanoemulsion	ALA-rich (56% by weight) flaxseed oil	138–144	92–98	CER and/or 17- β E	32.30 \pm 1.81 (CER) 33.80 \pm 2.45 (17- β E)	To improve 17- β E and/or CER internalization in vascular cells	Human EC and VSMC in vitro	Increased CER and 17- β E uptake by cultured cells; increased EC and decreased VSMC proliferation (p38 MAPK inhibition)	17
ALA-rich* flaxseed oil-based oil-in-water nanoemulsion	ALA-rich (56% by weight) flaxseed oil	176	94.6	CREKA and 17- β E	33.80 \pm 2.45 (17- β E); NR (CREKA)	To improve 17- β E internalization in vascular cells	Human EC in vitro; IV administration to apoE ^{−/−} high-fat diet mice	Increased NO production by EC in vitro; decreased atherosclerotic plaque size in mice; improved plasma lipid profile in mice; reduced ICAM-1, VCAM-1, IL-6, TNF in mice atherosclerotic plaque	18

Note: *56% by weight.

Abbreviations: ALA, α -linolenic acid; CER, ceramide; CREKA, cysteine–arginine–glutamic acid–lysine–alanine peptide; 17- β E, 17 β -estradiol; EC, endothelial cells; EE, encapsulation efficiency; IV, intravenous; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NR, not reported; PUFA, polyunsaturated fatty acid; VSMC, vascular smooth muscle cells; TNF, tumor necrosis factor.

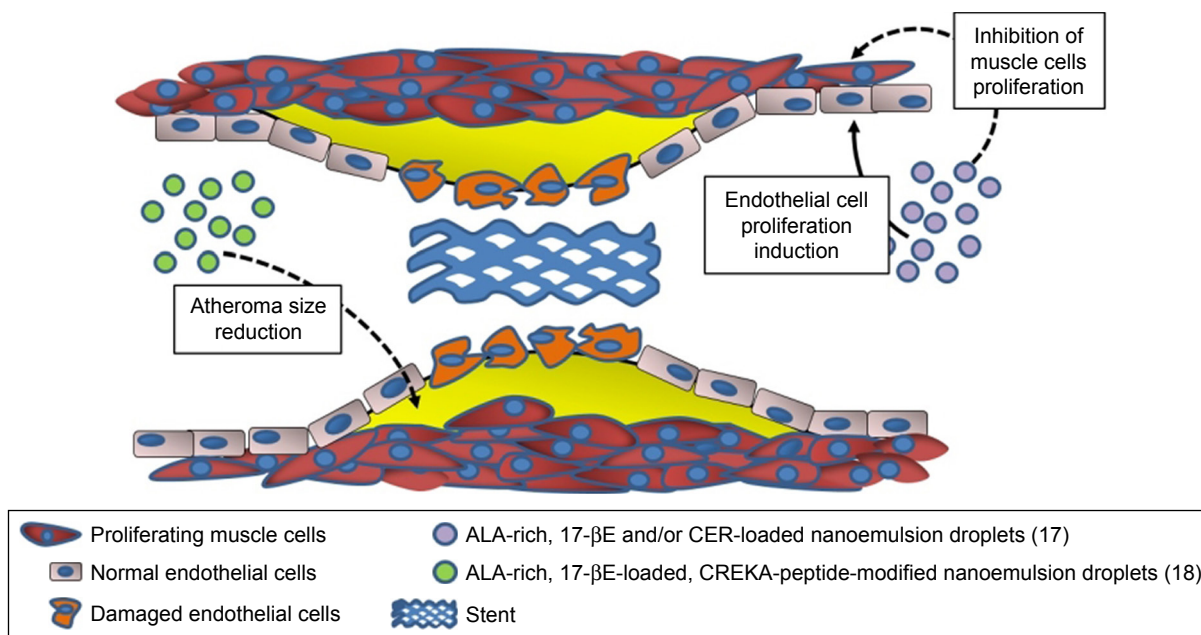


Figure 1 Potential use of ALA-containing nanoemulsions against the development of restenosis and atheroma.

Note: The biological activity of nanoemulsions were evaluated *in vitro* or *in vivo*.

Abbreviations: ALA, α -linolenic acid; 17- β E, 17 β -estradiol; CER, C6-ceramide; CREKA, cysteine–arginine–glutamic acid–lysine–alanine.

The study was based on the recent observations demonstrating that ω -3 PUFAs and some of their bioactive metabolic derivatives (ie, the specialized proresolving mediators, including resolvins, protectins, and maresins) are crucial endogenous signals to maintain vascular homeostasis, either by exerting anti-inflammatory properties,¹⁹ or by modulating the resolving phase of vascular injury and accelerating repair.²⁰

In particular, ω -3 PUFAs and their derivatives have been considered potential anti-restenosis agents, and Deshpande et al¹⁷ included an ALA-rich oil (flaxseed oil) in an oil-in-water nanoemulsion system they engineered for 17 β -estradiol (17- β E) and C6-ceramide (CER) (Table 1; Figure 1). This combination appeared remarkably encouraging, since ALA has been largely reported to improve CV health, and both 17- β E and CER were previously demonstrated to be cardio-protective and vasoactive.^{21,22} However, it has been reported that their effect *in vivo* may be markedly reduced by a scarce release due to their high hydrophobicity and capability to bind with circulating proteins.^{23,24} In addition to the observation that the two cell systems related to restenosis (human aortic endothelial cells [ECs] and vascular smooth muscle cells [VSMCs]) showed a high *in vitro* uptake of 17- β E and CER, the most remarkable finding was a greater biological activity of the nanoemulsion compared to each single component administered alone (ie, increased or decreased platelet-derived growth factor-induced proliferation in EC and VSMC, respectively).¹⁷ The authors explained the results on the basis of the

more efficient delivery of 17- β E and CER to vascular cells when they were encapsulated in ALA-based nanodroplets. For the muscular cells, they associated the findings with the increased inhibitory effect exerted by encapsulated 17- β E on the p38 MAP-kinase activity, known to be involved in VSMC proliferation. In a more recent work by the group,¹⁸ the same flaxseed oil-based nanoemulsion encapsulating 17- β E was used with the clot-binding peptide cysteine–arginine–glutamic acid–lysine–alanine, for its ability to selectively bind to the atherosclerotic plaque (Table 1; Figure 1). This strategy was aimed to specifically target atherosclerotic lesions and, thus, better counteract their development and progression. The nanoemulsion was administered either to EC cultured *in vitro* or, intravenously, to a mouse model of atherosclerosis (apoE^{-/-} mice fed a high-fat diet). The nanoemulsion components 17- β E and ALA were considered responsible for the increased production of nitric oxide by the EC cultured *in vitro*. They also minimally reduced the levels of plasma cholesterol and decreased the atherosclerotic plaque size, as well as the expression of atherosclerosis-related markers (intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), IL-6, tumor necrosis factor [TNF], etc.) within the aorta of the nanoemulsion-treated apoE^{-/-} mice. The apoE^{-/-} mice showed significantly increased levels of both triglycerides and cholesterol after a 10-week high-fat diet administration. Interestingly, the treatment with 17- β E and ALA containing nanoemulsion

was able to slightly decrease, yet significantly, the level of plasma cholesterol which, conversely, remained unchanged when 17- β E was administered alone as a solution. The authors suggested that the marginal change in cholesterol induced by 17- β E and ALA nanoemulsion could partially contribute to the protective anti-atherosclerotic effects of the nanoparticles. However, they were induced to hypothesize that it was not responsible for the lesion-size reduction triggered by these 17- β E/ALA nanoformulations, since previous observations in the same mouse model of atherosclerosis had shown that the levels of circulating cholesterol were not related to the size and area of the atherosclerotic lesion.²⁵ Probably, the endothelial modifications induced by the nanoemulsion could be responsible for the size reductions of the atherosclerotic lesions.

It is worth pointing out that a recent meta-analysis did not detect any result of increased dietary intake of ALA in the blood concentrations of the same inflammation markers (soluble ICAM and VCAM, IL-6, and TNF).²⁶ This suggests that a more efficient delivery of ALA – such as the one demonstrated in the apoE^{-/-} mouse model – could allow this fatty acid to reach vascular walls at adequate amounts to induce CV health effects, particularly if the delivery occurs in combination with other cardioprotective compounds (such as 17- β E).

ω -3 PUFA-containing nanoformulations for cancer prevention and therapy

Developed ω -3 PUFA-containing nanoformulations for liver cancer prevention and therapy

Most of the studies on new nanotechnology-based strategies for a more efficient delivery of ω -3 PUFAs to cancer cells in vitro and in vivo have been conducted on liver cells. A relevant approach was used by the Corbin group.^{12,27–29} They created low-density lipoprotein (LDL)-based nanoparticles with DHA as bioactive cargo (Table 2; Figure 2A), and tested them on a normal mouse liver cell line, as well as on mice,^{12,28} rats,²⁹ or human liver cancer cell lines.²⁷ Interestingly, this DHA nanoformulation was successfully employed by the same group of authors also for the delivery of DHA to the brain.³⁰ In this case, they used the systemic administration of DHA-LDL nanoparticles combined with pulsed focused ultrasound exposures. In the LDL nanoparticles, DHA was placed inside the hydrophobic core, where its stability and biological activity can be preserved. The strategy of using

LDLs as ω -3 PUFA transporters to liver cells has several advantages, since LDLs have a great capacity of carrying active substances, and they are naturally present in circulation, so that they are not recognized and internalized by mononuclear circulating phagocytes.³¹ Moreover, LDLs carry and supply cells with lipids also in physiological conditions via LDL receptor-mediated endocytosis and seem particularly suitable to deliver bioactive lipid factors to cancer cells, which show higher avidity for lipids than normal cells, due to their sustained membrane turnover.^{32–34} Indeed, one particularly interesting feature of these engineered nanoparticles was their ability to be selectively cytotoxic to hepatocarcinoma cells, over the nonmalignant counterparts.^{12,28} This specificity for cancer cells is highly advantageous for antineoplastic agents, and, presumably, it was related to the fact that high concentrations of the cell growth inhibitor DHA were achieved only inside the cancer cells. Particularly, the selective growth-inhibitory effect of the LDL-DHA nanoparticles in liver cancer cells was explained on the basis of the specific and powerful anti-inflammatory and pro-resolving properties of the ω -3 PUFAs and their bioactive derivatives. Moreover, it was also related to increased levels of reactive oxygen species (ROS) and iron found in cancer cells, as well as to the reported reduced glutathione (GSH) cellular depletion.^{35,36} This pro-oxidative condition of cancer cells may easily induce peroxidation of a highly unsaturated compound, such as DHA.³⁷ In turn, the peroxidation of intracellular membranes is triggered, and that may lead to lysosomal, mitochondrial, and nuclear disruption, and, lastly, cell death.²⁸ Of great significance, a single intra-arterial injection of LDL-DHA in rats bearing transplanted orthotopic hepatocarcinoma markedly reduced tumor growth by altering redox balance and inducing tumor cell death.²⁹ The most recent application of LDL-DHA particles by these authors convinced them to formulate the stimulating hypothesis that DHA enclosed in these particles could induce a recently described regulated type of necrotic cell death called ferroptosis in liver cancer cells.²⁷ This non-apoptotic iron-dependent form of cell death³⁸ is characterized by the simultaneous intracellular occurrence of increased lipid peroxidation, high levels of intracellular ROS originating from iron metabolism, and glutathione depletion. Moreover, it is critically and negatively regulated by the lipid antioxidant enzyme glutathione peroxidase-4 (GPX-4).²⁷ In line with this, LDL-DHA also substantially reduced the expression and activity of GPX-4 in liver cancer cells, along with causing all the pro-oxidative changes typical of ferroptosis.²⁷ Since it is usually reported that DHA – given as a free fatty acid – mainly causes apoptotic death

Table 2 Application of ω -3 PUFA nanoformulations in liver cancer

Nanoparticle (NP) type	Chemical form of ω -3 PUFA used	NP size (nm)	EE (%)	Cargo molecule	Zeta potential (-mV)	Function of the NP	Experimental model	Mechanisms involved in the NP effects	Reference
LDL-based nanoparticles	Unesterified DHA incorporated in LDL	18.3	13	DHA	22	To enhance physical, oxidative stability, and delivery of DHA to target cells	Normal TIB-73 and neoplastic TIB-75 murine hepatocytes in vitro	Enhanced oxidative and physical stability of LDL-based nanoparticles over free DHA; selective cytotoxicity toward cancer cells	12
LDL-based nanoparticles	Unesterified DHA incorporated in LDL	18.3	13	DHA	Not reported	To enhance physical, oxidative stability, and delivery of DHA to target cells	Rat and human HCC cell lines in vitro; mice transplanted with human HCC cells	Enhanced tumor cell death through ferroptosis; reduced tumor volumes in transplanted mice	27
LDL-based nanoparticles	Unesterified DHA incorporated in LDL	18.3	13	DHA	24 \pm 2.6	To enhance physical, oxidative stability, and delivery of DHA to target cells	Normal TIB-73 and neoplastic TIB-75 murine hepatocytes in vitro	Selective cytotoxicity toward cancer cells through an impairment of lysosomal, mitochondrial, and nuclear function	28
LDL-based nanoparticles	Unesterified DHA incorporated in LDL	18.3	13	DHA	25	To enhance physical, oxidative stability, and delivery of DHA to target cells	H4IIE rat hepatoma cells IV injected in rats	Reduced tumor volumes; tumor-specific necrosis induced by selective redox balance alteration within cancer cells	29
Tuftsins-tagged liposomes	ALA esterified to 2,6-di-isopropylphenol (propofol)	100–130	74	2,6-propofol ALA conjugate	43.23 \pm 1.4	To enhance the delivery and anticancer activity of ALA in target cells	Mice subject to DEN-induced hepatocarcinoma	Increased mice survival; reduced expression of COX-2 and Bcl-2; increased expression of Bax in hepatocarcinomas	41

Abbreviations: ALA, α -linolenic acid; DEN, diethylnitrosamine; DHA, docosahexaenoic acid; EE, encapsulation efficiency; HCC, hepatocellular carcinoma; IV, intravenous; LDL, low-density lipoproteins; PUFA, polyunsaturated fatty acid.

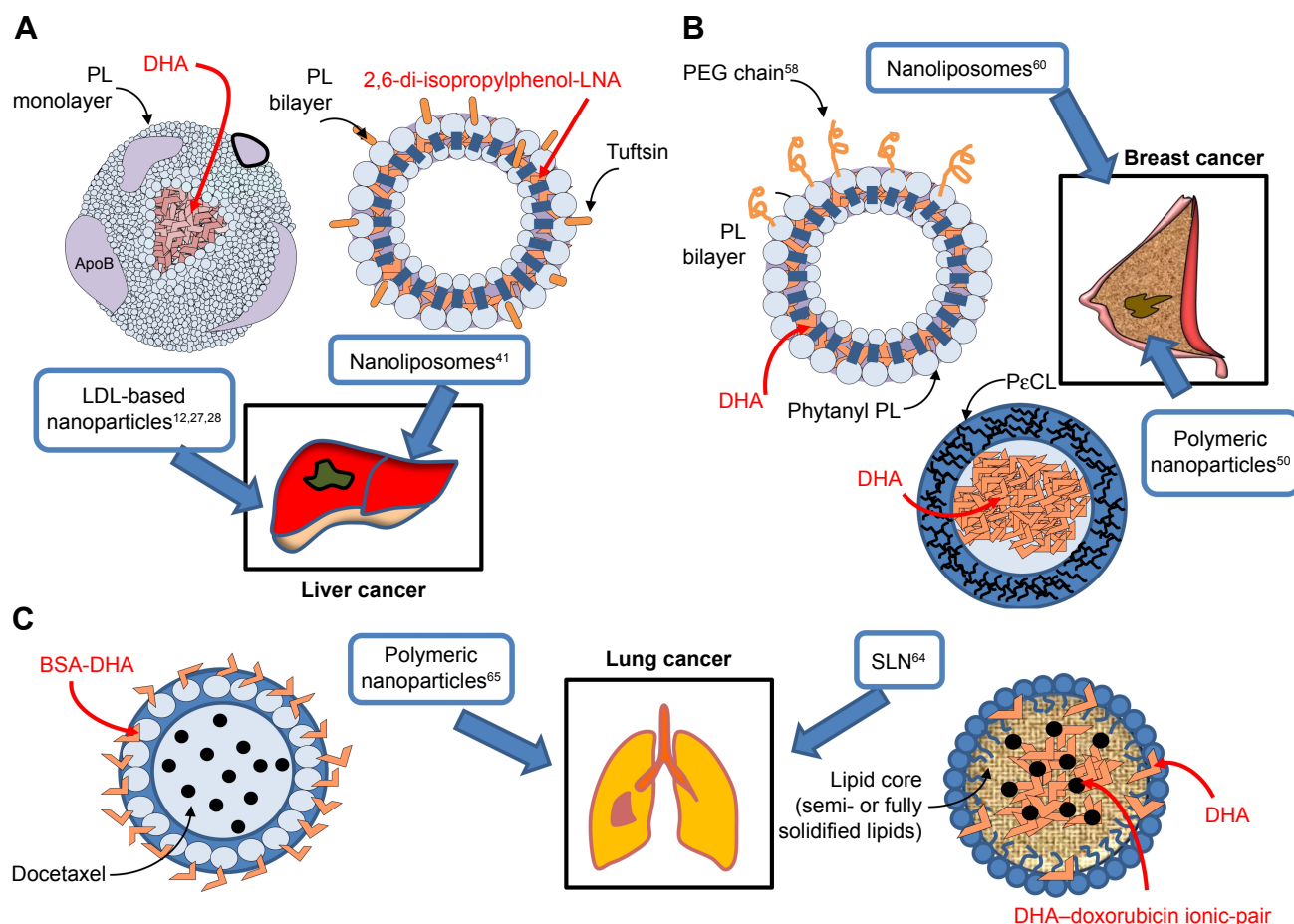


Figure 2 Omega-3 PUFA-containing nanomaterials developed and evaluated in vitro or/and in vivo for their potential use against liver (A), breast (B) and lung (C) cancer. **Abbreviations:** DHA, docosahexaenoic acid; LDL, low-density lipoprotein; LNA, linolenic acid; PEG, polyethylene glycol; PL, phospholipids; PεCL, poly-ε-caprolactone; PUFA, polyunsaturated fatty acid; SLN, solid lipid nanoparticle.

in cancer cells,³⁹ the finding that LDL-DHA, on the other hand, triggers ferroptosis would imply that, depending on the delivery system used, ω-3 PUFAs may induce different death pathway inside the cells (ie, apoptosis or ferroptosis). The authors associated this effect to the fact that – while free DHA enters the cells by diffusion or facilitated transport⁴⁰ – LDL-DHA nanoparticles are endocytosed in large amounts. This different entrance pathway into the cells could enable DHA to easily reach more internal subcellular structures and, thus, exert considerable effects on them, thus contributing to the ferroptotic pathway of cytotoxicity.²⁷

Another interesting approach for the nanodelivery of ω-3 PUFAs was recently used to deliver ALA to diethylnitrosamine-induced hepatocarcinomas in mice.⁴¹ In this case, ALA was conjugated with 2,6-di-isopropylphenol, an anesthetic agent being structurally similar to vitamin E. The chemical structure of this conjugate was reported in Khan et al.⁴² This compound, conjugated with DHA, was previously shown to possess antioxidant properties and the

ability to induce apoptosis and decrease the metastatic potential of human cancer cells.⁴³ For an efficient delivery, the 2,6-di-isopropylphenol-linolenic acid conjugate was enclosed in nanoliposomes tagged with the tetrapeptide tuftsin (Thr-Lys-Pro-Arg),⁴⁴ a fraction of the immunoglobulin G molecule (Table 2; Figure 2A). The use of tuftsin-tagged liposomes was previously and successfully used to deliver antibiotics in the area of infections,⁴⁵ due to the immunomodulatory properties of tuftsin.⁴⁴ It is worth highlighting that tuftsin was also previously administered in combination with antineoplastic drugs and shown to enhance their antitumor activity.^{46,47} The treatment with tuftsin-tagged liposomes containing the ω-3 PUFA conjugate⁴¹ increased the survival of the hepatocarcinoma-bearing mice, and this effect was partly related to the markedly reduced expression of COX-2 and Bcl-2, as well as the upregulation of Bax observed in hepatocellular carcinomas. In our opinion, the co-presence of tuftsin and ω-3 PUFAs inside the same nanoliposomes is significant, since ω-3 PUFAs also show both immunomodulatory and antineoplastic activities

at the same time, and their possible applications in the field of cancer immunotherapy have been recently suggested.^{48,49} Moreover, due to the safe and non-toxic nature of both ω -3 PUFAs and tuftsin and their common immunomodulating properties, the application of this liposomal delivery could be potentially useful, not only for the treatment of hepatocarcinoma, but also for its prevention in populations at high risk (such as hepatitis C virus (HCV)-positive patients, alcoholics, or patients with inborn errors of metabolism, including type I glycogenosis or type I tyrosinemia).

Developed ω -3 PUFA-containing nanoformulations for breast cancer prevention and therapy

Roy et al⁵⁰ developed polymeric nanoparticles (150–200 nm) encapsulating DHA (Table 3; Figure 2B) with the aim of using them in breast cancer. The polymeric wall of the nanocapsules (NCs) consisted of poly- ϵ -caprolactone, a polyester able to protect the small liquid droplets of DHA included in the internal part (core) from chemical and enzymatic degradation, protecting DHA against the adverse conditions present in the gastrointestinal tract. This nanoencapsulation approach was previously used for the delivery of different drugs achieving positive results, such as enhanced drug efficacy and decreased drug toxicity.⁵¹ Roy et al⁵⁰ observed a clear cytotoxic effect (measured by the MTT assay) when they administered 50–100 μ M DHA in the form of free fatty acid for 4 days to MDA-MB-231 cultured in vitro, whereas lower concentrations showed no effect. This finding was in line with the results of several papers^{52–56} analyzed by us in a recent review,⁵⁷ showing that treatments of the triple-negative MDA-MB-231 or MDA-MB-453 breast cancer cells with EPA or/and DHA in the 30–100 μ M range for 1–5 days were necessary to inhibit cell growth and proliferation, and to enhance apoptosis or breast cancer cell sensitivity to docetaxel (DTX). Unexpectedly, however, Roy et al⁵⁰ found that, when lower concentrations of DHA (1–10 μ M) were enclosed in NCs, they induced the proliferation of MDA-MB-231 cancer cells. On the other hand, higher concentrations (30–50 μ M) of encapsulated DHA reduced breast cancer cell proliferation, similarly to what was obtained with free DHA. The simultaneous presence of vitamin E inside the NCs eliminated the growth inhibiting effect of DHA. Moreover, encapsulated DHA became more toxic than free DHA if H₂O₂ was added inside the NCs. Based on these results, the authors hypothesized that DHA should be oxidized and, thus, release secondary toxic products of lipid peroxidation which may be responsible for

Table 3 Application of ω -3 PUFA nanoformulations in breast cancer

Nanoparticle (NP) type	Chemical form of ω -3 PUFA used	NP size (nm)	EE (%)	Cargo molecule	Zeta potential (–mV)	Function of the NPs	Experimental model	Mechanisms involved in the NP effects	Reference
Polymeric P ϵ CL nanocapsules	DHA-free fatty acid	183	NR	DHA	29.7 \pm 0.7	To protect DHA from oxidation; to provide a delivery system to be used orally (through a higher resistance to adverse environmental conditions of GI tract)	Human breast epithelial MCF-10A and tumor MDA-MB-231 cancer cells in vitro	Inhibition of cell proliferation (MTT assay) (only in the presence of H ₂ O ₂ within the nanoparticle)	50
PEGylated liposomes	DHA-free fatty acid	99	81.4	DHA	15.7 \pm 2.5	To enhance cell permeability and retention and facilitate local delivery of DHA	Murine 4T1 breast cancer cells in vitro; murine RAW264.7 and human THP-1 monocytes in vitro	Inhibition of cancer cell proliferation (BrdU assay); decreased inflammation (reduced MCP-1 and TNF- α production by monocytes)*	58
Phytanyl lipid-based liposomes	DHA-sodium salt	130–160 (depending on pH values)	60–80	DHA	1.6	To enhance DHA chemical and oxidative stability	Human MDA-MB-231 and MCF-7 breast cancer cells in vitro	Reduction of cell viability (MTT assay); increased apoptosis; reduced p-Akt expression [#]	60

Notes: *Compared to unloaded nanoparticles or free ω -3 PUFA. [#]Compared to free DHA.

Abbreviations: DHA, docosahexaenoic acid; EE, encapsulation efficiency; GI, gastrointestinal; NR, not reported; PEGylated, polyethyleneglycolated; P ϵ CL, poly- ϵ -caprolactone; PUFA, polyunsaturated fatty acid; TNF, tumor necrosis factor.

its antiproliferative effects. However, the authors suggested that H_2O_2 could have also acted by disrupting the nanoparticle wall, releasing DHA outside the nanoparticles and, therefore, increasing its biological activity. Otherwise, due to its hydrophobic nature, this fatty acid tends to remain bound to the oily core of these nanoparticles and lose its bioactivity. Overall, however, the delivery of DHA to scarcely differentiated breast cancer cells through this kind of polymeric nanoparticles does not appear promising for breast cancer therapy, as the antineoplastic activity of this fatty acid appears to decrease when it is encapsulated at high concentrations. Moreover, when it is encapsulated at lower concentrations, it seems to even promote breast cancer cell growth.⁵⁰

On the other hand, Alaarg et al⁵⁸ enclosed DHA into liposomes by using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, cholesterol, and distearoyl phosphatidylethanolamine (PE); and polyethylene glycol (PEG) and these liposomes were used for the delivery of DHA to murine breast cancer cells in vitro (Table 3; Figure 2B). PEGylated liposomes were chosen for their acknowledged ability to stay in the circulation for a longer period (about 1 day half-life) compared to non-PEGylated particles that are cleared from the circulation in ≤ 1 hour.⁵⁹ This implies that a bioactive compound (in this case, DHA) enclosed in these nanoparticles can have more chances to be taken up by cells and tissues. DHA-loaded liposomes were able to inhibit the proliferation of murine 4T1 breast cancer cells, and other kinds of epithelial cells. However, it is worth underlining that, in this study, the effects induced by the DHA-loaded liposomes were compared only to those induced by the unloaded liposomes. For a clearer understanding, however, it would have been essential also to evaluate the effects of DHA administered in its free fatty acid form.

Skibinski et al⁶⁰ evaluated the improvement in the bioactivity of DHA included in a different kind of liposomes administered to MCF-7 and MDA-MB-231 breast cancer cells in vitro. Their DHA-loaded liposomes contained 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine (Table 3; Figure 2B), phytanyl lipids similar to those present in Archaea prokaryotes and known to be highly resistant to pH and temperature changes. The authors observed that DHA loaded into the liposomes reduced more the viability of breast cancer cells and induced apoptosis more efficiently in both the cell lines. Inside the liposomes, only DHA was responsible for these effects, since the empty phytanyl lipid liposome did not significantly alter cell viability and apoptosis. Moreover, the authors found that DHA enclosed in the

liposomes could more effectively decrease the expression of the activated form of Akt (p-Akt). They hypothesized that this effect could explain the increased antineoplastic activity showed by DHA loaded in the liposomes. This hypothesis is particularly interesting, since p-Akt represents a biomarker associated with poor prognosis in breast cancer, and used to evaluate the activity of the tyrosine kinase receptor signaling, often deregulated in breast cancer carcinogenesis.^{57,61}

A possible future ω -3 PUFA-containing nano-based approach for breast cancer may be suggested on the basis of the above analyzed remarkable results, obtained by both Alaarg et al⁵⁸ and Skibinski et al⁶⁰ with DHA-loaded nanoliposomes. Since ω -3 PUFAs are known to potentially act as an adjuvant therapy to strengthen the activity of conventional chemotherapeutics or targeted drugs in the therapy of breast cancer,^{57,62} it would be remarkable to develop nanoliposomal structures that are able to deliver simultaneously both ω -3 PUFAs and drugs with previously acknowledged activity in breast cancer. This liposomal combined approach could also be experimented in the clinical setting, since some liposomes are known to be safe and have already been clinically approved. Moreover, liposomes are able to encapsulate both hydrophobic and hydrophilic compounds and show a high capacity, making them particularly suitable to transport combinations of antineoplastic agents with different activities (conventional, targeted, or immunoactive agents),⁶³ including also safe nutritional factors with multiple anticancer functions, such as ω -3 PUFAs.

Developed ω -3 PUFA-containing nanoformulations for lung cancer prevention and therapy

To date, only two studies^{64,65} have evaluated the anticancer properties of ω -3 PUFA-containing nanoformulations on lung cancer cells (Table 4; Figure 2C), and their main aim was to improve the delivery and antitumor efficacy of doxorubicin (DOX) and DTX, two chemotherapeutic agents widely used in the therapy of solid tumors. A variety of nanotechnological approaches have been investigated so far to improve the limited uptake and distribution of DOX in solid tumors.^{66,67} Indeed, DOX is a weakly basic compound that becomes protonated when exposed to the slightly acidic pH of the microenvironment surrounding cancer cells, making its cellular uptake difficult.⁶⁶ Liposomes, solid lipid nanoparticles (SLNs), and polymeric micelles are among the different types of DOX nanocarriers designed to overcome this problem. A PEGylated liposomal formulation containing DOX (Doxil) has also been approved for the

Table 4 Application of ω -3 PUFA nanoformulations in lung and prostate cancer

Nanoparticle (NP) type	Chemical form of ω -3 PUFA used	NP size (nm)	EE (%)	Cargo molecule	Zeta potential (-mV)	Function of the NP	Experimental model	Mechanisms involved in the NP effects	Reference
SLN	DHA-free fatty acid	94 (0.4% DHA)	99 (0.4% DHA)	DOX \pm DHA	34 \pm 0.2 (0.4% DHA)	To reduce SLN size; to induce negative charges in SLN; to enhance DOX EE, release in medium and solubility in lipids	Human A549 lung adenoCa cells in vitro	Enhanced DOX cytotoxicity [§] (TB exclusion assay)	64
DHA-BSA polymeric nanoparticles	DHA-free fatty acid	146.9 \pm 22.9	98.6 \pm 9.3	DTX	31.5 \pm 1.5	To specifically target cancer cells	Murine LLC cells and human A549 adenoCa in vitro; murine LLC cells transplanted in C57BL/6 mice	[§] In vitro: enhanced cancer cell targeting; enhanced cell uptake efficiency; enhanced DTX antiproliferative effect (MTT assay); decreased cancer cell migration. [@] In vivo: decreased lung cancer metastases to bone; increased mice survival	65
Oil-in-water nanoemulsion	DHA-SBT-1214 conjugate	228 \pm 7	97	DHA-SBT-1214	24.9 \pm 4.3	To enhance permeability and retention of SBT-1214 in cancer cells	Human CSC-enriched PPT2 prostate cancer cells in vitro and transplanted in NOD/SCID mice	Enhanced drug cytotoxicity in vitro [*] ; enhanced tumor growth suppression in vivo [*]	87
Oil-in-water nanoemulsion	DHA-SBT-1214 conjugate	213.2	97	DHA-SBT-1214	28.9 \pm 4.3	To improve the targeted delivery of SBT-1214 to cancer cells	Human CSC-enriched PPT2 prostate cancer cells transplanted in NOD/SCID mice	Slower in vitro drug release; improved pharmacokinetic properties in plasma and tissues; enhanced stability [#]	88

Notes: [§]Compared to free DHA + DOX and to empty SLN. ^{*}Compared to free DTX or to DTX-BSA nanoparticles. [@]Compared to DTX-BSA nanoparticles. ^{*}Compared to both Abraxane[®] and placebo nanoemulsion formulation. [#]Compared to aqueous solution.

Abbreviations: EE, encapsulation efficiency; LLC, Lewis lung carcinoma; NOD, nonobese diabetic; SCID, severe combined immunodeficiency; SLN, solid lipid nanoparticles; adenoCa, adenocarcinoma; CSC, cancer stem cells; DHA, docosahexaenoic acid; DOX, doxorubicin; DTX, docetaxel; PUFA, polyunsaturated fatty acid.

treatment of some cancers (ovarian cancer, AIDS-related Kaposi sarcoma, and multiple myeloma).⁶⁸ Mussi et al⁶⁴ designed DOX-loaded SLN for lung cancer, where the oily phase included DHA and surfactants, such as Compritol, Tween 80, and triethanolamine (Table 4; Figure 2C). The SLN formulation allows a site-specific release of DOX in a slightly acidic environment, such as tumor tissues.^{66,69} Moreover, the SLN solid matrix allows a more controlled release, and the lipophilicity of these nanoparticles improves the interaction with the cellular membranes. Furthermore, the SLNs obtained by encapsulating 0.4% DHA (w/v) were of smaller size (94 ± 1 nm), which is advantageous, being the nanoparticles in the range of 100–200 nm taken up and retained at a higher concentration inside cancer cells compared to particles with a higher size.^{70–72} The simultaneous encapsulation of DOX with a lipophilic anion, such as DHA in the SLN, was aimed to overcome the low level of incorporation in lipidic nanostructures that DOX usually exhibits, due to its cationic amphiphilic nature. Moreover, the SLN exhibited negative charges due to the presence of DHA, and, inside the SLN, DOX formed an ion-pairing with DHA that could explain the high DOX encapsulation efficiency (almost 100%), as well as the enhanced cytotoxicity shown by the DHA-DOX-loaded SLN against A549 lung adenocarcinoma cells, as compared to free DOX and DHA administered in combination.

The second study was performed by Zu et al,⁶⁵ who evaluated the anticancer activity of ω -3 PUFA-containing polymeric nanoparticles on lung cancer cells. The nanoparticle surface contained DHA covalently bound to BSA, a natural biodegradable polymer often used to prepare polymeric nanoformulations⁷³ (Table 4; Figure 2C). Also in this case, the size of the newly developed nanoparticle (141.7 ± 7.9 nm) was within the range of sizes allowing a more efficient uptake by cancer cells.^{70–72} The DHA-BSA nanoparticles were designed to enclose DTX, an approved semisynthetic antineoplastic drug belonging to the taxane family and, at present, largely used for the treatment of a variety of solid cancers, including lung and breast cancer.^{74,75} Its inclusion in the nanoparticle was aimed to overcome the scarce aqueous solubility of the drug. Numerous formulations of taxanes have been developed over the last decades in order to increase the aqueous solubility of the hydrophobic taxanes, such as cyclodextrin, liposomes, and albumin-bound nanoparticles (Abraxane) formulation.^{73,76} In the nanoformulation analyzed,⁶⁵ the addition of DHA to the albumin on the surface of the DTX-BSA nanoparticle was aimed to specifically target cancer cells, since it was previously observed that DHA preferentially accumulates in tumor cells

and is considered a target ligand for cancer cells.^{77–79} This tumor targeting ability of DHA has been typically associated with the increased fluidity shown by cancer cell membranes which contain high levels of this fatty acid.^{77–79} In turn, the enhanced fluidity was related to their higher content in phospholipids, and, particularly, in PE, which was found to have a specific affinity for DHA.⁷⁷ Above all, targeting lung cancer cells with these nanoparticles seems mainly appropriate since PE has been found to be overexpressed not only in the cancer cells used in this study (murine Lewis Lung Carcinoma [LLC] cells)⁷⁷ but also, more in general, in the human non-small-cell lung cancers.⁸⁰ Furthermore, the addition of an antineoplastic natural agent, such as DHA, to polymeric DTX-BSA nanoparticle⁶⁵ was also related to the results of several human studies demonstrating that the therapeutic efficacy of this taxane can be increased after its conjugation to DHA, which is also able to decrease the side effects usually induced by taxane therapy, and improve the taxane delivery to the tumor tissues.^{81–86} Only the choice of BSA to prepare these polymeric nanoformulations could be controversial, since BSA was reported to antagonize the effect of DHA.¹³ The new DTX-DHA-BSA nanoparticles were administered to lung cancer cells cultured *in vitro* and to mice injected with syngeneic LLC cells in the tibia of the right limb, which mimicked lung cancer metastasis in the bone.⁶⁵ The finding demonstrated that the simultaneous presence of DHA in the nanoparticles enhanced the anticancer activity of taxanes both *in vitro* and *in vivo*, and also increased the mean survival time of the mouse.

Developed ω -3 PUFA-containing nanoformulations for prostate cancer prevention and therapy

Similarly to the results of the work by Zu et al,⁶⁵ where nanoformulations containing DHA bound to a taxane were tested in lung cancer, two studies performed on prostate cancer used an oil-in-water nanoemulsion formulation,^{87,88} where the starting oil phase consisted of fish oil with a novel DHA-taxoid conjugate (DHA-SBT-1214) (Table 4; Figure 3A). The oil phase was mixed with the water phase formed by egg phosphatidylcholine (Lipoid E80[®]), polysorbate 80 (Tween80[®]), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino[polyethylene glycol]-2000) (DSPE-PEG2000). Both the empty nanoemulsion (not containing drugs) and the nanoemulsion containing DHA-SBT-1214 showed spherical shape and a very similar particle size (225 ± 7 and 228 ± 7 nm, respectively). The surface charge of the oil droplets in the two formulations did not change even using the maximum encapsulation of DHA-SBT-1214 within

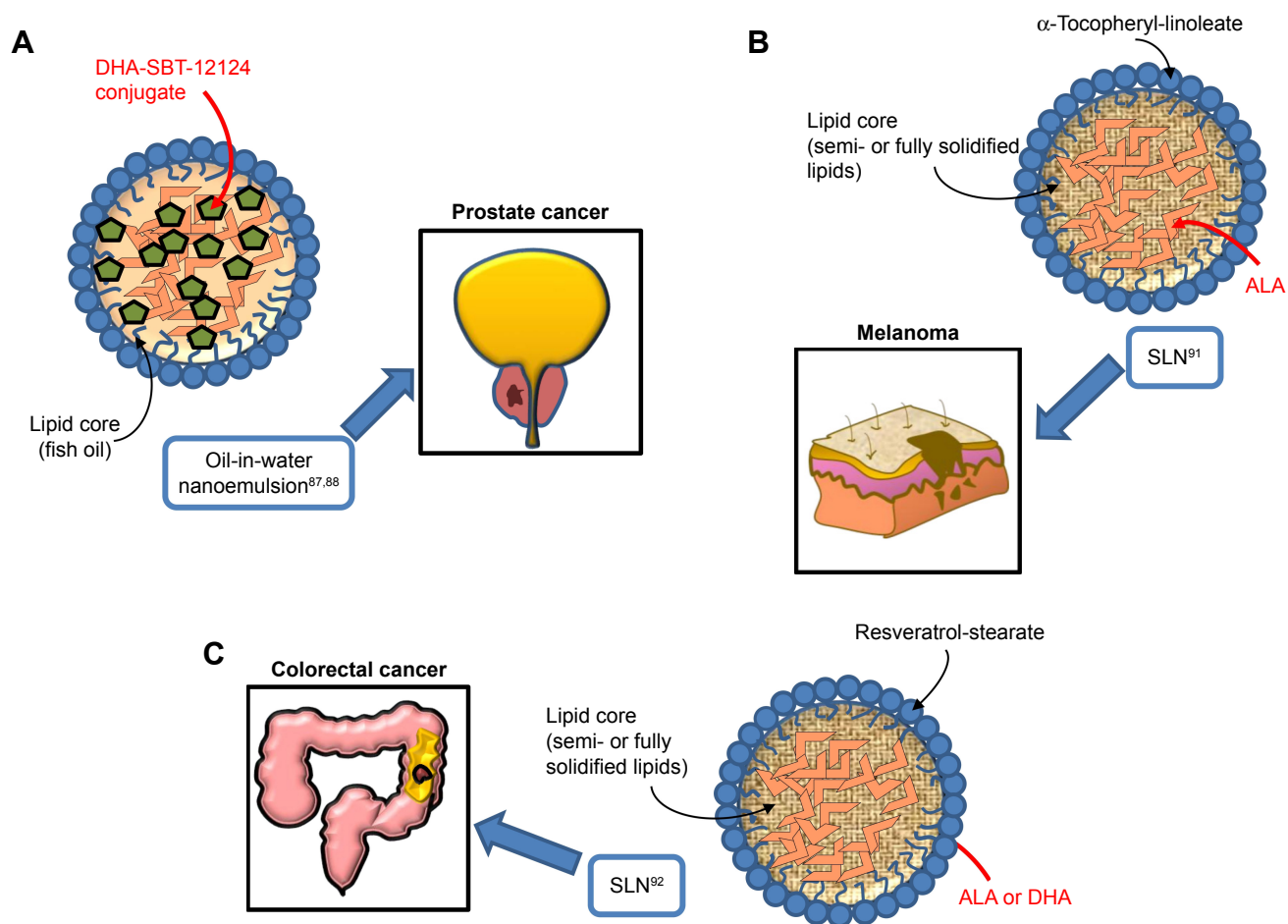


Figure 3 Omega-3 PUFA-containing nanomaterials developed and evaluated in vitro or/and in vivo for their potential use against prostate (A), melanoma (B), and colorectal cancer (C).

Abbreviations: PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; SLN, solid lipid nanoparticle; ALA, α -linolenic acid.

the oil droplets. The two studies are remarkable, since they benefit from a new-generation taxoid (SBT-1214), bound to DHA, and avoid the use of albumin, which can reduce the anticancer effect of DHA.¹³ In particular, the use of SBT-1214 was aimed to overcome the mechanism through which paclitaxel⁸⁹ or DTX⁹⁰ was observed to induce drug resistance in prostate cancer (by mechanisms related to the induced expression of either P-glycoprotein or transforming growth factor- β superfamily components, respectively). On the other hand, the rationale of SBT-1214 binding to DHA was related to the above-mentioned ω -3 PUFA ability to exert cancer-specific toxicity, to the synergistic antineoplastic effects that these fatty acids exert when bound to anticancer drugs, and to their ability to protect non-transformed prostate cells from cytotoxicity and reduce the secondary systemic harmful effects of conventional anticancer drugs, including taxanes. Moreover, the use of the non-ionic emulsifying agent Tween 80 in the preparation of this nanoemulsion is relevant, as it allows to obtain optimal oil droplet size and charge by inhibiting aggregation through competitive

absorption to interfaces. This surfactant was also used for the preparation of other nanoemulsions containing ω -3 PUFAs and herein analyzed.^{64,91,92} The newly designed nanoemulsion of DHA-SBT-1214 (NE-DHA-SBT-1214) was tested for its antineoplastic activity against human prostatic cancer PPT2 cells cultured in vitro, or transplanted subcutaneously in NOD/SCID mice.^{87,88} These prostate cancer cells have the features of stem-like cells and are considered a good model for testing new drugs for prostate cancer. This is a highly recurrent tumor, which has been related to the presence of high levels of cancer stem cells with the potential of self-renewing the tumor.^{93–95} The NE-DHA-SBT-1214 exerted higher cytotoxicity toward the PPT2 cells cultured in vitro, compared with DHA-SBT-1214 in solution. In addition, intravenous administrations of this nanoemulsion carrier system containing DHA-SBT-1214 in fish oil droplets to the transplanted mice markedly suppressed prostate cancer cell growth in vivo compared to a placebo nanoemulsion formulation previously approved for the treatment of metastatic human breast cancer (AbraxaneTM, ie, paclitaxel

albumin-bound particles). Moreover, the prostate cancer cells that still remained viable after the *in vivo* treatment with NE-DHA-SBT-1214 had reduced neoplastic potential, which was shown by their decreased ability to give rise to colonies and spheres when analyzed *ex vivo* for clonogenic and sphere-forming capacities. These results suggest that the use of NE-DHA-SBT-1214 for the treatment of prostate cancer could be a promising strategy.

Developed ω -3 PUFA-containing nanoformulations for melanoma and colorectal cancer (CRC) prevention and therapy

We have studied the antineoplastic potential of ω -3 PUFAs in CRC and melanoma for many years⁹⁶⁻¹⁰¹ and investigated the possible mechanisms underlying the beneficial action of these fatty acids. We have recently developed new SLNs for the delivery of ω -3 PUFAs to C32 melanoma cells⁹¹ and HT-29 and HCT116 CRC cells,⁹² to reduce possible ω -3 PUFA oxidative degradation of these compounds, and, thus, prolong their shelf-life, enhance their bioavailability, and improve their antineoplastic activity (Table 5; Figure 3B and C). In particular, the matrix of the SLN tested in melanoma cells contained ALA esterified to α -tocopherol and encapsulated ALA itself. ALA carried in these nanoformulations was always more cytotoxic than ALA or α -tocopherol administered alone to melanoma cells.⁹¹ On the other hand, the lipid matrix of the SLN formulation tested in CRC cells contained stearic acid esterified to the natural dietary antioxidant resveratrol (RV). Furthermore, these RV-based SLN (RV-SLN) carried either ALA or DHA inside.⁹² Compared with the other SLN tested on melanoma cells, the substitution of ALA in the matrix with stearic acid was aimed to further increase the hydrophobicity of the encapsulated ω -3 PUFAs, thus potentially improving the efficiency of their delivery. Even though stearic acid is a saturated fatty acid, it has been shown that it may lower serum LDL cholesterol levels,¹⁰² and also protect from the development of prostate cancer.^{103,104} In particular, this protective action appears remarkable, since it has been recently hypothesized that the incidence of prostate cancer may be associated with a high intake of ω -3 PUFAs. Thus, the combination stearic acid/ ω -3 PUFAs inside the SLN could neutralize possible prostate-specific adverse effects of these fatty acids.¹⁰⁵ Moreover, the encapsulation of both ALA and DHA makes them more efficient in inhibiting CRC cell growth, by enhancing their inhibitory effect on cell proliferation rather than further increasing their already high ability to induce apoptosis.⁹² These increased

Table 5 Application of ω -3 PUFA nanoformulations in melanoma and colon cancer

Nanoparticle (NP) type	Chemical form of ω -3 PUFA used	NP size (nm)	EE (%)	Cargo molecule	Zeta potential (-mV)	Function of the NP	Experimental model	Mechanisms involved in the NP effects	Reference
α -Tocopheryl linoleate-based SLN	ALA-free fatty acid	181	77	ALA	ND	To improve the delivery and stability of ALA	Human C32 melanoma cells <i>in vitro</i>	Enhanced antioxidant efficiency* [§] ; enhanced cytotoxicity [#]	91
Resveratrol-based SLN	ALA- or DHA-free fatty acids	842 (DHA-containing SLN); 1000 (ALA-containing SLN)	77 (ALA); 100 (DHA)	DHA or ALA	ND	To protect ω -3 PUFA from degradation and to increase their incorporation in cancer cells	Human HT29 and HCT116 colon cancer cells <i>in vitro</i>	Increased ω -3 PUFA incorporation in tumor cells; increased cancer cell growth inhibition; reduced cancer cell proliferation [§]	92

Notes: *Compared to the empty SLN. [#]Compared to free ALA. [§]Compared to free DHA and ALA.

Abbreviations: ALA, α -linolenic acid; DHA, docosahexaenoic acid; EE, encapsulation efficiency; ND, not determined; SLN, solid lipid nanoparticles; PUFA, polyunsaturated fatty acid.

antineoplastic effects were strictly related to the enhanced cellular incorporation observed when DHA and ALA were enclosed in the RV-SLN to CRC cells. It was remarkable to find that, only after the encapsulation, ALA induced an evident cellular increase in both EPA and DHA and that encapsulated DHA was more efficiently retroconverted to EPA. This implies that the encapsulation of either DHA or ALA into RV-based SLN could also markedly increase the CRC content of EPA, which, according to several recent reports, seems to protect against CRC risk better than all the other main ω -3 PUFAs.^{106–108} The more efficient conversion of SLN-encapsulated ALA is particularly interesting when considering that ALA is present at high levels in a variety of seeds and vegetables, and its increased intake could be achieved more easily and in a more sustainable way compared to an increased consumption of EPA itself.

In conclusion, it is important to underline that the possibility to further enhance the protective activity of these dietary compounds against CRC is appealing, since the possible prevention and treatment of CRC with PUFA has involved many researchers in the past two decades.^{96,109–112} In particular, possible successful future investigations in the field of CRC nanomedicine-based therapy could be related to the powerful modulator effect recently demonstrated for ω -3 PUFAs on the intestinal microbiota.^{107,113} Indeed, since the microbiome may deeply affect the individual response to cancer immunotherapy, it would be worth investigating the approach of combined nanodelivery of immunomodulating innovative drugs, already used for CRC^{114,115} with ω -3 PUFAs against CRC *in vivo*. Moreover, co-delivering powerful anti-inflammatory agents, such as ω -3 PUFAs, and these drugs in nanoparticles could also have the potential to overcome the severe colon immune-related adverse inflammatory events induced by these innovative treatments in CRC patients.¹¹⁶

Mixed and contrasting outcomes of recent human studies and the well-known benefits of ω -3 PUFAs in CVD and cancer

We cannot avoid focusing hereinafter on the topic outlined by the following question: are the contrasting outcomes of recent large human trials weakening the accepted dogma of ω -3 PUFA healthy effects? In our opinion, this topic deserves particular attention, particularly by the researchers who have been studying for decades the beneficial effects of ω -3 PUFAs in the prevention of CVD and cancer.^{1,39,57,62,96,98,117} Even though this issue cannot be tackled exhaustively in just one paragraph, it is essential to outline some considerations.

As far as the ω -3 PUFA cardioprotective role is concerned, the beneficial outcomes of early human studies (epidemiological studies, prospective cohort studies, and randomized controlled trials) appear in contrast with the mixed or less positive results of some recent prospective trials, and meta-analysis of clinical trials.¹¹⁷ Indeed, they provided scarce or no evidence that ω -3 PUFA may reduce CV events or mortality (for a review see Bowen et al¹¹⁸). It has been hypothesized¹¹⁷ that these discrepancies may be attributed to the difficulty of ω -3 PUFA to induce healthy effects in a completely changed scenario. In fact, in recent years, the enrolled patients have often been simultaneously subject to the administration of statins and/or other pharmacologic agents widely used to prevent CVD. Moreover, higher levels of ω -3 PUFA containing foods have been generally added to the diet following the numerous recommendations formulated over the last two decades. The results of the recently published VITAL study¹¹⁹ also obtained mixed and less promising results. It had the novelty of being a large, 5-year randomized, double-blind, placebo-controlled trial of a daily fish oil supplementation (1 g, Omacor[®] fish oil) for primary prevention in healthy subjects at baseline. On the contrary, the earlier trials mostly evaluated whether a supplementation with fish oil or purified ω -3 PUFAs could prevent CV adverse events in patients with a history of CVD or at high risk for CVD. Interestingly, the primary outcomes of the VITAL study indicated only a small but non-significant decrease in the rate of a major adverse CV event (ie, myocardial infarction [MI] or stroke) in the ω -3 PUFA group with respect to the placebo group. However, a secondary analysis in those patients that were specifically affected by stroke or MI revealed that the intervention was able to significantly reduce the risk of MI (by 28%) and the death rate of MI (by 50%). Interestingly, a much higher reduction in MI risk (by 77%) was observed in the African subpopulation, thus enforcing the notion that the ω -3 PUFA beneficial effects may be modified depending on the individual genetic background.¹²⁰ Remarkably, the need for angioplasty was also significantly reduced (by 22%) by the fish oil supplementation. The accuracy of this trial was also demonstrated by the evaluation of the basal plasma ω -3 index (EPA + DHA as a percentage of total fatty acids) of the subjects, which was evaluated also after 1-year treatment to show its increase (by about 50%). The outcomes of another recently published randomized double-blind, placebo-controlled trial (REDUCE-IT) reported more positive results. However, it evaluated the effects of simultaneous treatment with a very high dose (4 g/day) of purified EPA and statin therapy for

almost 5 years in patients with CVD or at high risk for CVD and high fasting triglyceride levels.¹²¹ The study showed that the EPA treatment reduced the levels of serum triglycerides, CV events, and CV death. In particular, the rates of emergency or urgent revascularizations or MI were reduced by the EPA supplementation, even though the high dose used slightly increased the rate of bleeding events (see next paragraph), as well as the onset of atrial arrhythmias. It can be underlined that many differences do exist between these two most recent and largest trials, which can however help us to better understand the actual potential of ω -3 PUFAs in preventing CVD. For instance, the use of fish oil at a quite low dose may lack in showing the induction of health CV effects. On the contrary, EPA administered alone at a very high dose, even though in the presence of a statin therapy, may elicit beneficial effects, increasing, however, also the risk of some detrimental properties. Overall, the debate on the ω -3 PUFA-induced CV benefits is ongoing, also considering the manifold data from *in vitro* studies and *in vivo* animal studies supporting a variety of different biological and molecular mechanisms whereby ω -3 PUFA may confer coronary protection and reduce the risk of CVD.¹²² Therefore, further large interventional trials comparing different doses and types of fatty acid (EPA and DHA) are needed for a better knowledge of this topic. Moreover, it would be more advantageous that the subjects/patients would be stratified according to the ω -3 index expressed as the content of EPA and DHA in red cell membranes, which is considered a more reliable and stable indicator of the real ω -3 PUFA intake than their plasma level.^{123,124} Moreover, it would be important to evaluate the ω -3 index several times along the long-term studies.

With regard to cancer, plenty of evidence obtained from *in vitro* and *in vivo* preclinical studies has clearly indicated the anticancer activity of omega-3 PUFA.¹²⁰ On the contrary, as we have widely discussed in a recent critical review of the literature,¹²⁰ the results of the human studies are more controversial. In particular, most of the recent results confirmed the beneficial effects of the preclinical studies. However, there are studies where no effect was observed, or the antineoplastic effects were confined only to specific subpopulations (obese subjects, with specific dietary habits or with a particular fatty acid pattern, or having a specific T-cell infiltration in the tumor microenvironment). Only a few reports found positive associations between the development/progression of cancer (mainly for prostate cancer), and the high level of ω -3 PUFA

introduced with the diet, present in blood, or accumulated in tissues. In our opinion, to clarify the issue, it would be essential to know the actual levels of ω -3 PUFA intake and the efficiency of their incorporation in the tissues of each subject along the study period. To this end, we recommended repeated evaluations of biomarkers reflecting the intake of ω -3 PUFAs, and in particular, the ω -3 index expressed as the content of EPA and DHA in red cell membranes.¹²⁰ Moreover, we suggested, whenever possible, not to overlook some characteristics of the subjects being studied, such as the race/genetic background, and the metabolic/nutritional status.

Interestingly, the outcomes of the more recent interventional VITAL study¹¹⁹ indicated that the daily supplementation with 0.8 g/day ω -3 EPA + DHA was unable to lower the incidence of different kinds of invasive cancer of any type, as well as that of colorectal, breast, and prostate cancer, or the incidence of death from cancer. These findings were consistent with the results of two recent meta-analyses of ω -3 PUFA trials of CVD that had shown either no association between the supplementations with ω -3 PUFA and the risk of cancer in general¹²⁵ or a slightly increased but not significant risk.¹²⁶ Again, however, attention should be focused on the doses supplemented and the consequent ω -3 PUFA tissue enrichment, that is, in our experience, the most related parameter to the antineoplastic activity of these fatty acids.¹²⁴ It is possible that the daily dose of about 1.0 g (generally recommended for the secondary prevention of CVD) may not be sufficient to induce any anticancer effect. In a recently published review,¹²⁴ we critically analyzed the doses of ω -3 PUFA that had been able to induce anticancer effects both in the animal studies and in the few available interventional human studies. We found that supplementation to humans with safe doses of EPA + DHA of \sim 2.0 g/day produced a plasma and tissue enrichment in these fatty acids of about 2–5 folds, which was associated with significant anticancer effects.^{127–129} Higher doses were not able to further increase the amount of EPA and DHA incorporated in tissues. In mice or rats the dietary doses of EPA + DHA that have clear antineoplastic effects were variable in relation to their body mass and surface. However, these doses always produced an increase of \sim 2–5 folds of these fatty acids in the plasma, as well as in the normal and neoplastic tissues. Altogether, these observations prompted us to suggest that a daily dose of about 2 g could be the most appropriate for future human studies evaluating the anticancer effects of ω -3 PUFAs.

Possible adverse effects of ω -3 PUFAs: are they real obstacles to their use?

Increased bleeding risk

It is not possible to completely overlook here the controversies regarding the possible adverse effects of high doses of ω -3 PUFAs. In particular, high intakes of ω -3 PUFAs have been suggested to exert adverse effects on bleeding risk, since, in an earlier study, the diet with high levels of ω -3 PUFAs of the Greenland Inuit population was associated with an increased bleeding time.¹³⁰ The finding that these fatty acids were able to displace the ω -6 PUFA arachidonic acid from cell membranes and decrease its metabolic conversion to the powerful pro-thrombotic platelet-aggregating factor thromboxane A₂, further substantiated this hypothesis.⁹⁶ Moreover, increased intakes of ω -3 PUFAs were also reported to be associated with reduced blood levels of several clotting factors such as factor V, prothrombin, and von Willebrand factor.¹³¹ However, a recent review of the literature¹³² examining this issue supported the safe consumption of EPA and DHA even in highly vulnerable and sensitive populations, such as patients having gastrointestinal cancer or hospitalized in Intensive Care Units and consuming fish oil-enriched medical nutrition. No increased risk of bleeding-related event was identified in the analyzed reports, even if the patients were simultaneously treated with platelet aggregation inhibitors. On the contrary, the results concurred to support the safety of either very high daily doses of EPA + DHA given for a short period (up to 10 g/day) or much lower doses consumed for 1 year. Results obtained more recently by the large VITAL study having a 5-year median follow-up confirmed these findings.¹¹⁹ In this study, healthy subjects supplemented with 1 g/day fish oil (providing 460 mg EPA + 380 mg DHA) did not show increased major bleeding episodes as compared to the subjects of the placebo group. The other recent large REDUCE-IT study¹²¹ was performed on patients already receiving statin therapy and showing high levels of serum triglycerides, patients with a previously diagnosed CVD or at high risk of developing CVD. They were supplemented for almost 5 years with a high daily dose (4 g) of purified EPA. This study reported a modestly but significantly higher overall rate of adverse events, but not fatal bleeding events in the EPA group (2.7% vs 2.1% in the placebo group). However, it was observed that there were no significant differences between the placebo and EPA groups in severe bleeding events, such as hemorrhagic

stroke, serious central nervous system bleeding and gastrointestinal bleeding. Moreover, CV patients are often subject to chronic antithrombotic therapies, and this factor was not considered in the study. In any case, the inclusion of ω -3 PUFA in nanoformulation and the consequent increased efficiency of their delivery and absorbance at specific sites may prevent the induction of adverse bleeding effects at the circulatory level.

Immunosuppression

Concern has also been expressed/raised over the possibility that ω -3 PUFAs could have the potential to suppress any possible basal immunoreactivity against cancer, and decrease the impact of cancer immunotherapy.¹³³ This alarming possibility is related to the widely acknowledged anti-inflammatory and immunosuppressive activities of ω -3 PUFAs, and it is clear that their inclusion in nanoformulations to increase their bioactivity could also intensify these effects. However, in the last decades, a great deal of information has been gathered supporting the anti-cancer activities of ω -3 PUFAs, which oppose the preconceived alarm of a possible ω -3 PUFA cancer-promoting activity.¹²⁰ Moreover, it should be underlined that, whereas acute inflammation is a response to antagonize infections, repair, and resolve tissue damage, chronic inflammation has been involved in the promotion of cancer, by inducing immunosuppression in tumor microenvironment.^{134,135} Per se, it is considered a hallmark of cancer. To this respect, ω -3 PUFAs reveal their multifaceted nature, on one hand, by inhibiting persistent inflammation, thus preventing the promotion of cancer, and by also exerting pro-inflammatory actions to inhibit the immunosuppression existing in the tumor environment.¹³⁶ In fact, recent findings have shown that one of the mechanisms through which ω -3 PUFAs may inhibit tumor development *in vivo* is the induction of a local antitumor immune response.^{137,138} In particular, Liang et al¹³⁷ demonstrated a decreased expression of markers of tumor-associated macrophages in the tumor microenvironment of immunocompetent mice bearing prostate cancer and fed a fish oil-enriched diet. They also observed a decreased expression of a chemokine (CCL-2) that specifically recruits monocytes and macrophages to inflammatory sites.¹³⁷ It was also hypothesized that more than the dietary intake of ω -3 PUFAs by themselves, an increased dietary ω -3 PUFA/ ω -6 PUFA ratio could maximally and beneficially influence the immune characteristics of the tumor microenvironment and reduce the risk of developing some forms of cancer.¹³⁹ This was demonstrated by Gevariya et al¹³⁸ that used mice

transplanted with syngeneic prostate transgenic cell line and supplemented with a high ω -3 PUFA/ ω -6 PUFA ratio diet (3.3). They found increased levels of cytokines associated with Th1 and Th2 immune response in the tumors growing in these mice compared to those of mice fed a diet with a much lower ω -3 PUFA/ ω -6 PUFA ratio (0.002). These findings are in line with recent *in vitro* studies showing that ω -3 PUFAs are able to stimulate the innate and the adaptive immune response, both in the humoral and in the cellular branch.^{140–142}

Finally, considering the immunosuppressive activity of ω -3 PUFAs, it could be argued that they may have the potential to inhibit the immune response to infections. Again, this detrimental effect could be augmented by their inclusion in nanoformulations aimed to increase their efficacy. On the contrary, however, according to recent relevant results comprehensively analyzed by Costantini et al,¹⁴³ dietary ω -3 PUFA can positively modulate the immune response to microbe infections. In particular, evidence has been provided that the strict relationship between ω -3 PUFAs, gut microbiota, and immune system may influence the activity of the host immune cells.¹⁴³ In particular, plenty of animal studies and some evidence in humans have demonstrated that dietary supplementations with ω -3 PUFAs are able to normalize the gut microbial imbalance.¹⁴³ This appears interesting, since the intestinal microbiota has been reported to exert a crucial role in the development of the systemic and gut immune response.^{144,145}

Conclusion

To date, only a limited number of recent studies performed *in vitro* or in animals have directly investigated the biological effects of newly developed ω -3 PUFA-containing nanomaterials in CVDs and cancer. To date, none of these nanoformulations have been tested in the clinical setting. The studies have almost univocally demonstrated that a more efficient delivery of ω -3 PUFAs through these newly developed nanosystems may markedly enhance the therapeutic potential of nutraceuticals and other co-delivered drugs toward inflammatory lesions and cancer. Different strategies have been used, and these fatty acids have generally been included in the newly developed systems because of their ability to induce healthy effects. However, in some cases, they were also used just as a component of the lipidic phase to obtain nanoparticles carrying different bioactive products. Moreover, their position in the external part of some nanoparticles was also thought to specifically target cancer cells, which are avid of lipids necessary for their high-level metabolism and

high proliferative turnover rate. Thus, through this expedient, such ω -3 PUFA-coated nanoparticles could be preferentially taken up by cancer cells.

Notably, the inclusion of these fatty acids – showing both anti-inflammatory and anti-neoplastic activities – in nanoformulations could be significant in making them more bioavailable and to better deliver them to cancer tissues. Therefore, ω -3 PUFAs could better inhibit cancer cell growth, but simultaneously decrease the adverse inflammatory effects induced by the currently used conventional and innovative antineoplastic agents and, also, reduce the cachexia associated with the advanced cancer stages.

It is worth observing that ALA becomes more bioactive when enclosed in some of the nanoformulations analyzed in this review. This happened since ALA in this form was more efficiently delivered to the target cells and converted into its more bioactive products (EPA and DHA). This implies that these ALA-containing nanoformulations could allow to easily enrich our tissues with EPA and DHA, which, unlike ALA, largely found in vegetables and nuts, are mainly obtained from fish that indeed are not considered a sustainable food source for the entire world population.

Altogether, the reports analyzed univocally suggest that multiple potential advantages could be obtained with the administration of nanoformulations containing ω -3 PUFAs for the prevention and cure of both CVDs and cancer. In particular, the inclusion in nanoparticles has been often designed to offer protection to these quite unstable compounds from oxidative insults and to increase and enhance their delivery to the sites of the pathological lesions, resulting in an increased efficacy. Moreover, the safety of ω -3 PUFAs at the doses generally used in human studies,¹²⁴ their inexpensiveness, and the results so far obtained made them optimal candidates to be enclosed in nanoparticles in combination with other pharmacological agents already used in the clinical setting. The potential of these therapeutic combinations is to deliver all the agents enclosed successfully, increase their activity, and make the therapy more effective.

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Disclosure

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