Review

OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS AS SENSITIZING AGENTS AND MULTIDRUG RESISTANCE REVERTANTS IN CANCER THERAPY

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Abstract: The efficacy of chemotherapy depends on sensitivity and intrinsic or acquired drug resistance of cancer cells. The n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) are considered chemosensitizing agents and revertants of multidrug resistance by pleiotropic mechanisms. The specific mechanisms are not fully understood, but nowadays, it is widely accepted that there are a complex network of mechanisms, including alteration in gene expression, modulation of cellular proliferation and differentiation, induction of apoptosis, generation of reactive oxygen species and lipid peroxidation. A crucial mechanism in the control of cell drug uptake and efflux is related to n-3 LCPUFA influence on membrane lipid composition. The incorporation of docosahexaenoic acid in the lipid rafts produces significant changes in their physical-chemical properties affecting content and functions of transmembrane proteins, such as growth factors, receptors and ATP-binding cassette transporters. Of note, n-3 LCPUFAs often impact on the lipid compositions more in chemoresistant cells than in chemosensitive cells, suggesting their adjuvant role in cancer treatment.

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1. Introduction

Epidemiological studies highlight the association between long chain n-3 polyunsaturated fatty acids (n-3 LCPUFAs) and reduction of different tumours such as breast [1], colon [2], prostate [3], liver [4] and pancreas [5], suggesting a sensitizing n-3 LCPUFA effect. Moreover, the n-3 PUFAs improve the efficacy of chemotherapy and radiation against cancer. For example, the efficacy of doxorubin [6], epirubicin [7], 5-fluorouracil [8], mitomycin C [9], arabinosylcytosine [10], tamoxifen [11], and irinotecan/CPT-11 [12], and of radiation therapy [13] has been shown to be enhanced by n-3 PUFA association.

Recently, the data of randomized controlled clinical trials involving eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation during cancer chemotherapy and radiotherapy have been summarized by de Aguiar Pastore Silva *et al.* [14]. Those studies demonstrate that the combinations between n-3 LCPUFAs and conventional chemotherapy is beneficial: fish oils induce weight maintenance or gain, and immunomodulation that reduces inflammation, even when associated with cellular immune system suppression caused by radiotherapy and chemotherapy.

The success of chemotherapy always depends on intrinsic or acquired drug resistance of cancer cells. In fact, tumours are able to modulate signalling pathways causing drug resistance [15]: different resistance mechanisms might operate such as increased drug efflux, mutations of the drug target, DNA damage repair and cell death evasion.

This review will resume the evidences that sustain n-3 LCPUFAs as useful chemosensitizing agents and efficient multidrug resistance revertants. Moreover, it will discuss the proposed mechanisms for these actions, highlighting the n-3 LCPUFA impact on membrane architecture and its consequences on drug uptake and protein activity.

2. Evidences of n-3 PUFA positive effects on chemosensitization.

In vivo and *in vitro* studies indicate that n-3 LCPUFAs enhance the sensitivity of cancer cells to chemotherapy [10,16,17,18].

As early as 1979, Burns *et al*. [19] observed that in L1210 leukemia cells, n-3 PUFA feeding changes plasma membrane fatty acid composition with consequences on methotrexate transport. The cells isolated from animal treated with n-3 PUFA-enriched oil had a lower Km value for methotrexate transport than those isolated from animals fed a saturated-enriched oil.

Later, Guffy *et al.* [20] demonstrated that L1210 leukemia cells, grown in medium with DHA, were more sensitive to the adriamycin (ADR) cytotoxicity than cells grown in medium with oleic acid or without fatty acid. In the same period, Ziljlstra *et al.* [21] showed that also in human small-cell lung carcinoma cell line GLC4 the intracellular level of adriamycin increases when the cancer cells are

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cultured in medium supplemented with DHA. Furthermore, the adriamycin uptake increases in ADR-resistant cells to level equal to that of sensitive GLC4 cells. ADR content and cytotoxicity seemed linked to the higher levels of DHA, and to significant modifications in membrane phospholipid composition. In particular, Guffy *et al.* [21] sustained that when cell membrane phospholipids are enriched with n-3 PUFAs become more sensible to lipid peroxidation causing membrane damage.

On the same line, Ikushima *et al.* [22] and Das *et al.* [23] demonstrated that DHA is also able to increase vincristine cytotoxicity and its uptake in neuroblastoma and cervical cell lines.

In addition, Sturlan *et al.* [24] showed that DHA strongly increases arsenic trioxide (As_2O_3)-mediated apoptosis in the acute myeloid leukemia. As_2O_3 has been used for the treatment of acute promyelocytic leukemia (APL) HL60. Many mechanisms have been suggested for anti-leukemic activity of arsenic trioxide, but the generation and accumulation of ROS are most responsible of cytotoxicity. In fact, ROS intracellular accumulation determines the disruption of the mitochondrial membrane potential, release of cytochrome c, activation of caspase cascade and finally the apoptosis process. Sturlan's research indicated that the cotreatment of HL-60 cells with As_2O_3 and DHA causes the increase of ROS and thiobarbituric acid reactive substances (TBARS) content, a reduction of the mitochondrial potential, and activation of caspase-3 with subsequent apoptosis.

As_2O_3 was also used to treat solid cancers such as neuroblastoma, head and neck cancer, gastric, prostate and renal cell carcinoma. Baumgartner *et al.* [25] tested several cancer cell lines: breast (MDA-MB-468, SKBR-3, MCF7), cervical (HeLa), ovarian (SKOV-3, ES-2), colon (HT29, SW-620, LS-174T), prostate (PC-3), and pancreatic (PANC1) cancers. These cell lines showed resistance to treatment with either As2O3 or DHA alone, but the co-treatment leaded to a reduction of cell viability in SKBR-3, HT29, SW-620, LS-174T, SKOV-3 and PC-3 cells in association to a significant increase of TBARS.

In 2005, Menendez *et al.*[26] demonstrated that n-3 PUFAs enhance chemosensitivity through their peroxidation process, but also by regulating expression of oncoproteins. In fact, the results demonstrated that γ-linolenic acid (GLA) is the most potent PUFA in increasing paclitaxel toxicity followed by alpha-linolenic acid, EPA and DHA, while linoleic acid (LA) does not have any effect. Menendez sustained that there is a strong synergistic interaction between DHA and paclitaxel or docetaxel on the cytotoxic effects in MDA-MB-231 cells. The exposure of BT-474 or SK-Br3 cells to DHA for 24h reduced p185Her/neu oncoprotein expression up to 78% in BT-474 and to 38% in SK-Br3 cells compared to untreated cells.

The co-treatment DHA and cisplatin was used on human small lung carcinoma cell line (GLC4) and its cisplatin-resistant cells (GLC4-cisplastin). Timme-Bosscha *et al.*[27] demonstrated that DHA

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reduces the resistance from 11 to 4 in the cisplatin resistant cell lines. The authors suggested that the modulation of DHA incorporation could be the result of an increase of cisplatin content, which can determine an enhancement of DHA platinization and adduct formation.

3. Mechanisms proposed for chemosensitizing effects of n-3 LCPUFAs.

The specific mechanisms involved in n-3 LCPUFAs chemosensitizing effects are not fully understood, but nowadays, it is widely accepted that there are a complex network of mechanisms, including alteration in gene expression [28], modulation of cellular proliferation [29] and differentiation [30], induction of apoptosis [31], increase in drug transport across the cell membrane, generation of reactive oxygen species (ROS), and lipid peroxidation (Figure 1). For example, lipid peroxidation is the major mechanism exerted by doxorubicin cytotoxicity, mainly correlated to the topoisomerase II inhibition and to the ROS production [32]. Moreover, the cardiotoxicity caused by anthracyclines could be mediated through ROS produced during their metabolism. Since DHA, with its 6 double bonds, is inclined to peroxidation, the increase of membrane unsaturation index produced by DHA incorporation would enhance the ROS content generated from doxorubicin metabolism [33]. This hypothesis is sustained by *in vivo* and *in vitro* studies that highlight the correlation between DHA supplementation and oxidative stress resulting from a higher peroxidation.

In this context, Vibet *et al.* [34] demonstrated that sensitization of breast cancer MDA-MB-231 cells to doxorubicin by DHA is related to a marked decrease in glutathione peroxidase (GPx), a major antioxidant enzyme that uses glutathione as a reductive agent. In particular, the decrease of GPx1 activity in MDA-MB-231 cells was linked to a decreased protein level but not to a decreased mRNA, suggesting a DHA effect at post-transcriptional events. One hypothesis is that GPx might be damaged by lipid peroxidation products produced by DHA feeding in breast cancer cells treated with doxorubicin. These products lead a loss of GPx activity, probably by a modification of the selenocysteine residue at the active site of the enzyme [35]. Finally, the inactivated enzyme is degraded by proteases. In the same study, GPx1 activity decreased also in rat tumours after supplementation with EPA/DHA or DHA alone and this reduction was associated to an increase of chemosensitivity to anthracyclines.

The most important biological function of n-3 LCPUFAs is to be precursors of bioactive lipid mediators such as eicosanoids (Figure 2). N-3 LCPUFAs and their metabolites exert a second messenger action when inserted in the cell membrane. Following the binding of growth factors and hormones to membrane receptors, phospholipase A_2 (PLA_2) is activated and releases dihomo-γ-linolenic acid (DGLA, C20:3, n-6), arachidonic acid (AA, C20:4, n-6), EPA (C20:5, n-3) and DHA

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(C22:6, n-3) from sn-2 position of phospholipids. These fatty acids become substrates for eicosanoid biosynthesis, depending on the cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 monoxygenase (CYP) activities. High levels of omega-6 derived prostaglandins (PG) and/or high level of cyclooxigenase 2 (COX2) are linked to many human cancer, including breast, cervix, lung, skin, colon and prostate [36,37], so that the COX2 inhibitors, such as celecoxib, indomethacin, aspirin, and piroxicam, may be used to reduce carcinogenesis. A novel approach combining these drugs at low concentrations with dietary elements has been suggested to improve their effects and decrease side effects. Negi K. *et al.* [38] showed that a combination of celecoxib and n-3 PUFAs is more effective in the treatment of experimental mammary carcinogenesis, and this effect can be attributed to the modification of redox signalling, with decreased c-myc, p53 expression, apoptosis, and proliferation. In addition, Reddy BS *et al.* [39] showed that low-dose level of celecoxib in association with a diet containing 10% mixed lipids and 10% fish oil determined a significant inhibition of COX-2 activity and expression, and colon cancer incidence compared with low dose of celecoxib in a Western-style diet high in mixed lipids, including saturated fats of animal origin as well as n-6 PUFAs.

Moreover, it has been shown that the carcinogenesis inhibition induced by n-3 PUFAs is also mediated through the activation of retinoid X receptors (RXR) and peroxisome proliferator activated receptor (PPAR) [40]. In fact, Narayanan *et al.* [41] suggested that combination of DHA and celecoxib inhibit the carcinogenesis process in several prostate cancer cell lines through multiple pathways that involve PPARγ, RXRα and Nuclear Factor Kappa-B (NF-kB) activity. NF-kB is an inducible transcription factor responsible for the expression of a vast number of inflammation and cancer related genes. The co-treatment with DHA and celecoxib significantly reduces NF-kB-p65 translocation from the cytoplasm to the nucleus, blocking its transcriptional activity for genes related to cancer progression. Moreover, n-3 LCPUFAs inhibit the cleavage of inactive to active sterol response element binding protein 1c (SREBP-1c), modulating fatty acid synthesis; indeed, in prostate cancer, fatty acids are the main energy source and androgens upregulate fatty acid synthase enzyme (FASN). SREBP-1c is a positive regulator of FASN expression through binding elements in the FASN promoter [42]. The inhibition of SREBP-1c causes the accumulation of cholesteryl esters within the cells, resulting in cell cycle arrest [43].

Noteworthy, PUFAs greatly affect cell membrane fluidity and structure, especially in membrane microdomains or lipid rafts. The plasma membrane regulates many cell biology aspects, such as morphogenesis, proliferation, migration, differentiation, secretion, and apoptosis. Numerous studies indicate that n-3 LCPUFA incorporation in the membrane bilayer might determine dramatic changes in physical–chemical properties, a significant lowering of cholesterol solubility [44], and

changes in the activity of transmembrane proteins such as growth factors and the G-protein coupled membrane receptors [45].

4. n-3 PUFA impact on cell membrane function and lipid raft organization

Recently, the new therapeutical targets in cancer treatment are increasingly specific membrane proteins whose activity is modulated by changes in membrane environment. This novel approach, defined "membrane lipid therapy" [46], is based on the hypothesis that the use specific lipids might alter cancer membrane composition and structure, dismantling lipid raft architecture, with consequences on localization and activity of tumour crucial membrane-associated proteins, and their downstream pathways (Figure 1). In fact, the chemical-physical properties of cellular membrane not only influence protein functions, but also modify the recruitment and activity of peripheral, amphitropic membrane proteins that interact with membrane lipids [47]. Membrane lipids interact with hydrophobic moieties and residues of membrane proteins by lipid-lipid and lipid-protein interactions, respectively. For instance, the ABC (ATP Binding Cassette) transporter activity is closely related to membrane lipid environment. Changes in phospholipid (PL) and cholesterol content and PL fatty acid composition might modify the membrane surface properties, then modulating specific cell functions [48].

Indeed, biological membranes represent two-dimensional solutions where lipids are packed with transmembrane proteins [49] and interact with extrinsic membrane proteins. The main membrane lipid components, including sterols (especially cholesterol), sphingolipids (in particular, sphingomyelin - SM), phospholipids (PLs), such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), contribute to stabilize membrane architecture.

In order to provide new membrane generation to sustain neoplastic proliferation, cancer cells are characterized by an intense lipid biosynthesis [50]. Data present in literature highlight that membrane fatty acid and phospholipid profiles of breast cancer are different compared to normal tissues: breast tumours are characterized by a striking increase in membrane PC and PE and in PL-induced cell signalling. In addition, an increase of saturated fatty acid-containing PC (16:0/16:0) was correlated to poorer overall survival [51]. Saturated fatty acids make the cell membrane less fluid and their higher content is associated to most aggressive tumours and chemotherapy resistance [52,53]. In fact, the length and unsaturation degree of FA might modulate membrane fluidity, phase behavior, permeability, membrane fusion, lateral pressure and flip-flop dynamics, disturbing the protein-lipid interactions in plasma membrane. The unsaturation degree increase of phospholipid

198 acyl chains improves their flexibility due to rapid isomerisation [54]; moreover, their fatty acid acyl
199 chain composition mediate growth/survival signalling pathways.
200 Cancer cells might acquire fatty acids, not only through *de novo* synthesis, but also through uptake
201 of exogenous fatty acids obtained by diet or released by cancer-associated adipocytes. Exogenous
202 fatty acids may alter membrane organization either if integrated in membrane as free fatty acids
203 (FFA) or as constituents of phospholipids.
204 Our researches have demonstrated that n-3 LCPUFA incorporation into cell membrane
205 phospholipids may alter membrane fluidity, modulate cell signalling [44,55], and enhance the ROS
206 production and lipid peroxidation [34]. The PUFA-enriched membranes are thinner and have a
207 major fluidity compared to saturated membrane. Moreover, n-3 and n-6 PUFAs display significant
208 differences related to their different saturated chain length, longer in n-6 PUFAs [56,57].
209 n-3 PUFAs may alter the optimal protein conformation by changes of the membrane biochemical-
210 biophysical properties. In the last few years, our group has accumulated evidences showing that n-3
211 PUFAs, especially DHA, are incorporated in breast cancer membranes with different specificity for
212 each PL moiety: the enrichment is significant, especially in PE, PI and PC [44]. Biochemical and
213 biophysical approaches have confirmed that DHA incorporation causes morpho-dimensional
214 changes in plasma membrane, in particular in detergent resistant domains or lipid rafts [55,58].
215 Lipid rafts are dynamic structures characterized by a relative rigidity and reduced fluidity compared
216 with the surrounding plasma membrane, and may rapidly assemble and disassemble, leading to a
217 dynamic segregation of proteins [59]. In fact, they are enriched in cholesterol and sphingo- and
218 glycerol-lipids containing saturated fatty acids, and contain several proteins, such as receptors,
219 channels and transporters, whose localization in raft or non-raft regions modulates their function. In
220 cancer cells, many signalling proteins and receptors regulating pro-oncogenic and apoptotic
221 pathways during the early, advanced and metastatic stages of tumorigenesis are isolated in lipid
222 rafts [60]. Furthermore, lipid rafts and their main component, cholesterol, are enhanced in
223 membrane of cancer cells [61].
224 n-3 LCPUFAs and their metabolites are inserted in lipid rafts with different yield and they alter
225 fatty acid composition without decreasing the total percentage of saturated fatty acids that
226 characterize these structures. Especially in estrogen insensible breast cancer cells (MDA-MB-231),
227 that display the highest content of cholesterol and saturated fatty acids, it was demonstrated the
228 lowest incorporation of DHA, probably for sterical reasons; nevertheless DHA was able to decrease
229 cholesterol concentration in lipid rafts (Figure 3). Moreover, in two cell breast cancer lines (MCF-7
230 and MDA-MB-231) the DHA treatment determined a decrease of the lipid rafts in the order of
231 about 20–30 %. Worth of note, after DHA incorporation lipid rafts exhibit different height ranges

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[44]. These alterations influence resident protein conformation turning on and/or off signalling proteins, and modulate cellular events [62].

In conclusion, n-3 LCPUFAs might dismantle lipid raft structure and thereby protein lateral distribution (Figure 3). The poor affinity between n-3 PUFAs and cholesterol determines a shift of cholesterol out of the raft, inducing de-clustering of membrane microdomains. n-3 LCPUFA incorporation in membrane microdomains determines a re-localization of raft-localized proteins, for example from rafts into non-rafts or in cytosolic compartment. Shaikh SR *et al.* [63] have demonstrated that n-3 PUFA acyl chain enrichment in membrane microdomains and subsequent their de-clustering, force raft-localized major histocompatibility complex (MHC) class I proteins from rafts into non-rafts.

Furthermore, the specificity of PUFA incorporation for the PL moiety might be relevant to the PUFA metabolite synthesis (prostaglandins, prostacyclins, leukotrienes, resolvines and protectines) and signal transduction activation.

The impact of n-3 PUFAs, especially DHA, on membrane organization affect anticancer drug uptake not only increasing sensitization of cancer cells but also modulating chemoresistance.

5. n-3 LCPUFAs as revertants of multidrug resistance: *in vitro* evidences

Chemoresistance, in particular the simultaneous resistance towards different chemotherapeutic agents known as multidrug resistance (MDR), is one of the biggest problem encountered by chemotherapy. MDR can be present at the diagnosis or can be induced by the selective pressure of chemotherapy and includes different mechanisms, such as the increased drug efflux, the reduced drug uptake owing to changes in lipid membrane composition, the increased drug sequestration within endo-lysosomes followed by exocytosis, the enhanced metabolic inactivation of the drug, the quantitative or qualitative changes in the drug target [64]. The most common event characterizing MDR cells is the overexpression of ABC transporters, such as P-glycoprotein (Pgp), MDR related proteins (MRPs) and breast cancer resistance protein (BCRP). Together, they efflux classical chemotherapeutic agents (e.g. anthracyclines, taxanes, Vinca alkaloids, epipodophyllotoxins, topotecan, methotrexate) and new targeted drugs (e.g. imatinib, dasatinib, lapatinib, gefitinib, sorafenib, erlotinib), limiting their intracellular accumulation and cytotoxicity [65].

Since n-3 LCPUFAs induce a good chemosensitization in drug sensitive cancer cells, some works started to analyze whether and how they have any benefits as MDR reversing agents. Interestingly, n-3 LCPUFAs effect appeared rather selective for chemoresistant cells, because a lower or no chemosensitization at all was often reported in the chemosensitive parental clones [21,66] and in non transformed cells [67]. Different cell lines, even if derived from the same tissue, have different

metabolic pathways for n-3 PUFAs [68]; such variability may explain the discrepancies obtained by using different PUFAs and different cancer cells.

n-3 LCPUFAs act as MDR reversing tools by pleiotropic mechanisms.

Since they are well incorporated in plasma membrane phospholipids and in particular in lipid rafts, this was one of the first mechanism investigated and was correlated with the increased ratio between drug uptake and efflux exerted by n-3 LCPUFAs [23]. Interestingly, n-3 LCPUFAs often changed the lipid compositions more in chemoresistant cells than in chemosensitive cells [21], likely as a consequence of the different membrane composition that characterizes these two cell populations [69]. An increased incorporation of saturated FAs due to the *de novo* lipogenesis has been associated with an increased resistance to doxorubicin [53]; opposite effects should be expected when unsaturated FAs are incorporated in tumour cells plasma membranes. Indeed, for drugs entering cells by passive diffusion, such as anthracyclines, Vinca alkaloids and purine analogues, an increased membrane fluidity favors the drug uptake [23,70,71]. In Pgp-overexpressing vincristine resistant neuroblastoma cells, DHA and γ-linolenic acid (GLA) increased the intracellular retention of the drug by inverting the PUFAs/mono-unsaturated fatty acids (MUFAs) ratio in plasma membrane, without changing vincristine efflux [23]: this data suggests that an enhanced uptake more than a reduced efflux is responsible for the higher accumulation of vincristine. This conclusion, however, was partially in contrast with the experimental evidences gathered in doxorubicin resistant breast cancer cells and in vinblastine resistant nasopharyngeal cancer cells: in both models, DHA reduced the efflux of the Pgp substrate rhodamine 123 [72], leading to hypothesize that the higher accumulation of doxorubicin and vinblastine detected in DHA-treated cells was due to their reduced efflux.

As noted above, several ABC transporters mediating MDR are highly sensitive to the changes in lipid plasma membrane. Pgp activity for instance is activated by saturated fatty acid (SFA)-rich environment and is inhibited by increased levels of MUFAs and PUFAs in plasma membrane [73]. The depletion of SFAs from drug resistant cell membranes also decreased the amount of Pgp in lipid-rafts compartment [73], where the protein is abundant and active [74,75]. We recently reported that DHA and EPA were highly incorporated in the lipid rafts of MDR colon cancer cells [66]: by doing so, they reduced the amount of total membrane- and lipid rafts-associated Pgp and MRP1 (another ABC transporter enriched in rafts [76]), restoring the chemosensitivity to doxorubicin and irinotecan. These MDR-reversing effects were peculiar of n3 FAs, but not of the n6 arachidonic acid (AA): n3 FAs are indeed highly flexible structures and can produce a greater disassembly of the ordered lipid rafts structure, which impairs the activity of many lipid raft-associated proteins. Not all the ABC transporters contained in lipid rafts, however, are inhibited by n-3 LCPUFAs: lipid

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rafts-associated BCRP, for instance, was increased in DHA-treated cells [66]. BCRP has a less hydrophobic structure than Pgp and MRP1 [64]; in this case, the enrichment of n-3 LCPUFAs may favour the retention of BCRP in rafts compartment instead of promoting its shift in non-rafts fractions. According to these data, n-3 LCPUFAs should be considered able to reverse the resistance towards substrates of Pgp and MRP1, but ineffective towards substrates of other ABC transporters not localized in lipid rafts and not dependent upon the membrane fluidity for their activity. In this perspective, n-3 PUFAs are not general ABC transporters inhibitors, but their efficacy appears restricted to selected groups of chemotherapeutic drugs and ABC transporters. Since each drug can be effluxed by more than one ABC transporter [64] and resistant tumour cells often express more than one transporter, this consideration freezes the enthusiasms of using n-3 LCPUFAs as a *panacea* for MDR tumours.

Besides SFAs, cholesterol is a second component abundant in lipid rafts. Of note, it is higher in the plasma membrane of chemoresistant cells than of chemosenitive cells [66,77]. Pgp activity is strictly dependent on membrane cholesterol: cholesterol depletion induces the shift of Pgp from lipid rafts to non-lipid rafts compartment [78,79] and reduces its efflux activity [77]. DHA and EPA, which were well incorporated in lipid rafts, displaced cholesterol from the raft fractions of MDR colon cancer cells [66]: in agreement with other experimental observations [78,79], this event displaced Pgp from lipid rafts and lowered its activity [66]. MDR cells have a basally higher rate of synthesis of cholesterol and isoprenoids, which increase the expression of Pgp by activating the transcriptional axes Ras/ERK/HIF-1α and RhoA/RhoA kinase/HIF-1α [80]. Interestingly, besides reducing the cholesterol amount in lipid rafts, DHA and EPA also reduced the endogenous synthesis of cholesterol in MDR colon cancer cells: they allosterically activated the E3-ubiquitin ligase Trc8, which promotes the degradation of the cholesterol-pacemaker enzyme 2-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) [66]. This effect was specific of n-3 LCPUFAs, because arachidonic acid (AA) was ineffective: this may be due to the different tridimensional conformation of n-3 and n-6 LCPUFAs that makes only the former suitable allosteric activators of Trc8. The reduction of the endogenous cholesterol synthesis further contributed to the depletion of cholesterol from plasma membrane, reduced the activity of Pgp and overcame the resistance to the Pgp substrates doxorubicin and irinotecan [66] (Figure 4).

The impact of DHA and EPA on the endogenous cholesterol synthesis, however, is rather variable [81,82,83], depending on tumour types and species, and on the presence or absence of a MDR phenotype. Therefore, notwithstanding the promising results obtained in single cell lines *in vitro*, we hardly believe that the effects of DHA and EPA as Pgp inhibitors through the modulation of cholesterol synthesis can be valid for all tumours.

Only few works describe PUFAs as direct inhibitors and down-regulators of Pgp. For instance, n-3 and n-6 LCPUFAs decreased the transcription of Pgp in colorectal cancer cells: such a decrease, however, was very small if compared with the marked increased efficacy of paclitaxel induced by PUFAs in these cells [84], leading to hypothesize that the changes in the activity or distribution of Pgp, more than in the expression of the protein, are the main responsible for chemosensitization. Indeed, a side-effect of PUFAs in colon cancer cells is the increased expression of the transcription factors CAR and PXR [84], which are Pgp inducers [85]: this side-effect may attenuate the down-regulation of Pgp. On the other hand, PUFAs reduce the activity of NF-kB, which also induces Pgp [86], adding an additional mechanism that may contribute to decrease Pgp expression [87]. The complex balance between Pgp transcriptional inducers and repressors is not always easy to unveil, and makes hard to predict *a priori* whether PUFAs work as Pgp down-regulators in a specific tumour model.

Although the changes in drug uptake and efflux have been primarily considered responsible for n-3 LCPUFAs-induced chemosensitization, other biochemical mechanisms inducing MDR, such as the changes in detoxifying and in activating/inactivating enzyme, can be modulated by n-3 PUFAs. Many chemotherapeutic drugs, including anthracyclines and platinum salts, are metabolized and effluxed as glucuronic acid-, GSH- or sulphate-conjugates via MRP1 [88]. The gene profiling analysis of colon cancer cells pointed out that DHA and EPA down-regulated specific isoforms of glucuronyltransferase, glutathione S-transferase (GST) and sulfotransferase, as well as members of the cytochrome p450 family [89]: such reduction of phase-I and phase-II enzymes is expected to reduce the metabolic inactivation of chemotherapeutic drugs and their efflux, and to increase the intracellular accumulation of the active agents/metabolites. A robust expression pattern of anti-oxidant enzymes is also protective towards the oxidative damages induced by agents such as doxorubicin and cisplatin, and is associated to the MDR phenotype [90]. Interestingly, DHA has been reported to decrease the activity of GPx and to sensitize MDA-MB-231 breast cancer cells to doxorubicin cytotoxicity [34]. Also this effect, however, was not generalized, since GPx was unaffected by DHA in other breast cancer cell lines like MCF7 [34] and it was increased, together with superoxide dismutase, catalase and GST-π, in non small cell lung cancer A549 cells [91]. The different rate and pathways of DHA uptake and metabolism, as well as the plethora of different transcriptional factors, co-activators and co-repressors affecting the expression of anti-oxidant enzymes, may account for the variable effects of the same n-3 PUFAs in different cell models.

From the data analyzed above, it seems clear that more than acting as general MDR reversing agents, n-3 PUFAs overcome the resistance to single chemotherapeutic drugs in selected tumour models, by reactivating specific mechanisms of drug toxicity or by targeting specific pro-

apoptotic/pro-survival pathways. For instance, DHA reversed the resistance to cisplatin in a small cell lung carcinoma cell line by enhancing the formation of DNA interstrand cross-links [92], thereby enhancing the typical pharmacodynamic effect of platinum salts. The resistance to gemcitabine in pancreas cancer is specifically associated with the increased ratio between the pro-survival NF-kB transcription factor and the pro-apoptotic protein PARP: since n-3 PUFAs inhibited the former and activated the latter, they were particularly suitable in restoring gemcitabine cytotoxicity in this model [93].

The effects of n-3 PUFAs on specific tumour subpopulations and/or on tumour stromal cells may represent additional factors contributing to MDR reversion. Cancer stem cells are the most resistant component of the tumour bulk and are often responsible for tumour relapses. Recently, EPA has been found to increase cell differentiation and deplete cancer stem cells from the colorectal COLO 320 DM cell line: this change restored the efficacy of oxaliplatin and 5-fluorouracile in the whole cells population, and chemosensitized stem cells subpopulation to 5-fluorouracile [13]. It has not been investigated yet whether such effect on cancer stem cells occurs also in other tumour types; in the case of a positive answer, this could represent a general mechanism of chemosensitization induced by n-3 PUFAs.

Aggressive tumours have often a fast and disordered growth that is not adequately supported by the tumour vasculature: the reduced supply of blood and oxygen limits the chemotherapy delivery and activity. By modulating the endothelial synthesis of nitric oxide, n-3 PUFAs increased tumour vasculature, improved the delivery and extravasation of docetaxel in rats bearing drug resistant mammary tumours [94]. A similar restoration of chemotherapy efficacy was observed in rats with epirubicin resistant mammary tumours, treated with DHA [95]: in contrast with the findings of Kornfeld *et al.* [100], however, in this model n-3 PUFAs decreased tumour vascularization, a mechanism that is suggestive of a direct anti-angiogenic effect. Since n-3 PUFAs were administered before starting the chemotherapy, two sequential mechanisms may occur in this case: the reduced angiogenesis exerted by n-3 PUFAs can decrease the tumour bulk; such a reduction can make the tumour more eradicable by the subsequent administration of epirubicin. In a neuroblastoma xenograft model, fish oil did not reduce the microvesssels density when administered alone, but it did so when co-administered with sunitinib [96]; in the same condition, fish oil altered the production of local eicosanoids and decreased tumour-associated inflammatory cells, which may include pro-tumoral populations such as tumour associated macrophages type I. The sum of all these effects produced a significant reduction of tumour growth [96].

Although contrasting in mechanisms, these studies suggest that n-3 PUFAs share the properties of reversing drug resistance *in vivo*, by targeting both tumor cells and microenvironment.

6. n-3 LCPUFAs as revertants of multidrug resistance: preclinical and clinical studies

Curiously, several studies demonstrated the chemosensitizing efficacy of PUFAs in pre-clinical models before that *in vitro* studies investigated the molecular mechanisms of such chemosensitization. Despite the differences in the amount, type and proportion of n-3 and n-6 PUFAs, all the *in vivo* studies reported that the dietary supplementation with n-3 PUFAs improved the efficacy of chemotherapy in solid and hematologic xenograft tumors [6,10,12,18,96,97], and in endogenous tumours [94,95]. Dogs fed with menhaden fish oil and arginine before and after remission of stage III lymphoblastic lymphoma also showed prolonged disease free interval and prolonged overall survival compared with animals fed with a standard diet [98].

When given separately and not in a mixture like fish oil, the effects of n-3 PUFAs are however a bit more controversial: for instance DHA alone enhanced the efficacy of epothilone but not the efficacy of 5-fluorouracile and cyclophosphamide, EPA produced the opposite effect, whereas neither DHA nor EPA increased the antitumor efficacy of gemcitabine in mice bearing colon cancers [99].

Compared with the supplementation with a single n-3 PUFA, the supplementation with a mixture alters more deeply the balance between SFAs and PUFAs within tumour tissues. The changes in membrane lipid microenvironment, the increase of lipid peroxidation products, the modifications in the spectrum of eicosanoids produced in tumour stroma - three possible mechanisms of chemosensitization - are produced more by n-3 PUFA mixture than by a single n-3 PUFA. This difference may explain why the dietary supplementation with fish oil improves the efficacy towards many different chemotherapeutic drugs, whereas the supplementation with single n-3 PUFA has sometimes-doubtful efficacy.

The presence of other nutritional supplements may represent another confounding factor: for instance, the supplementation with n-3 PUFAs or glutamine alone increased the efficacy of 5-fluorouracile in colon cancer bearing mice, but this event was surprisingly reduced by their simultaneous administration [100]. Moreover, the schedule of n-3 PUFA administration widely varies between each work, making the comparison between preclinical studies not so easy. Most protocols gave n-3 PUFAs immediately after the tumour implantation and before starting chemotherapy, and continued with a combined administration of n-3 PUFAs and chemotherapy [6,10,12,18,94,95]. Although in neuroblastoma xenografts the administration of n-3 PUFAs before or together sunitinib did not produce significant differences in tumour growth [96], this comparison has not been performed for other tumour models and other chemotherapeutic drugs, leaving several issues uninvestigated. Whether the preventive supplementation of n-3 PUFAs reduces the onset of chemoresistance, whether the administration of n-3 PUFAs at tumour diagnosis or during tumour

recurrences are equally effective in overcoming MDR, are still unsolved questions. Only when these questions will be solved, more precise information about the most effective administration scheme of n-3 PUFAs can be inferred.

An interesting results of the *in vivo* studies is that the supplementation with n-3 PUFAs increased the benefits of chemotherapy in both drug sensitive [97,100,101] and drug resistant tumours [101,102] [6,18,95,99]: in the latter, they usually produced stronger benefits in terms of tumour regression or stabilization. Chemosensitive and chemoresistant tumours often differ for the metabolic pathways targeted by DHA or EPA, as exemplified by the different rate of cholesterol synthesis and by the different membrane lipid composition that affects ABC transporters expression and activity [66]: these and other metabolic differences, which are targeted by n-3 PUFAs, may amplify PUFA effects on drug resistant tumours.

Moving to clinical settings, a first study reporting a direct correlation between the response to chemotherapy and the level of n-3 LCPUFAs, in particular DHA, in adipose breast tissue [103], suggested the possibility that raising the concentration of PUFAs might improve chemotherapy efficacy.

A phase II trial in patients with metastatic breast cancer showed that the daily supplementation with DHA was well tolerated in patients receiving anthracycline-based chemotherapy and produced a significant increase in the overall survival, which was correlated with the blood DHA concentration [104].

Non-small cell lung cancer is often refractory to standard chemotherapy: interestingly, the supplementation with fish oil in patients received first-line chemotherapy improved the clinical response and the overall survival, without increasing the burden of chemotherapy-induced side effects [105]. Since the fish oil supplements used in the study included 2.2 g EPA and 240-500 mg DHA, the therapeutic benefit was likely attributable to these two PUFAs. The adequate intake of n-3 PUFA ranges from 1.1 to 1.6 g/day in adults, with at least 10% of DHA and EPA. Although the amount of DHA and EPA given in the study of Murphy *et al.* were higher than the average adequate intake recommended in a standard Western diet, this amount was well tolerated also by debilitated patients, such as patients undergoing chemotherapy treatment. Indeed, the lack of adverse effects of n-3 PUFAs and/or the attenuation of the chemotherapy side-effects [103,105] made relatively high the compliance of patients in the clinical studies. The amount and types of PUFAs that are optimal to achieve therapeutic benefits in cardiovascular diseases and dyslipidemic syndromes are well known by clinicians. Such experience makes easier translating the administration of PUFAs to other clinical settings, like oncological diseases. The low cost of n-3 PUFAs [106] compared with the costs of the most recent targeted-therapies used in patients unresponsive to conventional

chemotherapy is another appealing factor that makes n-3 PUFAs supplementation particularly suitable for large population studies.

An alternative to the DHA administration and chemotherapy as single agents is the use of a multitarget conjugate of DHA and chemotherapeutic drug. Two independent trials reported that a DHA-paclitaxel conjugate induced less side-effects than free paclitaxel in patients with resistant solid tumours, owing to the different pharmacokinetic profile of paclitaxel released from DHA [107,108]; the conjugate also produced a good stabilization of the tumour in patients refractory to previous chemotherapeutic regimens [108]. It has not been investigated in these studies whether and how DHA dissociates from paclitaxel: since the two agents are bound by an acyl link [109], it is likely that plasma esterases released DHA from paclitaxel and that the observed tumour stabilization was due to the chemosensitizing effect of DHA on resistant cells. Compared with single agents, multitarget drugs have a lower risk of drug-drug interaction, a better compliance for patients and a more predictable pharmacokinetic profile: given the good chemosensitization efficacy achieved by the DHA-paclitaxel conjugates, this approach may represent a useful tool for future phase II and phase III trials.

7. Concluding remarks

Despite a certain variability in the action mechanisms, types, doses and timing of n-3 LCPUFA administration, most studies agree that DHA and EPA improve the efficacy of chemotherapy *in vitro* and *in vivo*. Noteworthy, higher is the chemoresistance, higher is the chemosensitizing effect, a feature that is uncommon for other MDR reversing agents and ABC transporters inhibitors. Moreover, n-3 LCPUFA supplementation was generally well tolerated and did not increase the side-effects of chemotherapy: some studies reported indeed a reduction of tumour-related or chemotherapy-related side-effects, such as cachexia [10], osteoporosis [110], neutropenia [18], cardiotoxicity [111], diarrhea [12,100]. PUFA uptake by tumour and non tumour cells is highly variable [104,112], leading to exclude that the selectivity of PUFAs for tumour cells and the protection of non transformed cells are due to a different incorporation of these compounds in tumour and non tumour cells, respectively. On the other hand, it is known that PUFAs change lipid membrane composition of transformed and non transformed cells in a different way [113], and that the membrane composition of drug sensitive and drug resistant cells is different [69]. It is likely that the incorporation of n-3 LCPUFAs, which widely alters the cholesterol-rich and lipid rafts-rich plasma membranes of MDR cells, impairs the activity of membrane proteins more in drug resistant cells than in drug sensitive cells or in non transformed cells. This may explain why the chemosensitizing effects of n-3 PUFAs were often more pronounced in MDR cells.

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In addition, n-3 PUFAs may target specific metabolic pathways that are necessary for MDR phenotype maintenance [66]. The search for compounds exerting a selective cytotoxicity in MDR cells – an event known as "collateral sensitivity" [114]- is very active. ROS inducers, ATP depleting agents, detergents increasing membrane fluidity are the most promising agents in this new generation of MDR reversing tools [114]; however, their potential toxicity in non-transformed cells raises some doubts about their extensive use *in vivo*. n-3 PUFAs are a step over these "collateral sensitivity" inducers, because they are more effective in MDR cells and well tolerated by patients.

A considerable number of the *in vivo* studies showed a good chemosensitizing effect of n-3 PUFAs in tumours resistant to anthracyclines and taxanes [18,94,95,107,104,108], two drug classes widely used in both haematological and solid malignancies. This enlarges the potential number of oncological patients who may benefit from n-3 PUFA supplementation and makes the realization of phase III trials easier, compared with other MDR reversing compounds.

On the other hand, although *in vitro* and *in vivo* studies highlighting the therapeutic benefits of n-3 PUFAs have been abundant in the last two decades, several issues must be clarified, before proposing their extensive use in clinical practice. First, most attention has been focused on the effects of n-3 PUFAs on lipid plasma membrane and plasma membrane associated proteins. PUFAs can be theoretically incorporated in all the cell membranes, thus affecting the lipidomic/proteomic profile of endoplasmic reticulum, Golgi apparatus, endosome/exosome vesicles, mitochondria and nucleus. In all these organelles transmembrane proteins regulate crucial biological functions; the extent of PUFAs incorporation in these intracellular membranes and the impact on the physiological organelles activity is a subject largely unexplored. Specific investigations in the field may unveil new mechanisms at the basis of the MDR reversing efficacy of n-3 PUFAs.

In contrast with most evidences showing that PUFAs chemosensitize cancer cells, a recent work reported that cisplatin-treated colon carcinoma became resistant to different chemotherapeutic drugs following the cisplatin-induced production of two endogenous PUFAs, namely 12-oxo-5,8,10-heptadecatrienoic acid and hexadeca-4,7,10,13-tetraenoic acid (16:4, n-3) acid, by mesenchymal stem cells [115]. This work is the only one reporting that endogenous n-3 PUFAs, in contrast with the exogenously administered ones, have a deleterious effect on chemotherapy efficacy, opening a second field of investigation that is actually unexplored.

A third issue poorly known is represented by the inter-individual differences in PUFA absorption, by the genetic polymorphisms in the enzymes involved in FA uptake, transport and metabolism, by the amount and types of other FAs present in the patients diet. In the light of these factors, a careful optimization of the n-3 PUFA supplementation protocol, tailored on single patients, might be required. At the present how such inter-individual differences affect n-3 PUFA efficacy is not

known; only large population studies will likely clarify these points. The safety and the low cost of n-3 PUFA supplementation may be advantageous in realizing such studies, increasing the confidence that most of the open questions concerning mechanisms and benefits of PUFAs as chemosensitizing agents will be solved soon.

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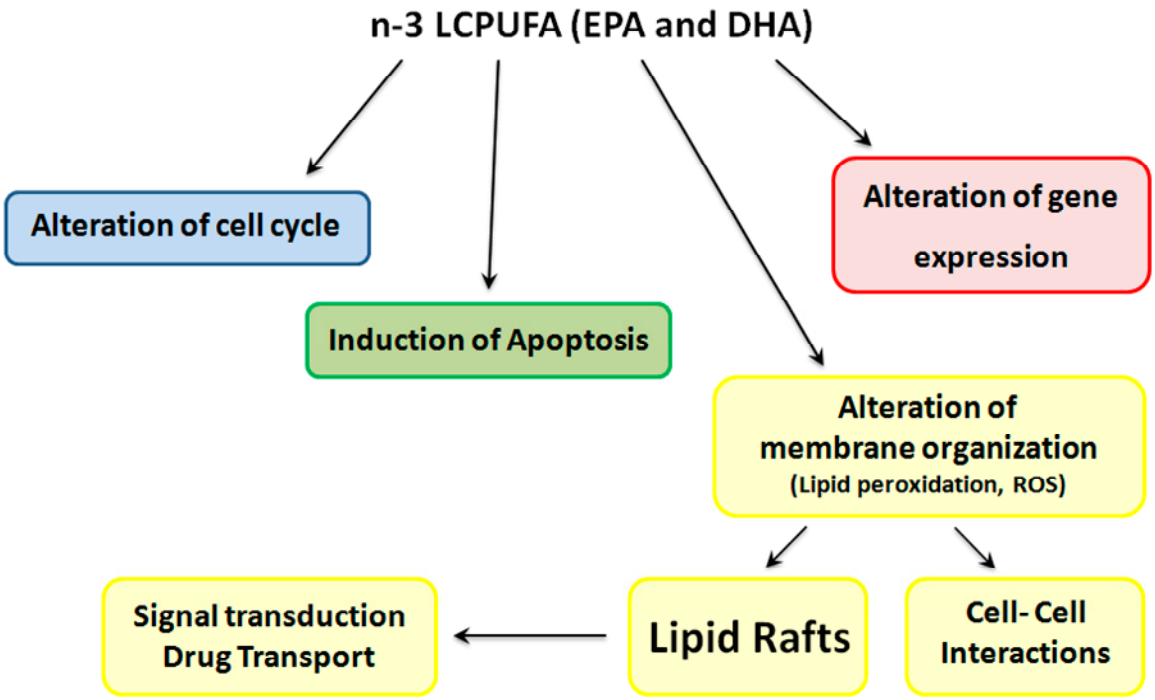
550 **Figures and captions**



551

552 **Figure 1. Proposed mechanisms for anticancer n-3 LCPUFA effects.**

553 LCPUFA – long chain polyunsaturated fatty acids; EPA – eicosapentaenoic acid; DHA –

554 docosahexaenoic acid.

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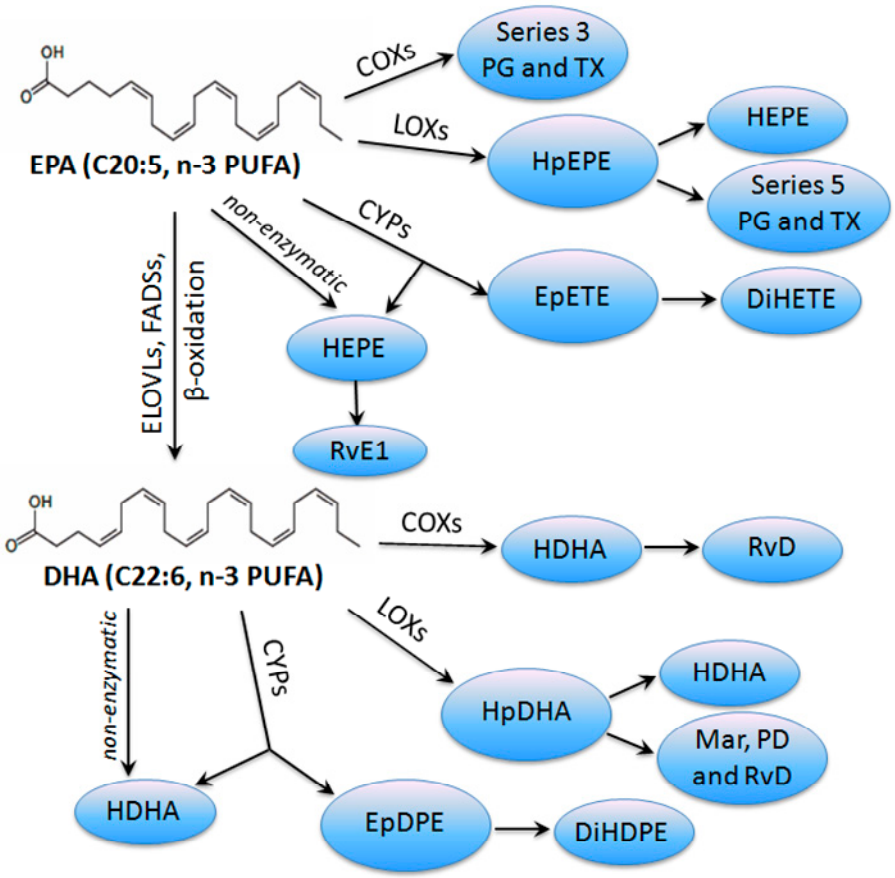

555

556 **Figure 2. Overview of the key COX, LOX and CYP-derived metabolites of EPA and DHA.**

557 COX – cyclooxygenases; LOX – lipoxygenases; CYP – cytochrome P450; PG – prostaglandin; Tx
558 – thromboxane; HpETE – hydroperoxy eicosatetraenoic acid; HpEPE – hydroperoxy
559 eicosapentaenoic acid; EpETE – epoxy eicosatetraenoic acid; DiHETE – dihydroxy eicosatetraenoic
560 acid; HEPE – hydroxy eicosapentaenoic acid; HpDHA – hydroperoxy docosahexaenoic acid;
561 HDHA – hydroxy docosahexaenoic acid; EpDPE – epoxy docosapentaenoic acid; DiHDPE –
562 dihydroxy docosapentaenoic acid; Lx – lipoxin; LT – leukotriene; Mar – maresin, PD – protectin;
563 RvD – D series resolvins.

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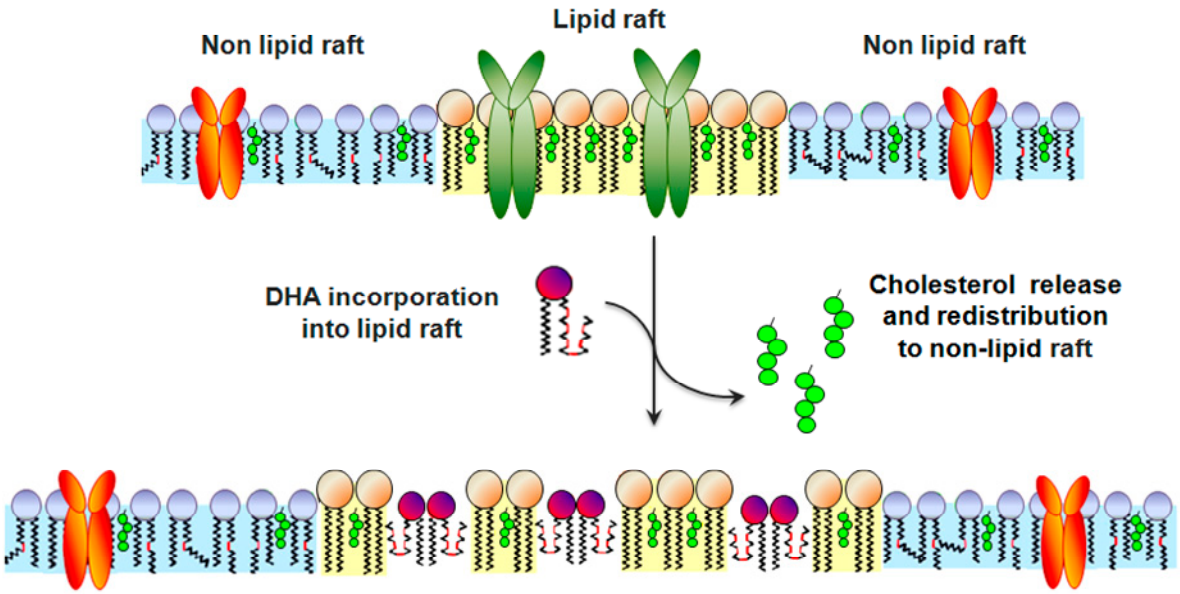

565

566 **Figure 3. DHA impact on lipid raft structure**

567 DHA incorporation in membrane affects lipid raft organization inducing a shift from
568 cholesterol/saturated fatty acid-rich domains to n-3 LCPUFA-rich/cholesterol-poor domains, that
569 exhibit different height ranges.

570

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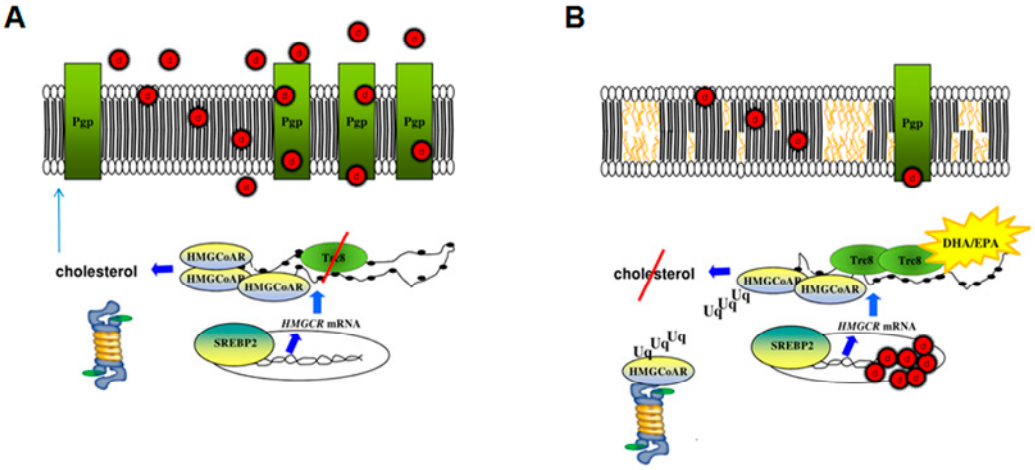

572

573 **Figure 4. n-3 LCPUFAs reverse chemoresistance induced by P-glycoprotein by modulating**
574 **cholesterol synthesis and altering membrane lipid microenvironment**

575 **Panel A**. MDR cells have a high synthesis of cholesterol, owing to the constitutive over-expression
576 of the enzyme 3-hydroxy-3-methlyglutaryl coenzyme A reductase (HMGCoAR). This is
577 independent from the activation of the transcription factor sterol regulatory binding protein-2
578 (SREBP2), but is due to the lower activity of the E3-ubiquitin ligase Trc8. A high cholesterol
579 content in plasma membrane sustains the activity of P-glycoprotein (Pgp), which effluxes several
580 chemotherapeutic drugs (d). **Panel B**. DHA and EPA are allosteric activators of Trc8; by doing so,
581 they increase HMGCoAR ubiquitination (Uq), reduce HMGCoAR amount and cholesterol
582 synthesis. The cholesterol depletion, together with the incorporation of DHA/EPA, in plasma
583 membrane alters the cholesterol rich/saturated fatty acids rich lipid microenvironment, reduces Pgp
584 surface level and activity. As a result, n-3 LCPUFAs increase the intracellular retention of Pgp
585 substrates, chemosensitizing resistant cells.

586

587

588 **References**

589

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