



Mesoporous silica particles functionalized with newly extracted fish oil (Omeg@Silica) inhibit lung cancer cell growth

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Aim: To assess whether Omeg@Silica microparticles – fish oil from anchovy fillet leftovers (AnchoisOil) encapsulated within mesoporous silica particles – are effective in promoting antitumor effects in lung cancer cells. **Methods:** Three human non-small-cell lung cancer cell lines (A549, Colo 699 and SK-MES-1) were used. Cells were treated with AnchoisOil dispersed in ethanol (10 and 15 $\mu\text{g/ml}$) or encapsulated in silica and further formulated in aqueous ethanol. Cell cycle, reactive oxygen species, mitochondrial stress and long-term proliferation were assessed. **Results & conclusion:** Omeg@Silica microparticles were more effective than fish oil in increasing reactive oxygen species and mitochondrial damage, and in altering the cell cycle and reducing cell proliferation, in lung cancer cells. These *in vitro* antitumor effects of Omeg@Silica support its investigation in lung cancer therapy.

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Lung cancer is the leading form of cancer worldwide in terms of incidence and death rate, with non-small-cell lung cancer (NSCLC) accounting for 85% of lung cancer cell types [1]. Despite advances in detection and improvements to standard of care, NSCLC is often diagnosed at an advanced stage and bears a poor prognosis. There is a widespread, urgent and global need to implement therapies to improve the prognosis of NSCLC. Regular intake of omega-3 ($n-3$) marine lipids rich in long-chain polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA, C22:6 $n-3$) and eicosapentaenoic acid (EPA, C20:5 $n-3$) is recommended by most of the world's national and international health authorities for the prevention of many chronic diseases, including cancer [2].

Following associative epidemiological evidence (changing with time) originating from Dyerberg and Bang's discovery of the very low incidence of ischemic heart disease and the complete absence of diabetes mellitus in Greenland's Inuit population in the 1960s [3], detailed physiological, pharmacological and pathophysiological evidence has given clear insights into the benefit from balancing the $n-6$ with $n-3$ lipid molecular mediators in the arachidonic acid cascade [4].

A plethora of studies have demonstrated that omega-3 PUFAs exert a therapeutic role against certain types of cancer [5], including lung cancer [6]. These essential lipids exert inhibitory effects on lung cancer growth by reducing tumor cell proliferation or by increasing cell apoptosis [7]. In 2014, Newman *et al.* discovered that in lung carcinoma cell lines, fish oil-derived EPA reduces NSCLC cellular proliferation through PGE2 formation by COX-2 enzymes and the downregulation of PI3K pathways in NSCLC cells [8]. Subsequent investigations on the inhibitory role of DHA on NSCLC *in vitro* and on fat-1 transgenic mice identified resolvin D1, an eicosanoid metabolite of DHA, as the molecule responsible for significantly inhibiting lung cancer cell growth and invasion by increasing the expression of a miR-138-5p precursor [9]. Concomitant results suggest that miR-138-5p is indeed a tumor suppressor in NSCLC cells [10]. Preclinical studies have shown evidence also that omega-3 PUFA metabolites

modulate pivotal pathways underlying complications secondary to cancer, upregulating anti-inflammatory lipid mediators such as protectins, maresins and resolvins [11].

Unfortunately, the near-global dietary imbalance between n-3 and n-6 essential lipids which has emerged since the early 1950s has led to the present very low to low range of blood EPA + DHA for most of the world's population (6.5 billion out of 7 billion people in 2015), increasing global risk for chronic disease [12]. This omega-3 lipid deficit in the diet of most industrialized countries originated the development of the omega-3 dietary supplement industry based on chemically refined fish oil, with a significant impact on increasing overfishing across the oceans and in the Mediterranean Sea [13].

These trends originate two urgent needs: to develop sustainable sources of marine lipids [14] and to create formulations that may potentiate and expand the physiological activity of marine oils; for example, by avoiding refinement, which eliminates from fish oil the lipophilic natural phlorotannins that crucially protect fish oil omega-3 from oxidation and autoxidation [15].

The present study reports the *in vivo* anticancer effects of a new formulation (Omeg@Silica) comprised of a whole fish oil (AnchoisOil) rich in omega-3 lipids, vitamin D₃ and zeaxanthin, extracted with biobased limonene from anchovy fillet leftovers [16] and further microencapsulated within mesoporous silica particles (Supplementary Figure 1) [17]. Experiments were carried out testing both the newly sourced AnchoisOil whole fish oil and Omeg@Silica microparticles suspended in aqueous ethanol (fish oil in silica; FOS) in limiting lung cancer cell growth using a panel of lung cancer cell lines: human lung adenocarcinoma cell lines (A549 and Colo699) and squamous carcinoma cell line (SK-MES-1).

Materials & methods

Extraction of AnchoisOil

Fish oil was obtained from anchovy fillet leftovers kindly donated by an anchovy fillet company based in Sicily (Agostino Recca Conserve Alimentari, Sciacca, Italy) according to a published procedure introduced in 2019 [16]. In detail, 204 g of frozen anchovy waste in the blender jar of an electric blender was added with a first aliquot of 106 g of D-limonene (Acros Organics, 96%, Antwerpen, Belgium) refrigerated at 4°C. After grinding twice (for 15 s each time) to mix and homogenize the anchovy leftovers, a semi-solid gray mixture was obtained. This mixture was extracted with limonene, a high-boiling-point citrus-derived natural solvent with many health-beneficial properties [18]. An aliquot (50.7 g) of this mixture was transferred into a glass beaker and another aliquot (51.4 g) of limonene was added. The solid-liquid extraction proceeded by magnetically stirring the latter mixture at 700 r.p.m. and room temperature in the beaker, closed with aluminum foil and parafilm. After 21 h, the yellow supernatant obtained was transferred to the evaporating balloon of a rotary evaporator (Büchi Rotovapor R-200, Sankt Gallen, Switzerland) equipped with a V-700 vacuum pump and V-850 vacuum controller) to remove the solvent under reduced pressure (40 mbar) at 90°C. An oily extract weighing 3.0 g and colored deep orange (AnchoisOil) remained in the evaporating balloon after limonene evaporation. Besides a significant fraction of DHA (12.4%) and EPA (5.4%) PUFAs [16], the oil contains a good amount (81.5 µg/kg) of vitamin D in the form of bioactive isomer vitamin D₃ (cholecalciferol), in good agreement with the typical amounts of vitamin D₃ found in fish oils [19]. The limonene solvent was almost entirely recovered thanks to the recirculation chiller supporting the rotary evaporator with sufficient cooling to condense the vaporized solvent. The recovered solvent can be used in a subsequent extraction run.

Preparation of Omeg@Silica

The Omeg@Silica particles used throughout this study were MCM-41 silica particles loaded with 50 wt% sustainably sourced anchovy fish oil. For their synthesis, we used an optimized reproducible synthesis method described recently in the literature [20]. After dissolving 1 g of hexadecyltrimethylammonium bromide (CTAB, ≥99% pure; Sigma-Aldrich, Milan, Italy) and 280 mg of sodium hydroxide (Analyticals, Milano, Italy) in 480 ml of deionized water, an aliquot (5.4 ml) of tetraethylorthosilicate (TEOS, ≥99% pure; Sigma-Aldrich) was added dropwise to the solution. The resulting mixture was kept under continuous mechanical stirring (400 r.p.m.) at 80°C for 2 h. The solid precipitate was recovered by filtration, washed with abundant deionized water and methanol (99.8% pure, Sigma-Aldrich) and dried at 60°C for 48 h. The residual surfactant entrapped in the silicate was removed via calcination in air at 550°C for 6 h.

AnchoisOil was added dropwise to the resulting MCM-41 mesoporous silica, kept in a glass flask under mild mechanical agitation. Addition of a first 60-µl aliquot of fish oil to FMCM-41 (100 mg) was followed by subsequent

additions of 20- μ l aliquots. After 8 min, the addition of oil was complete and a material with a 50% w/w fish oil load was left under agitation for 24 h. [Supplementary Figure 1](#) displays the resulting Omeg@Silica next to the free AnchoisOil. Scanning electron microscopy photographs of the Omeg@Silica microparticles, their textural and thermal properties and the vibrational spectroscopy analysis have been lately published along with the ζ potential of their suspensions in water [17]. In brief, the material has a low polydispersity index (0.3) and a large negative value of the ζ potential (-37.6 mV). These are both important characteristics to develop stable drug-delivery applications [21], particularly in the aqueous phase, as the negatively charged particles repel each other, preventing aggregation with the dispersion remaining electrostatically stable. The Z-average size (measure of the average particle size distribution resulting from dynamic light scattering) indicates a moderate increase of the average particle size from 217 to 269 nm when going from empty mesoporous silica to silica filled with 50 wt% fish oil [17].

Preparation of the aqueous formulations

The aqueous formulations of different concentrations used throughout this study were obtained by proper dilution of stock suspensions of Omeg@Silica, AnchoisOil and MCM-41 silica in 10% (v/v) ethanol/PBS (Dulbecco's phosphate-buffered saline) purchased from EuroClone (Pero, Italy). The Omeg@Silica stock suspension was prepared by adding 10.1 mg of material to 10 ml of 10% ethanol/PBS solution (1 ml ethanol in 9 ml PBS). The AnchoisOil and MCM-41 stock suspensions had a concentration of the single components equivalent to that of the Omeg@Silica stock suspension. In detail, a 5 mg sample of AnchoisOil was suspended in 10 ml of 10% ethanol/PBS, while a 5.1 mg sample of MCM-41 was suspended in 10 ml of 10% ethanol/PBS.

Cell cultures

Three different human NSCLC cell lines (A549, Colo 699 and SK-MES-1) were used in this investigation. SK-MES-1 cells were cultured in Eagle's minimum essential medium, with 10% heat-deactivated (56°C, 30 min) fetal bovine serum (FBS), 1% nonessential amino acids, 2 mM L-glutamine and 0.5% gentamicin. A549 and COLO699 were cultured in RPMI-1640 medium containing heat-deactivated (56°C, 30 min) 10% FBS, streptomycin and penicillin, 1% nonessential amino acids and 2 mM L-glutamine (all from EuroClone). The cells, maintained in an incubator at 37°C with a humidified atmosphere with 5% CO₂, were kept as adherent monolayers.

Treatment of the cells

Cells were seeded on six-well plates and cultured to confluence. Then the serum was reduced from 10 to 1% in the medium and the cells were treated with three different concentrations (5, 10 and 15 μ g/ml) of AnchoisOil dispersed in ethanol, silica sub-micron particles or FOS. At the end of stimulation, cells were collected for further evaluations. At least four replicates were performed for each experiment.

Cell viability/metabolic assay

To assess the right concentration of the stimuli to add to the culture, we used the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, WI, USA), a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. Cells plated in 96-well plates were treated for 24 h in triplicate with AnchoisOil dispersed in ethanol (5, 10 and 15 μ g/ml), silica sub-micron particles (5, 10 and 15 μ g/ml) or FOS (at 5, 10 and 15 μ g/ml concentration). Then 20 μ l of One Solution reagent containing MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulphophenyl)2H-tetrazolium] was added to each well, and the cells were incubated for 20 min at 37°C under a 5% CO₂ atmosphere. Absorbance was read at 490 nm on the microplate reader Wallac Victor2[™] 1420 Multilabel Counter (Perkin Elmer, MA, USA). Results are given as percentage of absorbance compared with nontreated control cells (NT).

Clonogenic assay

Clonogenic assay was performed as previously described [22]. In six-well plates, a lower layer was prepared using complete medium in 0.5% agarose. The plates were stored at 4°C for 24 h. Subconfluent cell monolayers were cultured with the different treatments in 1% FBS-supplemented medium for 24 h. The cultured monolayers were harvested and 5×10^4 cells mixed in complete medium with 0.3% agarose, seeded and incubated for 21 days at 37°C in an atmosphere containing 0.5% CO₂. To count the colonies, an inverted phase-contrast microscope

(Leitz, Wetzlar, Germany) was used. Only cell aggregates with at least 40 cells were considered as colonies. All the experiments were carried out in triplicate. Results are expressed as percentage of NT result.

Cell cycle

The cell lines in 1% FBS-supplemented medium were stimulated with the different treatments for 24 h as previously described. After harvesting, the cells were washed twice with ice-cold PBS and resuspended at 1×10^6 cells/ml in hypotonic fluorochrome solution (0.1% sodium citrate, 0.03% Nonidet P-40 and 50 $\mu\text{g}/\text{ml}$ propidium iodide) for 30 min at room temperature in the dark. Then the cells were acquired and analyzed by flow cytometry. On the basis of their DNA content, apoptotic cells were identified as M1 (sub-G1 phase), M2 (G0/G1 cells), M3 (S cells) or M4 (G2/M cells). Data are expressed as a percentage of cells.

Evaluation of mitochondrial stress

Mitochondrial stress was evaluated by the MitoSOX Kit (Molecular Probes, MA, USA). The cell lines were stimulated in 1% FBS-supplemented medium with the previously described different treatments for 3 h. After stimulation, the cells were collected, the MitoSOX reagent added at a concentration of 3 μM , and the cells incubated for 15 min at 37°C. At the end of the incubation, the cells were washed twice in PBS with 1% FBS, followed by flow cytometric analysis. Data are expressed as percentage of cells.

Evaluation of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) were measured by the conversion of the non-fluorescent dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) into a highly fluorescent dye (DCF) by monitoring the cellular esterase activity in the presence of peroxides, as described in previous work [23]. The cell lines were stimulated in 1% FBS-supplemented medium with the previously described different treatments for 3 h. After stimulation, the intracellular generation of ROS was assessed by the cells' uptake of 1 μM DCFH-DA, following cell incubation for 10 min at room temperature in the dark. At the end of the incubation, the cells were washed twice in PBS and acquired on a FACSCalibur flow cytometer, supported by CellQuest acquisition and data analysis software (Becton Dickinson, CA, USA). Data are expressed as percentage of cells.

Statistics

Data are expressed as mean \pm standard deviation (SD) and analyzed by paired t-test. A p-value of <0.05 was considered statistically significant.

Results

Effects of fish oil, silica & FOS on cell viability/metabolic activity in NSCLC cell lines

Initially, the effects of fish oil (5, 10 and 15 $\mu\text{g}/\text{ml}$), silica (5, 10 and 15 $\mu\text{g}/\text{ml}$) and FOS (5, 10 and 15 $\mu\text{g}/\text{ml}$ load) on cell viability/metabolic activity after 24 h were assessed in NSCLC cell lines (A549, Colo699 and SK-MES-1).

In A549, silica at 5, 10 and 15 $\mu\text{g}/\text{ml}$ and FOS at 10 and 15 $\mu\text{g}/\text{ml}$ were able to reduce cell viability/metabolic activity when compared with control. FOS at 10 $\mu\text{g}/\text{ml}$ exerted a significantly stronger activity on cell viability/metabolic activity than fish oil (Figure 1A).

In Colo699, only FOS at 10 $\mu\text{g}/\text{ml}$ was able to reduce cell viability/metabolic activity when compared with control (Figure 1B).

In SK-MES-1, neither silica nor fish oil or FOS at all the tested concentrations significantly modified cell viability/metabolic activity (Figure 1C).

Effects of fish oil, silica & FOS on cell cycle events in NSCLC cell lines

The effects of fish oil alone (10 and 15 $\mu\text{g}/\text{ml}$), silica (10 and 15 $\mu\text{g}/\text{ml}$) and FOS (10 and 15 $\mu\text{g}/\text{ml}$) on cell cycle events in the NSCLC cell lines (A549, Colo699 and SK-MES-1) were tested.

In A549, fish oil alone at both the tested concentrations was able to significantly reduce M3 (fish oil at 10 and 15 $\mu\text{g}/\text{ml}$ vs NT: $p = 0.0129$ and $p = 0.02$) without any effects on M1, M2 or M4. Silica at 10 $\mu\text{g}/\text{ml}$ (but not at 15 $\mu\text{g}/\text{ml}$) was able to increase M1 (silica at 10 $\mu\text{g}/\text{ml}$ vs NT: $p = 0.014$), whereas silica at 15 $\mu\text{g}/\text{ml}$ significantly reduced M3 (silica 15 $\mu\text{g}/\text{ml}$ vs NT: $p = 0.006$). FOS at 10 $\mu\text{g}/\text{ml}$ (but not at 15 $\mu\text{g}/\text{ml}$) was able to significantly increase M1 (FOS at 10 $\mu\text{g}/\text{ml}$ vs NT: $p = 0.019$) without any effects on M2, M3 or M4. This effect

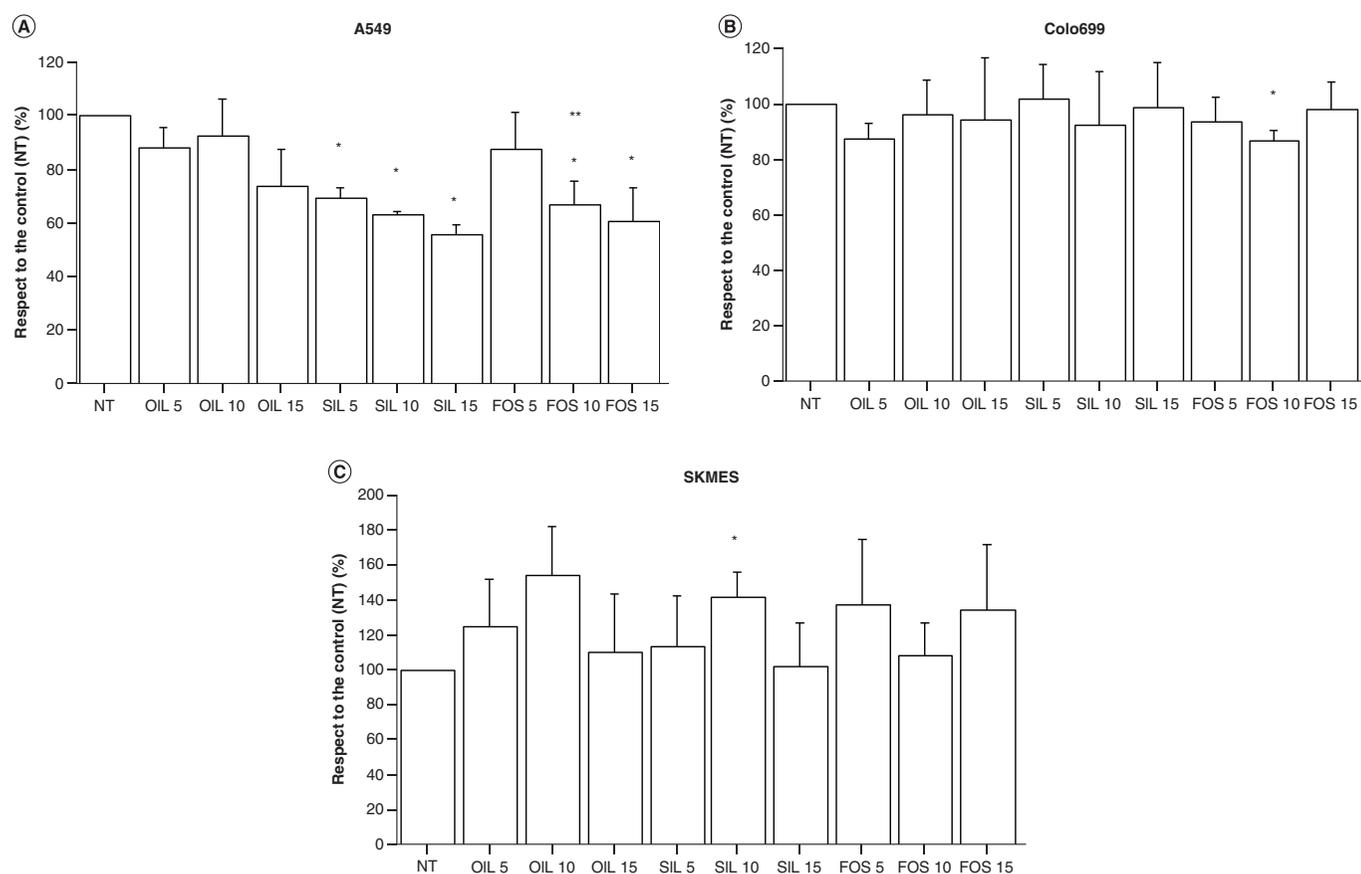


Figure 1. Effect of OIL, SIL and FOS on cell viability/metabolic activity in non small-cell lung cancer cell lines. Non-small-cell lung cancer cell lines (A) A549, (B) Colo699 and (C) SSK-MES-1 were cultured for 24 h with OIL (5, 10 and 15 $\mu\text{g/ml}$), SIL (5, 10 and 15 $\mu\text{g/ml}$) or FOS (5, 10 and 15 $\mu\text{g/ml}$) and cell viability/metabolic activity was assessed by MTS assay. Data are expressed as % of NT and represent mean \pm standard deviation ($n = 3$).

* $p < 0.05$.

FOS: Fish oil in silica; NT: Nontreated (control); OIL: Fish oil; SIL: Silica.

was significantly higher than the effects mediated by fish oil alone at the same concentration (FOS at 10 $\mu\text{g/ml}$ vs fish oil at 10 $\mu\text{g/ml}$: $p = 0.014$) (Figure 2A & B).

In Colo699 cells, fish oil alone at both the tested concentrations was able to significantly reduce M3 (fish oil at 10 and 15 $\mu\text{g/ml}$ vs NT: $p = 0.0012$ and $p = 0.005$) without any effects on M1 or M2. Fish oil at 10 $\mu\text{g/ml}$ significantly reduced M4 (fish oil at 10 $\mu\text{g/ml}$ vs NT: $p = 0.007$). Periodic mesoporous silica at 15 $\mu\text{g/ml}$ significantly reduced M4 (silica at 15 $\mu\text{g/ml}$ vs NT: $p < 0.003$) without any effects on M1, M2 or M3. FOS at 10 $\mu\text{g/ml}$ (but not at 15 $\mu\text{g/ml}$) was able to significantly increase M1 (FOS at 10 $\mu\text{g/ml}$ vs NT: $p = 0.008$). This effect was significantly higher than the effects mediated by fish oil alone at the same concentration (FOS at 10 $\mu\text{g/ml}$ vs fish oil at 10 $\mu\text{g/ml}$: $p = 0.037$). FOS at 10 $\mu\text{g/ml}$ significantly reduced M4 (FOS at 10 $\mu\text{g/ml}$ vs NT: $p = 0.025$) without any effects on M2 or M3 (Figure 3A & B).

In SK-MES-1 cells, fish oil alone at both the tested concentrations was able to significantly reduce M3 (fish oil at 10 and 15 $\mu\text{g/ml}$ vs NT: $p = 0.005$ and $p = 0.001$) and M4 (fish oil at 10 and 15 $\mu\text{g/ml}$ vs NT: $p = 0.02$ and $p = 0.003$), while it was only able to increase M2 at 10 $\mu\text{g/ml}$ (fish oil at 10 $\mu\text{g/ml}$ vs NT: $p = 0.03$). Silica at 15 $\mu\text{g/ml}$ significantly increased M2 and reduced M3 (silica at 15 $\mu\text{g/ml}$ vs NT: $p = 0.002$). FOS at 10 $\mu\text{g/ml}$ significantly increased M2 (FOS at 10 $\mu\text{g/ml}$ vs NT: $p = 0.01$) and reduced M3 (FOS at 10 $\mu\text{g/ml}$ vs NT: $p = 0.0009$), whereas FOS at both the tested concentrations significantly reduced M4 (FOS at 10 and 15 $\mu\text{g/ml}$ vs NT: $p = 0.03$ and $p = 0.03$). FOS did not significantly increase M1 in SK-MES-1 at either 10 or 15 $\mu\text{g/ml}$ (Figure 4A & B). Supplementary Table 1 provides means and SD values of all the data regarding cell cycle experiments in the three cell lines.

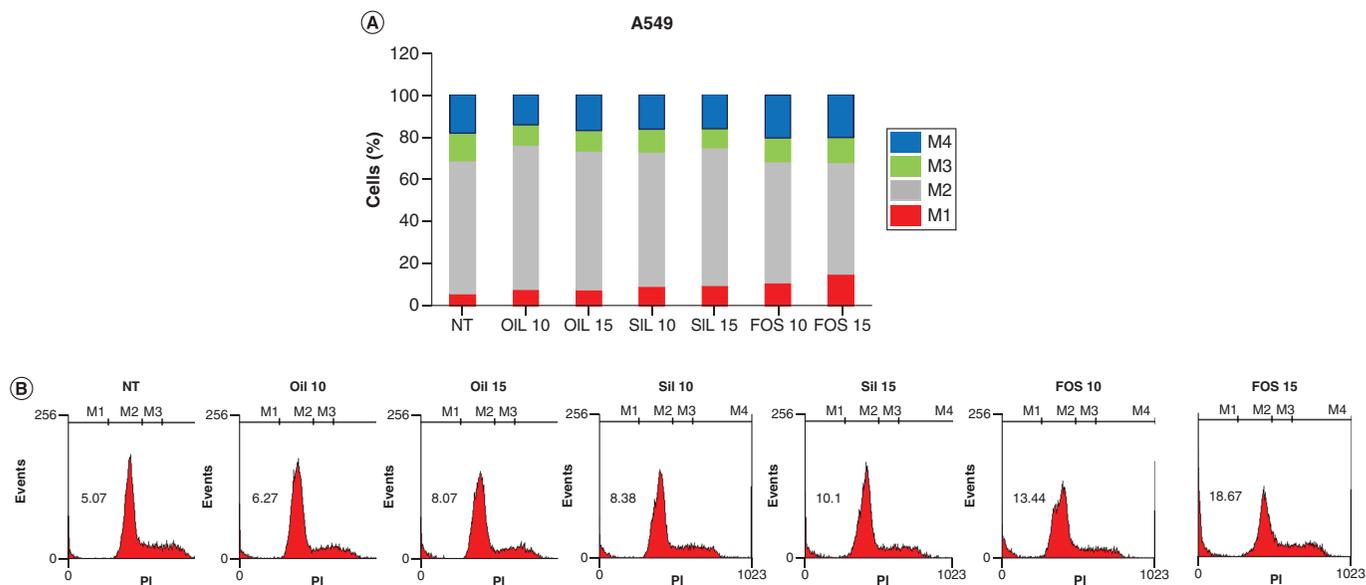


Figure 2. Effect of OIL, SIL and FOS on cell cycle events in A549 cells. A549 cells were cultured for 24 h with OIL (10 and 15 $\mu\text{g/ml}$), SIL (10 and 15 $\mu\text{g/ml}$) or FOS (10 and 15 $\mu\text{g/ml}$) and cell cycle was assessed by flow cytometry. **(A)** Data expressed as percentage of cells (mean \pm standard deviation). **(B)** Representative histograms.

* $p < 0.05$.

FOS: Fish oil in silica; NT: Nontreated (control); OIL: Fish oil; PI: Propidium iodide; SIL: Silica.

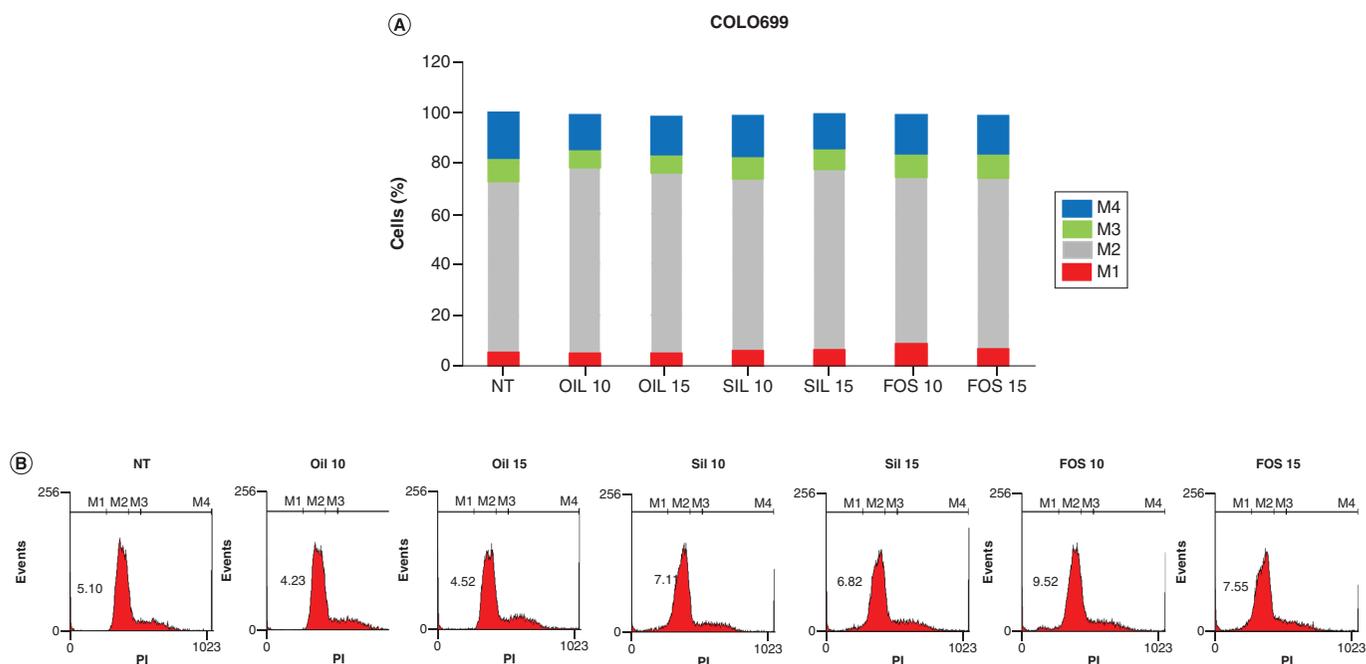


Figure 3. Effect of OIL, SIL and FOS on cell cycle events in Colo699. Colo699 cells were cultured for 24 h with OIL (10 and 15 $\mu\text{g/ml}$), SIL (10 and 15 $\mu\text{g/ml}$) or FOS (10 and 15 $\mu\text{g/ml}$) and cell cycle was assessed by flow cytometry. **(A)** Data expressed as percentage of cells (mean \pm standard deviation). **(B)** Representative histograms.

* $p < 0.05$.

FOS: Fish oil in silica; NT: Nontreated (control); OIL: Fish oil; PI: Propidium iodide; SIL: Silica.

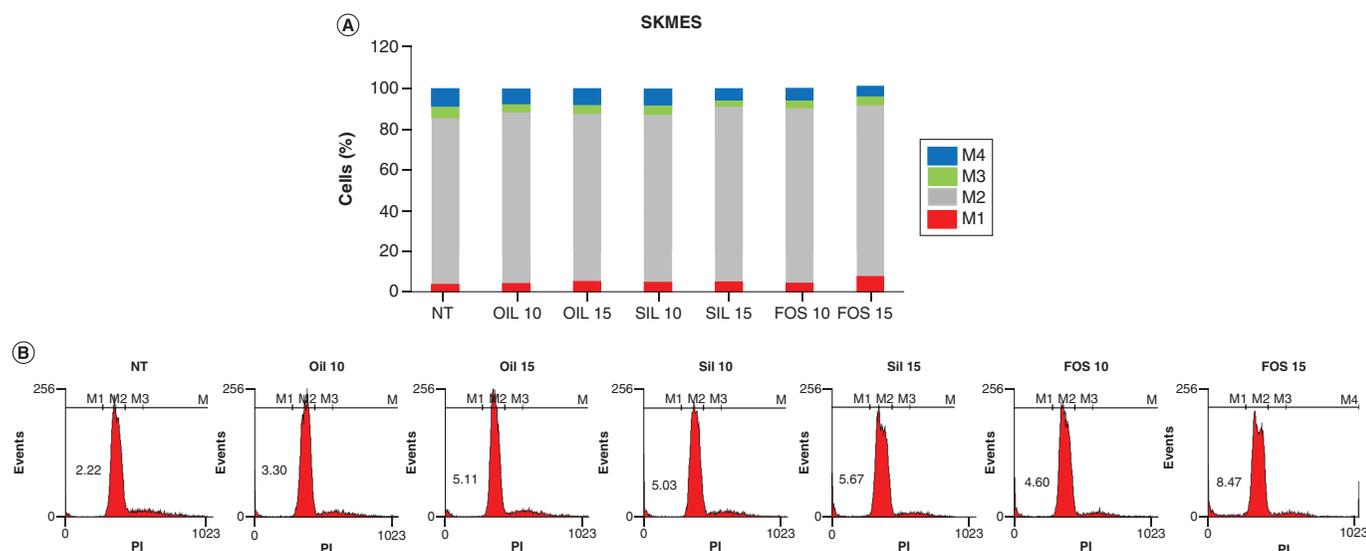


Figure 4. Effect of OIL, SIL and FOS on cell cycle events in SK-MES-1. SK-MES-1 cells were cultured for 24 h with OIL (10 and 15 $\mu\text{g/ml}$), SIL (10 and 15 $\mu\text{g/ml}$) or FOS (10 and 15 $\mu\text{g/ml}$) and cell cycle was assessed by flow cytometry. **(A)** Data expressed as percentage of cells (mean \pm standard deviation). **(B)** Representative histograms.

* $p < 0.05$.

FOS: Fish oil in silica; NT: Nontreated (control); OIL: Fish oil; PI: Propidium iodide; SIL: Silica.

Effects of fish oil, silica & FOS on colony formation ability in NSCLC cell lines

In A549 (Figure 5A), Colo699 (Figure 5B) and SK-MES-1 (Figure 5C) cells, FOS at 10 and at 15 $\mu\text{g/ml}$ significantly reduced colony number when compared with untreated cells or the other experimental conditions, including fish oil alone at the same concentration.

Effects of fish oil, silica & FOS on mitochondrial stress in NSCLC cell lines

Playing an essential role in the survival and viability of cancer cells, mitochondria have been considered a target for anticancer agents [24].

In A549 cells, mitochondrial stress was increased upon exposure to fish oil, to silica and to FOS at both tested concentrations, with both concentrations of FOS exerting a significantly stronger activity on mitochondrial stress than fish oil or silica alone (Figure 6A & B).

In Colo699 cells, mitochondrial stress was increased upon exposure to silica at 15 $\mu\text{g/ml}$ and to FOS at both tested concentrations, with both concentrations of FOS exerting a significantly stronger activity on mitochondrial stress than fish oil or silica alone (Figure 6C & D).

In SK-MES-1 cells, mitochondrial stress was increased upon exposure to fish oil at 10 $\mu\text{g/ml}$ and to silica and FOS at both tested concentrations. Both concentrations of FOS exerted a significantly stronger activity on mitochondrial stress than fish oil alone (Figure 6E & F).

Effects of fish oil, silica & FOS on ROS content in NSCLC cell lines

Upregulation of mitochondrial superoxide leads to increased intracellular ROS levels [25].

In A549 cells, a significant increase in ROS levels was observed upon exposure to fish oil at both tested concentrations, to silica at 15 $\mu\text{g/ml}$ and to both concentrations of FOS. Both concentrations of FOS exerted significantly stronger activity on ROS than fish oil alone at the same concentrations (Figure 7A & B).

In Colo699 cells, a significant increase in ROS levels was noted upon exposure to fish oil and to FOS at both tested concentrations. FOS at 10 $\mu\text{g/ml}$ exerted a significantly stronger activity on ROS than fish oil alone at the same concentration (Figure 7C & D).

In SK-MES-1 cells, a significant increase in ROS levels was measured upon exposure to FOS at 10 $\mu\text{g/ml}$, with FOS exerting a significantly stronger activity on ROS than fish oil or silica alone at the same concentration (Figure 7E & F).

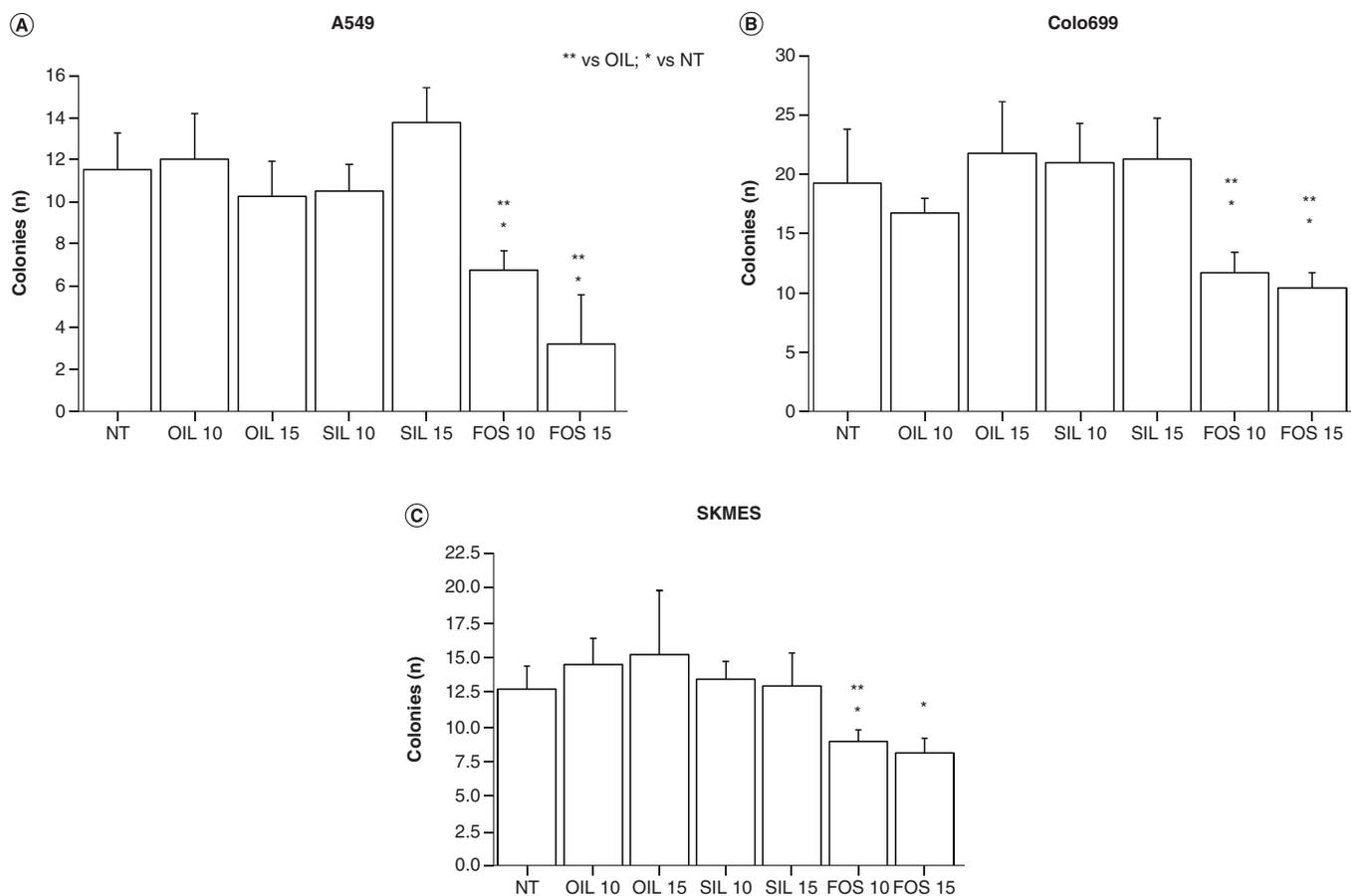


Figure 5. Effect of OIL, SIL and FOS on colony formation ability in non small-cell lung cancer cell lines. NSCLC cell lines (A) A549 (B) Colo699 and (C) SK-MES-1 were cultured for 24 h with OIL (10 and 15 µg/ml), SIL (10 and 15 µg/ml) or FOS (10 and 15 µg/ml) and colony-forming ability was assessed by clonogenic assay. Data are expressed as percentage of control (mean ± standard deviation). *p < 0.05.

FOS: Fish oil in silica; NT: Nontreated (control); OIL: Fish oil; SIL: Silica.

Discussion

The use of whole fish oil, either alone or encapsulated in silica particles, reported in the present study is new because the majority of the *in vitro* studies on the anticancer activity of omega-3 PUFAs have assessed the effects of the two major single EPA and DHA components in non-esterified acid form [26].

The toxic effects of specific compounds on cancer cells can be evaluated by analyzing early events such as cell cycle alterations, cell viability/metabolic activity and oxidative stress, or late events (by assessing colony-forming ability). Data reported herein demonstrate that FOS altered cell cycle phases in all the tested cell lines. At ultra-low concentration of 10 µg/ml, FOS was able to increase the number of apoptotic cells (identified as cells with reduced DNA content in sub-G1 phase) in adenocarcinoma cell lines and to reduce colony-forming ability in all the tested cancer cell lines. FOS was more effective than AnchoisOil fish oil alone at the same concentration. FOS was able to reduce the cell viability/metabolic activity of A549 and Colo699 cells but not of SK-MES-1 cells (MTS data). It is worth noting that MTS reduction is a marker reflecting viable cell metabolism and relies on a mitochondrial NAD(P)H-dependent oxidoreductase enzyme to convert the tetrazole to formazan. The amount of signal generated is dependent on several parameters, including the number of viable cells and their metabolic activity.

Single components of fish oil, EPA and DHA, are able to induce toxic effects (cell apoptosis or reduced cell proliferation) on lung cancer cells, but at higher concentrations of 50–75 µg/ml [27]. The findings of the present study therefore further support the effectiveness of this new silica-based formulation comprised of whole fish oil heterogenized in mesoporous silica. However, FOS at both tested concentrations did not increase cell apoptosis but reduced colony-forming ability in the squamous carcinoma cell line (SK-MES-1). Taken together, these data

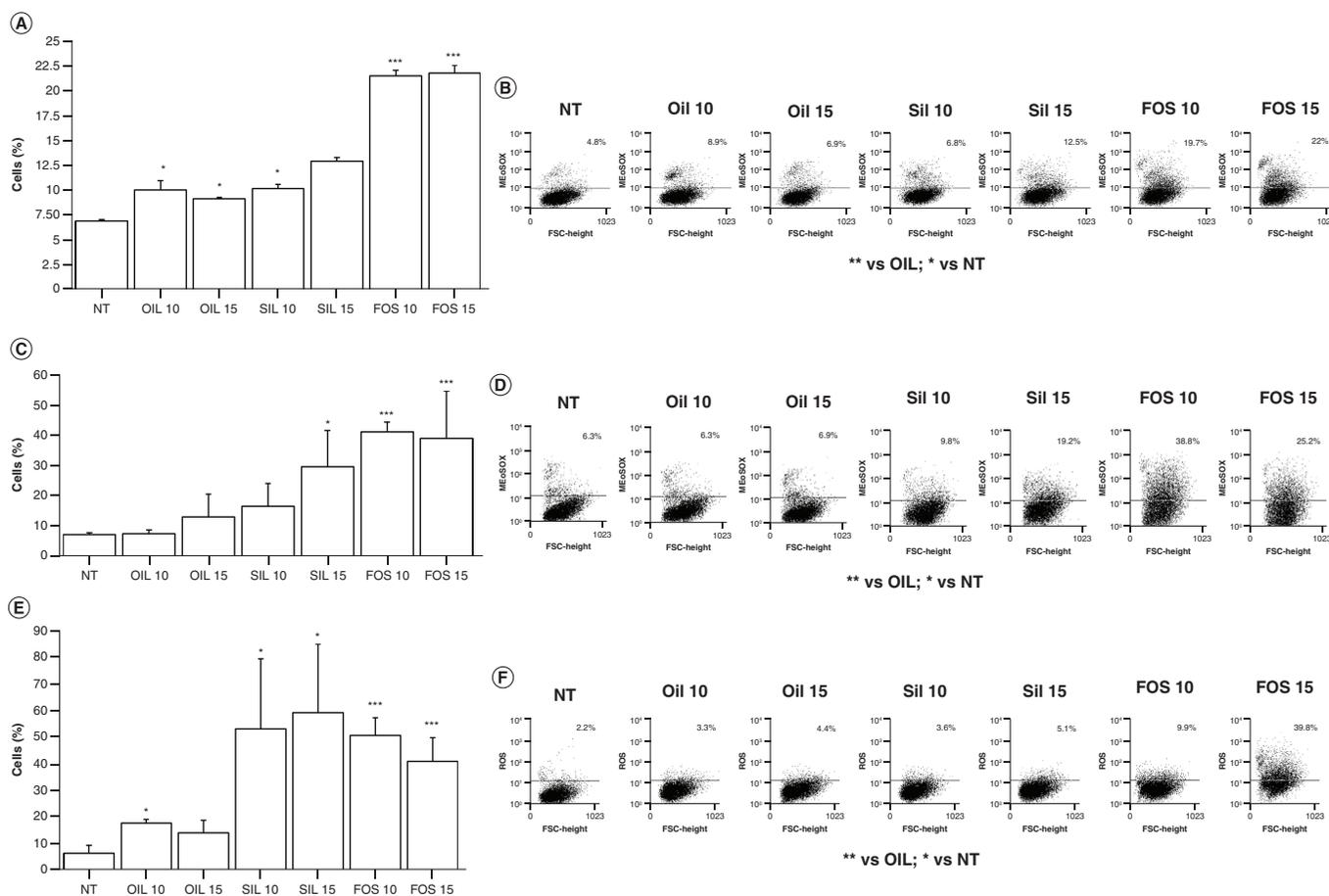


Figure 6. Effect of OIL, SIL and FOS on mitochondrial superoxide production in A549, Colo699 and SK-MES-1 cells. A549, Colo699 and SK-MES-1 cells were cultured for 24 h with OIL (10 and 15 $\mu\text{g/ml}$), SIL (10 and 15 $\mu\text{g/ml}$) or FOS (10 and 15 $\mu\text{g/ml}$) and mitochondrial superoxide production was assessed using MitoSOX by flow cytometry. (A) Mitochondrial superoxide production in A549: data expressed as a percentage of positive cells (mean \pm standard deviation). (B) Representative dot plots for A549. (C) Mitochondrial superoxide production in Colo699: data expressed as percentage of positive cells (mean \pm standard deviation). (D) Representative dot plots for Colo699 are shown. (E) Mitochondrial superoxide production in SK-MES-1: data expressed as a percentage of positive cells (mean \pm standard deviation). (F) Representative dot plots for SK-MES-1 are shown.

* $p < 0.05$.

FOS: Fish oil in silica; FS: Forward scatter; NT: Nontreated (control); OIL: Fish oil; ROS: Reactive oxygen species; SIL: Silica.

suggest that adenocarcinoma cells arising from distal airway epithelium are more sensitive in terms of some early events (cell apoptosis and cell viability/metabolic activity) than cancer epithelial cells from the proximal airways. Further, we observed some different responses in the NSCLC cell lines characterized by a different histotype; A549 and COLO699 are lung adenocarcinoma cell lines, while SK-MES-1 is a lung squamous carcinoma cell line. Experimental and clinical evidence indicates enormous differences among NSCLC subtypes. Studies *in vivo* showed divergent tumor progression mechanisms inducing transformation in adenocarcinomas versus squamous cell lung cancers, and these progressions are linked to a great variance of cellular control networks [28].

Further studies are needed to investigate whether higher concentrations of FOS may induce early cell apoptosis also in lung squamous carcinoma.

With regard to late anticancer events, FOS reduced the colony-forming ability in all the tested cancer cells. This could be related to the alteration of arachidonic acid (AA) metabolism. Indeed, the increased concentration of omega-3 PUFAs in cancer cells, associated with a statistically significant reduction in AA concentrations, suppresses AA-derived eicosanoid biosynthesis, thereby decreasing PGE2 concentration [29]. As mentioned above, a further mechanism employed by omega-3 lipids to decrease PGE2 levels is linked to their inhibitory effect on COX-2, the cyclooxygenase enzyme responsible for PGE2 synthesis [30,31]. PGE2 promotes tumor growth by multiple autocrine and paracrine mechanisms that increase cell proliferation and tissue invasion, reducing cancer cell apoptosis and

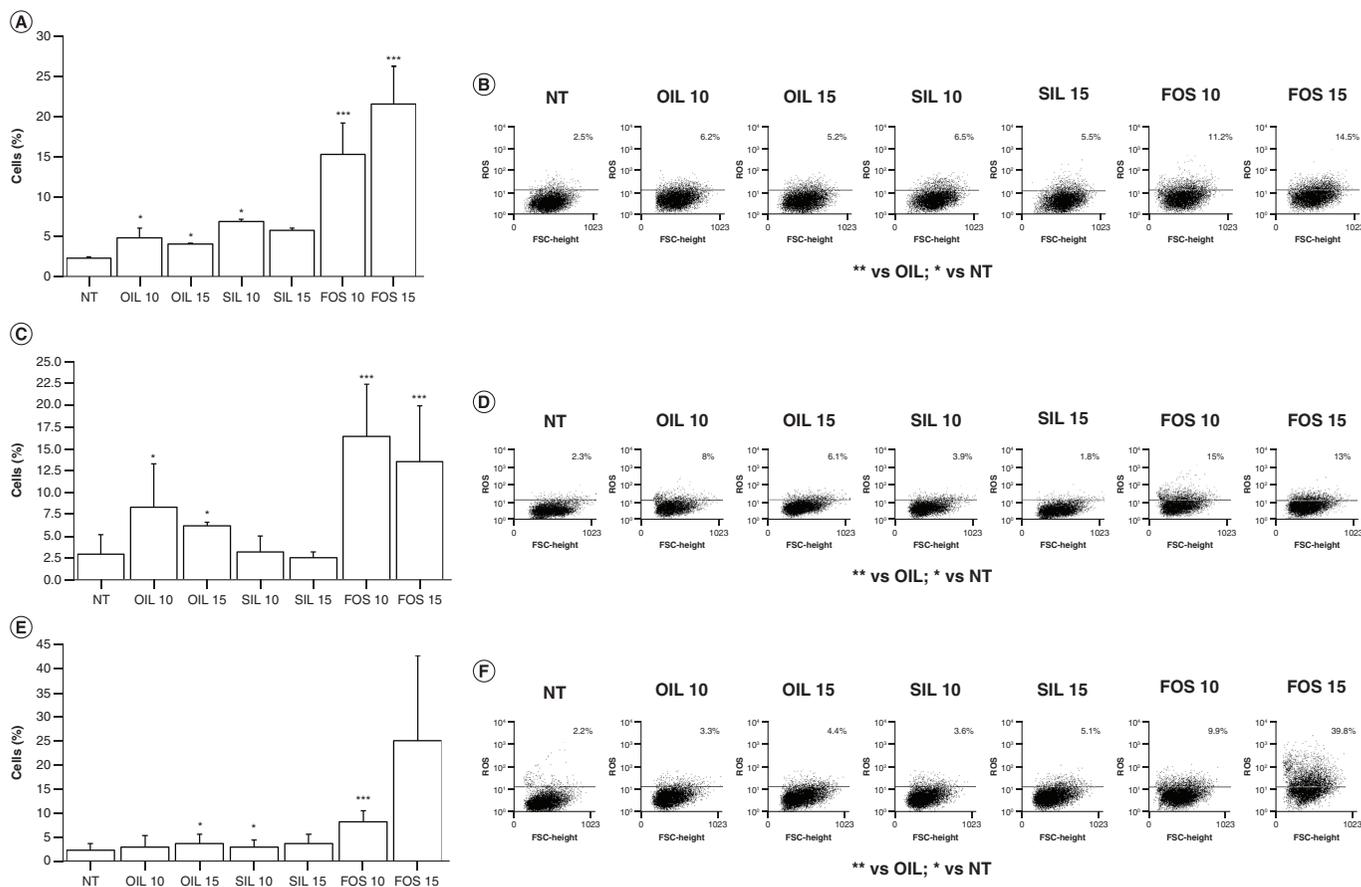


Figure 7. Effects of OIL, SIL and FOS on ROS production in A549, Colo699 and SK-MES-1 cells. A549, Colo699 and SK-MES-1 were cultured for 24 h with OIL (10 and 15 $\mu\text{g/ml}$), SIL (10 and 15 $\mu\text{g/ml}$) or FOS (10 and 15 $\mu\text{g/ml}$) and ROS production was assessed using dichlorofluorescein diacetate by flow cytometry. (A) ROS production in A549: data expressed as percentage of positive cells (mean \pm standard deviation). (B) Representative dot plots for A549. (C) ROS production in Colo699: data expressed as percentage of positive cells (mean \pm standard deviation). (D) Representative dot plots for Colo699. (E) ROS production in SK-MES-1: data expressed as percentage of positive cells (mean \pm standard deviation). (F) Representative dot plots for SK-MES-1. * $p < 0.05$.

FOS: Fish oil in silica; FSC: Forward scatter; NT: Nontreated (control); OIL: Fish oil; ROS: Reactive oxygen species; SIL: Silica.

the efficiency of anticancer immune responses [32]. In this context, it has been demonstrated that PGE contained in exudative pleural effusions from lung adenocarcinoma patients is able to increase the colony-forming ability of Colo699 [33].

Moreover, compounds capable of further increasing the oxidative stress within cancer cells have anticancer potential due to their ability to induce cell apoptosis and inhibit cell proliferation and tissue invasiveness. With their crucial role in ATP production and biosynthesis of macromolecules, mitochondria exert a relevant role in increasing intracellular ROS production. Mitochondrial metabolism is crucial for tumor proliferation, tumor survival and metastasis [24]. Compounds exerting antiproliferative and apoptotic activities in A549 human lung cancer cells alter mitochondrial function [34]. Defects in mitochondrial ATP synthesis and mitochondrial membrane potential increase superoxide production, thus leading to cell apoptosis. Multiple mechanisms may account for increased mitochondrial superoxide production. In this regard, inhibition of PIM kinases cause excessive mitochondrial fission and significant upregulation of mitochondrial superoxide [35].

In addition, BCL2 levels are essential to maintain mitochondrial integrity and prevent cell apoptosis. DHA downregulates BCL2 and upregulates BAX [36]. When BAX transfers to the cell membrane and binds to BCL2, it alters the mitochondrial membrane potential and leads to increased superoxide production and the release of cytochrome C, inducing cell apoptosis. Given that FOS increases mitochondrial superoxide production and induces cell apoptosis, it is also conceivable that it could promote the interaction between BAX and BCL2. Moreover, fish

oil can directly increase ROS levels. In this regard, it has been demonstrated that DHA increases ROS production by downregulating catalase [35].

In addition, mitochondrial NADH/NAD⁺ ratio levels can affect superoxide production [37]; FOS can reduce this enzymatic activity in A549 and in Colo699, as confirmed by our MTS data.

We ascribe the enhanced anticarcinogenic activity of Omeg@Silica compared with that of whole fish oil alone to the hydrophilic nature of the embedding silica matrix and to its large inner mesoporosity, which allows it to encapsulate within the hexagonal channels a high load of bioactive AnchoisOil. Cancer cells are known to be acidic and to efficiently take up mesoporous silica nanoparticles functionalized with hydrophobic cancer drugs into their acidic organelles [38]. Delivery of the omega-3 lipid molecules into the cancer cells led to growth inhibition and cell impairment on all lung cancer cell lines examined. Mesoporous SiO₂ submicron particles are not toxic to human cells and end up preferentially localized in (acidic) lysosomes of cancer cells [39], which makes them ideally suited, biocompatible carriers for a host of hydrophobic anticancer drugs [38].

Conclusion

In this study, we report the high *in vitro* anticancer activity of a new material comprised of whole fish oil (AnchoisOil) extracted from anchovy fillet leftovers with biobased limonene, sequestered within the inner mesoporosity of periodic mesoporous MCM-41 silica submicron particles. Tested on lung adenocarcinoma A549 and Colo699 cells, a hydroalcoholic formulation of these Omeg@Silica submicron particles in aqueous ethanol, FOS, is significantly more active than the fish oil alone in terms of its effects on cell apoptosis, long-term proliferation and mitochondrial superoxide ROS production. Less pronounced effects were observed when lung squamous cancer cells (SK-MES-1) were treated with both fish oil and FOS.

Several aspects support further preclinical investigation of this new functional material in the treatment of lung cancer. First, the whole oil, rich in omega-3 lipids, vitamin D₃ and zeaxanthin, is directly obtained in natural form and at low cost from a freely available and abundant fishery by-product using a biobased and health-beneficial solvent (limonene) that is widely used in the food industry [18]. No refinement of the resulting fish oil is needed to obtain the omega-3 lipids in diethylester form, as happens with most commercial omega-3 supplements [14].

Second, the encapsulant material, comprised of low polydisperse, submicron (269 nm) silica particles of large negative ζ potential (-32 mV) and devoid of toxicity for healthy tissues [38,39], is well suited to deliver the hydrophobic molecules of the AnchoisOil. These include EPA and DHA in natural (and highly bioavailable) [40] form (DHA in position 2 and EPA in positions 1 and 3 of the triglyceride molecules), as well as vitamin D₃ and natural zeaxanthin. Both cholecalciferol [41] and zeaxanthin [42] are known to exert anticancer activity. In conclusion, this study shows evidence that FOS exerts antitumor effects on NSCLC by affecting mitochondrial function and cell growth ability. Further studies to identify the molecular mechanisms originating the anticancer activity of FOS are needed to support the use of this new formulation in the therapy of lung cancer.

Future perspective

The data herein provided open up new frontiers for cancer treatment by using fish waste in a circular economy perspective.

Summary points

- Omeg@Silica consists of whole fish oil rich in omega-3 lipids, vitamin D₃ and zeaxanthin encapsulated within mesoporous silica particles.
- Omeg@Silica is extracted with biobased limonene from anchovy fillet leftovers (AnchoisOil).
- Omeg@Silica is effective in modulating oxidative stress.
- Omeg@Silica is effective in inducing mitochondrial damage.
- Omeg@Silica is effective in reducing the reproductive potential of lung cancer cells.
- Omeg@Silica is effective in altering cell cycle phases in lung cancer cells.
- Omeg@Silica is effective in inducing cell apoptosis in lung cancer cells.
- Omeg@Silica exerts stronger antitumor effects than integral fish oil in lung cancer cells.
- The anticancer activities of this new formulation of fish oil support its further investigation in lung cancer therapy.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2021-0202

Author contributions

C Di Sano and C D'Anna conducted the largest number of experiments, participated in the interpretation of the data, contributed to writing the manuscript and declare that they accept responsibility for the accuracy of the data analysis. A Scurria and C Lino extracted fish oil, synthesized mesoporous silica particles and incorporated fish oil in mesoporous silica, affording the Omeg@Silica material. M Pagliaro revised the original manuscript and designed the Omeg@Silica structural investigation. R Ciriminna conceived the Omeg@Silica material and participated in the interpretation of the data. E Pace designed the study, performed the statistical analysis of the data, contributed to the interpretation of the data, contributed to writing the manuscript and declares that she has had access to and takes responsibility for the integrity of the data. All authors approved the final version of the manuscript.

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Data availability statement

Data used to support the findings of this study are included in the present article.

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