EUR. J. ONCOL.; Vol. 22, n. 1, pp. 5-20, 2017

Shark derivatives (Alkylglycerols, Squalene, Cartilage) as putative nutraceuticals in oncology

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Summary. The capability of sharks to resist infection and the low incidence of tumors found in different species of sharks (e.g. spiny dogfish shark, tiger shark) suggested the presence into their tissues of active compounds, provided of anticancer activities, such as Alkylglycerols (from the shark liver), Squalene (from the shark mesenchyme and skin), and Cartilage (from the shark skeleton). The Alkylglycerols, highly concentrated in the shark liver oil, have several biological activities: stimulation of hematopoiesis, immune-modulation, anti-tumors. The Squalene is an anticancer, antioxidant, detoxifier, skin hydrating, drug carrier and emollient agent. The Cartilage has anti-inflammatory properties commonly used in the folk medicine for the treatment of arthritis, osteoarthritis, diabetic retinopathy, psoriasis, and supposedly also anticancer.

Key words: alkylglycerols, shark liver oil, squalene, shark cartilage, urea, anti-squalene antibodies, cancer, lipids, anti-tumoral activity, oncology

1. Introduction

Several studies show a low incidence of cancer in sharks (1-3). Ostrander and colleagues (4) supposing a possible role of the cartilage components in tumor prevention, examined the incidence of cancers in the class of Chondrichthyes (e.g. sharks, skates, rays, and chimaeroids as a common phylogeny reputed by the experts the very first monophyletic groups (5), and all chondrichthyans) share at least 17 primary characteristics, such as a cartilaginous endoskeleton devoid of bone-producing osteoblasts. Forty-two cases of malignant or benign chondrichthyan tumors were found in the literature and the Registry of Tumors in Lower Animals (6-11). Some shark-related compounds, such as cartilage and its extracts, e.g. urea, shark liver oil (SLO) have vascular effect, improving central arterial elasticity and peripheral microvascular function, and play also a protective role in oncogenesis, inducing a resistant background (12, 13).

The genotoxic effect of oil of three species of Mediterranean sharks: two benthic sharks, *Centrophorus granulosus* and *Galeus melastomus*, and one pelagic species, *Prionace glauca*, was examined by a in *vitro* micronucleus assay (MNvit) which detects simultaneously micronucleus induction by cytotoxic agents (e.g. mitomycin C, bleomycin) that induce dose-response increases in micronuclei frequency and by aneugenic agents (e.g. colchicines, diethylstilbestrol, etoposide, griseofluvine) that increase the number of multinucleated cells (14). The MNvit is an indicator for fixed genomic damage in cells: the accumulation of genetic changes causes a genetic instability, which may result in cancer. The micronuclei frequencies, besides chromosome aberration frequencies are a cytogenetic endpoints, are the supposed link between chromosomal changes and cancer risk (15, 16).

The incubation of human cells with the hepatic crude oils of *Centrophorus granulosus* increases the rate of the binucleated micronucleated cell in a dose de-

pendent manner. Similar results have been obtained with other crude hepatic oils, enclosing three genotoxic sharks species at carcinogenic risk.

The antiproliferative effect of Alkylglycerols (AKGs) was investigated in human ovarian carcinoma (OVP-10), mammary carcinoma (MCF-7), and prostate cancer (DU-145, PC-3 and PCa-2b) cell lines (17). The cells were exposed to Ecomer® (American Nutriceuticals Corp, Deland, USA), a commercially available SLO containing 20% AKGs and 3% methoxy-derivates in a dose of 0.1 mg/ml. The results showed an increased percentage of apoptotic OVP-10 cells (24.9±12.2) and apoptotic DU-145 cells (18.0±1.4), and increased percentage of necrotic MCF-7 cells (20.8±6.4) after exposure to shark liver oil (SLO). The OVP-10 cells displayed the least sensitivity, MCF-7 cells a moderate sensitivity and all three lines of prostate cancer cells showed a high sensitivity to Ecomer®. The observed anti-proliferative effects of SLO could be both cytostatic and cytotoxic. In fact, the OVP-10 cells developed smaller colonies and displayed a higher percentage of apoptotic cells, supporting the cytostatic and apoptotic hypothesis at the same time. The remaining cell lines mainly showed a cytotoxic effect. The MCF-7 cells displayed mainly necrotic cell death, while DU-145 cells underwent simply apoptotic cell death. These effects of AKGs on different cell lines may be due to the differences in the affinity of AKGs to the cell membrane of different neoplastic cells (18). Several studies showed that polyunsaturated fatty acids, long chain fatty acids with two or more double bonds and the first double bond in either the n3 (omega-3) or n6 (omega-6) position have anti-carcinogenic, anti-inflammatory and anti-proliferative effects (19-24). The n3 fatty acid supplementation inhibits the progression stages of carcinogenesis (25), increases the efficacy of various anticancer drugs, chemotherapy and radiotherapy. In contrast, n6 fatty acids has both anti and pro-inflammatory effects, stimulates cell proliferation of some cell types and increases the incidence of some types of cancer, particularly mammary (26). The majority of SLO contains a range of lipid classes, including AKGs, triacylglycerols, Squalene (SQ) and fatty acids (mostly n3 polyunsaturated fatty acids, but also saturated, monounsaturated and n6 polyunsaturated fatty acids) (27). Their composition depends by shark species, shark size, sex, diet, growth rate, swimming depth and reproductive status, as well as the season (28, 29).

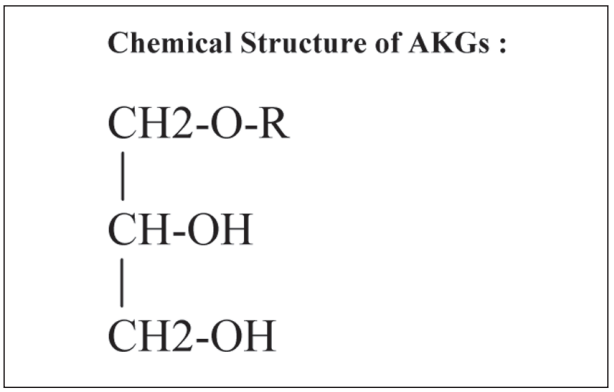

Figure 1. AKGs are glyceryl lipids composed by an ether linkage of a fatty acid attached to the chain length and by the number of double bonds. The principal AKGs are: chimyl (hexadecyl), batyl (octadecyl), and selachyl (octadecenyl) alcohols (30). The AKGs are present, in small quantity, in the living cells of hematopoietic organs (e.g. bone marrow, spleen, liver) and lymphatic tissues and blood (31) and, in highest content in the liver oil of Greenland sharks, the gray dogfish, and the ratfish (25). Glyceryl ether lipids are also found in human colostrum, human milk, and sheep's milk. An experimental study (32) showed that Human milk contains about 10 times more AKGs than cow's milk. The common composition of alkyl chains in AKGs from SLO of *Centrophorus squamosus* is as follow: chain lengths of 12 carbon atoms: 0 double chain, 1–2%; chain lengths of 14 carbon atoms: 0 double chain, 1–3%; chain lengths of 16 carbon atoms: 0 double chain, 9–13%; chain lengths of 16 carbon atoms: 1 double chain n-7, 11–13%; chain lengths of 18 carbon atoms: 0 double chain, 1–5%; chain lengths of 18 carbon atoms: 1 double chain n-9, 54–68%; chain lengths of 18 carbon atoms: 1 double chain n-7, 4–6%, and minor species (<1%) (33).

SLO extracted by different Indian Ocean shark species (*Carcharhinus obscures*, *Carcharhinus brevipinna*, *Carcharias Taurus*, *Carcharodon carcharias*) was examined in *vitro* on human colon Adenocarcinoma (Caco2) and P3X63.Ag8.653 mouse myeloma (653 cells) and on the normal cell lines, immortalized mouse fibroblasts (3T3) and human lung embryonic cells (MRC5), confirming an anti-proliferative effect. All the normal cell lines and most of the cancer cell lines, don't stop growing with some SLO concentrations (0, 40, 80, 120, 160 and 200 mg oil/l culture medium) and haven't significant effects on cell growth in response (1). Further studies are required to evaluate this hypothesis, given the highly complex nature of a natural oil.

The aim of this review is to describe the experimental (in *vitro,* in *vivo*) and clinical trials, published between 1962 and 2015, employing AKGs and derivates for treatment of cancer.

We searched Pubmed/Medline, Embase, Web of Science and Scopus search using the keywords "AKGs", "cancer", "squalene", "shark cartilage", "anti-squalene antibodies", "breast cancer", "HCC carcinoma", "chemotherapy", "radiotherapy".

2. Anti-tumor effect of Alkylglycerols

The anti-tumor effects of SLO and AKGs were evaluated in a model of solid tumor, Lewis lung carcinoma (3LL) murine tumor model, grafted in ten C57B1/6 mice (34). 3LL cells were inoculated intramuscularly into the leg of mice, that later were treated orally with olive oil (as control) and AKGs (100 mg/die per mouse for 10 days). The results showed that both treatments reduced significantly the cancer growth and the number of pulmonary metastases. The authors observed also that the most active antitumor AKGs molecules were 18:1, 14:0, and 16:1, but not the 16:0. The AKGs administration induced a significant reduction of the tumor blood vessel endothelial marker (–26 ± 9%) compared with olive oil–treated mice, with reduction of cancer neovascularization. Other possible mechanism for anti-tumor effect of AKGs is anti-neoangiogenic activity. AKGs reduced also the major angiogenesis stimulator (35), Fibroblast Growth Factor (Bfgf), on endothelial cell proliferation (36).

Six constituents of natural AKGs' mix, namely: 1) AKG 12:0=1-O-Dodecyl-***sn***-glycerol, 2) AKG 14:0=1-O-Tetradecyl-***sn***-glycerol, 3) AKG 16:0=1-O-Hexadecyl-***sn***-glycerol (chimyl alcohol), 4) AKG 18:0=1-O-Octadecyl-***sn***-glycérol (batyl alcohol), 5) AKG 16:1 =1-O-(Z)-90-Hexadecenyl-***sn***-glycerol, 6) AKG 18:1=1-O-(Z)-90-Octadecenyl-***sn***-glycerol (selachyl alcohol) were synthesized and their activities were compared with the in *vivo* model of solid tumors grafted in mice (37). The results showed that AKG 16:1 and 18:1 were the most potent compounds on tumor growth and lung macrometastasis number, the AKG 18:0 did not reduce but increased tumor growth and metastasis number.

In the experimental study, 27 Wistar rats divided in 3 groups (9 rats per group), supplemented with SLO (1 g/kg body weight), fish oil (FO) (1 g/kg body weight), SLO+FO (1g/kg body weight), respectively, for 8 weeks and inoculated subcutaneously with 1 mL of a sterile suspension of 3×10^7 Walker tumor cells (38). Later, the rats maintained the supplementation along additional 2 weeks. The biochemical and molecular assays (Serum glucose, lactate and triacylglycerol tests, HPLC, annexin V-FITC) showed that the SLO Supplementation reduced tumor growth (40%), proportionately to the increase of lipid peroxidation, apoptosis, and reduction of cancer cell proliferative capacity (35%). The long-life exposure to SLO increased the nitrite production by the peritoneal macrophages. The nitrite production determines the NO production, that could contribute to the reduction of cancer growth in SLO animals (39). The FO and FO-SLO supplementations reversed the cachexia parameters (anorexia, asthenia, anemia, weight loss, weakness, and intense peripheral catabolism with depletion of carbohydrate, lipid, and protein stores) to the control baseline. The levels of glycemia, triacylglycerolemia, lactatemia, and liver glycogen were similar in all the groups of non-tumor-bearing animals ($p>0.05$). While, the tumor-bearing rats presented a reduction of glycemia, hypertriacylglycerolemia, hyperlactatemia values and reduced liver glycogen content characterizing cachexia state.

The Association of SLO with FO showed results similar to those found in animals supplemented with FO alone, probably due to competition between n-3 polyunsaturated fatty acids (n-3 PUFA) and AKGs for incorporation in the cancer cell membrane.

The n-3-PUFAs display protective effects against some common cancers in animal models, such as Walker 256-bearing rat, suitable to investigate metabolic markers of anti-cancer activity against different histotypes (40). Female Wistar rats were fed with coconut oil (CO), oil composed by saturated fatty acids, or FO, at the value of 1g/kg body weight per day, and injected in the right flank with a sterile suspension of 2×10^7 Walker 256 tumor cells. In FO-supplemented rats, the tumor growth decreased by 60%, with consequent reduction of serum lactate, serum glucose, liver glycogen concentration at normal values. The

decrease of tumor growth might be due to enhanced lipid peroxidation and/or eicosanoid production within the tumor and/or altered host immune responses. The FO supplement may reduce cachexia reducing the inflammatory mediators that cause cachexia (41), or decreasing the arachidonic acid levels in host tissues and cancer (42): this would decrease the formation of prostaglandin E2 (PGE2) and related eicosanoids (43, 44). The n-3 PUFA concentration inhibits arachidonic acid metabolism (45, 46), and may suppress the PGE2 production within the tumor. Further studies are needed to analyze the antitumor and anticachectic effects of FO supplement.

3. Anti-tumor effect of Alkylglycerols mediated by immune response

The AKGs and non-adherent (B and T) cells have a crucial role of on the activation of macrophages (47). AKGs (10-100 ng) were injected intraperitoneally to Female BALB/c mice, 4-5 days before harvesting the peritoneal cells, whose Dodecylglycerol (DDG) was the AKG with the most potent effect of macrophage activation (48, 49), at the dose of 5 ng/mouse. In the same way, a similar dose range of a longer carbon chained AKGs, sn-3-octadecylglycerol (batyl alcohol), and BTA (10-100 ng/mouse) activated the macrophages. No pathological functions in the animals fed with different doses of DDG and BTA (50, 51) were observed. Although high doses of DDG are immunosuppressive (44), the animals were protected against infectious agents due to antibacterial activity of DDG. In the *vitro* study, however, Peritoneal cells are cultured and treated in *vitro* with a low concentration (50 ng/ml) of DDG, with subsequent macrophages activation in 2-3 hours. These data suggest that DDG treatment of B-cells determines the development of macrophage ingestion capacity (47). The effect of SLO on DTH test, tumor volume, percentage of $CD4^+$ and $CD8^+$T of tumor infiltrating lymphocytes and cytokine profile is also increased accordingly with Hajimoradi and coworkers (52).

Yamamoto *et al* (48) showed macrophage stimulation by lysophospholipids, and found that alkyl-analogues of lysophospholipids and neutral lipids for macrophage activation are potent macrophage-stimulating agents. 35 normal female BALB/c mice were injected subcutaneously with 1×10^8 sheep red blood cells (sRBCs) and divided into 6 groups. Five groups were administered intraperitoneally with progressive concentrations (50, 10, 5, 2.5 and 0.1mg/kg/day) of SLO for 5 days, and the control group was injected with Tyrode buffer and cooking oil (sunflower) in the same volume and time to the SLO-groups. Significant changes in immune function and tumor growth in SLO-groups were detected, suggesting a possible causal link between increased CD8+TILs and decreased tumor growth. The T-cell infiltration into the tumor usually decreases along tumor progression (49). The SLO can strongly increase $CD8^+$T cells infiltration, rather than than $CD4^+$ TILs which reduced the T ($CD4^+/CD8^+$) ratio. The possible benefit of high dose SLO-supplement for prophylaxis and treatment of disease in immune-compromised patients is worth of further investigation.

4. Protective effect of Alkylglycerols in patients who received radiation therapy

The AKGs are structurally enclosed into 1-*O*-alkyl-2-acyl-***sn***-glycero-3-phosphoethanolamine (RAcylGro*P*Etn) and 1-*O*-alkyl-2-acyl-***sn***-glycero-3-phosphocholine (RAcylGro*P*Cho) in rat intestinal mucosal cells and in several major organs (e.g. liver, kidney, lung tissues) (50, 51). The RAcylGro*P*Cho is the precursor for biosynthesis of platelet-activating factor, 1-O-alkyl-2-acetyl-***sn***-glycero-3-phosphocholine (PAF), a mediator involved in human pathophysiology (e.g. septic shock, asthma and allergy) and in biological activities (neuronal functions, circulation, inflammation, reproduction and fetal development) (50, 51, 53). The PAF is a modification of the ether-linkage of the fatty alkyl group at the ***sn-1*** position of the glycerol backbone; the chain acyl residue (acetate) at the ***sn-2*** position, or the phosphocholine head group at the *sn-3* position reduces the biological activity of the molecules (54). It has been found in the plasma of rabbits undergoing an anaphylactic reaction (55), in rat (56, 57) and human (56, 58) blood. In addition to the activation of platelets, PAF is a potent agonist for the activation

of polymorphonuclear leukocytes (59) and monocytes (60), it has marked vasocostrinction effects (61), and it stimulates glycogenolysis in perfused rat liver (62). PAF is synthesized, by various cells (including platelets, endothelial cells, neutrophils, monocytes, and macrophages), in two pathways: the remodeling and *de novo*: The first way is crucial in various inflammatory and allergic responses: an arachidonate-specific phospholipase A2 (PLA2) hydrolyzes arachidonate from 1-O-alkyl-2-arachidonoyl-***sn***-glycero-3-phosphocholine, producing lyso-PAF and free arachidonic acid. The latter is acetylated by acetyl coenzyme A (lyso-PAF acetyltransferase) in PAF. In the second pathway, 1-O-alkyl-***sn***-glycero-3-phosphate is acetylated by l-O-alkyl-***sn***-glycero-3 phosphate (acetyl coenzyme A acetyltransferase). Then, 1-O-Alkyl-2-acetyl-***sn***-glycero-3-phosphate phosphohydrolase produces 1-O-alkyl-2-acetyl-***sn***-glycerol that is converted to PAF by a dithiothreitol-insensitive CDP-cholinephosphotransferase (Figure 2).

PAF is degraded, in whole human blood, by hydrolysis of the acetyl residue at the ***sn-2*** position (54), being this reaction catalyzed by a specific phospholipases, the Plasma PAF acetylhydrolases (PAF-AH).

This enzyme, known also as LDL-PLA_2, associated mainly with low-density lipoprotein (LDL) and, to a lower amount, with high density lipoprotein (HDL) (63, 64), inhibits the proinflammatory activity of PAF. It might be crucial in atherosclerosis, since it is a Ca^{2+}independent PLA_2 belonging to group VII, which also degrades the short-chain ***sn-2***-analogs of phosphatidylcholine generated upon oxidation of LDL (65). PAF-AH activity and a panel of inflammatory mediators were measured in plasma of 496 patients with coronary artery disease (CAD), whose 276 patients suffering from stable angina pectoris (SAP) and 220 patients suffering from acute coronary syndrome (ACS), as well as in 477 healthy control patients, confirming the correlation of plasma PAF-AH activity with different markers of inflammation (e.g. acute phase reactants or proinflammatory cytokines) (66). The results showed that the plasma PAF-AH activity increased gradually ($p<0.0001$) in both SAP and ACS patients as compared with healthy controls. The CAD patients had a lower values of HDL, LDL and total cholesterol HDL, apolipoprotein A1 (apo A-I), tryglicerides, and elevated levels of inflammatory markers, including C-reactive protein (CRP), interleukin-6 (IL-6), and fibrinogen. The CAD patients with hypertension exhibited lower PAF-AH activity, a result that was unexplained, probably due to the type of treatment (e.g. ACE inhibitors or other anti-hypertensive drugs) or to the decrease in LDL values. The PAF-AH can be associated with various subclasses of LDL; however, it is more abundant in small, dense LDLs (64). This is probably the reason that in hypercholesterolemia, the PAF-AH levels are elevated in LDLs, however, they show a normal value in HDLs

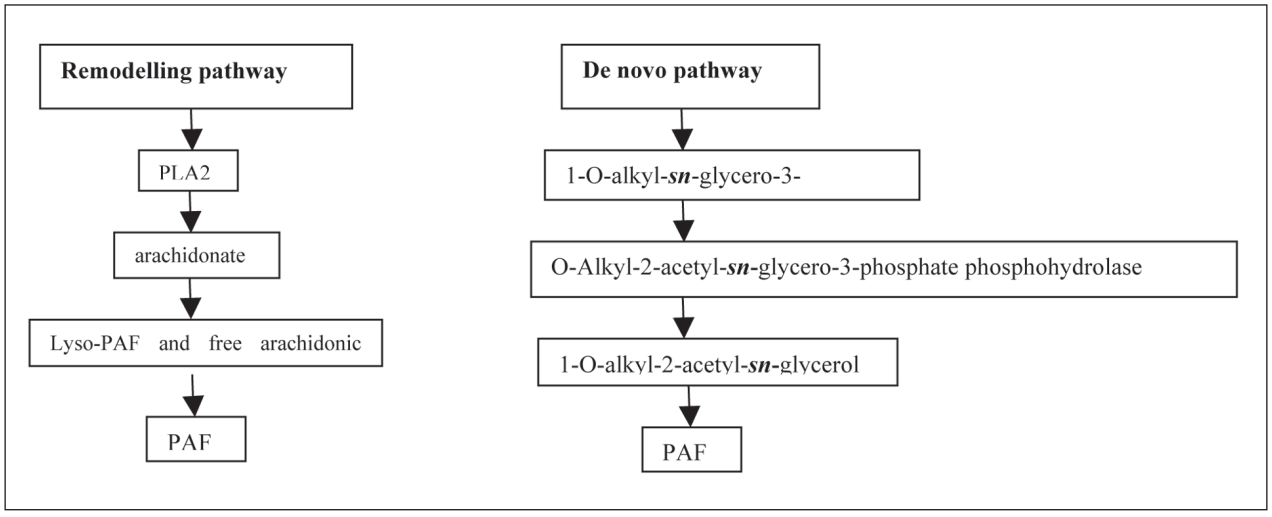

Figure 2. Description of the pathways for synthesis of PAF: Remodelling pathway and De novo pathway

(67). The association of PAF-AH with HDL is due to a glycosylation, which does not include its enzymatic activity, thus excluding the possibility that the altered activity of the enzyme is dependent on the transporting particle (64). Actually there are not trials supporting the hypothesis that PAF-AH associated with HDL would be more protective against atherosclerosis than that associated with LDL. Some studies (68-70) warn whether PAF-AH is simply a marker of risk or directly promotes atherosclerosis. In fact, in mild inflammatory and oxidative condition, PAF-AH supposed to prevent enzymatically against lipid oxidation and, in severe stress, to be converted in a proatherogenic factor that by releasing excessive levels of fatty acids or their oxidative products, increasing the inflammatory reaction. However, no association could be demonstrated between PAF-AH activity and inflammatory markers.

Further studies reported beneficial effects in cancer treatment, such as preventive action of AKGs on radiotherapy side effects, including leukopenia and thrombocytopenia (71-73).

Hichami and coworkers (74) analyzed the effect of AKGs incorporated in human promonocyte leukemia cell line THP-1 and its influence on PAF synthesis. THP-1 cells ($5x10^5$ cells/ml) were incubated for 48h with AKGs (10 μM, 24.82 mCi/mmol) and for 24h with AKGs (10 μM, 92.13 mCi/mmol), containing the C18:1 hydrocarbon chain, respectively. The cells were stimulated with the calcium ionophore A23187 (5Mm, 10 min) and treated with methanol C18:0 [^{14}C]PAF (10 000 dpm, 55 mCi/mmol) and 50 μg PAF were added as internal standard and carrier, respectively, to stop the stimulation. After 24h incubation and incorporation into phospholipids, the THP-1 cells produced 1.85±0.54 pmol [^{3}H]PAF/$2x10^6$ cells under resting conditions. After 10-min calcium ionophore, stimulation the [^{3}H]PAF increased significantly ($p<0.001$) to 3.58±0.7 pmol [^{3}H]PAF/$2x10^6$ cells. The AKGs incorporation induced the formation of three distinct [^{3}H]PAF molecular species: C16:0, 16:1, and C18:1 PAF and represented 15.2, 12.5 and 72.3% in resting cells, and 16, 15.4 and 68.6% in stimulated cells. The results showed, after 48h, a valuable decrease (by 32%) in RAcylGro*P*Cho-associated radiotherapy and an increase (by 15%) in RAcylGro*P*Etn-associated radiotherapy. An increase in C18:1 PAF represents the 9.07% of total PAF, and the fraction of C16:0 PAF drops to 88.3%. The authors suggest a conversion of RAcylGro*P*Cho into RAcylGro*P*Etn, confirming that AKGs increase the biosynthesis of PAF produced by THP-1 cells under resting and stimulated conditions.

The inadequate progress in chemotherapy of childhood brain tumors requires new approaches to overcome the blood-brain barrier (75). Recently evidence has emerged showing that AKGs increase the permeability of the blood-brain barrier (76). Antineoplastic agents, such as 4mg/kg of cisplatin (CDDP), 5mg/kg of methotrexate (MTX) and antibiotics (10 mg/kg of vancomycin and 3 mg/kg of gentamicin) were injected into the right internal carotid artery of 138 male Wistar tumor-free rats and of 12 C6 astroglioma bearing rats, in the absence and presence of 1-*O*-pentylglycerol (0.3 M) (77). The normal rats, showed, in the absence of AKGs, a low brain tissue concentration of each drug with no regional differences between the right hemisphere, the left hemisphere and the cerebellum and an increase of the tissue concentration in the ipsilateral hemisphere: 230-fold (MTX), 125-fold (CDDP), 15-fold (vancomycin) and 12-fold (gentamicin), after the administration of AKGs. In rats with C6 tumors, the 1-*O*-pentylglycerol increased the MTX concentration ($p<0.05$): 18-fold in the tumor and in the contralateral brain , 28-fold in the surrounding brain, and 19-fold in the cerebellum compared to MTX controls in the absence of AKGs. Hematological and serum analyses (sodium, potassium, calcium, glucose, total protein, aminotransferases, lactate dehydrogenase, bilirubin, and creatinine) turned out no acute toxic side effects of the monoglycerol analogues up to 0.3 M. However, the intracarotid administration of AKGs might be a new and very promising approach to increase drug delivery to brain tissue. AKGs are thus able in increasing cerebrovascular permeability in both normal and tumor tissues.

5. Cytotoxic and cytostatic effect of Alkylglycerols

The cytotoxicity and cytostaticity of AKGs were investigated *in vitro* in human OVP-10; MCF-7 and in DU-145, PC-3, and PCa-2b cell lines, propagated in Minimal Essential Medium (MEM) supplemented with 7% fetal calf serum (FCS) and antibiotics (17).

After 24 hours, the cells were exposed to Ecomer® (Natumin Pharma AB, Sandefjord, Norway): a SLO, with its standardized concentration of AKGs (20%) and methoxy-derivates (3%), for other 24h. The cells were also trypsynized to obtain a single-cell suspension, stained with Anexin V and propidium iodine (Apoptosis detection kit, Caltag Laboratories) for 20 min in darkness and subjected to cytometry (FACS VANTAGE device, Becton-Dickinson, USA). All the prostate cells showed a decrease in the colony number, even after low doses of Ecomer® (0.5 and 0.1 mg) per 1ml medium. The OVP-10 cells showed a minimal sensitivity, the MCF-7 displayed a moderate sensitivity, whereas all three lines of prostate cancer cells showed a high sensitivity to Ecomer®. However, the Ecomer- treated OVP-10 cells indicated cytostatic and apoptotic effects: they developed smaller colonies and displayed a higher percentage of apoptotic cells. The remaining cell lines underwent cytotoxic effect. Necrotic death of MCF-7 cells and apoptotic death of DU-145 prostate cancer cells were observed. The SLO contains, in addition to AKGs, also methoxy-derivates of AKGs and SQ, provided of anticancer effect (36). Wang *et al* (78) showed a differentiation-promoting effect of methoxyalkylglycerol (2 methoxyhexadecyl glycerol or MHG) in human colon cancer cells. These data asserted the therapeutic effect of AKGs, but additional studies are yet needed.

6. Different Alkylglycerols uptake in cancer cells

The main features of the lipid composition of cancer cells are: increase in AKGs and a difference in the composition of glycosphingolipids in the cell membranes (79, 80).

The lipid quality difference between cancerous and non cancerous cells form identical specimen remains to be defined (81). Lin and coworkers (82) compared the AKGs concentration of human hepatocellular carcinomas (HCC), with the corresponding composition of non-cancerous livers. They analyzed 30 tissue obtained, surgically at autopsy and by partial hepatectomy, from 18 specimens (9 cases of noncancerous liver and 9 cases of liver specimens with HCC). The results showed that all the 9 HCC-cases contain greater amounts of neutral O-AKGs than the normal tissues. The ratio of hexadecylglycerol, octadecylglycerol, and octadecenylglycerol was 1:2:2 in the non-cancerous livers and 2:1:1 in the tumor group. Compared to the noncancerous livers specimens, the main molecules found in the tumor group were: Hexadecylglycerol (16:0), octadecylglycerol (18:0), and octadecenylglycerol (18:1). HCC cells contained higher proportions of hexadecylglycerol (46.8±18.0) yet lower proportions of both C_{18} glyceryl ethers (26.0±10.9 and 23.4±8.8). The accumulation of glyceryl ether lipids in neoplastic tissues may be due to the active synthesis within the tumor (83, 84). The increase in neutral O-AKGs could be due to the loss of α-glycerol phosphate dehydrogenase activity in tumors (85), but it would not explain the observed change in their composition.

7. Toxicity study in rats with high doses of Alkylglycerols

The first standard toxicology study (2010) on an AKGs extract from SLO (11.9% fatty acid ethyl esters, 53.6% nonesterified alkoxyglycerols, 18.8% monoesterified alkoxyglycerols, 6.7% triacylglycerols, and 9.0% free fatty acids) evaluated 40 rats (20 males, 20 females) divided into 2 groups (20 each group): 1) the control received distillate water orally, 2) the treated group a single oral dose of AKGs once a day (1000 mg kg-1 of body weight), for 28 days (86). The rats of the second group were kept alive further 14 days for a follow up of the possible toxic effects. No treatment-related changes of organ weight, macroscopic clinical findings, hematological and clinical test parameters and no changes in the general fitness and body appearance in each group we were detected.

Further safety studies of AKGs in rats (e.g. a subchronic study, 91 days of daily oral gavage treatment) will fulfill the requirements for dietary supplement recommended dose in humans.

8. Squalene

Squalene (SQ), or 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane is a triterpenoid hydrocarbon oil ($C_{30}H_{50}$), a polyprenil compound

structurally similar to β-carotene. It is a symmetrical 30-carbon polyprenyl compound containing six isoprene units. SLO (*Squalus spp.*) is the main source of SQ, but it is also found in olive oil, palm oil, wheat-germ oil, amaranth oil, and rice bran oil (87). SQ, and essential fatty acids (88) have various pharmacological benefits, including chemoprotective properties against reactive oxygen species, anti-inflammatory, antibacterial, antifungal, and anticancer activity (89). It is also synthesized in the human liver and skin, transported in the blood by very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), and secreted by sebaceous glands (90, 91). It is a component of vaccine adjuvants, including MF59, an oil-in water emulsion developed by Chiron (92) and well-known to significantly enhance the immune response to a various vaccine antigens. The results showed that vaccines with the SQ-containing MF59 adjuvant emulsion do not induce any increase either in the titer or in the proportion of subjects with anti-squalene antibodies (ASAs) (93).

However, some studies focused on possible immunotoxic effects of squalene and reported that a single i.p. administration of squalene, even in the absence of antigens, can induce chronic autoimmune arthritis or lupus-like autoimmunity in non-autoimmune BALB/c mice and rats (94-98). Dupuis and coworkers, using squalene-based adjuvant MF59, emphasized a key role of macrophage trafficking and apoptosis and suggested that dendritic cells acquire antigen and adjuvant by uptake of the apoptotic macrophages (99). Another study showed that adjuvant enhanced survival of bone marrow-derived macrophages and even induced DNA synthesis (100). It could be supposed that squalene induces cytokine production via interactions with toll-like receptors (TLRs), monophosphoryl lipid A (MLA) and other lipid A mimetics via TLR4, poly I:C via TLR3, and CpG DNA via TLR9. The stimulation of different TLRs leads to dendritic cell maturation and induction of distinct Th responses (101).

Nevertheless, SQ's antiproliferative capacity and oxidative DNA damage protection was investigated in human MCF7 (highly invasive, oestrogen and progesterone receptor-positive) and MDA-MB-231 (minimally invasive, oestrogen and progesterone receptor-negative) cell lines, and an immortalized non-tumourigenic human mammalian epithelial cell line (MCF10A) (102). These cell lines were treated with different SQ-doses (0,3,6,12, 25, 50, 100, 200, 400 μM) for 24 h. The radical assays (DPPH, ABTS and ORAC assays) showed no SQ antiradical activity without any crucial effect on MCF10A, MCF7 and MDA-MB-231 cell turnover, except a slight not significant increase in MDA-MB-231 cell proliferation. The incubation of these cells for 24 h with SQ did not alter the cell cycle parameters, nor did it induce cell apoptosis. The SQ acted as an antioxidant only on mammary epithelial cells. Its antioxidant sensitivity can be due to an increase of glutathione (GSH) concentration in normal cells but not in breast cancer cells (103); or to differences in cellular uptake and accumulation of SQ (104); or to the variable regulation of antioxidant systems in normal cells (105). SQ can reduce oxidative stress by decreasing ROS levels and protect against oxidative DNA damage in mammary epithelial cells. However, it might be helpful in human breast cancer prevention. The SQ potentiates the citotoxicity and antitumoral activity of anticancer agents, including adriamycin (ADM), 5-fluorouracil (5-FU), bleomycin (BLM) and cis-dichlorodiamminoplatinum (CDDP). Combinations of these anticancer agents and SQ showed synergistic antitumoral effect in sarcoma 180 (S180) ascites cells (106).

The putative role of ASAs, found in high percentage in the Gulf War Syndrome (GWS) veterans and civilian personnel as an experimental immunological adjuvant, was analyzed (107). The GWS, unknown its origins, affected approximately 100,000 American and British individuals of the 700,000 veterans deployed in the Persian Gulf War (1990-1991). It is characterized by variable and non specific symptoms, including fatigue, emotional disorders, muscle and joint pains, headaches, memory loss, post-traumatic stress, recurrent fevers, allergies where none existed before (108, 109). Previous institutional studies run on Gulf War veterans have confirmed a strong link between Gulf War service and the occurrence of GW Syndrome (GWS) (110-114). 152 GWS patients who worked in the USA or the UK military forces (48 blood donors, 40 systemic lupus erythematosus patients, 34 silicone breast implant recipients, 30 chronic fatigue syndrome patients) were recruited. These patients had polymorphous autoimmune connective tissue, neurological symptoms and

arthritis (94%), fibromyalgia (94%), lymphadenopathy (94%), rashes (94%), weakness (86%), fatigue (81%), chronic headaches (78%), and memory loss (72%). The serum samples were treated with the ASA assay, which measures the binding of serum immunoglobulin (IgG) to SQ immobilized on nitrocellulose. The results showed that GWS patients had ASA responses in the range of intensity 1-4. The reactivity to SQ (range 2-3) was observed at a serum dilution of 1:400. The 95% of symptomatic deployed patients with GWS were positive on the ASA assay. The authors examined two volunteers for a vaccine trial including the SQ as adjuvant and they observed that also two patients developed a similar multisystem disease. One received a single injection and became sick within a few weeks with arthritis, fibromyalgia, lymphadenopathy, photosensitive rashes, fatigue, headaches, and fasciculations. He was found positive to ASA assay. Another patient displayed similar signs and positivity for ASA (3+). These data confirmed that the ASA reactivity is a marker of GWS. The pathogenetic role of ASAs is still unclear. SQ is an adjuvant to induce autoimmune diseases in experimental models (94, 115-119). It increases the levels of interleukin-5 (IL- 5), IL-6, and interferon-γ (120). The majority of SQ (60%) is absorbed naturally from food (121). It is synthesized as a precursor for cholesterol, myelin, and hormones within the hepatocytes and is processed into cholesterol in the endoplasmic reticulum (122). Further studies are required to define the role of ASAs. However, it is excluded any statistic meaningful link between ASAs and chronic multisymptoms reported by 579 Navy mobile construction battalion personnel (Seabees) that have been among the most symptomatic GWS veterans. Statistically, there was no significant association (p=0.465) between SQ status and chronic multisymptom illness status (123). According to the study of Asa and coworkers (107), SQ in pre-military vaccines probably induced an autoimmune disease that could explain many of the symptoms exhibited by GWS veterans.

9. Shark Cartilage

Shark Cartilage (SC) is an anti-inflammatory folk medicine remedy, used mainly for treatment of arthritis, osteoarthritis (124, 125), diabetic retinopathy and psoriasis (126). In a phase II open-label trial, 49 psoriatic patients fed with high SC-dose (240-mL/day) showed reduction of psoriatic plaques in a dose-dependent manner and a decrease (26%) of mean psoriasis area and severity index score (PASI) (127). SC, chemically, encloses proteins (e.g. troponin-I) tetranectin-type protein, collagenases, cartilage-derived inhibitor (CDI), tissue inhibitors of metalloproteinases (TIMPs); glycoproteins (shyrnastatin-1 and -2, galactosamine, glucosamine) and, glycosaminoglycans (chondroitin sulfate-D, chondroitin-6-sulfate, keratan sulfate). SC is extracted from spiny dogfish and hammerhead sharks in the Pacific Ocean, cleaned, shredded, dried, pulverized and sterilized. SC dust can be mixed with water or fruit juice and taken orally or it can be administered rectally as a retention enema. Some manufacturers reduce the cost, combining the supplement with bovine cartilage, that is also claimed to be an effective cancer treatment (128) and other manufacturers supply a products, composed by a small amount SC-extract and a large amount of fillers (129, 130).

A SC-fraction implanted between the V2 carcinoma and the limbus of the eye in 53 corneas of Wistar rats with pain and inflammation, by intramuscular and subcutaneous injection of acetic acid and formalin, displayed a weak anti-inflammatory and significant analgesic activity (131). In *vitro* the SC protects the cells pre-treated by hydrogen peroxide and carcinogens (e.g. 2-aminofulorene and sodium azide) against DNA damage and mutagenesis (132). Furthermore, the SC inhibits the cell migration, produces a dose-dependent decline in thymidine incorporation, the angiogenesis, and blocks collagenase-induced collagenolysis in human umbilical vein endothelial cell cultures (133, 134). The SC has also an anti-angiogenic effect and inhibits the tumor growth, such as carcinoma, sarcoma and melanoma (135-137).

In the follow-up of 21 advanced cancer patients fed with SC: 17 patients reported improvements in quality of life, whose 6 became tumor free, 6 exhibited reductions in tumor size, and 7 patients with prostate cancer reported reductions in prostate specific antigen (PSA) levels (138). In other study by Lane *et al* (139): 8 advanced cancer (stage III and IV) patients resistant

to previous treatments with life expectancies less than six months received SC (*Cartilage Technologies* Inc. Port Chester, NY) dosages (30g/day) subdivided to two or three oral or rectal doses, for 7 weeks. The results showed that 6 patients had an 80% or greater reduction in tumor size within 11 weeks of treatment; and no evidenced significant toxicities.

10. Potential toxicity of Shark Cartilage

Some studies suggest SC as a potential cause of allergic occupational asthma (140, 141). Ortega *et al* (141) reported the case of 38-year-old male workers died of asthma after 10 months of exposure to respirable dust. The co-workers, during shark industrial processing, were exposed to high breath dust concentration (0.92-5.14 mg/m^3) being "non-toxic" dust (limits: 5 mg/m^3). The symptoms of workers were: wheezing, coughing spells, and episodes of dyspnea during exertion at work. No immune imbalance or other toxic symptoms were detected in blood analysis.

In 2004, it was reported the first case of IgE-mediated occupational asthma induced by SC (140). A 29-year-old man, without history of asthma or respiratory disorders, worked in a shark based dietetic product industry for 7 years. After 2 years, he developed asthma, with chest tightness, cough, and dyspnea. These data suggest that SC dust is a potential asthma-inducing agent but the results of tests, including skin prick test, bronchial challenge test, and specific IgE results were available in this clinical note (141).

10.1 Putative anticancer effect of shark Cartilage extract: Urea

Several papers suggest a possible link between an high intracellular and extracellular level of urea in the sharks, and cancer immunization. The sharks use urea, in high intra and extracellular concentration, as osmotic adaptationin order to survive in hypertonic sea water; urea on the other side has an antimitotic effect: it disrupts DNA transcription and inactivates some proteins in marine elasmobranchs. Furthermore the sharks have higher amino-acid sequence preservation than the mammals (2). Some shark's proteins need urea for adaptation to cellular stress. According to the comparison between rates and patterns of amino-acid replacement between sharks and mammals, the shark's amino-acid replacement rates are 6 times slower than in mammals by sequencing mitochondrial cytochrome b gene (18). In elasmobranch fishes, the high level of urea (350-600 mM) is crucial in osmoregulation. Some reports have suggested that urea may be a novel and effective anti-tumor drug but without consistent demonstration (3, 4, 18).

Danopoulos and Danopoulou tried the urea treatment for primary and metastatic human malignancies of the liver (142, 143), for skin (144) and eye epibulbar malignancies (145, 146). In the cases in which the tumor has invaded the deeper layers of the bulbus oculi, urea was ineffective. The other drawback of this treatment is an corneal opacity if not protected. Recovery of normal transparence occurs 2-4 weeks after the treatment. Danopoulos (145) treated 3 patients with malignant epibulbar tumours: the first patient with 27 subconjunctival injections of 10% urea solution around the tumor, the second and the third with sterilized urea powder to the surface of the diseased eye, avoiding the cornea for 5-7 treatment sessions. He underscored that one of the main conditions for the hypothetical anticancer action of urea is that the tissue surrounding the tumor must be healthy. In fact, the edema of conjunctiva does not absorb urea, preventing any therapeutic effect. However, in all cases the regression of cancer decreased slightly after 2-3 urea treatment sessions.

On the other side, the urea and its derivatives (e.g. hydroxyurea, dimethylurea, and thiourea) are potential antioxidant cardioprotective agents against myocardium oxidative stress-induced damage including the post-ischaemia reperfusion-induced injury (147). In the 1999, Wang *et al* (147) examined *ex vivo* a dogfish shark heart under oxidative stress, induced by electrolysis and post-ischaemia Reperfusion, and the data compared with those obtained from 20 isolated rat hearts. It was argued that urea protects both the tissue homogenates and whole organ from the oxidative damage. This cardioprotective capacity of urea may be due to its activity to inhibit the production of the gaseous free radical nitric oxide (148, 149).

11. Conclusions

In the last ten years a worldwide skyrocketing use of herbs, brewer's yeast, algaes, bee pollen and royal jelly, fish oil, omega 3, essential fatty acid supplements, colost, psyllium seed husks, wheat germ, wheatgrass, and mushrooms (mainly shiitake and reishi varieties) was observed.

In the oncological setting, 56–73% of cancer patients regularly use multivitamin-multimineral, and herbal supplements (150-152). The most common claim of these herbal extracts and botanical products (including Echinacea, cranberry, ginseng, milk thistle, Astragalus, Kombucha tea, Spilanthes Acmella, garlic extract and the medicinal mushrooms) is the immune system stimulation (153-158). Several clinical trials confirm the anti-cancer activity of natural products, e.g. Lycopene, carotenoids, green tea, Resveratrol, allium sativum (159-164). SLO, a well-known dietary supplement, and it's main component AKGs, are further potentially useful nutraceuticals widely self prescribed, particularly in the Northern European Regions with the claim of many clinical benefits in dermatology, immunology and oncology popular medicine area; the compounds are generally very safe, and even if the clinical evidence based is quite scanty, the biochemical and preclinical experimental investigation are encouraging: anecdotal follow up of the self prescribing patients might be a further useful step to verify the treatment effectiveness.

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Received: 12.2.2016
Accepted: 9.5.2017
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