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# Influence of different lipid emulsions on specific immune cell functions in head and neck cancer patients receiving supplemental parenteral nutrition: An exploratory analysis

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ABSTRACT

*Objectives:* The effect of diet on immune responses is an area of intense investigation. Dietary lipids have been shown to differently influence and fine-tune the reactivity of immune cell subsets, thus potentially affecting clinical outcomes. Patients with head and neck squamous cell carcinoma face malnutrition, due to swallowing impairment related to the tumor site or to treatment sequalae, and may need supplemental parenteral nutrition (SPN) in addition to oral feeding when enteral nutrition is not feasible. Additionally, immune depression is a well-known complication in these patients. Parenteral nutrition (PN) bags contain amino acids, minerals, electrolytes and mostly lipids that provide calories in a concentrated form and are enriched with essential fatty acids. The aim of this study was to investigate multiple parameters of the immune responses in a cohort of patients with head and neck squamous cell carcinoma undergoing supplemental PN with bags enriched in ω-3 or ω-9 and ω-6 fatty acids.

*Methods:* To our knowledge, this was the first exploratory study to investigate the effects of two different PN lipid emulsions on specific immune cells function of patients with advanced head and neck squamous carcinoma. ω-3-enriched fish-oil-based- and ω-6- and ω-9-enriched olive-oil-basedSPN was administered to two groups of patients for 1 wk in the context of an observational multicentric study. Polychromatic flow cytometry was used to investigate multiple subsets of leukocytes, with a special focus on cellular populations endowed with antitumor activity.

*Results:* Patients treated with olive-oil-based PN showed an increase in the function of the innate (natural killer cells and monocytes) and adaptive (both CD4 and CD8 cells) arms of the immune response.

*Conclusion:* An increase in the function of the innate and adaptive arms of the immune response may favor antitumoral responses.



## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, representing 3% of all new tumor diagnoses in the general population [1,2]. HNSCC is associated with several alterations of the immune system, which ultimately lead to markedly depressed antitumor immunity [3]. In addition to a direct immune-suppressive effect of HNSCC, a complicating factor in this set of patients is malnutrition, due to swallowing impairment related to the tumor site (oral cavity; oro-hypopharynx) or to treatment sequalae (surgery; chemo-radiotherapy) [4,5].

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Due to malnutrition, patients experience a further decline of their immune defenses, which increases the incidence of infections, mortality, and treatment-related complications reducing, at the same time, the compliance of the patient to treatment [4,6]. Improving patients' nutritional status can help restore immunocompetence with consequent recovery of antitumor activity in patients with cancer [5–8]. Patients with HNSCC receive parenteral nutrition (PN) during chemoradiotherapy, when oral intake is compromised and enteral nutrition is not feasible [7,8]. PN bags contain mostly lipids that provide calories in a concentrated form and are enriched with essential fatty acids such as ω-3, ω-6 (polyunsaturated fatty acids [PUFAs]), and ω-9.

Dietary lipids, hence, also PUFAs, are not immunologically inert [9]. ω-3 fatty acids (FAs) have been shown to be immunosuppressive as they induce expansion of T regulatory cells (Tregs) while restraining natural killer (NK) cell activity [10]; also, they favor the differentiation of T-helper (Th)2 lymphocytes at the expense of the Th1 antitumoral subset. On the other hand, ω-6 FAs induce lymphoproliferation and production of proinflammatory interferon (IFN)-γ, interleukin (IL)-17A, and tumor necrosis factor (TNF)-α [11–13].

Therefore, lipid emulsions administered as PN to patients may also influence the immune response, with different mechanisms and effects [8,13]. Indeed, the clinical guidelines suggest caution in the use of lipid emulsions based on ω-6 class FAs in patients with uncontrolled inflammatory responses; these may instead be advisable in patients unable to develop an adequate immune (e.g., antitumoral) reaction. Similarly, several studies have shown beneficial effects of a diet enriched in ω-3 FAs in animal models of autoimmune disease confirming the ω-3 immunosuppressive effect [14]. Epidemiologic studies on the effect of ω-3 dietary supplementation have been mostly performed on the general population rather than on those with cancer, and the evidence points to a protective effect on the development of cancer. Indeed, these FAs interfere with chronic inflammation, favoring tumor prevention [15–18].

Moreover, in some clinical trials, the administration of ω-3-rich supplements to patients with cancer has shown to be associated with better outcomes [19,20], and in general the ratio between ω-6 and ω-3 PUFAs seems to play a role in cancer risk [21].

Given the effect that even small shifts in immune responses often determine in an organism, enhanced activation of cells known to be involved in anticancer responses may be exploited to further support antineoplastic therapies. The choice of an appropriate nutritional support regimen is a decision, which should take in account also the immunologic effects of lipids, some of which seem to facilitate desired proinflammatory responses. Hence, we performed the present exploratory study to investigate multiple parameters of the immune responses in a cohort of patients with HNSCC undergoing supplemental PN with bags enriched with ω-3 or ω-9 and ω-6 FAs.

## Methods

### *Patients*

Nine patients with HNSCC were recruited in the context of an observational study conducted at the Policlinico San Matteo of Pavia Italy, the Oncology Institute Veneto of Padova Italy, and at the Geriatric Hospital of Verona Italy, which assessed the 1-wk effects of tailored early supplemental PN (SPN) in hypophagic cancer patients at nutritional risk [22]. SPN was prescribed and infused to satisfy energy-protein needs according to estimated requirements, clinical, biochemical conditions, and residual oral feeding upon admission and was reassessed and possibly modified after 3 d to guarantee appropriate intakes [22]. Hence, an adequate amount of calories and amino acids was provided to the nine patients, whose PN treatment differed substantially only with regard to the lipid emulsions content. After signing the informed consent, blood samples were taken before (T0) and 7 d after (T1) the start of PN. Patients were given two different types of PN: Six patients were treated with bags containing FAs derived mainly from olive oil (80% olive oil - 20% soybean oil; Olimel N4 E, by Baxter Healthcare Corporation, Deerfield, IL, USA) and three were treated with bags containing FAs derived partially from fish oil (43% soybean oil, 35% olive oil, 22% fish oil enriched with ω-3; Peri-Smofven, by Fresenius Kabi, Italy,). Daily lipid intake for each patient ranged between 1 and 1.5 g/kg. To assess the immune cell responses to purified FAs in vitro, we recruited three healthy donors.

### *Cell stimulation*

Peripheral blood mononuclear cells (PBMCs) were prepared by standard Ficoll-density gradient centrifugation and suspended in RPMI 1640 complemented with penicillin and streptomycin (100 μg/mL and 100 μg/mL, respectively), L-glutamine (2 mM), and 5% autologous human serum. Maximal stimulation of NK and T cells was obtained following incubation of $2 \times 10^6$ PBMCs with phorbol 12-myristate 13-acetate (25 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (200 ng/mL, Sigma-Aldrich) for 6 h at 37°C- 5% carbon dioxide in the presence of brefeldin A (10 μg/mL, Sigma-Aldrich). After 6 h, PBMCs were washed and prepared for flow cytometric analysis.

To assess cytokine production by monocytes, 1 mL of whole blood was stimulated for 3 h at 37°C- 5% carbon dioxide with the Toll-like receptor agonist lipopolysaccharide (1 μg/mL, Sigma-Aldrich), in the presence of Brefeldin A (10 μg/mL, Sigma-Aldrich).

### *Cell stimulation with fatty acids*

To study the effects of individual FAs on immune cells in vitro, we incubated $1 \times 10^6$ PBMCs with α-linolenic acid (ALA; 0.01 μM), linoleic acid (LA; 0.01 μM), oleic acid (OA; 0.01 μM), and palmitic acid (PA; 0.01 μM; all by Sigma-Aldrich) dissolved in dimethyl sulfoxide (Sigma-Aldrich) for 24 h at 37°C- 5% carbon dioxide. After 24 h, PBMCs were washed and stained for flow cytometric analysis.

### *Flow cytometry*

Cells were washed in Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich) and labeled with antibodies directed to cell surface proteins along with a dead-cell discrimination reagent for 20 min at room temperature in the dark. All antibodies were titrated to determine optimal concentrations. After surface staining, cells were fixed in 4% formaldehyde for 5 min and made permeable with 0.5% saponin buffer before staining for intracellular cytokines. Samples were then incubated at room temperature for 15 min, washed in PBS and finally suspended in 100 μL of PBS for flow cytometric analysis. The following antibodies were used: CD39, CD25, CD3, CD38 (all by Biolegend, San Diego, CA, USA); HLA-DR, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, INF-γ, CD4, CD19, CD69, CD80, CD8 (all by BD Biosciences, Franklin Lakes, NJ, USA); CD16, CD56, CD127, CD11c (all by Beckman Coulter, Brea, CA, USA); IL-17, CD14, (all by ebioscience, San Diego, CA, USA); TNF-α (by Miltenyi Biotec, Bergisch Gladbach, Germany); live dead (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The fluorochromes used in the panels were fluorescein isothiocyanate, PerCP cyanine 5, PE, electron coupled dye, PC-5, PC-5.5, PC-7, allophycocyanin (APC), APC Alexa700, APC Vio770, Brilliant Violet (BV)421, BV480, BV605, BV650, BV785, Brilliant Ultraviolet (BUV)395, BUV496, and BUV661 [23]. Samples were acquired on either a CytoFLEX S or a LX flow cytometer (Beckman Coulter), equipped with three and six lasers, respectively, and able to measure up to 13 or 18 parameters simultaneously. For each sample, ~300 000 cells were selected based on scatter parameters and the analysis was conducted after exclusion of dead cells and doublets. Single fluorochrome-labeled beads (Versacomp Beads, Beckman Coulter) were used to set the compensation matrix for fluorescence spillover. The data was analyzed using FlowJo_V10 software (TreeStar, Ashland, OR, USA).

### *Immunogenicity of fatty acids*

We tested the capacity of polyunsaturated (ALA-ω3 and LA-ω6), monounsaturated (OA-ω9), and saturated (PA) FAs to stimulate cells of the immune system. Lipids were added to short-term cultures of freshly isolated PBMCs, and the expression of activation markers was evaluated on the surface of the different cell populations by flow cytometry. Following treatment with lipids, dead cells were always <5%.

### *Effects of PN on innate immune responses*

Freshly obtained PBMCs were challenged with maximal stimulation and cytokine production by different cell subtypes was measured by polychromatic flow cytometry. Given that patients were undergoing chemotherapeutic regimens; blood cell counts were very low. In order to obtain maximal information from these limited samples, we designed 13- and 18-color panels that enabled the simultaneous investigation of multiple cell subsets, including the measurement of activation markers and/or of cytokine production. With this comprehensive analysis, we could interrogate the phenotype and function of all major leukocyte subsets.

*Statistical analyses*

For multiple comparisons (Fig. 1), the two-way analysis of variance with Sidak correction was used. Otherwise, the Student's *t* test was applied (GraphPad Prism 7). $P \leq 0.05$ was considered statistically significant.

## Results

*Immunogenicity of fatty acids*

Exposure to FAs induced the expression of activation markers in cells of both the innate and adaptive arms of immunity (Fig. 1). Interestingly, the tested FAs do not uniformly stimulate all subsets: ALA and LA activate only NK cells, as detected by the statistically significant upregulation of CD25, whereas OA induces expression of the CD80 costimulatory marker on monocytes. PA, on the other hand, effectively activated monocytes and NK cells, and also induced expression of the activation marker CD69 on B cells (not shown).

Cumulatively, these data indicate that indeed FAs may modulate the activation of immune cell subsets, and that different cells respond to distinct lipid molecules.

*Effects of PN on innate immune responses*

NK cells from patients treated with olive-oil-based PN produced higher amounts of IFN-γ at T1 than at T0, showing a more vigorous response (Fig. 2A). In contrast, NK cells from patients treated with fish-oil-based PN did not show any difference between the two time points.

Within the cells of the innate immune system, the data shows that both classical and non-classical monocytes from patients treated for 1 wk with olive oil-based PN produced significantly higher amounts of TNF-α (Fig. 2B, C), a cytokine that stimulates the production of IL-1β and IL-6, enhances the action of NK cells, and induces apoptosis of neoplastic cells [24]. In contrast, in patients receiving fish oil-enriched PN, both subsets produced lower amounts of TNF-α at T1 compared with T0, although this reduction was not statistically significant.

This data shows that indeed the composition of the bags for PN may influence the quality of the immune cells response. One week of PN enriched in ω-9 FAs was sufficient to affect the functional response of NK cells and both classical and non-classical monocytes, inducing a significant increase in the release of proinflammatory cytokines; fish oil-enriched formulations, as could be expected given the high content of ω-3 FAs, conversely determined a reduction in the frequency of cytokine-producing cells. This suggests that olive-oil-based PN fosters protective antitumor responses mediated by IFN-γ and TNF-α.

*Effects of PN on adaptive immune responses*

Cells of the adaptive arm of the immune system play a crucial role in the control of cancer. Thus, we evaluated the effect of PN on the function of T lymphocytes. As shown in Figure 3, CD4+ from patients who had received olive-oil-based PN produced a greater quantity of IFN-γ at T1 compared with T0, thus showing an improvement in the immune response. In contrast, patients treated with fish-oil-enriched PN showed a decrease in the production of IFN-γ by CD4+ (Fig. 3A), even if not statistically significant. IFN-γ produced by CD4+ effector lymphocytes promotes the differentiation of Th0 to Th1 cells, which in turn produce IFN-γ with a positive feedback loop effect [25].

CD4+ cells of patients treated with olive oil-based PN also produced higher amounts of GM-CSF compared with T0, whereas no changes were observed in patients treated with fish oil-enriched PN (Fig. 3B). This cytokine promotes the activation and differentiation of antigen-presenting cells and increases the survival of monocytes, macrophages, neutrophils, and eosinophils. It exerts proinflammatory functions by increasing antigen presentation and the release of inflammatory cytokines [26], thus the finding of higher levels of GM-CSF in this setting may indicate a beneficial effect of olive-oil-based PN in patients in need of an active an efficient immune system.

We also measured the production of IL-2 by CD4+ lymphocytes (Fig. 3C). After 1 wk of treatment, IL-2 was increased in patients treated with olive-oil-based PN, whereas no differences were noted before and after the treatment with fish oil-enriched PN. Downregulation of IL-2 represents a characteristic of the HNSCC immune escape mechanism. IL-2 is necessary for the differentiation and expansion of T cells (especially for cytotoxic T lymphocytes [CTL] cytotoxic T cells), but also stimulates NK cells' proliferation and increases their cytotoxic activity [27].

Finally, we measured production of cytokines by CD8+ T lymphocytes (Fig. 3D). In patients treated with olive oil-based PN, we found a statistically significant increase in IFN-γ after 1 wk of SPN, whereas patients treated with fish-oil-enriched PN showed a slight but measurable decrease in IFN-γ production.

Healthy individuals produced comparable levels of cytokines as patients before olive- or fish oil-enriched PN, except for CD8+ T cells, which produced higher levels of IFN-γ; in this case, however, treatment with olive-oil-based PN restored IFN-γ production.

Taken together, these data point to a stimulatory effect on the immune system by ω-6-enriched olive oil-based PN compared with ω-3-enriched fish-oil-based PN. In the setting of cancer patients with aggressive disease, the increased activation and/or production of immune effector cells and molecules may represent an extra support in the fight against cancer.

*Effects of PN on Th1 and Treg cells*

Immune responses comprise cells that downregulate and shut down effector cells, to contain overshooting or unnecessary reactions. Tregs, characterized as CD4+CD25+CD127−, are powerful mediators of immune tolerance, and have been shown also to be involved in the suppression of antitumor responses. We found that

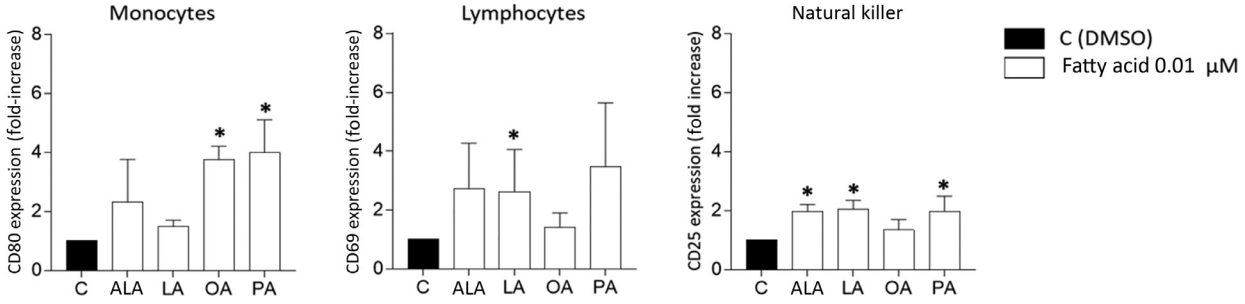


**Fig. 1.** Immunogenicity of lipid components. Activation markers were evaluated upon lipid challenge on innate and adaptive immune cells. ALA (ω-3), LA (ω−6), OA (ω-9), PA. Two-way analysis of variance multiple comparisons. *$P < 0.05$. n = 3. ALA, α-linolenic acid; DMSO, dimethyl sulfoxide; LA, linoleic acid; OA, oleic acid; PA, palmitic acid.

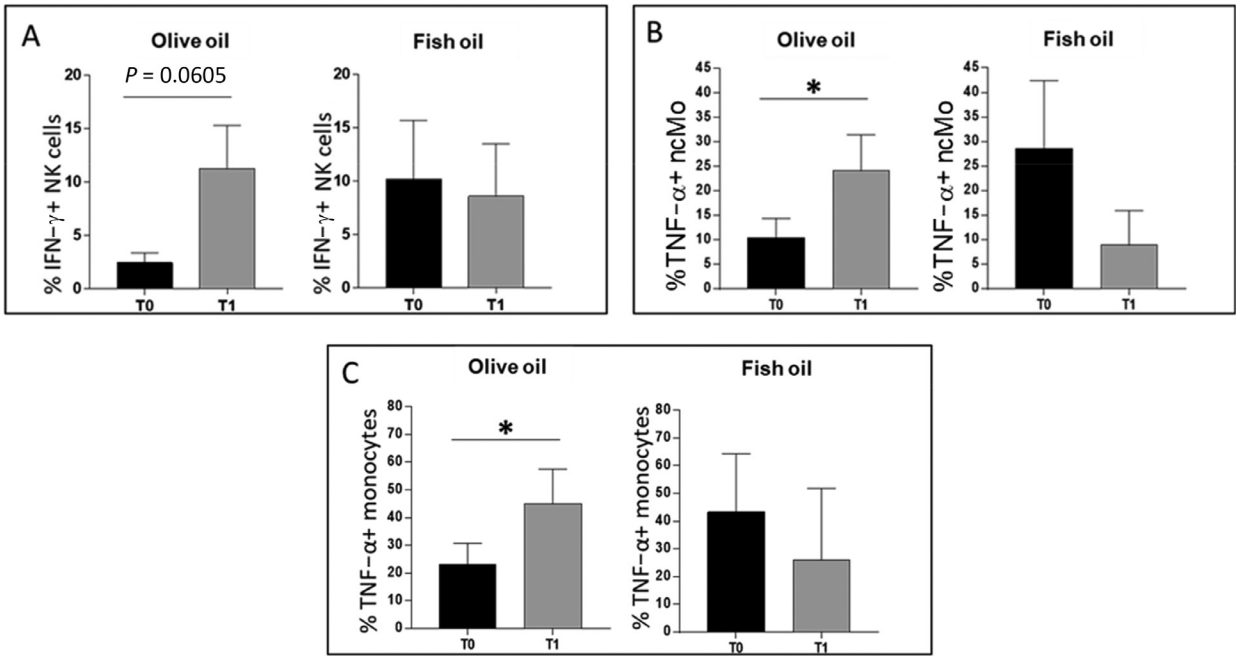


**Fig. 2.** Innate immune responses in patients with head and neck squamous cell carcinoma before and after administration of olive or fish oil PN. (**A**–**C**) IFN-γ and TNF-α production by NK, monocytes, and ncMos after stimulation with PMA and ionomycin before (T0) and after (T1) PN treatment. Analysis: Student's *t* test $^{*}P < 0.05$. IFN, interferon; ncMo, non-classical monocytes; NK, natural killer; PMA, phorbol 12-myristate 13-acetate; PN, parenteral nutrition; TFN, tumor necrosing factor.

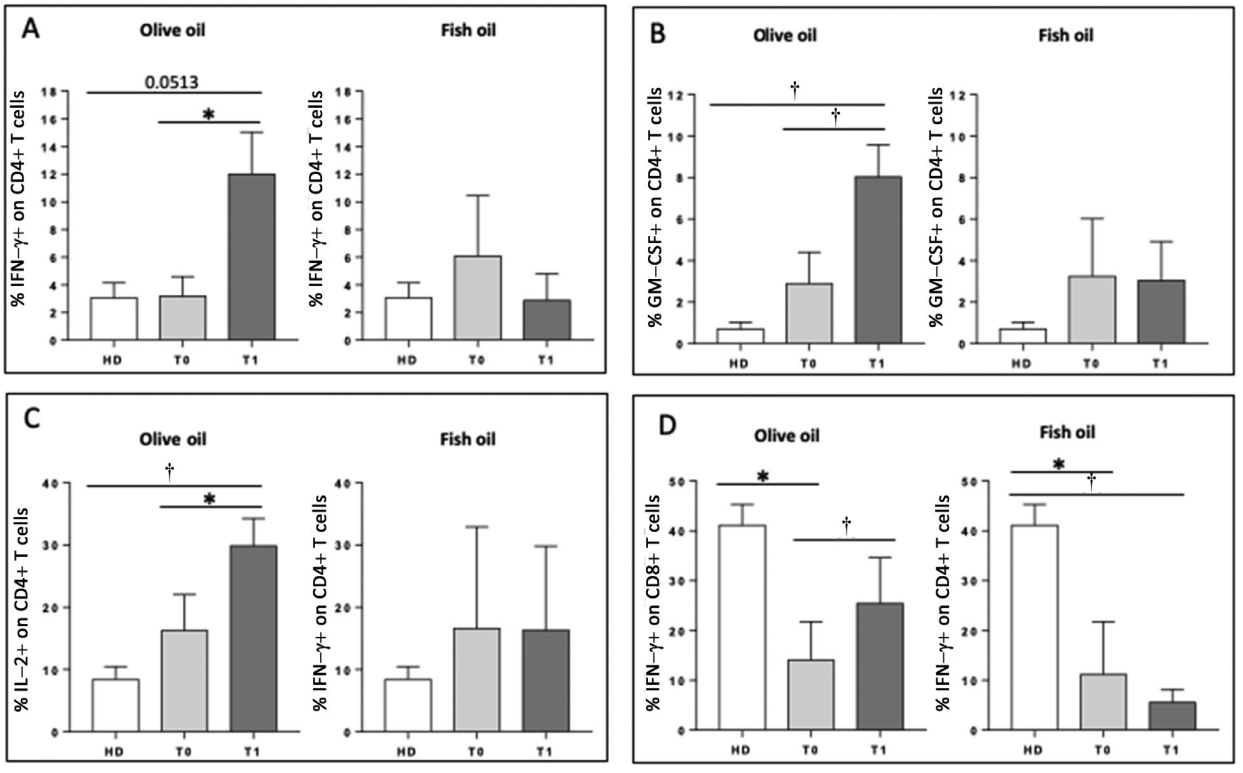


**Fig. 3.** Adaptive immune responses following stimulation with phorbol 12-myristate 13-acetate and ionomycin in HDs and in patients with head and neck squamous cell carcinoma before (T0) and after (T1) 1 wk of treatment olive oil- and fish oil-based parenteral nutrition. (**A**–**C**) Fraction of IFN-γ-, GM-CSF-, and IL-2-producing CD4+ cells. (**D**) Fraction of IFN-γ-producing CD8+ cells. Analysis: Student's *t* test $^{*}P < 0.05$; $^{\dagger}P < 0.01$. GM-CSF, granulocyte macrophage colony-stimulating factor; HD, healthy donor; IFN, interferon, IL, interleukin.

patients treated with olive oil-based PN showed a decrease in the frequency of Tregs cells after 1 wk of treatment, whereas in patients treated with fish-oil-enriched PN, Tregs frequency was unchanged (Fig. 4A). Interestingly, the Th1/Treg ratio, which indicates the tendency of the immune system to carry out an active proinflammatory response (Fig. 4B), showed that in patients treated with olive oil-based PN there was a clear imbalance in favor of the Th1 population, whereas in patients treated with fish-

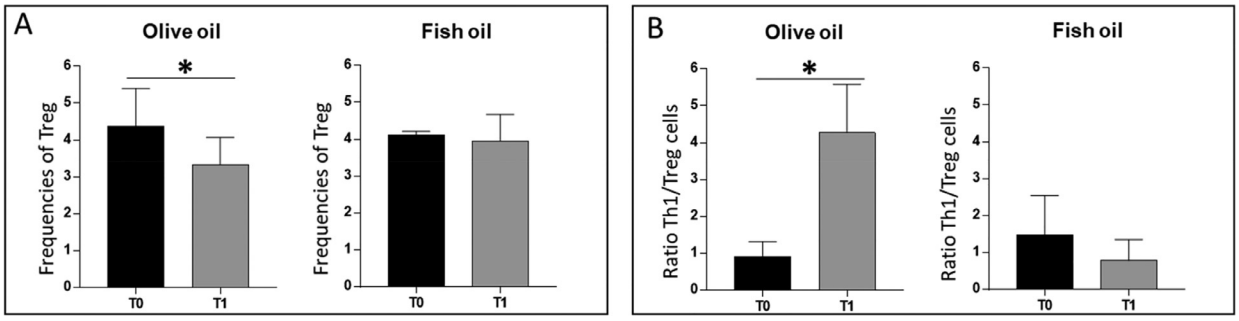


**Fig. 4.** (**A**) Treg cell frequencies before (T0) and after (T1) 1 wk of PN treatment. (**B**) Th1/Treg ratio before (T0) and after (T1) PN treatment. Analysis: Student's *t* test $^{*}P < 0.05$. PN, parenteral nutrition; T helper; Treg, T regulatory.

oil-enriched PN, the ratio was inverted in favor of Tregs. Thus, treatment with olive oil-based PN was able to increase the quota of IFN-γ-producing lymphocytes, while simultaneously reducing the frequency of immunosuppressive Tregs, potentially leading to a strengthened immune response [28].

## Discussion

Given the improvement in cancer therapies, patients now can survive for extended periods and some may need to be sustained with PN, to avoid malnutrition leading to cachexia with associated deterioration of the immune response. The components of the bags for PN contain glucose, amino acids, and lipids. Interestingly, the currently available PN bags differ mainly in the composition of lipids, and these have now been shown to be immunologically active [9]. The immune response plays a crucial role in the fight against cancer, thus the effects of lipids on the activity of immune cells are an important aspect that needs to be investigated. We performed an exploratory study using two different PN bags differing in the composition of FAs in a cohort of patients with HNSCC, in whom the issue of malnutrition is particularly prominent, also due to the cancer localization, which often involves swallowing impairment.

ω-3 FAs have been shown to have a mainly immunosuppressive effect through the modulation of molecular pathways leading to inflammation or through the inhibition of the production of proinflammatory cytokines [29–31], and they have been shown to be protective in inflammatory conditions in animal models of disease [32,33]. On the contrary, ω-6 and ω-9 FAs have a rather proinflammatory effect, as exemplified by the increase in inflammatory diseases in contemporary populations following diets rich in animal fat [34,35]. This proinflammatory activity may be useful to favor the functions of antitumor responses. Indeed, we found that peripheral blood cells from healthy individuals incubated in vitro with ω-6 and ω-9 FAs showed increased levels of activation. We found that monocytes upregulated CD80, a molecule that is necessary to provide co-stimulation to responding T cells and that is rapidly exposed on the cell surface after activation [36,37]. Activation of T lymphocytes was instead detected, in the same cultures, through the increased expression of CD25, the IL-2 receptor, which binds the IL-2 required for their proliferation and expansion [38].

The study of cells isolated ex vivo from patients undergoing different regimens of SPN was also highly informative, and revealed that indeed cell populations with antitumor activity may have significantly enhanced functions already 1 wk after administration of olive oil-based PN. NK cells are main players in cancer immunosurveillance, a task they achieve through the release of cytotoxic granules and through the rapid production of IFN-γ [39], thus an increase in the release of this cytokine denotes enhanced antitumoral competence. Indeed, the best known effect mediated by IFN-γ is the reinforcement of the cytotoxic function of NK cells and CTLs as potent effectors of antitumor responses. It is also known that IFN-γ improves the antigenicity of tumor cells through the upregulation of the expression of major histocompatibility complex class I (MHC) molecules. Because tumor cells express antigens that differ from their untransformed counterparts (such as neo-antigens resulting from gene mutations, overexpressed cellular antigens, or viral antigens), IFN-γ-induced expression of MHC molecules may increase the immunogenicity of cancer cells making them susceptible to immune recognition and destruction. IFN-γ also shows direct antitumor activity through the inhibition of tumor cell proliferation [25].

TNF-α was identified as a cytokine able to induce tumor regression (hence its name) [40], through the induction of apoptosis and necrosis on tumor cells and the activation of immune cells. GM-CSF, on the other hand, acts mainly on myeloid cells favoring their differentiation and activation and promoting the function of proinflammatory cells of the immune system [41]. We found that these cytokines were produced at higher levels in patients who received olive oil-based PN bags with higher ω-9 FA content. These subtle but measurable shifts in the proinflammatory "habitus" of the immune system secondary to the intake of different FAs confirm the ability of dietary components to influence immune, potentially anti tumoral, responses. Indeed, several epidemiologic studies have shown the beneficial effects of ω-3 on cancer prevention in different disease settings [15–18].

## Conclusion

These exploratory data point to an activation of cells involved in the antitumoral immune response induced by PN with lipid emulsions enriched in ω-6 and ω-9 FAs. It is indeed possible to foresee that, in the near future, the individualized choice of specific nutrients could allow the patient to deal with chemotherapy/immunotherapy/radiotherapy/surgery with less toxicity, but also to obtain an advantage of response and therefore of survival. In this respect, it is not clear how clinical nutrition with different lipids could be synergistic or detrimental for the novel drugs that stimulate the immune response to fight cancer cells. Thus, further, properly sized similar studies in different cancer types are warranted.

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