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ORIGINAL CONTRIBUTION

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Short-term fish oil supplementation improved innate immunity, but increased *ex vivo* oxidation of LDL in man – a pilot study

Summary *Background:* Fish and fish oils are rich in the two long-chain polyunsaturated fatty acids (LCPUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The n-3 LCPUFAs have been reported to have beneficial effects on cardiovascular functions, but their role in relation to immune functions is still controversial. *Aim of the study:* The objectives of this study were to determine the effects of supplementation with fish oil on immune cell functions in human subjects. We have also assessed the effects on plasma lipids, antioxidant status and susceptibility of low-density lipoproteins (LDL) to oxidative stress. The antioxidant status was determined by measuring plasma vitamin C, tocopherols and carotenoids in plasma and LDL, and superoxide dismutase (SOD) in red blood cells. *Design:* For 30 days, 10 volunteers ingested 25 g/d of either fish oil, providing n-3 LCPUFAs (7.5 g), or high-oleic sunflower oil, providing monounsaturated fatty acids mainly as oleic acid (22 g). The oils contained similar profiles of tocopherols. At day 0 and day 30, blood samples were drawn by venipuncture for plasma lipid and antioxidant analyses and lipoprotein isolation, and for isolation and functional tests of mononuclear cells and granulocytes. Fatty acid profiles of immune cells and LDL were also determined. *Results:* Fish oil supplementation resulted in an accumulation of n-3 LCPUFAs (EPA, DHA) in LDL and immune cells. The phagocytic activity, a measure of immune cell activity, was increased in both groups. Whereas the plasma and LDL antioxidant status do not appear to be affected by fish oil supplementation, an increased susceptibility of LDL to oxidation was observed in these healthy volunteers. *Conclusions:* The optimal amounts of n-3 fatty acids required to modulate immune functions remain to be established. In addition, adequate levels of antioxidant protection need to be provided during fish oil supplementation.

Key words Fish oil – High-oleic acid oil – Immune – Lipoproteins – Phagocytosis – Respiratory burst – Human

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Introduction

In recent years, interest has focused on the role of n-3 fatty acids on immune function. The functional changes in response to dietary fat treatments are thought to be mediated partially via changes in cell membrane phospholipid fatty acid composition. Results in the literature are not consistent as both immunostimulation and immunosuppressive effects of n-3 polyunsaturated fatty acids (PUFAs) have been reported as reviewed by Erickson and Hubbard [1]. Neutrophils and mononuclear cells, which play a role in the processes of chronic inflammatory disorders and on atherosclerotic disease, exhibit reduced activity following fish/fish oil ingestion. Clinical benefits on inflammatory diseases, such as rheumatoid arthritis and psoriasis, have been reported; however, these benefits are mainly observed with high doses of fish oil.

Although the immunosuppressive effect of fish/fish oil may be beneficial for the prevention and treatment of atherosclerotic and inflammatory diseases, it may be

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detrimental during infection [2]. The mortality rate of mice given a very low oral challenge with *Salmonella typhimurium* was higher when the animals were fed fish oil rather than corn or hydrogenated coconut oils, or low fat chow diet [3, 4]. Furthermore, Chang et al. [3] and Mayatepek et al. [5] have demonstrated bacterial translocation to the spleen following fish oil ingestion. In contrast, Barton et al. [6] observed a decreased mortality in a rat model of chronic sepsis following menhaden oil ingestion.

The PUFAs are highly susceptible to lipid peroxidation and as such may contribute to an overall enhancement of prooxidative stress in the body [7–9]. A number of studies have shown that enrichment of n-3 PUFAs in the diet, even in low amounts, may result in lower concentrations of endogenous antioxidants such as vitamin E, and may indirectly affect other labile micronutrients such as retinol [10]. The susceptibility of n-3 fatty acids to oxidation may be modulated by concomitant administration of antioxidants such as vitamin E [8, 11, 12].

The aethiology of cardiovascular diseases is multifactorial; plasma total cholesterol and LDL cholesterol levels as well as LDL oxidation are all risk markers for developing CVD. Low-density lipoproteins (LDL) that have been modified by oxidation have been implicated not only in the development of atherosclerosis, but also in inflammatory processes. Esterbauer et al. [13] and Steinberg et al. [14] have demonstrated the importance of evaluating the resistance of LDL particles to an oxidative stress. The susceptibility of LDL towards *ex vivo* oxidation is used to determine the influence of different dietary fatty acid patterns on oxidative status.

In this study, a fish oil supplement that provided n-3 PUFAs similar to that found in immuno-modulatory clinical products was compared to a high-oleic sunflower seed oil supplement (Trisun) in human volunteers. Both supplements had a similar content and profile of tocopherols, provided at nutritional levels. Changes in plasma lipids, immune cell functions, antioxidant status and susceptibility of low-density lipoproteins (LDL) to oxidative stress were examined. The fatty acid compositions of immune cells and LDL were also determined.

Materials and methods

Materials

High-oleic sunflower oil (Trisun) was supplied by Oleificio SABO (Manno, Switzerland), and the fish oil of the ROPUFA "30" n-3 EPA oil type was from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Covi-ox® T70 was from Chemische Fabrik Grünau GmbH (Illertissen, Germany), d-alpha-tocopherol 1000 was from Eisai Pharma-Chem Europe Ltd. (London, UK). The microvettes were purchased from Sarstedt (Sevenen, Switzerland). Adenosine diphosphate (ADP), and Histopaque 1119 and 1017 were purchased from Sigma (St. Louis, MO, USA). Collagen was supplied by Horm-Chemie München GmbH (Germany), the adrenaline was from Sintetica (Switzerland), and heparin came from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Phosphate buffered saline 10 x (Gibco/BRL-Life technologies, Paisley, Scotland) was diluted with sterile distilled water prior to use.

Methods

Study design

Ten healthy men, with an average age of 50 yr. (range 46–55 yr.) and a body mass index (BMI) of 25 ± 1 kg/m2 (mean ± SEM), were randomised into two groups (5 subjects/group). Exclusion criteria included the existence of pathologies interfering with immune functions (i. e. inflammatory diseases), haemophilia, hypertension, hypercholesterolaemia, and hypertriglyceridaemia. None of the subjects was taking antioxidant supplements or were on drug therapy from two weeks before starting the study until its completion. Volunteers consumed either high-oleic acid (18:1n-9) sunflower seed oil (Trisun) or fish oil, rich in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), at a dose of 25 g per day for 30 days at lunch time. One subject from the Trisun supplemented group had to withdraw from the study due to drug therapy. The Human Ethical Committee of Nestlé Ltd. granted approval for the study.

Trisun oil supplied 20.7 g of oleic acid per day, while the fish oil supplement supplied 4.3 g and 2.8 g per day of EPA and DHA, respectively. The α-, β-, γ-, and δ-tocopherol contents of the oils were adjusted, using Covi-ox T70 and d-alpha tocopherol 1000, to provide similar amounts in the two treatment groups, and corresponded to an intake of 1.4 mg and 1.2 mg of RRR-α-tocopherol equivalents per g of Trisun oil and fish oil, respectively. The ratio of tocopherol to unsaturated fatty acids (mg/g) was 10.9 mg and 3.3 mg for the Trisun oil and the fish oil supplemented groups, respectively. Both values are higher than 0.4 mg/g, which is the US RDA for PUFA protection against oxidation [15]. The fatty acid composition and tocopherol profiles of both supplements are presented in Tables 1 and 2.

At day 0 and day 30, samples of blood were drawn by venipuncture after a 12 h overnight fast. The blood was subsequently used for plasma tocopherols and carotenoids measurements, plasma lipid analyses, lipoprotein preparation, and for isolation and functional tests of mononuclear cells and granulocytes. The fatty acid compositions of immune cells and LDL were also assessed.

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Tab. 1 Fatty acid composition of the high-oleic sunflower seed oil (Trisun) and the fish oil supplements[a]

Fatty acids (weight%)	Trisun	Fish oil
C14:0	ND	6.9
C16:0	3.7	17.1
C16:1n-7	0.1	8.2
C18:0	3.9	3.3
C18:1n-9	78	11.0
C18:2n-6	12.4	2.7
C18:4n-3	*ND*	2.2
C20:3n-6	*ND*	1.0
C20:5n-3	*ND*	16.1
C22:0	1.0	0.1
C22:5n-3	*ND*	1.9
C22:6n-3	*ND*	10.6
Sum SAT	9.2	29.1
Sum MUFA	78.3	25.3
Sum PUFA	12.5	36.6
Sum (n-6)	12.5	5.0
Sum (n-3)	0.0	31.6
PUFA/SAT ratio	1.4	1.3
n-6/n-3 ratio	–	0.2

[a] Only fatty acids representing 1.0 weight% or more are presented.
ND not detected

Tab. 2 Tocopherol content of the high-oleic sunflower seed oil (Trisun) and the fish oil supplements

Tocopherols (mg/100 g)	Trisun	Fish oil
α-Tocopherol	129.0	114.0
β-Tocopherol	1.2	0.7
γ-Tocopherol	60.3	65.4
δ-Tocopherol	17.5	18.5
Total tocopherols	208.0	198.6
TE[a]	136.3	121.6
TE/PUFA (mg/g)	10.9	3.3

[a] *TE* RRR-α-tocopherol equivalents

Phagocytic activity of peripheral blood cells

Phagocytic activity of monocytes and granulocytes was quantitatively determined in whole blood using flow cytometry and fluorescein isothiocyanate-labeled opsonised *Escherichia coli* sp. according to the manufacturer's procedure (PHAGOTEST™; Becton-Dickinson, Basel, Switzerland). Briefly, 100 µl of fresh heparinised whole blood were mixed with 20 µl of an *E. coli* bacterial suspension (1 x 10^9/ml). This mixture was then incubated for 10 min at 37 °C in a controlled shaking water bath, followed by quenching to remove free floating or bacterial cells attached to, but not ingested by, the leukocytes. The blood was then lysed and fixed, and DNA staining with a propidium iodide solution (100 µl) performed. Measurements were with a FACScan™ flow cytometer using a blue-green excitation light (488 nm). During data acquisition with LYSIS II™ software (Becton-Dickinson), a live gate was set in the red fluorescence histogram such that the only events considered were those positive for propidium iodide staining, corresponding to human diploid cells.

Oxidative burst of leukocytes

Oxidative burst of leukocytes was quantitatively determined in whole blood using flow cytometry. Cells were exposed to opsonised *E. coli* sp. and to a non-fluorescent substrate (dihydrorhodamin 123), which becomes oxidised via oxidation by cellular enzymes and superoxide ion to a fluorescent product (rhodamin 123). The test was performed according to the manufacturer's procedure (BURSTTEST™; Becton-Dickinson, Basel, Switzerland).

Briefly, 100 µl of fresh heparinised whole blood were mixed with 20 µl of an *E. coli* bacterial suspension (1 x 10^9/ml). This mixture was then incubated in a controlled shaking water bath for 10 min at 37 °C. The substrate solution (20 µl) was then added and the mixture further incubated for 10 min to allow oxidation of the substrate. The blood was then lysed and fixed, and DNA staining with a propidium iodide solution (100 µl) was subsequently performed. Measurements were as for the PHAGOTEST procedure.

Leukocyte separation

Blood (9 ml), collected in a vacu-tube containing Na-citrate, was centrifuged at 150×g, 20 min at room temperature. The supernatant (platelet-rich plasma) was collected and then centrifuged at 1000×g for 15 min at room temperature. PBS was added to the red blood cells and leukocytes to a final volume of 14 ml, and the mixture gently mixed. Leukocytes were separated according to Boyum [16] and to English and Andersen [17] with slight modifications. The diluted cell suspension was carefully layered on a Histopaque gradient consisting of 7.5 ml of each of Histopaque 1119 and Histopaque 1077, respectively. The gradient tube was centrifuged at 700×g, 30 min at 22 °C. Bands containing the mononuclear cells and the granulocytes were then aspirated and transferred into 15 ml Falcon tubes. The cells were washed in 10 ml PBS and centrifuged at 200×g, 10 min at 22 °C.

The granulocytes were resuspended by gentle aspiration with a plastic Pasteur pipette prior to the addition of a 5 ml NaCl solution (0.2 %) to create an osmotic shock to lyse the contaminating red blood cells [18]. After 20 s of gentle mixing, a 5 ml NaCl solution (1.6 %) was added followed by 2 ml of PBS. Once the cells were well resuspended, the tube was centrifuged at 200×g, 10 min at 22 °C. Cell pellets, from both granulocytes and mononuclear cells, were washed once more as above, then resuspended in 1 ml of PBS before lipid extraction.

Lipid extraction

Lipids from granulocytes and mononuclear cells were extracted by the method of Bligh and Dyer [19] with slight modifications. Chloroform:methanol (1:2, by vol.; 3.75 ml) was added to the cell suspension (1 ml) to which 3.5 µg of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine standard had previously been added. Chloroform (1.25 ml) and 0.9 % KCl solution (1.25 ml) were sequentially added with thorough mixing between each step. The mixture remained at 4 °C overnight. The tube was then centrifuged and the lower chloroform phase containing the lipids was collected. This organic phase was dried under N2, the lipids resupended in 200 µl of chloroform:methanol (2:1) and then stored at –80 °C under N2.

Total phospholipid fatty acid analyses

Phospholipids were separated from neutral lipids by thin layer chromatography using hexane:diethylether: acetic acid (70:30:1, by vol.) as the solvent system [20]. Fatty acids from the phospholipids were converted to their metyl esters in a 6 % H_2SO_4 methanolic reagent (3 ml). Fatty acid methyl-ester separation was performed by automated gas-liquid chromatography (FISON GC 8000 series) using a DB-Wax column (30 m × 0.32 mm I. D.; J&W Scientific, Folsom, California, USA) with a 2 m retention gap. Hydrogen (55 Kpa) was used as the carrier gas with an on-column injection mode. The initial oven temperature of 40 °C, maintained for 2 min, was increased by 15 °C/min up to 145 °C, and held for 1 min; then increased by 4 °C /min up to 227 °C and held for 5 min prior to a further increase of 1.5 °C/min up to 250 °C. This final temperature was held for 24 min. Fatty acid methyl-esters were detected by FID (320 °C); authentic standard mixtures of fatty acid methyl-esters were injected to identify fatty acid methyl-esters peaks.

LDL total lipid analyses

Total lipids from LDL suspensions (400 µl) were extracted using triheptadecanoyl-*sn*-glycerol (100 µg) as an internal standard followed by trans-esterification similarly to the procedure described above. Fatty acid methyl-esters were resuspended in hexane (300 µl) prior to analysis by capillary gas-liquid chromatography.

Plasma antioxidant analyses

Vitamin C was determined in plasma, which was previously deproteinised with perchloric acid, by RP-HPLC (Hewlett Packard 1090 LC) using a Nova-Pak column (3.9 × 300 mm) with a mobile phase of potassium dihydrogen phosphate (5.6 g/L, pH 2.5), and quantified using an electrochemical detector LC–4A at 0.7 V and 500 nA (Bioanalytical System Inc.) by the method of Kimoto et al. with slight modifications [21].

The α-, γ-tocopherols and carotenoids were assayed in plasma, in lipoproteins and in the oils by RP-HPLC [22] using a Nova-Pak column (3.9 × 300 mm) and a mobile phase consisting of acetonitrile/tetrahydrofuran/methanol/ammonium acetate 1 % (533.5/193.6/53.7/28; by weight). Tocopherols were detected at 297 nm and carotenoids at 450 nm.

The superoxide dismutase (SOD) activity in RBC was assayed as reported by Belsten and Wright [23].

Plasma analyses of triacylglycerols, total cholesterol and HDL cholesterol

Triacylglycerols, total plasma cholesterol and HDL cholesterol, were measured enzymatically using the "Triglycerides Enzymatique PAP 150" kit, the "Cholesterol Enzymatique PAP" and the "HDL cholesterol/ Phospholipids" kits (bioMérieux, Lyon, France), respectively, according to the manufacturer's protocol. All analyses were performed using a Centrifugal autoanalyser (Cobas Fara; Roche Diagnostica, Basel, Switzerland).

Lipoprotein isolation

Plasma VLDL (density < 1.006 g/ml), LDL (1.006 < d < 1.063 g/ml) and HDL (1.063 < d < 1.21 g/ml) were isolated by sequential ultracentrifugation [24]. Lipoproteins were aliquoted and stored at –80 °C until required for the determination of their antioxidant content as well as for the characterisation of the LDL fatty acid profile.

Determination of LDL susceptibility to oxidation

For evaluating the susceptibility of LDL to an *ex vivo* oxidation, LDL isolation was rapidly performed in order to minimise the formation of oxidized products. LDL isolation was performed by ultracentrifugation with a discontinuous gradient density [25]. An aliquot of LDL (80 µg cholesterol) was immediately desalted and subjected to *ex vivo* oxidation initiated with cupric ions (1.7 mM final concentration) at 37 °C. The formation of conjugated dienes (CD) was recorded at 234 nm [26].

Statistical analyses

Comparisons of the group means, as well as the means of the difference between day 30 and day 0 (degree of change or net change), were analysed by Student's t-test. The latter comparison is particularly important; the mean values for a given time point may be statistically different between groups, while the degree of change occurring in each group with dietary treatment may be

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similar. Comparisons of the means within a group were analysed by paired t-tests. A p value ± 0.05 was considered significant. Statistics have been performed using Systat® version 7.0.1 for Windows®.

Results

There were no difference between groups for any of the parameters measured at the beginning of the study (data not shown). No change in body weight occurred during the course of the study (data not shown).

Phagocytic activity and oxidative burst of peripheral blood cells

Monocyte phagocytic activity was increased post-treatment by 24% and 12% in the Trisun and fish oil fed groups, respectively; Granulocytes from the fish oil supplemented subjects exhibited a small but significant, 3% increase in phagocytic activity (Fig. 1A and B). The respiratory burst activity was not altered by dietary treatment (data not shown).

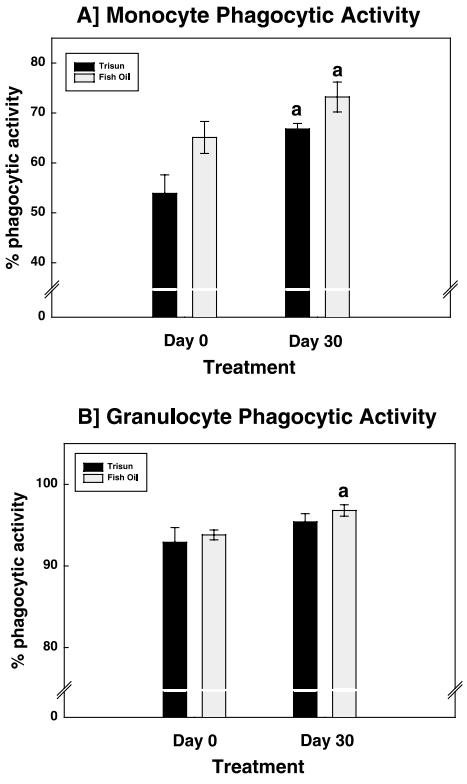

Fig. 1 Phagocytic activity of peripheral blood cells from subjects who consumed either high-oleic sunflower seed oil (Trisun) or fish oil supplements.
Values represent the means ± SEM; Trisun supplemented group, n=4; fish oil supplemented group, n=5. Significantly different from respective pre-treatment values, $p < 0.05$ (paired Student's t test).

Total phospholipid fatty acid composition

Monocyte

The percent distribution of total PUFAs in the monocyte phospholipids was not influenced by the fish oil supplementation; however, there was a replacement of n-6 PUFAs by n-3 PUFAs. Total n-6 series PUFAs decreased from 29.05 ± 0.99 mole% to 22.86 ± 0.89 mole%, whereas the total n-3 series PUFAs increased from 5.04 ± 0.41 mole% to 10.00 ± 0.48 mole% (means ± SEM; Table 3A). These changes were reflected in the n-6/n-3 fatty acid ratio that decreased by 61% from 5.85 ± 0.29 to 2.30 ± 0.11 (means ± SEM). At the end of dietary treatment, the fish oil supplemented group exhibited a 21% lower total n-6 PUFAs and a 120% higher level of total n-3 PUFAs, accounting for a 74% lower n-6/n-3 ratio, than the Trisun supplemented group. These effects were also observed when comparing the degree of change (day 30 minus day 0) in total n-3 PUFAs and n-6/n-3 ratio between groups (not shown). Trisun oil supplementation did not affect monocyte membrane fatty acid composition (Table 3A).

Granulocyte

Fish oil intake did not change the percent distribution of total PUFAs in granulocyte phospholipids, whereas the sum of n-3 series PUFAs increased from 2.70 ± 0.67 mole% to 6.23 ± 0.42 mole% (180%) (means ± SEM; Table 3B). These changes were reflected in the n-6/n-3 fatty acid ratio, which decreased by 63% from 7.34 ± 0.44 to 2.71 ± 0.15. At the end of dietary treatment, the fish oil supplemented group exhibited a 22% lower total n-6 PUFAs and a 137% higher level of total n-3 PUFAs, and thus a 78% lower n-6/n-3 ratio, than the Trisun supplemented group. These effects were also observed when comparing the degree of change (day 30 minus day 0) in total n-3 PUFAs and n-6/n-3 ratio between groups (not shown). Trisun oil supplementation did not affect monocyte membrane fatty acid composition (Table 3B).

LDL

After one month of fish oil supplementation, n-3 PUFAs were significantly incorporated into LDL (Table 3C) leading to a 8.6% increase of total LDL n-3 PUFAs. The participation of individual n-3 PUFAs in LDL was EPA (5.4%), DHA (2.5%) and DPA (0.7%). Linoleic (LA, 18:2 n-6) and arachidonic (AA, 20:4 n-6) fatty acid concentration remained unchanged following fish oil supplementation. In contrast, Trisun oil supplementation did not affect the fatty acid composition of LDL (Table 3C).

Tab. 3 Fatty acid composition (mole %) of monocyte and granulocyte phospholipids, and LDL from subjects who consumed either high-oleic sunflower seed oil (Trisun) or fish oil supplements

Fatty acids[a]	Trisun		Fish oil	
	Day 0	Day 30	Day 0	Day 30
A) Monocytes				
Sum SAT	53.66 ± 0.91	52.59 ± 1.06	53.33 ± 1.80	54.68 ± 1.29
Sum MUFA	12.74 ± 0.40	13.77 ± 0.35	12.47 ± 0.64	12.14 ± 0.51
Sum PUFA	12.74 ± 0.40	13.77 ± 0.35	12.47 ± 0.64	12.14 ± 0.51
Sum (n-6)	28.79 ± 0.73	29.03 ± 1.37	29.05 ± 0.99	22.86 ± 0.89[b,c]
Sum (n-3)	4.87 ± 0.52	4.53 ± 0.25	5.04 ± 0.41	10.00 ± 0.48[b,c]
PUFA/SAT ratio	0.63 ± 0.02	0.64 ± 0.04	0.65 ± 0.04	0.61 ± 0.03
n-6/n-3 ratio	6.13 ± 0.66	6.47 ± 0.50	5.85 ± 0.29	2.30 ± 0.11[b,c]
B) Granulocytes				
Sum SAT	43.36 ± 0.20	42.45 ± 0.66	50.16 ± 9.22	47.35 ± 3.59
Sum MUFA	32.18 ± 1.00	33.67 ± 0.89	28.05 ± 5.03	30.40 ± 2.51
Sum PUFA	24.56 ± 1.02	24.30 ± 0.40	22.34 ± 5.73	23.04 ± 1.75
Sum (n-6)	21.97 ± 0.94	21.67 ± 0.58	19.65 ± 5.09	16.81 ± 1.40[b]
Sum (n-3)	2.59 ± 0.19	2.63 ± 0.24	2.70 ± 0.67	6.23 ± 0.42[b,c]
PUFA/SAT ratio	0.57 ± 0.02	0.57 ± 0.01	0.57 ± 0.18	0.50 ± 0.07
n-6/n-3 ratio	8.62 ± 0.62	8.51 ± 1.01	7.34 ± 0.44	2.71 ± 0.15[b,c]
C) LDL				
Sum SAT	33.45 ± 1.11	32.93 ± 0.87	35.86 ± 1.24	33.78 ± 0.65
Sum MUFA	20.45 ± 0.89	21.25 ± 0.55	20.02 ± 1.54	18.28 ± 1.0
Sum PUFA	46.08 ± 1.99	45.75 ± 1.33	44.14 ± 0.80	47.94 ± 1.42[c]
Sum (n-6)	43.80 ± 2.27	43.80 ± 1.62	41.40 ± 0.84	36.68 ± 1.47[b,c]
Sum (n-3)	2.30 ± 0.36	2.00 ± 0.28	2.72 ± 0.37	11.28 ± 0.49[b,c]
PUFA/SAT ratio	1.38 ± 0.10	1.40 ± 0.09	1.24 ± 0.05	1.46 ± 0.07
n-6/n-3 ratio	20.70 ± 3.61	23.83 ± 3.71	16.42 ± 2.61	3.26 ± 0.22[b,c]

[a] Values represents the means ± SEM expressed in mole%; Trisun supplemented group, n=4; fish oil supplemented group, n=5. Abbreviations: *SAT* saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids.
[b] Significantly different ($p \leq 0.05$) from Trisun supplemented group using a two-tailed t-test.
[c] Significantly different ($p \leq 0.05$) from day 0 (treatment initiation) using a paired t-test.

Plasma lipids

Although a lower plasma triacylglycerol was observed in the fish oil supplemented group compared to the Trisun fed group at day 30 (Table 4), the net triacylglycerol change (day 30 minus day 0) did not differ significantly between groups. A slight decrease in triacylglycerols in the fish oil fed group combined with a slight increase in triacylglycerols in the Trisun fed group accounts for the significant effect at day 30.

There was no difference in the total plasma cholesterol or HDL cholesterol between groups pre- or post-treatment. Subjects ingesting the fish oil supplement exhibited increased plasma HDL cholesterol at the end of treatment.

Antioxidant status

Vitamin C concentration was not modified by either oil supplementation (Table 5). Furthermore, there was no significant change in either plasma or LDL α-tocopherol. Similarly, plasma and LDL concentrations of the different carotenoids such as α- and β-carotene, and lycopene were not influenced by the oil intake. No change in the activity of red blood cell superoxide dismutase was detected in either treatment group.

Susceptibility of LDL to oxidation

It is well recognised that PUFAs are prone to oxidation. Since oil supplementation, especially fish oil, modified the fatty acid composition of LDL, the theoretical susceptibility of the LDL to oxidation (Th-Ox) was calculated using the equation reported by Esterbauer and Jürgens [27]. In this formula, the concentration of each PUFA in the LDL is first expressed in mmol/L, then multiplied by a number which is one less than the number of double bonds in the molecule. The Th-Ox is the sum of all these values:

$$\text{Th-Ox} = (\text{C18:2} \times 1) + (\text{C18:3} \times 2) + (\text{C20:4} \times 3) + (\text{C20:5} \times 4) + (\text{C22:6} \times 5)$$

This equation allowed an estimation of the number of oxidative sites in each fatty acid molecule (i. e. the number of methylenic groups enclosed between two double

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Tab. 4 Plasma lipid levels from subjects who consumed either high-oleic sunflower seed oil (Trisun) or fish oil supplements

Variables (mmole/L)	Subjects	Trisun		Fish oil	
		Day 0	Day 30	Day 0	Day 30
Triacylglycerols	1	0.86	1.53	0.48	0.38
	2	0.81	0.87	0.66	0.76
	3	1.43	0.88	0.73	0.55
	4	1.45	1.89	0.88	0.57
	5			1.51	1.04
	mean ± SEM	1.14 ± 0.17	1.29 ± 0.25	0.85 ± 0.18	0.66 ± 0.11[a]
Total cholesterol	1	6.02	5.82	5.21	5.22
	2	6.45	6.01	5.51	5.99
	3	5.24	5.36	5.26	5.54
	4	5.93	6.92	3.94	4.07
	5			6.62	6.34
	mean ± SEM	5.91 ± 0.25	6.03 ± 0.33	5.31 ± 0.43	5.43 ± 0.39
HDL cholesterol	1	1.42	1.36	1.55	1.67
	2	1.37	1.24	1.23	1.32
	3	1.16	1.25	1.17	1.26
	4	0.98	1.18	1.00	0.99
	5			1.38	1.48
	mean ± SEM	1.23 ± 0.10	1.26 ± 0.04	1.27 ± 0.09	1.34 ± 0.11[b]

[a] Significantly different ($p \leq 0.05$) from Trisun supplemented group using a two-tailed t-test.
[b] Significantly different ($p \leq 0.05$) from day 0 (treatment initiation) using a paired t-test.

Tab. 5 Vitamin C, tocopherol and carotenoid concentrations in plasma and LDL from subjects who consumed either high-oleic sunflower seed oil (Trisun) or fish oil supplements

		Trisun		Fish oil	
		Day 0	Day 30	Day 0	Day 30
Vitamin C[a]	Plasma	60 ± 13	54 ± 8	73 ± 5	65 ± 5
α-Tocopherol	Plasma	12.5 ± 0.9	13.4 ± 1.3	10.5 ± 1.0	12.1 ± 1.1
	LDL	5.8 ± 0.6	6.2 ± 0.9	4.7 ± 0.5	5.6 ± 0.4
β-Tocopherol	Plasma	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
	LDL	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
α-Carotene	Plasma	0.09 ± 0.04	0.06 ± 0.02	0.15 ± 0.08	0.12 ± 0.05
	LDL	0.07 ± 0.02	0.06 ± 0.02	0.11 ± 0.05	0.10 ± 0.05
β-Carotene	Plasma	0.37 ± 0.12	0.27 ± 0.06	0.45 ± 0.16	0.45 ± 0.15
	LDL	0.30 ± 0.09	0.24 ± 0.05	0.34 ± 0.11	0.36 ± 0.13
Lycopene	Plasma	0.36 ± 0.07	0.25 ± 0.03	0.32 ± 0.07	0.33 ± 0.04
	LDL	0.29 ± 0.05	0.21 ± 0.02	0.25 ± 0.05	0.27 ± 0.03
SOD	RBC	921 ± 40	889 ± 23	907 ± 34	869 ± 38

[a] Vitamin C is expressed in μM; tocopherols and carotenoids in mg/L, and superoxide dismutase activity (SOD) in units per gram haemoglobin. Values represent the means ± SEM; Trisun supplemented group, n=4; fish oil supplemented group, n=5.

bonds). At the end of fish oil supplementation, the ThOX increased markedly from 64.8 ± 30 to 125.6 ± 21.7 while in the Trisun treated group, it remained unchanged (78.1 ± 16.7 vs 78.5 ± 15.1).

To experimentally confirmed this predicted model, LDL were subjected to an *ex vivo* oxidation initiated by cupric ions. The kinetics of LDL oxidation were characterised by three parameters a) the lag time or the time during which antioxidants were consumed; b) the rate of oxidation and c) the maximum production of conjugated dienes (CD) (Table 6). Noteworthy is that the inter-individual variation was quite low for all three parameters, suggesting that the chosen population was rather homogenous. After fish oil supplementation, the lag time was significantly reduced by 25 % (from 64.6 ± 1.9 to 47.6 ± 1.0 min, means ± SEM) and the rate of oxidation by 20 % (from 11.9 ± 0.4 to 9.6 ± 0.9 μmol / mg LDL cholesterol / min, means ± SEM). In contrast, the maximum CD produced remained stable. In agreement with the literature, a correlation between measured lag time and calculated susceptibility of LDL to oxidation was observed ($y=84.96–0.27x$; $r^2=0.58$). LDL from subjects supplemented with Trisun oil which did not exhibit any change in their fatty acid composition, did not show any change in the lag time prior to oxidation but exhibited a decreased rate of oxidation post-treatment similar to that of fish oil.

Tab. 6 LDL susceptibility to oxidation in subjects who consumed either high-oleic sunflower seed oil (Trisun) or fish oil supplements

	Subjects	Trisun		Fish oil	
		Day 0	Day 30	Day 0	Day 30
Lag time[a]	1	67.3	70.1	61.3	46.8
	2	57.8	56.8	71.0	47.4
	3	68.2	76.7	65.4	51.2
	4	68.6	60.5	60.0	47.5
	5			65.3	45.2
	mean ± SEM	65.5 ± 2.6	66.0 ± 4.6	64.6 ± 1.9	47.6 ± 1.0[b]
Oxidation rate	1	13.7	13.0	13.6	13.0
	2	12.0	8.3	11.4	8.3
	3	11.1	9.1	11.5	9.1
	4	11.2	8.3	11.4	8.3
	5			11.5	9.1
	mean ± SEM	12.0 ± 0.6	9.7 ± 1.1[b]	11.9 ± 0.4	9.6 ± 0.9[b]
CD max	1	0.783	0.763	0.783	0.763
	2	0.731	0.739	0.677	0.739
	3	0.687	0.740	0.708	0.740
	4	0.731	0.740	0.677	0.740
	5			0.708	0.745
	mean ± SEM	0.733 ± 0.020	0.746 ± 0.06	0.711 ± 0.019	0.745 ± 0.004

[a] Lag time is expressed in min; oxidation rate in µmol/ min/mg LDL cholesterol, and maximum conjugated dienes (CD) in absorbance units.
[b] Significantly different from respective pre-treatment values, $p < 0.05$ (paired Student's t test).

Discussion

Supplementation with lipids influences cell membrane composition and plasma lipids. These changes modulate the functions of the immune and the cardiovascular system. Both systems are well integrated and closely interrelated. In this study, the effects of a dietary supplement of fish oil, at levels similar to that found in immunomodulatory clinical products, was investigated in human volunteers.

The phospholipid fatty acid composition of both monocytes and granulocytes reflected dietary fatty acid intake. The fatty acid changes were smaller in the granulocytes compared to the monocytes. The increased n-3 fatty acid content in the monocytes from subjects supplemented with fish oil was associated with an enhanced phagocytic activity (12%). Monocytes from subjects supplemented with Trisun also exhibited an increase (24%) in phagocytic activity. The latter cannot be explained by changes in the membrane fatty acid composition as no changes were observed in this group following dietary treatment. As the functional tests are performed with whole blood, some components of the plasma such as opsonins, cytokines, lipoprotein modulation of lipopolysaccharide (LPS) may be influenced by dietary treatments and thereby contribute to changes in immune cell activity. The influence of minor unidentified components of the oils may also be involved. In this regard, the inclusion of a wash-out period or a non-supplemented control group would help conclude whether or not this is a dietary effect. It is noteworthy that even though the phagocytic activity was initially high, a further increase in activity could be detected. On the other hand, the granulocytes did not show enhanced phagocytic activity. In contrast, the peripheral blood cell respiratory burst activity was not influenced by either of the dietary treatments. Our results are in agreement with those of a recent study by Halvorsen et al. [28] in which the monocytes from healthy men supplemented with highly purified EPA or DHA for 7 weeks maintained their phagocytic and respiratory burst activities. A major difference between our study and those of other groups is that the tocopherol content and profile of both supplements has been adjusted to be similar in the present work. A role of tocopherols on immune functions has been suggested by Meydani et al. [2] who reported an enhancement of cell-mediated immunity by vitamin E supplementation in healthy elderly subjects. Kramer et al. [29] also found that supplements of vitamin E prevented the decreased blastogenesis of mitogen-stimulated T-cells seen following fish oil treatment. The vitamin E levels in these two studies were 21 and 6 times higher, respectively, than in the present work. Our supplements provided more than 3 times the recommended dietary allowance (RDA) for Americans [15].

Elevated plasma triacylglycerols, a high level of total cholesterol and LDL cholesterol, as well as low level of HDL cholesterol are all risk markers for developing CVD. LDL oxidation also plays an important role in the development of CVD. N-3 fatty acids are known to affect lipid metabolism by lowering plasma triacylglycerols through the inhibition of VLDL synthesis in the liver

[30–32] and by increasing HDL cholesterol [33]. Plasma triacylglycerol levels were lower in the fish oil fed group post-treatment compared to the Trisun supplemented group. However, the net change of triacylglycerols did not reach significance possibly due to the small sample size and the fact that individuals were not hypertriglyceridaemic when entering the study. An increase (5.5 %) in HDL cholesterol occurred in the fish oil supplemented group. This is in agreement with the literature which reports that fish oil has little or no effect on HDL cholesterol [34]. We did not observe a change in plasma cholesterol following Trisun supplementation.

Nair et al. [10] reported that fish oil supplementation decreased vitamin E levels in plasma, platelets and red blood cells, whereas others [12, 35] have reported no changes. In the present study, the fish oil administration provided 9.7 g PUFAs together with 30 mg of RRR-α-tocopherol equivalents. Under these conditions, the plasma vitamin E, vitamin C or carotenoid levels, and the superoxide dismutase activity in red blood cells were not altered. Comparisons with previously reported data in the literature are difficult to make as different doses of PUFAs and vitamin E have been used.

In addition, n-3 fatty acid accumulation in LDL increased their susceptibility to oxidative stress, in agreement with previous reports [7, 36–38]. Therefore, the incorporation of an adequate antioxidant supply into oils need to be carefully evaluated to avoid the potentially adverse effect of an increased LDL susceptibility to oxidation. This issue might be critical for patients submitted to, or suffering from, a stressed condition.

In conclusion, immune cell activities were not decreased following 25 g/d fish oil ingestion for 30 days. On the contrary, a potentially beneficial increase in phagocytic activity was seen in blood monocytes. The long-chain n-3 PUFAs, derived from fish oil were readily incorporated into LDL particles and cell membranes with no apparent adverse effect on the plasma antioxidant status as measured by tocopherols and carotenoids, or SOD activity. On the other hand, an increased susceptibility of LDL to *ex vivo* oxidation was observed following fish oil supplementation even though the tocopherol contents and profiles were similar between both treatments. Further studies in patients are required to determine optimal levels of n-3 PUFAs and oleic acid, together with a combination of antioxidants, needed to modulate immune functions while minimising the oxidative susceptibility.

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