The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/15131

Please be advised that this information was generated on 2017-07-30 and may be subject to change.
Pro- and anti-inflammatory cytokines in healthy volunteers fed various doses of fish oil for 1 year


*Department of General Internal Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands; †Department of Endocrinology, University Hospital Ghent, Belgium; ‡Department of Human Nutrition, Agricultural University, Wageningen, The Netherlands

Abstract. Dietary supplementation with n-3 fatty acids from fish oil alleviates inflammation in various chronic inflammatory disease states. Reductions in the production of pro-inflammatory cytokines interleukin 1β (IL-1β), tumour necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) have been seen in humans after short-term n-3 fatty acid supplementation. We investigated long-term effects of dietary n-3 fatty acids on circulating cytokine concentrations and on ex vivo stimulated whole-blood production of IL-1β, TNF-α and interleukin 1 receptor antagonist (IL-1Ra), the naturally occurring antagonist of IL-1. A total of 58 monks with a mean age of 56 years were randomized into four groups and their diets were supplemented with 0, 3, 6, or 9 g of fish oil, providing 0, 1, 0.6, 2, 1.3 or 3.19 g of n-3 fatty acids per day. Subjects received equal amounts of saturated fatty acids, vitamin E and cholesterol. Compliance was excellent and erythrocyte fatty acid profiles closely reflected the amounts of n-3 fatty acids ingested. In the group receiving 9 g of fish oil per day, no influence of n-3 fatty acids on circulating cytokine concentrations was observed relative to placebo. Endotoxin-stimulated whole-blood cytokine production was measured at 26 and 52 weeks after the start and at 4, 8 and 26 weeks after cessation of supplementation. In all groups, the production of IL-1β and IL-1Ra was higher during supplementation than afterwards. However, no differences in cytokine production were noted between the placebo group and does not affect results suggest that long-term supplementation of fish oil...
blood pressures were below 160 mmHg and 95 mmHg respectively; concentrations of serum total cholesterol were below 7.0 mmol L\(^{-1}\) and triglycerides below 3.0 mmol L\(^{-1}\).

Ten monks were Trappists and thus vegetarians. During the study period, the subjects did not take medications influencing lipid metabolism or non-steroidal anti-inflammatory drugs. The study was approved by the Ethics Committee of the Department of Human Nutrition, and all subjects gave informed consent.

**Experimental design**

The study consisted of a 2-week baseline period, followed by a 1-year treatment and a 6-month follow-up period. Within each monastery, the subjects were randomly and blindly divided into four groups, which received zero (\(n = 14\)), three (\(n = 15\)), six (\(n = 15\)) or nine (\(n = 14\)) fish oil capsules per day. In each dosage group, the total number of capsules was made up to nine by adding nine, six, three or zero placebo capsules respectively. Periodically, every subject received three bottles with dark-coloured capsules. Depending on the dosage group, none, one, two or all three of the bottles contained fish oil capsules, whereas the remaining bottles contained placebo capsules. At each meal one capsule was taken from every bottle. At regular intervals compliance was established by counting the remaining capsules.

A clinical examination including weight, height, waist–hip ratio, thigh circumference and blood pressure was performed eight times during the trial: on days -14, 0 (start of the supplementation), 56, 182, 363 (end of the supplementation), 396, 424, and 536. At these times blood was also drawn. The conditions used for whole-blood ex vivo production of IL-1\(\beta\), TNF-\(\alpha\), and IL-1Ra turned out to have been inappropriate until 26 weeks after the start of the dietary supplementation. Thus, these data are available only at 26 and 52 weeks of supplementation and at 4, 8 and 26 weeks after cessation of the supplementation. In weeks 26 and 52 the subjects recorded their habitual food consumption by writing down what they had eaten during 3 days. These data were completed by a trained dietitian, who interviewed the subjects, weighed portion sizes and checked recipes.

All subjects received a diary to record the use of medicine, illness, alcohol intake, inadvertent omission of capsule intake, and other relevant facts.

**Oil supplements**

Eicosapentaenoic (EPA)-rich fish oil capsules were a kind gift from Labaz (Brussels, Belgium). The placebo capsules contained olive oil (Puget, Marseille, France), and palm oil (Loders-Krooklaan, Wormerveer, The Netherlands). Cholesterol (Merck, Darmstadt, Germany) and vitamin E (Organon, Oss, The Netherlands) were added. The placebo capsules were also manufactured by Labaz. Fatty acids composition and cholesterol content of the capsules were determined in our laboratory using capillary gas–liquid chromatography. The vitamin E content was measured by Dr J. Schrijver (CIVO-TNO, Zeist, The Netherlands) using a high-performance liquid chromatographic (HPLC) method. The composition of the fish oil and placebo capsules is shown in Table 1. EPA accounted for 72% of all \(n\)-3 fatty acids in the fish oil. The fish oil and the placebo capsules contained equal amounts of saturated fatty acids, vitamin E and cholesterol. There was a small difference in the concentration of \(n\)-6 fatty acids. The \(n\)-3 fatty acids of the fish oil capsules were replaced by neutral monounsaturated fatty acids (largely oleic acid) plus some linoleic acid in the placebo capsules. Both capsules contained 1 g of fat; thus every subject had 9 g of extra fat per day. The daily intake of eicosapentaenoic acid (EPA, \(C_{20:5}\) \(n\)-3), docosahexaenoic acid (DHA, \(C_{22:6}\) \(n\)-3), and \(C_{22:5}\) \(n\)-3 in the various treatment groups is shown in Table 2.

**Measurement of fatty acids**

After an overnight fast, venous blood was drawn into a 10-mL vacutainer tube with EDTA as anticoagulant. The tubes were centrifuged on the spot at low speed at 4°C to separate red blood cells. The cells were washed twice with ice-cold saline, transferred into stoppered plastic tubes (Greiner, Nuttingen, Germany), and haemolysed by freezing at -80°C for at least 2 h. The haemolysates were stored at -20°C for a maximum of 7 days. Fatty acids of the haemolysates were measured by capillary gas chromatography.

**Table 1.** Mean fatty acid composition, mean cholesterol and mean vitamin E content of fish-oil and placebo capsules

<table>
<thead>
<tr>
<th></th>
<th>Fish oil capsule</th>
<th>Placebo capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids (% mass of methylesters)</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>26</td>
<td>65</td>
</tr>
<tr>
<td>(n)-3 fatty acids (% mass of methylesters)</td>
<td>37</td>
<td>0.6</td>
</tr>
<tr>
<td>(n)-6 fatty acids (% mass of methylesters)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol (mg/100 g)</td>
<td>115</td>
<td>119</td>
</tr>
<tr>
<td>Vitamin E (IU/100 g)</td>
<td>63</td>
<td>63</td>
</tr>
</tbody>
</table>

**Table 2.** Daily \(n\)-3 fatty acid (g day\(^{-1}\)) intake in the various treatment groups

<table>
<thead>
<tr>
<th>(n)-3 fatty acid</th>
<th>Low-dose fish oil group (3 g day(^{-1}))</th>
<th>Medium-dose fish oil group (6 g day(^{-1}))</th>
<th>High-dose fish oil group (9 g day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA ((C_{20:5}) (n)-3)</td>
<td>0.81</td>
<td>1.62</td>
<td>2.43</td>
</tr>
<tr>
<td>(C_{20:4}) (n)-3</td>
<td>0.09</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td>DHA ((C_{22:6}) (n)-3)</td>
<td>0.16</td>
<td>0.33</td>
<td>0.49</td>
</tr>
<tr>
<td>Total</td>
<td>1.06</td>
<td>2.13</td>
<td>3.19</td>
</tr>
</tbody>
</table>

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 3. Concentration of eicosapentaenoic acid (EPA) (C₂₀₅, n-3) in erythrocyte lipids as a function of duration and level of fish oil supplementation. Values are represented as grams of EPA per 100 g fatty acids

<table>
<thead>
<tr>
<th>Week of experiment*</th>
<th>Daily ingestion of fish oil (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0.79 ± 0.21</td>
</tr>
<tr>
<td>2</td>
<td>0.85 ± 0.19</td>
</tr>
<tr>
<td>4</td>
<td>0.83 ± 0.16</td>
</tr>
<tr>
<td>8</td>
<td>0.86 ± 0.30</td>
</tr>
<tr>
<td>26</td>
<td>1.12 ± 0.24</td>
</tr>
<tr>
<td>52</td>
<td>0.83 ± 0.28</td>
</tr>
<tr>
<td>56</td>
<td>0.88 ± 0.28</td>
</tr>
<tr>
<td>60</td>
<td>0.78 ± 0.19</td>
</tr>
<tr>
<td>78</td>
<td>0.83 ± 0.17</td>
</tr>
</tbody>
</table>

*Fish oil supplementation from week 0 until week 52. From week 56 on, post-supplementation values are represented.

Cytokine measurements

Venous blood was drawn into three 4-mL evacuated γ-sterilized blood collection tubes (Becton Dickinson, Vacutainer Systems, Rutherford, NJ, USA) containing 6 mg of EDTA. The tubes were filled with blood by vacuumaspiration. An aliquot (250 μl) of aprotinin with an activity of 10-000 kalikrein-inactivating units per mL (Trasylof, Bayer, Leverkussen, Germany) was added per tube. One tube was centrifuged immediately and the resultant plasma was drawn off, stored in dry ice, transported to the laboratory and stored at -80 °C until assay of circulating cytokines. One of the remaining two samples was stimulated by the addition of 50 μL of endotoxin (lipopolysaccharide, LPS, Escherichia coli serotype O55:B5, Sigma, St Louis, MO, USA), concentration 800 μg mL⁻¹, final concentration 10 μg mL⁻¹. The control sample did not have endotoxin added. The two samples were inverted gently to mix and incubated for 24 h at 25 °C in a circulating water bath. The samples were incubated within 5 min after drawing, and incubation at 25 °C was secured by keeping the samples in a portable stove throughout transportation to the laboratory. After the incubation, samples were centrifuged at 1250 g for 10 min. For removal of platelets, the resultant plasma was spun in an Eppendorf centrifuge at 13 000rpm for 5 min. Platelet-poor plasma, containing the secreted products of endotoxin-stimulated blood cells, was drawn off and stored at -80°C until assay of cytokines.

IL-1β, TNF-α and IL-1Ra were determined by radioimmunoassay as described previously [19-21]. The intra-assay variability was less than 10%. The detection limit of IL-1β was 50 pg mL⁻¹, of TNF-α 50 pg mL⁻¹ and of IL-1Ra 100 pg mL⁻¹. Circulating concentrations of IL-6 were measured using an enzyme-linked immunosorbent assay (ELISA) [22], with a detection limit of 6 pg mL⁻¹. To avoid experimental fluctuations, one batch of LPS and one batch of blood-collecting tubes was used throughout the study. All samples of one subject were determined in the same series and in each series an equal number of samples of each treatment group was analysed to avoid bias due to assay variability.

Statistical analysis

Cytokine concentrations of the various diet groups were compared using the multivariate rank test according to Koziol et al. [23] and Crepeau et al. [24]. This is a non-parametric test for the comparison of growth curves, which accounts for missing data at certain time points.

Results

Compliance

The interviews taken by the dietitians and the counting of the capsules revealed an excellent adherence to the experimental protocol throughout the 78 weeks that this study lasted. The determinations of fatty acid profiles of red blood cells closely reflected the amount of n-3 fatty acids ingested by the four experimental groups (Table 3) [25]. Twenty-six weeks after the cessation of the fat supplementation, the fatty acid profiles of the red blood cells had returned to pretreatment values. None of the subjects experienced adverse effects.

Circulating cytokines

The concentrations of circulating IL-1β, IL-6, TNF-α and IL-1Ra were determined only in the groups receiving 0 and 9 g of fish oil per day. As expected, these concentrations were low. The concentrations of IL-1β, TNF-α and IL-1Ra were well above the detection limit of the assay in all subjects, but IL-6 was below the detection limit in all samples. For the circulating cytokines that could be measured, there was no significant difference between the two treatment groups at any point in time, either in absolute concentrations or in changes relative to baseline (Fig. 1). Basal concentrations of circulating cytokines were not different between vegetarians and non-vegetarians (data not shown).

Ex vivo production of IL-1β, TNF-α and IL-1Ra after whole-blood stimulation

In all three dose groups, as well as in the placebo group,
endotoxin-stimulated secretion of IL-1β and IL-1Ra was significantly higher during oil supplementation than at 8 and 26 weeks after cessation of capsule intake (Fig. 2). A similar trend was observed in the secretion of TNF-α, although differences over time were not statistically significant. The ex vivo production of IL-1β, TNF-α and IL-1Ra was not significantly different between the four diet groups at any point in time. The inter-individual variations in cytokine production were large. When the values obtained at 78 weeks, i.e. 26 weeks after the oil supplementation, are taken as baseline values, percentage changes over time can be evaluated. Again, these values were not significantly different between the various experimental groups at any point in time (data not shown). Twenty-six weeks after cessation of the dietary supplementation, cytokine production capacity was not different between vegetarians and non-vegetarians (data not shown).

**Figure 1.** Circulating concentrations of IL-1β (top), TNF-α (middle) and IL-1Ra (bottom) at various time points before, during, and after dietary supplementation with placebo or 3-19 g n-3 fatty acids per day. Results expressed as mean ± SD.

**Figure 2.** Concentrations of IL-1β (top), TNF-α (middle) and IL-1Ra (bottom) after stimulation of whole blood with lipopolysaccharide (10 μg mL⁻¹) for 24 h. Subjects took 0, 1-06, 2-13 or 3-19 g of n-3 fatty acids per day. For IL-1Ra, only the groups receiving 0 or 3-19 g per day were analysed. Results expressed as means ± SD.

**Discussion**

The findings in this long-term placebo-controlled study disagree with the conclusions drawn in some previously published studies regarding the effects of dietary fish-oil supplementation on ex vivo cytokine production [13,15,16,26]. We found no effect of fish oil on the ex vivo production of pro-inflammatory cytokines, either during supplementation or after cessation of capsule intake, relative to placebo. The fluctuations in absolute levels in all groups, including the placebo group, demonstrate the need for a proper control group in studies of this nature, and for a proper analysis that compares the effect of fish oil with that of placebo. Statistical testing of changes from baseline within the fish oil group may lead to the faulty conclusion that fish oil does decrease cytokine levels or production [3,27]

In two controlled studies, no effect of dietary fish-oil on ex vivo production of IL-1β or TNF-α was found at 7 weeks and 4 months of supplementation [17,18]. Effects of n-3 fatty acids that are apparent at 6 weeks may thus
have disappeared at 26 weeks of dietary fish oil supplemen-
tation.

The question remains why the ex vivo production of IL-1βand IL-1Ra was higher during the dietary supple-
tmentation in both the placebo and the n-3 fatty acids-
supplemented groups. It is unlikely that the daily inges-
tion of placebo capsules containing 9 g of a mixture of
olive and palm oil exerts such profound biological effects
as the fatty acid composition of cholesterylesters, and
erthrocyte membranes did not change during intake of
the placebo capsules [25]. A seasonal effect on cytokine
production capacity is less likely because the measure-
ments after 26 weeks of supplementation (Fig. 2, first
time point) and after 26 weeks of washout (Fig. 2, final
time point) were obtained in the same season, 1 year
apart. Differences with other studies might also be
explained by the dose of fish oil used [13] or by dif-
ficiencies in background diet [26]; however, the
recent study of Schmidt et al. [28] yielded results
essentially similar to those reported here.

The changes in fatty acid profiles were similar to those
observed in the other studies mentioned. Therefore, the
dose of n-3 fatty acids ingested cannot account for the
lack of an effect on cytokine production.

We have evaluated the cytokine production capacity
of whole-blood samples, whereas others used in vitro
stimulation of mononuclear cells that were isolated by
density gradient centrifugation. The relative simplici-
ty of the whole-blood stimulation may render this method
less prone to experimental biases: manipulation of the cell
population studied is minimal, stimulation is started
immediately upon removal of the cells from the circula-
tion and experimental conditions can easily be kept
constant. Moreover, Nerad et al. [21] have shown a
good correlation between whole blood IL-1β measure-
ment and the method in which mononuclear cells are
separated by density gradient centrifugation and stimu-
lated ex vivo. Therefore, whole blood stimulation may
represent a reflection of cytokine production capacity
that is at least as relevant as a method using cell
separation techniques.

The concentrations of circulating cytokines were, as
expected, very low. Higher concentrations can be found
in severe disease states such as septic shock, burn trauma,
and after experimental administration of endotoxin [29–
32]. In rheumatoid arthritis patients, dietary fish oil
supplementation during 12 weeks resulted in decreased
circulating concentrations of IL-1β [33]. Our results
indicate that dietary fish oil supplementation does not
affect the low concentrations of circulating cytokines
that are found under physiological, unstimulated circum-
stances.

In conclusion, in this long-term controlled trial we
could not confirm that the fish oil induced reduction of ex
vivo stimulated cytokine production that has been
observed in most short-term studies until now, but the
results corroborate those of the medium-term study of
Cannon et al. [18]. Our findings emphasize the need for a
well-controlled intervention trial comparing short-term
and long-term effects of dietary fish oil supplementation.

Acknowledgements
We thank Gerard Pesman and Mariel Spruinenberg for
help with the measurements of cytokines.

References
1 Endres S, De Cauteria R, Schmidt EB, Kristensen SD. n-3 Poly-
2 Kremer JM, Michalek A, Lininger L. Effects of manipulation
of dietary fatty acids on clinical manifestations of rheumatoid arthritis.
3 Kremer JM, Lawrence DA, Jubiz W, DiGiacomo R, Rynes R,
Bartholomew LE, Sherman M. Dietary fish oil and olive oil
supplementation in patients with rheumatoid arthritis. Clinical and
4 Bitüner SB, Tucker WF, Cartwright I, Bleehen SS. A double-blind,
randomised, placebo-controlled trial of fish oil in psoriasis. Lancet
5 Stenson WF, Cort D, Rodgers J et al. Dietary supplementation with
6 Cora FB, Lehmann S, Konstantinides N et al. Improvement in
immune function in ICU patients by enteral nutrition supplemented
with arginine, RNA, and menhaden oil is independent of nitrogen
7 Cora FB, Holman RT, Bankey PE, Marxuzi JE, LiCari JJ. Omega 3
polyunsaturated fatty acids as modulators of cellular function in the
8 Gottschlich MM, Jenkins K, Warden GD, Baumber T, Havenes P,
Snook FT, Alexander JW. Differential effects of three enteral
dietary regimens on selected outcome variables in burn patients. J
9 Daly JM, Lieberman MD, Goldfain E et al. Enteral nutrition with
supplemental arginine, RNA, and omega-3 fatty acids in patients
after operation: immunologic, metabolic, and clinical outcome.
10 Needleman P, Raz A, Mintkes MS, Ferrendelli JA, Sprecher H.
Triene prostaglandins: prosacystein and thromboxane biosynthesis
and unique biological properties. Proc Natl Acad Sci USA
11 Kinless JE, Lokesh B, Broughton S, Whelan J. Dietary polyunsat-
urated fatty acids and eicosanoids: potential effects on the modula-
tion of inflammatory and immune cells: an overview. Nutrition
12 Lee TH, Hoover RL, Williams JD et al. Effect of dietary enrichment
with eicosapentaenoic and docosahexaenoic acids on in vitro
neutrophil and monocyte leukotriene generation and neutrophil
13 Endres S, Ghorbani R, Kelley VB et al. The effect of dietary
supplementation with n-3 polyunsaturated fatty acids on the synthe-
sis of interleukin-1 and tumor necrosis factor by mononuclear
14 Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood
1991;77:1627–32.
15 Meydani SN, Lichtenstein AH, Cornwall S et al. Immunologic
effects of National Cholesterol Education Panel step-2 diets with
and without fish-derived n-3 fatty acid enrichment. J Clin Invest
16 Cooper AL, Gibbons L, Horan MA, Little RA, Rohwell NJ. Effect
of dietary fish oil supplementation on fever and cytokine production
17 Molvig J, Pociot F, Worsaae H et al. Dietary supplementation with
omega-3-polyunsaturated fatty acids decreases mononuclear cell
proliferation and interleukin-1 beta content but not monokine
secretion in healthy and insulin-dependent diabetic individuals.
18 Cannon JG, Fiatarone MA, Meydani M et al. Aging and dietary
modulation of elastase and interleukin-1 secretion. Am J Physiol
19 Endres S, Ghorbani R, Lonnesmann G, van der Meer JVM,
Dinarello CA. Measurement of immunoreactive interleukin-1 beta


Results

Kinin (data not shown).

Kinin production was not different between vaccinated and non-vaccinated dogs at 6 weeks. Twenty-six weeks after cessation of the study, no vaccinated group showed a significant difference between the two groups in terms of plasma kininogen levels. The results of the study suggest that the cessation of the study after 6 weeks led to a significant decrease in addition of the treatment. The data show that the treatment was effective in reducing the levels of kinin in dogs vaccinated with the study. The results were compared with the controls in a non-parametric test with the statistical analysis.