n-3 Fatty Acids and Leukocyte Chemotaxis
Effects in Hyperlipidemia and Dose–Response Studies in Healthy Men
Erik Berg Schmidt, Jens Oluf Pedersen, Kim Varning, Erik Ernst, Casper Jersild, Niels Grunnet, and Jørn Dyerberg

Dietary supplementation with n-3 polyunsaturated fatty acids (n-3 PUFAs) has been shown to inhibit neutrophil and monocyte chemotaxis in healthy subjects and, with respect to neutrophils, also in various patient groups. We studied the effect of dietary supplementation with n-3 PUFAs on monocyte and neutrophil chemotaxis in patients with hyperlipidemia. Chemotaxis was investigated with the under-agarose assay, using autologous serum and N-formylmethionyl-leucyl-phenylalanine as chemoattractants. The patients were examined before and after 6 weeks of supplementation with 6 g n-3 PUFAs daily. Monocyte chemotaxis was reduced after n-3 PUFAs supplementation in type IIa patients but was unaffected in patients with type IV hyperlipidemia. Furthermore, monocyte chemotaxis was increased in untreated type IIa patients compared with normocholesterolemic controls. We also studied the dose–response effects of n-3 PUFAs on monocyte and neutrophil chemotaxis in healthy men given 1.3, 4, and 9 g n-3 PUFAs daily for 6-week periods. Monocyte and neutrophil chemotaxis was reduced after n-3 PUFAs supplements in a dose-dependent fashion, with the majority of the effect observed after the low dose. These results lend support to the notion of an antiatherosclerotic effect of n-3 PUFAs and may provide an explanation for the hitherto-unexplained effect of low doses of n-3 PUFAs in coronary heart disease. (Arteriosclerosis and Thrombosis 1991;11:429–435)

-n-3 Polyunsaturated fatty acids (n-3 PUFAs) of marine origin may be of value in the prevention and control of coronary artery disease (CAD). This has mainly been ascribed to the effects of n-3 PUFAs on plasma lipids and platelet function. However, it has recently been demonstrated that n-3 PUFAs decrease leukocyte reactivity. This may be relevant to CAD, as monocytes and macrophages are important cells in the development of atherosclerosis. Also, neutrophils may play a role in CAD since they contribute to cell injury in acute myocardial ischemia. Part of the effect of n-3 PUFAs in CAD could therefore be due to an effect on leukocytes.

We have previously reported that dietary supplementation with 5.3 g n-3 PUFAs daily for 6 weeks decreased monocyte and neutrophil chemotactic responsiveness in healthy men. In the present study, we investigated if the same effect of n-3 PUFAs could be found in hyperlipidemic patients with a high risk of CAD.

The effect of various doses of n-3 PUFAs on leukocyte reactivity is, at present, unknown. Therefore, we investigated whether a reduction in monocyte and neutrophil chemotaxis could be obtained with a low daily dose (1.3 g) of n-3 PUFAs and whether a dose–response effect of n-3 PUFAs—given in low (1.3 g), moderate (4 g), and high (9 g) daily amounts—to healthy volunteers could be observed.

Methods

Patients With Hyperlipidemia

Seventeen outpatients with hyperlipidemia were studied. Characteristics of the patients are given in Table 1. They were examined while still on their usual diet and after 6 weeks of supplementation with 10 ml daily of a reesterified fish oil triglyceride containing approximately 6 g n-3 PUFAs (Table 2). Monocyte chemotaxis using the patient’s own serum as a chemoattractant produced considerably higher values in patients with type IIa hyperlipidemia than is usually seen in this assay. Consequently, we compared these results in patients with type IIa and IV hyperlipidemias with their respective normolipidemic controls (Table 1).
TABLE 1. Characteristics of Patients With Type Ila and IV Hyperlipidemias and Respective Normolipidemic Controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Type Ila</th>
<th>Controls</th>
<th>Type IV</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>8/1</td>
<td>8/1</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>50</td>
<td>46</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Age range (yr)</td>
<td>28–63</td>
<td>25–66</td>
<td>28–49</td>
<td>31–55</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>On lipid-lowering drugs (n)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mean cholesterol level (mmol/l)</td>
<td>9.1</td>
<td>5.6</td>
<td>7.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Mean triglyceride level (mmol/l)</td>
<td>1.31</td>
<td>1.05</td>
<td>13.14</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Dose–Response Study in Healthy Volunteers

Ten healthy men from the hospital staff were investigated before and after three 6-week periods of supplementation with various doses of n-3 PUFAs. The fish oil used was the same as above but in an encapsulated form. For each 6-week period, a daily dose of 1.3 g (two capsules), 4 g (six capsules), and 9 g (14 capsules) of n-3 PUFAs was given, with at least 6 weeks of a washout period between each supplementation period. Half the participants were randomized to the high dose initially, while the rest started with the low dose.

Blood Sampling

Blood was drawn from an antecubital vein in the morning after an overnight fast and after 10 minutes of supine rest. EDTA was used as an anticoagulant at a final concentration of 0.003 M.

Cell Separation

Neutrophils were harvested after Hypaque/Ficoll (Nycomed, Oslo, Norway) discontinuous gradient centrifugation and then washed three times in tissue culture medium (RPMI 1640). The purity of neutrophils was more than 95%, and cell viability exceeded 98% as evaluated by the Trypan Blue-exclusion test. Cell counts were adjusted to $1 \times 10^8$ neutrophils/ml RPMI 1640.

Monocytes were purified from the crude mononuclear cell population by further centrifugation on a triple-layer (Pharmacia, Uppsala, Sweden) Percoll gradient. Monocytes were purified to more than 80%, the rest of the cells being lymphocytes. The viability of monocytes after the entire purification procedure was above 88%. Cell counts were adjusted to $5 \times 10^7$ monocytes/ml RPMI 1640 using esterase staining to identify monocytes.

Chemotactic Assays

Monocyte and neutrophil chemotaxis was investigated by the under-agarose technique. Chemo- taxis was examined in a 0.75 (wt/wt) agarose gel to which was added minimal essential medium (MEM), and the pH was adjusted to 7.10. The agarose gel was molded on gelatinized object slides. The buffer system used was N-(2-hydroxyethyl)piperazine-N'-(-ethanesulfonic acid) (HEPES) at a final concentration of 0.05 M. Six chemotactic systems, each comprising three wells, were punched out in the agar gel. Ten microliters of cell suspension ($1 \times 10^6$ neutrophils or $5 \times 10^5$ monocytes) was transferred to each of the wells in the middle row (Figure 1). Ten microliters of chemoattractant was placed in one row of wells, while 10 µl RPMI 1640 was placed in the opposite well as a control. The chemoattractants used were N-formyl-methionyl-leucyl-phenylalanine ($N$-formyl-Met-Leu-Phe) at a concentration of $10^{-8}$ mol/l RPMI 1640 and the patient's own fresh serum, activated by application to the agarose gel.

The agarose plates were incubated at 37°C in humid atmospheric air (2 hours for neutrophils and 20 hours for monocytes). Migration was then stopped, and after overnight fixation in glutaraldehyde, the agarose gels were removed and the slides were stained with Wright's stain. To further examine whether the changes in monocyte migration after intake of n-3 PUFAs in hypercholesterolemic patients were due to a cellular effect or an effect on chemotactic properties of the patient's serum, one additional experiment was undertaken. Chemotactic properties of sera from all hypercholesterolemic patients were compared before and after n-3 supplementation using monocytes from one healthy normo-
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FIGURE 1. Diagram showing setup for measurement of leukocyte migration. A, active migration by front-leading leukocytes toward chemoattractant; B, distance of spontaneous migration of leukocytes toward control medium (lowermost circle); C, location of leukocytes after incubation (shaded area); and D, cell density (number of leukocytes) in the 250x250-μm area.

cholesterolemic subject. This was done in one analytical run to exclude interassay variation.

Neutrophil and Monocyte Chemotaxis

Monocyte and neutrophil chemotaxis was assessed microscopically with a scale for measuring migration distances and a grid for counting the number of cells that had migrated into a predefined area of 250×250 μm (Figure 1). All readings were performed after the trial was completed with the investigator blinded to the n-3 PUFA dose. Each sample was examined in triplicate, and the mean values of the following measurements were recorded: 1) directed migration, as the distance migrated (in microns by the leading front of cells toward the chemoattractant; 2) spontaneous migration, as the distance migrated (in microns) by the the leading front of cells toward the control. For monocytes, this front was not considered spontaneous migration, since after 20 hours of incubation, the cells moving toward the culture medium would be affected by chemotactic factors diffusing from the well containing the chemoattractant; 3) cell density, as the number of cells that had migrated into the grid with its base at a distance defined as in the spontaneous migration front for neutrophils and half of that distance for monocytes.

Assay Variation

Intra-assay (well-to-well) coefficient of variation for the measured parameters was below 0.10 for neutrophil studies and below 0.23 for monocyte assays. Interassay (day-to-day) coefficient of variation was below 0.17 for neutrophils and below 0.24 for monocytes. In the crossed study (patient sera toward control cells), the intra-assay coefficient of variation was below 0.10 for all parameters.

Plasma Lipids

Plasma lipids were measured by routine enzymatic methods.

Platelet Fatty Acid Composition

Platelet fatty acid composition was determined in platelets obtained from platelet-rich plasma. Platelet lipids were extracted, dissolved in CH2Cl2, and transesterified to methyl esters. Analysis of platelet fatty acids was performed by gas chromatography using an HP Model 5700 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a flame ionization detector, a 50-m×0.53-mm capillary column isothermal at 250°C, and a flow rate of N2 of 10 ml/min. This approach permits quantification of fatty acid methyl esters with 16 and more carbon atoms.

Statistics

Pratt's test was used to evaluate the effect of n-3 PUFAs on chemotaxis in patients with hyperlipidemia and the effect of the low dose of n-3 PUFAs in healthy subjects. Page's test was used to study dose-response effects. Monocyte chemotaxis toward autologous serum in patients with type IIa and IV hyperlipidemias was compared with chemotaxis in normolipidemic controls by the Mann-Whitney U test for unpaired data. p<0.05 was considered significant.

Results

The n-3 PUFA oil was well tolerated, and no adverse effects were observed. The composition of platelet fatty acids was closely related to the dose of n-3 PUFAs given (Table 3), thereby documenting a high compliance.

Monocyte Chemotaxis

Before treatment, monocyte migration toward autologous serum was significantly increased in patients with type IIa hyperlipidemia when compared with that of normcholesterolemic controls (Figure 2). After dietary supplementation with n-3 PUFAs, monocyte migration was reduced in this group but significantly only toward autologous serum (Table 4). Furthermore, monocytes from one healthy volunteer showed a significant decrease in migration after

from the well containing the chemoattractant; 3) cell density, as the number of cells that had migrated into the grid with its base at a distance defined as in the spontaneous migration front for neutrophils and half of that distance for monocytes.
Table 3. Platelet Fatty Acid Composition Before and After Supplementation With n-3 Fatty Acids

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Before</th>
<th>1.3</th>
<th>4</th>
<th>9</th>
<th>Dose response</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2, n-6</td>
<td>8.2</td>
<td>6.5</td>
<td>6.2</td>
<td>5.9</td>
<td>( p&lt;0.01 )</td>
</tr>
<tr>
<td>(6.4–12.4)</td>
<td>(5.7–7.7)</td>
<td>(5.3–6.5)</td>
<td>(5.2–6.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4, n-6</td>
<td>23.8</td>
<td>26.7</td>
<td>23.9</td>
<td>22.3</td>
<td>NS</td>
</tr>
<tr>
<td>(23.4–25.4)</td>
<td>(24.2–29.1)</td>
<td>(20.9–27.1)</td>
<td>(21.3–25.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:5, n-3</td>
<td>0.4</td>
<td>1.2</td>
<td>2.3</td>
<td>4.1</td>
<td>( p&lt;0.001 )</td>
</tr>
<tr>
<td>(0.4–0.7)</td>
<td>(1.0–1.4)</td>
<td>(1.7–2.8)</td>
<td>(3.6–4.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:5, n-3</td>
<td>1.2</td>
<td>1.7</td>
<td>1.9</td>
<td>2.7</td>
<td>( p&lt;0.001 )</td>
</tr>
<tr>
<td>(0.8–1.5)</td>
<td>(1.5–2.0)</td>
<td>(1.4–2.3)</td>
<td>(2.4–2.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6, n-3</td>
<td>1.6</td>
<td>1.8</td>
<td>2.1</td>
<td>2.3</td>
<td>( p&lt;0.001 )</td>
</tr>
<tr>
<td>(1.4–2.1)</td>
<td>(1.4–2.2)</td>
<td>(1.5–2.5)</td>
<td>(2.0–2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total saturated</td>
<td>34.8</td>
<td>34.3</td>
<td>34.2</td>
<td>34.2</td>
<td>NS</td>
</tr>
<tr>
<td>(30.3–35.8)</td>
<td>(33.2–35.4)</td>
<td>(31.9–36.5)</td>
<td>(32.8–35.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total monounsaturated</td>
<td>22.0</td>
<td>20.2</td>
<td>20.5</td>
<td>21.8</td>
<td>NS</td>
</tr>
<tr>
<td>(20.0–23.0)</td>
<td>(19.8–20.9)</td>
<td>(19.8–21.3)</td>
<td>(20.6–22.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4/20:5 (ratio)</td>
<td>56.5</td>
<td>22.8</td>
<td>11.3</td>
<td>5.3</td>
<td>( p&lt;0.001 )</td>
</tr>
<tr>
<td>(37.2–62.7)</td>
<td>(17.0–26.6)</td>
<td>(7.6–13.6)</td>
<td>(4.6–7.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results, which are in percent of total fatty acid content, are medians with interquartile ranges in parentheses. NS, not significant.

Ingestion of fish oil compared with that before the oil supplement, when chemotaxis was examined using sera from type IIa patients as a chemoattractant (Table 5). In patients with type IV hyperlipidemia, monocyte chemotaxis did not differ from that of controls (median value, 2,221 \( \mu \)m; interquartile range, 2,111–2,450 \( \mu \)m) and was unaltered by n-3 PUFAs (Table 4).

The results from the dose–response studies in healthy volunteers are given in Table 6. Monocyte chemotaxis was reduced by n-3 PUFAs in a dose-related fashion. The decrease in monocyte chemotaxis was significant even after the low dose of 1.3 g daily.

**Neutrophil Chemotaxis**

In patients with hyperlipidemia (types IIa and IV), neutrophil migration was significantly inhibited after supplementation with n-3 PUFAs (Table 7). Cell density using N-formyl-Met-Leu-Phe as a chemoattractant was significantly reduced after the oil supplementation period in patients with type IV hyperlipidemia and in all patients with hyperlipidemia.

The effect of various doses of n-3 fatty acids on neutrophil chemotaxis in healthy men is given in Table 8. Neutrophil chemotaxis was reduced after ingestion of n-3 PUFAs in a dose-dependent manner. This decrease was significant even after intake of the low dose. No effect of n-3 fatty acids was observed on spontaneous neutrophil migration, either in hyperlipidemic patients or in healthy men (data not shown). Plasma cholesterol was unaltered (mean cholesterol values, 8.7 mmol/l) in patients with type IIa hyperlipidemia after fish oil supplementation.

**Discussion**

Untreated patients with type IIa hyperlipidemia showed an increased monocyte chemotactic response when compared with that of normolipidemic controls. This finding is in line with results from animal studies, where monocyte adhesion to endothelial cells and monocyte migration into the intima have been shown to be augmented in animals made hypercholesterolemic. These effects were attributed to the formation of chemoattractants in the atherosclerotic lesions and to functional changes in the circulating monocytes. In the present study, monocyte chemotaxis was reduced in type IIa patients by intake of n-3 PUFAs to a level similar to that of controls, despite unaltered plasma cholesterol levels. This was due, at least in part, to an effect of...
TABLE 4. Monocyte Chemotaxis in Patients With Type IIa and IV Hyperlipidemias Before and After Supplementation With 6 g n-3 Polyunsaturated Fatty Acids/Day for 6 Weeks

| Table 5. | TABLE 5. Monocyte Chemotaxis in One Normolipidemic Subject
<table>
<thead>
<tr>
<th>Variable</th>
<th>Directed migration</th>
<th>Cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Own serum</td>
<td>N-formyl-Met-Leu-Phe</td>
</tr>
<tr>
<td>Before fish oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIa</td>
<td>2,807 (2,214–3,100)</td>
<td>1,980 (1,770–2,379)</td>
</tr>
<tr>
<td>Type IV</td>
<td>1,992 (1,415–2,500)</td>
<td>1,700 (1,240–2,220)</td>
</tr>
<tr>
<td>All (types IIa+IV)</td>
<td>2,350 (1,992–2,943)</td>
<td>1,850 (1,634–2,224)</td>
</tr>
<tr>
<td>After fish oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIa</td>
<td>2,100 (1,914–2,765)*</td>
<td>1,797 (1,563–2,043)</td>
</tr>
<tr>
<td>Type IV</td>
<td>2,090 (1,920–2,300)</td>
<td>1,726 (1,667–2,093)</td>
</tr>
<tr>
<td>All (types IIa+IV)</td>
<td>2,100 (1,914–2,540)</td>
<td>1,780 (1,577–2,049)</td>
</tr>
</tbody>
</table>

Directed migration and cell density were measured using the A'-formyl-Met-Leu-Phe and N-formyl-Met-Leu-Phe chemotactic peptides, respectively. Results are medians with interquartile ranges in parentheses.

- *p<0.01.

TABLE 6. Effect of Various Doses of n-3 Polyunsaturated Fatty Acids on Monocyte Chemotaxis in Healthy Men

<table>
<thead>
<tr>
<th>Time/effect/p</th>
<th>Directed migration</th>
<th>Cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time/effect/p</td>
<td>Own serum</td>
<td>N-formyl-Met-Leu-Phe</td>
</tr>
<tr>
<td>Before n-3</td>
<td>2,370 (1,937–2,727)</td>
<td>1,760 (1,641–2,209)</td>
</tr>
<tr>
<td>After n-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3 g daily</td>
<td>1,753 (1,669–2,403)</td>
<td>1,570 (1,279–1,677)</td>
</tr>
<tr>
<td>4 g daily</td>
<td>1,627 (1,545–1,699)</td>
<td>1,485 (1,230–1,534)</td>
</tr>
<tr>
<td>9 g daily</td>
<td>1,533 (1,314–2,331)</td>
<td>1,460 (1,110–1,650)</td>
</tr>
<tr>
<td>Dose–response effect</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Before vs. after 1.3 g n-3 daily</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are medians with interquartile ranges in parentheses. Serum from each type IIa patient, obtained before and after fish oil supplementation, was used as a chemoattractant.
PUFAs have been shown to reduce mononuclear generation of proinflammatory leukotriene B4,26 platelet-activating factor,27,28 interleukin-1,29 and tumor necrosis factor.29 These effects of n-3 fatty acids on monocyte function may be important for the development of atherosclerosis, as monocytes (and macrophages) are known to be implicated in atherogenesis.7

In the present study, neutrophil chemotaxis was reduced after n-3 supplementation in hyperlipidemic patients, which is in agreement with previous observations in healthy volunteers and in various patient groups.4-10,26 Furthermore, we report here that the reduction in neutrophil chemotaxis in healthy persons is dose related and significant even after the intake of a low dose (1.3 g) of n-3 PUFAs per day. The decrease in chemotaxis when using N-formyl-Met-Leu-Phe as a chemoattractant suggests a direct effect of n-3 PUFAs on the reactivity of neutrophils, while spontaneous neutrophil migration was unaltered. In addition, other aspects of neutrophil function are suppressed after dietary intake of n-3 PUFAs.1-5,6

Neutrophils have been suggested to be important for the outcome of acute myocardial ischemic events.8,9 The reduction in myocardial infarct size33-34 and in ventricular tachyarrhythmias after coronary artery ligation and during reperfusion35 in animals given fish oil may therefore partly have been due to an effect of n-3 PUFAs on neutrophil function.

Finally, an attenuation of leukocyte reactivity exerted by n-3 PUFAs is in accordance with the low prevalence of chronic inflammatory diseases in Greenland Eskimos36 and with reports of clinical improvement in patients with inflammatory disorders after ingestion of n-3 fatty acids.37,38 Also, the hitherto-unexplained reduction in CAD after intake of very modest amounts of fish observed in some epidemiological studies39-41 and in secondary prevention after myocardial infarction42 may have been caused by the effect of n-3 PUFAs on leukocyte function. Our findings of a dose–response effect of n-3 PUFAs on monocyte and neutrophil chemotaxis—with a significant suppression even after a low dose of 1.3 g n-3 PUFAs daily—may be of considerable clinical interest as a dietary means to reduce leukocyte reactivity, provided that these results can be reproduced in placebo-controlled studies.

Acknowledgments

The fish oil used in the study was produced by Jahres Fabrikker A/S, Sandefjord, Norway, and delivered as Pikasol by Lube A/S, Hadsund, Denmark.
References


Key Words • n-3 fatty acids • fish oils • hyperlipidemia • dose response • monocyte chemotaxis • neutrophil chemotaxis
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