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Eicosapentaenoic acid (EPA) from highly concentrated \( n-3 \) fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stability.

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**Abstract**

**Objective:** To examine \( n-3 \) polyunsaturated fatty acid (PUFA) incorporation into atherosclerotic plaques and the association with plaque inflammation and stability.

**Methods and results:** Patients awaiting carotid endarterectomy (\( n=121 \)) were randomised to consume control capsules or \( n-3 \) PUFA ethyl ester capsules until surgery (median 21 days). The fatty acid compositions of plasma and carotid plaque phospholipids, plaque features, and expression of inflammatory genes were determined. The proportion of eicosapentaenoic acid (EPA) was higher (\( P<0.001 \)) in carotid plaque phospholipids in patients in the \( n-3 \) PUFA group. Plaques from patients in the \( n-3 \) PUFA group had fewer foam cells (\( P=0.0390 \)). There were no other differences between plaques in the two groups with regard to histological characteristics or morphology. Plaque stability was not different between the two groups. However, the EPA content of plaque phospholipids was inversely associated with plaque instability (\( P=0.0209 \)), plaque inflammation (\( P=0.0108 \)), the number of T cells in the plaque (\( P=0.0097 \)) and a summary score considering a range of plaque features (\( P=0.0425 \)). Plaques from patients who received \( n-3 \) PUFA had significantly lower levels of mRNA for matrix metalloproteinases (MMP)-7 (\( P=0.0055 \)), -12 (\( P=0.0048 \)) and -12 (\( P=0.0044 \)) and for interleukin-6 (\( P=0.0395 \)) and intercellular adhesion molecule 1 (\( P=0.0142 \)).

**Conclusions:** Atherosclerotic plaques readily incorporate EPA. A higher plaque EPA content is associated with a reduced number of foam cells and T cells, less inflammation and increased stability.

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tries, it is important to identify whether long chain \(n-3\) PUFA esters from ethyl esters enter advanced atherosclerotic plaques, whether this incorporation is associated with changes in the plaque indicative of increased stability, and the possible mechanism of increased stability. We hypothesised that \(n-3\) PUFA ethyl esters would result in incorporation of eicosapentaenoic acid (EPA; 20:5\(n-3\)) and docosahexaenoic acid (DHA; 22:6\(n-3\)) into plasma and atherosclerotic plaque phospholipids, in less plaque inflammation including lower expression of selected inflammatory genes, and in greater atherosclerotic plaque stability. Therefore, we undertook a randomised, double-blind, controlled study of \(n-3\) PUFA ethyl esters in patients destined to undergo carotid endarterectomy.

2. Patients, materials and methods

2.1. Study design

Permission for all procedures involving patients was obtained from the Southampton and South West Hampshire Research Ethics Committee, and all patients gave written informed consent. The study was registered at www.clinicaltrials.gov (ClinicalTrials.gov identifier NCT00294216), is known by the acronym OCEAN (Omacor Carotid Endarterectomy Intervention), and was conducted according to good clinical practice. Patients destined to undergo carotid endarterectomy in the Southampton University Hospitals NHS Trust, Southampton or at Queen Alexandra Hospital, Portsmouth during the period March 2003 to December 2004 were considered eligible for entry into the study. The two hospitals are about 30 km apart and serve a similar population. Inclusion criteria were awaiting carotid endarterectomy, >18 years of age, and ability to give written informed consent. Exclusion criteria were regular consumption of more than two oily fish meals per week, use of fish oil or evening primrose oil supplements, being pregnant or breastfeeding, participation in another clinical trial, or requiring surgery within 7 days. Those who agreed to participate and who met the inclusion/exclusion criteria were randomised, in a double-blind manner, to receive control capsules (oil) or \(n-3\) PUFA ethyl esters (Omacor \textsuperscript{®}), both supplied by Pronova BioPharma AS, Lysaker, Norway. Capsules were identical in appearance and contained 1 g of oil. Patients consumed two capsules per day until surgery. Thus patients in the control group consumed 1.55 g oleic acid/day from the capsules; habitual intake of oleic acid among adult males in the UK averages 30 g/day \[29\]. Patients in the \(n-3\) PUFA ethyl ester group consumed 0.81 g EPA and 0.675 g DHA/day from the capsules; habitual intake of EPA plus DHA among adults in the UK who do not regularly consume oily fish or take fish oil capsules is considered to be <0.2 g/day \[30,31\]. The dose of EPA plus DHA used here is similar to that used in our previous study \[28\]. Randomisation of patients to treatment group was according to a random number table and was performed by Pronova BioPharma AS. Capsules were provided to patients in sealed containers.

We hypothesised that consumption of \(n-3\) PUFA ethyl esters would result in incorporation of EPA and DHA into plasma and atherosclerotic plaque phospholipids and in improved atherosclerotic plaque stability, indicated by morphological examination, degree of leukocyte infiltration and inflammation, and expression of selected inflammatory genes. The primary endpoint of the study was a measure of plaque inflammation and stability that was a composite of histological features and immunohistochemical measurements (see below). Secondary endpoints were EPA and DHA contents of plasma and plaque phospholipids, plaque morphology assessed by the American Heart Association and modified American Heart Association classifications, plaque features determined by morphological examination and immunohistochemical staining, and levels of mRNA for a range of inflammatory genes.

121 patients entered the study; 61 were randomised to control and 60 to \(n-3\) PUFA ethyl esters (Fig. 1). At entry to the study, patients underwent a physical examination, had their blood pressure, height and weight measured and gave a fasting venous blood sample into EDTA. Patients then commenced consumption of capsules as described above. Patients continued with medications throughout the study period and were advised not to change their diet. Compliance was promoted by regular contact with patients and was assessed by counting returned capsules.

Surgical removal of carotid plaques was performed at a time consistent with current clinical practice in the Southampton University Hospitals NHS Trust, Southampton and Queen Alexandra Hospital, Portsmouth. This was between 7 and 102 days (median 21 days) after patients' entry into the study. On the morning prior to surgery a second fasting venous blood sample was taken. At surgery the carotid plaque was collected and rinsed. It was then cut into cross-sections 2 mm in width, starting at the common carotid artery. Sections for biochemical analysis were frozen in liquid nitrogen. Sections for histology were fixed in formaldehyde and then embedded in paraffin wax. Sections for immunohistochemistry were frozen in optimal cutting temperature embedding medium (Agar Scientific, Stansted, UK), and stored at \(-70^\circ\text{C}\).

2.2. Fatty acid composition analyses

The fatty acid compositions of the phospholipid fraction of plasma and of the frozen section closest to the bifurcation of the carotid plaques were determined. Total lipid was extracted, phospholipids isolated by solid phase extraction and the fatty acid composition determined by gas chromatography as described elsewhere \[32\].

2.3. Histological examination and morphology of carotid plaques

Paraffin embedded sections were stained with haematoxylin and eosin. Sections were examined in random order and without access to any patient information. The section closest to the bifurcation was assessed for size of the lipid core, number of foam cells, haemorrhage, inflammation, and cap inflammation; scoring was based upon the experience of one of the authors \[33\]. Lipid core and haemorrhage were scored 1 (none), 2 (small) or 3 (large). Foam cells were scored 0 (none), 1 (few), 2 (many). Inflammation and cap inflammation were scored 0 (little), 1 (some), 2 (moderate), 3 (heavy). In addition, the section was classified using the guidelines
2.4. Immunohistochemistry of carotid plaques

The plaque section second from the bifurcation was used for immunohistochemistry. Sections were stained for the presence of macrophages (distinguished by the presence of CD68 on their surface) and T lymphocytes (distinguished by the presence of CD3 on their surface). Cryostat sections of frozen plaque were mounted on organosilan-coated microscope slides. Endogenous peroxidase activity was blocked and then the sections were successively incubated with optimal dilutions of mouse anti-human CD68 (KP1; DAKO, Ely, UK), biotinylated goat anti-mouse immunoglobulin G (DAKO, Ely, UK), and streptavidin-horseradish peroxidase (DAKO, Ely, UK). Finally, peroxidase activity was visualised using hydrogen peroxide as substrate and 3-amino-9-ethyl carbazole (Sigma Chemical Co., Poole, UK) as chromogen. Stained sections were viewed using a microscope under 10× power of magnification. Staining was scored 1 (little staining), 2 (moderate staining) or 3 (heavy staining).

2.5. Composite measure of plaque inflammation and instability

The scores for lipid core, foam cells, haemorrhage, inflammation, cap inflammation, macrophage staining and macrophage staining in the plaque cap were summed and averaged to give an overall summary score representing plaque inflammation and instability, the primary outcome of the study.
2.6. Carotid plaque mRNA analysis

Total cellular RNA was extracted from plaques using an RNA-gents total RNA isolation system (Promega, Southampton, UK). The purity and integrity of the RNA samples were assessed by A260/A280 spectrophotometric measurements. RNA samples were converted to cDNA by reverse transcription using a standard reverse transcription system (Promega, Southampton, UK). Real-time PCR was performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). PCR primers and probes (see Table 1 for sequences) were designed using Primer Express Program Version 2.0 (Applied Biosystems, Warrington, UK), with the forward and reverse primers located in different exons and the probe spanning an intron-exon boundary. PCR products for matrix metalloproteinase (MMP)-1 were detected using the SYBR Green method and all other PCR products were detected using probes labelled with reporter dye 6-carboxy-fluorescein at the 5′ end and quencher dye 6-carboxy-tetramethyl-rhodamine at the 3′ end. The ΔΔCt method was used to analyse the results. In brief, the Ct (threshold cycle) value of a gene was subtracted from the Ct value of a reference housekeeping gene (36B4) to standardize for the amounts of RNA template and efficiencies of reverse transcription. The resulting change in Ct values was then converted to a linear form using 2−ΔΔCt and applied to the subsequent statistical analysis.

2.7. Statistical analysis

Sample size was based upon that of our previous study [28], which used a similar dose of EPA plus DHA to that used here and which reported similar outcomes to those investigated here. Data are shown only for patients who completed the study according to the protocol (n = 53 in the control group; n = 47 in the n-3 PUFA ethyl esters group). Continuous data are shown as mean ± SD; categorical data are shown as median and range or 95% confidence interval. Normality of data was determined by Shapiro-Wilk test. Comparisons of data within a treatment group were made using paired Student’s t-test or Wilcoxon signed ranks test, depending upon whether data were normally distributed or not. Comparisons of data between treatment groups were made using unpaired Student’s t-test or Mann Whitney U-test, depending upon whether data were normally distributed or not. Age, sex and duration of treatment were controlled for in the statistical analysis. Comparisons of distributions between groups were determined by χ2-test. Correlations were determined as Pearson’s or Spearman’s correlation coefficients (r). All analyses were performed using SAS for Windows version 8.2 (SAS Institute, Cary, NC, USA). In all cases a value for P < 0.05 was taken to indicate a statistically significant difference.

3. Results

3.1. Patient characteristics

Eleven patients withdrew from the study: four for clinical reasons, one because they could not comply with the study protocol and six for unspecified reasons (Fig. 1). A further 10 patients were excluded from the analysis study because they were identified as protocol violators, pre-defined as failure to consume more than 70% of the allocated capsules (Fig. 1). Thus, data were analysed for 53 patients in the control group and 47 in the n-3 PUFA ethyl esters group.

The characteristics of the patients who completed the study are shown in Table 2. There were no significant differences between the treatment groups at study entry with respect to sex mix, age, body mass index (BMI), fasting plasma lipid concentrations, degree of stenosis of the affected carotid artery, clinical history, and use of medications. Each group received the treatments for similar durations (median 21 days; Table 2).

3.2. Fatty acid composition of plasma phospholipids

There were no differences in fatty acid composition of plasma phospholipids between treatment groups at baseline (Table 2). Treatment with n-3 PUFA ethyl esters significantly affected the proportions of several fatty acids in plasma phospholipids (Table 3). The proportions of EPA, docosapentaenoic acid (22:5n-3) and DHA were all significantly increased in the n-3 PUFA ethyl esters group, such that they were higher than at baseline and higher than in the control group (Table 3). Increases averaged 153, 33 and 57% from study entry, respectively. These increases were associated with significant decreases in the proportions of several n-6 fatty acids (Table 3).

---

Table 2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>n -3 PUFA ethyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n)</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>Female (n)</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Age (years)</td>
<td>74 ± 9 (49-91)</td>
<td>71 ± 9 (41-88)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>26.5 ± 3.1 (17.9-36.4)</td>
<td>26.9 ± 4.8 (18.8-41.8)</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td>1.6 ± 0.8</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>4.8 ± 1.2</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Ex-smokers (n)</td>
<td>35</td>
<td>30</td>
</tr>
</tbody>
</table>

Data for age, body mass index, and blood lipids are mean ± SD (age and body mass index ranges are shown in parentheses). Data for stenosis and duration of treatment are median with range shown in parentheses.

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Please cite this article in press as: Cawood AL, et al. Eicosapentaenoic acid (EPA) from highly concentrated n-3 fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stability. Atherosclerosis (2010), doi:10.1016/j.atherosclerosis.2010.05.022
Table 3
Fatty acid composition of plasma phospholipids (% of total fatty acids).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>n-3 PUFA ethyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>End</td>
</tr>
<tr>
<td>14:0</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>30.8 ± 3.4</td>
<td>31.8 ± 2.3</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>0.6 ± 0.3</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>18:0</td>
<td>14.6 ± 2.2</td>
<td>14.2 ± 1.7</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>12.6 ± 7.6</td>
<td>11.6 ± 1.9</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>18.9 ± 3.2</td>
<td>18.0 ± 2.7</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>3.3 ± 1.0</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>9.6 ± 2.4</td>
<td>10.0 ± 2.8</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>1.3 ± 0.6</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>3.9 ± 1.2</td>
<td>4.2 ± 1.2</td>
</tr>
</tbody>
</table>

Data are mean ± SD.
* Significantly different from control: \( P<0.005 \).
** Significantly different from control: \( P<0.001 \).

3.3. Fatty acid composition of carotid plaque phospholipids

There was one significant difference in the proportions of fatty acids in carotid plaque phospholipids: the proportion of EPA was significantly higher (by an average of 100%) in the n-3 PUFA ethyl esters group (Table 4). DHA was higher by an average 13% in the n-3 PUFA ethyl esters group but this was not significantly different from the proportion of DHA in the control group (\( P=0.076 \)).

3.4. Carotid plaque morphologic characteristics and classification and macrophage and T lymphocyte numbers

Plaque features were not different between the two groups, apart from the number of foam cells which was significantly lower in the n-3 PUFA ethyl esters group (Table 5). The summation of seven selected plaque features and their average score, the primary outcome of the study, were not different between treatment groups (Table 5). However, using data from both groups there were significant inverse correlations between the proportion of EPA in carotid plaque phospholipids and plaque instability (\( r = -0.239 \); \( P=0.021 \)), plaque inflammation (\( r = -0.263 \); \( P=0.011 \)), the number of T cells in the plaque (\( r = -0.268 \); \( P=0.010 \)) and the median plaque feature summation score (\( r = -0.211 \); \( P=0.043 \)).

The distribution of lesion types determined using the AHA and modified classifications was not significantly different between the two groups (Table 6).

3.5. Levels of mRNA in carotid plaque

Plaques from patients who had received n-3 PUFA ethyl esters had significantly lower levels of mRNA for MMP-7 (\( P=0.006 \), Table 5).

Table 5
Plaque features determined by histology and immunohistochemistry.

<table>
<thead>
<tr>
<th>Plaque feature</th>
<th>Control</th>
<th>n-3 PUFA ethyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid core</td>
<td>2 (2,3)</td>
<td>2 (2,3)</td>
</tr>
<tr>
<td>Foam cells</td>
<td>2 (1,2)</td>
<td>1 (1,2)*</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>1 (1,1)</td>
<td>1 (1,1)</td>
</tr>
<tr>
<td>Overall inflammation</td>
<td>2 (2,3)</td>
<td>2 (2,3)</td>
</tr>
<tr>
<td>Cap inflammation</td>
<td>2 (2,3)</td>
<td>2 (2,1)</td>
</tr>
<tr>
<td>Macrophages in plaque</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
</tr>
<tr>
<td>Macrophages in cap</td>
<td>3 (2,3)</td>
<td>2 (2,3)</td>
</tr>
<tr>
<td>T lymphocytes in plaque</td>
<td>2 (2,3)</td>
<td>2 (2,3)</td>
</tr>
<tr>
<td>T lymphocytes in cap</td>
<td>2 (1,3)</td>
<td>2 (1,2)</td>
</tr>
<tr>
<td>Instability</td>
<td>2 (2,2)</td>
<td>2 (1,2)</td>
</tr>
<tr>
<td>Summary score of 7 plaque features</td>
<td>15 (12,16)</td>
<td>14 (12,16)</td>
</tr>
<tr>
<td>Median score of 7 plaque features</td>
<td>2 (2,3)</td>
<td>2 (2,3)</td>
</tr>
</tbody>
</table>

Data are median with 95% confidence interval shown in parentheses.
* Defined using anti-Cd68.
** Defined using anti-Cd3.
*** Lipid core, foam cells, haemorrhage, overall inflammation, cap inflammation, macrophages in plaque, macrophages in cap.
* Indicates significantly different from control: \( P<0.039 \).

MMP-9 (\( P<0.005 \)), MMP-12 (\( P=0.004 \)), interleukin-6 (\( P=0.040 \)), intercellular adhesion molecule (ICAM)-1 (\( P=0.014 \)) and tissue inhibitor of metalloproteinases (TIMP)-2 (\( P=0.014 \)) than those from patients in the control group (Fig. 2). Although plaques from the n-3 PUFA ethyl ester group also had less mRNA for MMP-1, MMP-3, MMP-8, MMP-13, TIMP-1, TNF-\( \alpha \) and IL-10, these were not significantly different from levels in the control group (Fig. 2).

Table 6
Plaque morphology according to American Heart Association and modified American Heart Association classifications.

<table>
<thead>
<tr>
<th>AHA classification</th>
<th>Control</th>
<th>n-3 PUFA ethyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>6 (12.2%)</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>IV</td>
<td>21 (42.9%)</td>
<td>19 (42.2%)</td>
</tr>
<tr>
<td>Va</td>
<td>13 (26.5%)</td>
<td>12 (26.7%)</td>
</tr>
<tr>
<td>Vb</td>
<td>5 (10.2%)</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>Vc</td>
<td>1 (2%)</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>Vla</td>
<td>3 (6.1%)</td>
<td>2 (4.4%)</td>
</tr>
</tbody>
</table>

Modified AHA classification
Pathological intima thickening 6 (12%) 5 (11.1%)
Fibrous cap atheroma 22 (44%) 19 (42.2%)
Thin fibrous cap atheroma 13 (26%) 13 (28.9%)
Erosion 0 (%) 1 (2.2%)
Rupture 3 (6%) 2 (4.4%)
Calculated nodule and fibrocalcific plaque 6 (12%) 5 (11.1%)

Data are n with % of patients shown in parentheses.
4. Discussion

Long chain n-3 PUFAs such as EPA and DHA are usually consumed in small quantities in Western diets [30,31], and are therefore found in relatively low proportions in plasma and tissue lipids [36]. However, increased consumption of these fatty acids is marked by an increase in their proportion in various blood and tissue lipid pools [36], as observed here for the plasma phospholipid fraction. A key observation from this study is that when EPA is consumed in the ethyl ester form at a modest dose it is readily incorporated into atherosclerotic plaque lipids. Two previous studies reported the effect of fish oil supplementation on the fatty acid composition of atherosclerotic plaques; fish oil provides EPA and DHA in triglyceride form. Rapp et al. [37] showed substantial incorporation of EPA and DHA into plaque lipids following consumption of a very high dose of fish oil (48-64 g/day providing 16-21 g EPA + DHA/day). Thies et al. [28] demonstrated that n-3 PUFAs provided at a much lower dose than used by Rapp et al. and at a similar dose to that used in the current study, resulted in higher EPA and DHA in carotid plaque lipids, including phospholipids. The average differences in EPA and DHA in plaque phospholipids reported by Thies et al. were 85% and 9%, respectively, compared with control [28]. This compares with differences of 100% and 13% for EPA and DHA in the current study, although DHA was not significantly different in plaque phospholipids between the two groups. Thus, it appears that substantial incorporation of EPA into advanced plaques occurs within a relatively short time frame. This suggests that atherosclerotic plaques are fairly dynamic, with some degree of lipid turnover, even at an advanced stage of atherosclerosis. Incorporation of DHA into atherosclerotic plaques is more modest.

This study demonstrates for the first time lower expression of selected inflammatory genes in plaques of patients receiving n-3 PUFA ethyl esters prior to surgery. Several MMPs were measured because these have been implicated in plaque cap thinning, so increasing vulnerability to rupture [38-42]. In addition mRNA for two MMP inhibitors, TIMP-1 and TIMP-2, two pro-inflammatory cytokines (TNF-α, IL-6), one anti-inflammatory cytokine (IL-10) and one adhesion molecule (ICAM-1) were measured. Markedly lower levels of mRNA for MMP-7, MMP-9 and MMP-12 were observed in plaques from the n-3 PUFA ethyl esters group with smaller effects on the other MMPs, and on the adhesion molecules and pro-inflammatory cytokines. Overall, these observations suggest a reduced inflammatory burden within the plaques in the n-3 PUFA ethyl esters group that might be expected to result in increased stability. However, it is important to note that mRNA for TIMP-2 was also lower in the n-3 PUFA ethyl esters group, and this effect might oppose the reductions in MMP mRNA levels. Cipollone et al. [43] reported a strong association between macrophages, and expression of cyclooxygenase-2, prostaglandin E synthase and MMPs within carotid plaques and identified that inhibition of cyclooxygenase-2 in cultured monocytes was accompanied by decreased production of MMPs, an effect that could be reversed by adding prostaglandin (PG) E2. Thus one mechanism by which n-3 PUFAs within the plaque could reduce inflammatory gene expression is through inhibition of PGE2 production as reported in various model systems [24]. An alternative mechanism of action could be down-regulation of nuclear factor kappa B activation, an effect of n-3 PUFAs reported in monocytes and macrophages [44-46]. Activated nuclear factor kappa B was not measured in the current study but future studies should investigate its relationship with plaque n-3 PUFAs.

There was no effect of n-3 PUFA ethyl esters on the primary outcome of the study (a composite of histological features and immunohistochemical measurements representing plaque inflammation and stability) or on the components of that outcome, apart from the number of foam cells. While the lack of differences between control and n-3 PUFA ethyl esters with respect to plaque histology, morphology and immunohistochemistry appears to contradict some of the findings from an earlier study where there was a difference in plaque morphology between control and n-3 PUFA groups [28], that study was of longer duration allowing for a greater time for incorporation of n-3 PUFAs and for their action within the vessel wall. Nevertheless, the current study identified significant inverse relationships between the content of EPA in plaque phospholipids and plaque inflammation and plaque instability. These relationships suggest that the more EPA there is in the plaque, the less inflamed and the more stable it is. Interestingly n-3 PUFA ethyl esters treatment resulted in fewer foam cells within the plaque, determined by histological examination, and EPA content was inversely associated with T cell content, determined by immunohistochemistry. T cells direct many of the inflammatory responses within the vessel wall and foam cells are responsible for generation of inflammatory mediators like MMPs that degrade and thin the plaque cap making it more prone to rupture [25-27]. Thus, these observations indicate a chain of effects of EPA from n-3 PUFA ethyl esters: fewer T cells and fewer (inflammatory) foam cells, resulting in less inflammation, resulting in more stability. Thus, modification of T cell and foam cell numbers and, perhaps, activity may be the mechanism by which EPA stabilises advanced atherosclerotic plaques. The impact of n-3 PUFAs on plaque stability has recently been examined in animal models: apoE-deficient or low density lipoprotein receptor-deficient mice were fed on a Western diet or this same diet also containing EPA [47]. Consistent with earlier studies EPA suppressed development of aortic lesions in both models; in addition EPA was found to decrease macrophage infiltration into lesions and to increase collagen and smooth muscle content indicative of increased stability. Interestingly EPA also resulted in lower MMP-2 and MMP-9 mRNA in aortic lesions, con-
sistent with the effect on MMP-9 mRNA seen in the current study.

Since the vulnerability of the plaque to rupture, rather than the degree of atherosclerosis, is the primary determinant of thrombosis-mediated acute cardiovascular events [26], our findings of increased stability with increased plaque phospholipid EPA content may be clinically relevant. If carotid plaques are stabilised by EPA from n-3 PUFA ethyl esters, then the risk of neurological events in patients with advanced carotid atherosclerosis could be reduced. Furthermore, if these effects occur in early atherosclerosis (as well as in advanced disease, as studied here) then it might be possible to slow the development of unstable plaques. This could apply to lesions throughout the vasculature, including coronary arteries, and might explain the significant protective effect of n-3 PUFA towards fatal MI [14-16], which is so far unexplained.

Most studies of the relationship between fish intake, n-3 PUFA intake or n-3 PUFA status and coronary heart disease have focused on mortality [1–8], as have secondary prevention trials in post-MI patients [14–16]. These studies demonstrate lower risk of mortality and protection against sudden death with intake of fish or n-3 PUFAs. As indicated above, a plaque stabilising effect of n-3 PUFA consumption could explain these findings. However, increased plaque stability would be expected to decrease all cardiovascular events, not just those that are fatal. Consistent with this, Hu et al. [7] found an inverse relationship between long chain n-3 PUFA intake and risk of non-fatal MI among women; the relationship remained highly significant after adjustment for age and many lifestyle, health and dietary variables. Furthermore, Siscovick et al. [9] reported that risk of primary non-fatal MI was significantly decreased with increasing dietary intake of long chain n-3 PUFAs and with increasing long chain n-3 PUFA content of red blood cells. Likewise, adipose tissue content of EPA and DHA, a marker of dietary intake of these fatty acids, was significantly lower in subjects suffering a primary non-fatal MI than in controls [11]. Another study found a significant inverse relationship between long chain n-3 PUFA intake and risk of non-fatal MI [10]. Thus, in addition to their well-described protective effects against mortality and sudden death [1–8,14,15], it appears that long chain n-3 PUFAs may protect against nonfatal MI [7.9–11]. This effect would be consistent with increased plaque stability, as proposed here.

One limitation of this study is the short duration of treatment (median = 21 days), which was imposed by the normal clinical routine for patients awaiting carotid endarterectomy. This treatment time was shorter than that of the previous study (median = 42 days) [28] and may have limited n-3 PUFA incorporation into the carotid plaques, so limiting their effectiveness. A second limitation of this study is that the dose of n-3 PUFAs used (approximately 1.5 g EPA plus DHA/day) may have been insufficient to strongly influence plaque morphology and characteristics, including inflammation, although the dose used was similar to that used in the previous study in this patient group [28]. A third limitation may be that the number of patients studied was not sufficient to observe the anticipated effects, although again sample size was similar to the earlier study [28]. A final limitation is that plaques from patients receiving n-3 PUFA ethyl esters did not show a higher DHA content, and it may be that DHA is an important anti-inflammatory fatty acid by virtue of its effects on inflammatory gene expression [48] and on synthesis of resolvins [49]. Thus, lack of significant enrichment of plaques with DHA may have reduced the likelihood of finding more marked effects on the plaque outcomes assessed here.

In conclusion advanced atherosclerotic plaques appear to readily incorporate EPA from n-3 PUFA ethyl esters and a higher content of EPA in carotid plaques is associated with a reduced number of foam cells and T cells, less inflammation and increased stability. Nevertheless, the primary outcome of the study was not different between the control and n-3 PUFA ethyl esters groups, perhaps because of the short duration of treatment, an insufficient dose of n-3 PUFAs, or lack of incorporation of DHA.

Disclosures

RV is an employee of Pronova BioPharma AS. OG is an employee of LINK Medical. PCC has received speaking fees from Solvay Pharmaceutilicals (Germany) and Solvay Healthcare (UK), both distributors of n-3 PUFA ethyl esters.

Conflict of interest

None of the other authors has a conflict of interest.

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