Development and Regression of Atherosclerosis in Pigs

Effects of n-3 Fatty Acids, Their Incorporation Into Plasma and Aortic Plaque Lipids, and Granulocyte Function


Fifty-one pigs were fed a low-cholesterol basal diet, to which either 10% (by weight) of lard fat (group I\textsubscript{INORM}, \(n=7\)), 2% cholesterol plus 8% lard fat (group II, \(n=33\)), or 2% cholesterol plus 4% lard fat plus 4% fish oil (group III\textsubscript{PREV}, \(n=11\)) was added. In all pigs, the left anterior descending coronary artery and the abdominal aorta were denuded at 1 month. In the first 24 hours thereafter, three animals in group II and two in group III\textsubscript{PREV} died suddenly. After 3 months, 0.5% bile acids was added to the diet in groups II and III\textsubscript{PREV}. After 8 months the degree of atherosclerosis was evaluated in groups I\textsubscript{INORM} and III\textsubscript{PREV} and in 14 animals from group II (HIND). At 4 months, one animal from Group II died of pneumonia. For the next 4 months (postinduction period), the remaining 15 animals from group II received the basal diet, to which either 10% lard fat (group II\textsubscript{IND}, \(n=6\)) or 5% lard fat plus 5% fish oil (group II\textsubscript{F0}, \(n=9\)) was added. The hypercholesterolemic diet increased plasma cholesterol from 2 to 9–12 mM after 8 months. Fish oil had no major effects on plasma lipids during both induction and postinduction. Superoxide production by granulocytes in response to the membrane receptor-dependent \textit{N}-formyl-methionyl-leucyl-phenylalanine (fMLP) gave a higher response in group I\textsubscript{INORM} than in group I\textsubscript{INORM}. In group III\textsubscript{PREV}, the response to phorbol myristate acetate (PMA) and fMLP was lowered, while in groups II\textsubscript{IND} and II\textsubscript{F0} the responses to PMA and fMLP were not affected. The response to serum-treated zymosan was similar in all groups. Abrasion caused increases in free cholesterol (40%) and phospholipids (46%) in the abdominal aortas of group I\textsubscript{INORM} animals. Hypercholesterolemia increased both free and esterified cholesterol in the entire aorta. Fish oil prevented accumulation of free cholesterol in the nonabraded ascending aorta during induction and further accumulation of free cholesterol and phospholipids in the abdominal aorta during postinduction. In the nonabraded ascending aorta, triglycerides were significantly (almost five times) lower in group II\textsubscript{IND} than in group II\textsubscript{F0}. During both induction and postinduction, a large incorporation of n-3 polyunsaturated fatty acids (up to 20%) occurred in plasma and aortic cholesterol esters and phospholipids of groups II\textsubscript{IND} and III\textsubscript{PREV}. In plasma lipids the major fatty acid was always 20:5\(\alpha\)-linolenic, but in aortic lipids the incorporation of its elongated product 22:5\(\alpha\)-linolenic was generally equal to 20:5\(\alpha\)-linolenic. Sudanophilia of the aorta was 0% for group I\textsubscript{INORM}, 33% for group II\textsubscript{IND}, 35% for group III\textsubscript{PREV}, 9% for group II\textsubscript{IND}, and 7% for group II\textsubscript{F0}. Luminal encroachment of the coronary arteries in groups I\textsubscript{INORM} and II\textsubscript{IND} was similar. Fish oil (group III\textsubscript{PREV}) had no effect on the development of coronary atherosclerosis. Despite the normocholesterolemia during the postinduction period in group II\textsubscript{IND}, coronary atherosclerosis progressed to 12% in the right coronary artery and to 18% in the left anterior descending coronary artery, while in group II\textsubscript{IND} these values were 5% and 10%, respectively. In conclusion, isoenergetic administration of n-3 fatty acid ethyl esters did not prevent the development of coronary atherosclerosis (luminal encroachment) or aortic atherosclerosis (sudanophilia and aortic lipid content). However, fish oil feeding during the postinduction period attenuated the progression of coronary atherosclerosis and the accumulation of lipids in the damaged aortic wall, while aortic atherosclerosis as measured by sudanophilia was not affected. This study shows that the criteria used to assess experimental atherosclerosis are essential in the evaluation of the effects of n-3 fatty acids.

\textbf{KEY WORDS} \cdot fish oil \cdot atherosclerosis development \cdot atherosclerosis regression \cdot coronary artery \cdot aorta \cdot blood lipids \cdot granulocyte function \cdot pigs

There is ample epidemiological evidence suggesting that n-3 fatty acids protect against atherosclerosis.\textsuperscript{1–3} However, the many experimental studies that have evaluated the effects of fish oil on the development of atherosclerosis have generated ambiguous results, possibly as a result of differences in species (nonhuman primates, swine, rabbits, Japanese quail),

\textsuperscript{1} Supported by grants from The Netherlands Heart Foundation (NHS) and The Netherlands Science Foundation (NWO).

\textsuperscript{2} Address for correspondence: P.D. Verdouw, PhD, Experimental Cardiology, Thoraxcenter, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands.

\textsuperscript{3} Received August 11, 1992; revision accepted January 12, 1993.
dose of eicosapentaenoic acid (varying from 7.5 to 330 mg/kg body wt per day), duration of the study (2 weeks–3 years), type of vessel studied (aorta, coronary arteries, pulmonary artery, iliac artery), or the method used to evaluate the effects (percentage of macroscopically visible lesions, luminal encroachment, and/or aortic lipids).4–23 The need for further investigation therefore remains.

Since the presence of atherosclerotic plaques may already be established in many patients when they start a dietary regimen or fish oil concentrate ingestion, the question of whether fish oil consumption can cause regression of established atherosclerosis appears to be even more clinically relevant. To date, only three experimental studies have reported on the effects of fish oil on the regression of atherosclerosis, again exhibiting conflicting results.23–25

In humans consuming a Western diet, eicosapentaenoic acid and docosahexaenoic acid are barely present in plasma and atherosclerotic plaques,26 while ingestion of n-3 fatty acids results in the incorporation of members of this lipid family into advanced plaques.27 This may be critical for the capacity of these fatty acids to affect the atherosclerotic process. For instance, changes in the function of macrophages due to a specific accumulation in these cells of docosapentaenoic acid (22:5n-3), resulting in a decreased leukotriene C4/leukotriene C5 ratio,28 could play a role in the antiatherogenic properties of fish oil.

Furthermore, changes in the physical and chemical state of plaque lipids as well as changes in the fluidity of plaque cellular components and infiltrating lipoproteins29 may be important. On the other hand, the n-3 fatty acids present in lipoproteins could promote plaque growth by being more susceptible to attack by oxygen-derived free radicals (ODFRs) and thereby causing macrophages to take up these peroxidized lipoproteins.

The current hypothesis of atherogenesis does not include granulocytes as initiators in diet-induced lesion formation,30 although their role in the pathophysiology of myocardial ischemia and infarction has been established.31,32 Furthermore, it has been found that ODFRs can induce transient adherence of granulocytes to the vascular endothelium,33 which is a prerequisite for the massive production of ODFRs by these leukocytes.34 Granulocytes produce ODFRs at a much higher rate than macrophages,35 and the rate in granulocytes can be modulated by diets enriched in n-3 fatty acids in normolipidemic humans36 and guinea pigs.37 Blood monocytes are activated by elevated levels of low density lipoprotein.38 It is not known whether granulocytes are also activated in hypercholesterolemia or whether fish oil–rich diets can attenuate their function under those conditions. This raised the question of whether diet-induced changes in the degree of atherosclerosis are correlated with the ability of granulocytes to produce ODFRs.

We studied the effects of isocaloric administration of fish oil on both the development and regression of atherosclerosis in swine. In addition to the effects on plasma lipid concentrations, luminal encroachment of the coronary arteries, and the amounts of lipids and sudanophilia in the aorta, we also investigated the distribution pattern of the fatty acids in cholesterol esters and phospholipids in plasma and vessel wall lesions. To discriminate between the development of lesions caused by hypercholesterolemia and that caused by mechanical injury, we also studied a group of normolipidemic animals that was subjected to endothelial denudation of the aorta and coronary artery. The present study also reports on the production of ODFRs under the various dietary conditions by granulocytes after their stimulation by various agents.

**Methods**

**Dietary Groups**

Fifty-one castrated male Landrace × Yorkshire pigs (2 weeks of age, 11.7±0.6 kg) were arbitrarily allocated to three groups. All groups received a basal diet (Hope Farms BV, Woerden, The Netherlands) to which 10–12% (wt/wt) of fat was added. Group INORM (n=7) received 10% lard fat; group II (n=32), 8% lard fat and 2% cholesterol; and group IIIFREV (n=11), 2% cholesterol, 4% lard fat, and 4% fish oil concentrate (Optimena as ethyl esters, Hastar Enterprises International, Soest, The Netherlands). To further increase plasma cholesterol levels during this induction period, 0.5% of bile acids was added to the diet in groups II and IIIFREV after 3 months because adding it earlier can cause diarrhea with subsequent malabsorption. At 8 months in all animals of groups INORM and IIIFREV and in 14 animals of group II (group IIIND), the level of coronary and aortic atherosclerosis was estimated (see below).

The remaining animals of group II were divided into groups IIIF and IIIF0, receiving 10% lard fat and 5% lard fat/5% fish oil, respectively. This diet was continued for another 4 months (postinduction period). For a schematic illustration of the dietary regimen and surgical intervention, refer to Figure 1. For the two fish oil groups (IIIF and IIIF0), the relative amounts (mole percent) of 16:0, 18:0, 18:1, 18:2n-6, 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3 fatty acids were =15.7%, 7.5%, 28.5%, 16.3%, 1.8%, 12.9%, 1.7%, and 8.7%, respectively. For the remaining diets (groups INORM, IIIND, and IIIF), the respective values were 24.7%, 9.5%, 41.8%, 17.7%, 1.3%, 0%, 0%, and 0%. The daily amount of 20:5n-3 and 22:6n-3 fatty acids in group IIIF0 was 309 mg/kg body wt at the beginning of the postinduction period and 193 mg/kg at the end of the postinduction period and 220 mg/kg (beginning) and 137 mg/kg (end), respectively. In group IIIFREV, daily 20:5n-3 intake was 1.040 mg/kg at the beginning of the dietary period and 242 mg/kg at the end of the dietary period, while the daily 22:6n-3 intake was 743 mg/kg (beginning) and 175 mg/kg (end). All experiments were performed in accordance with the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society and under the regulation of the Committee on Animal Experimentation of the Erasmus University of Rotterdam.

**Endothelial Denudation of the Aorta and Left Anterior Descending Coronary Artery**

After 1 month all animals were subjected to endothelial denudation of the left anterior descending coronary artery and of the abdominal aorta to enhance development of atherosclerosis.39 In the first 24 hours after the denudation procedure, five of the piglets died suddenly (three animals in group II and two in group IIIFREV).
NORM 10% LF
II IND 2% chol + 8% LF
II LF 2% chol + 8% LF 10% LF
II FO 2% chol + 8% LF 5% LF + 5% FO
III PREV 2% chol + 4% LF + 4% FO

Time (months)

Plasma Lipids
After 0, 4, and 8 months (all groups) and at 9, 10, and 12 months (groups II LF and II FO), the animals were fasted for 24 hours, the subclavian vein was punctured, and blood samples were drawn for the measurement of total cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides.

Dissection and Macroscopic Staining of the Aortic Wall
At the end of the dietary period the animals were killed with an overdose of pentobarbitone sodium, and the aorta was removed, dissected free of adventitia, and split longitudinally. Intima–media preparations were excised from the ascending aorta and from the macroscopically visible lesions in the abdominal part of the aorta. The samples were stored at −80°C until analysis. After the samples were taken, the aorta was stained for lipid by using Sudan IV. Sudanophilia of the aorta was determined by projecting the aortic image onto a TV monitor and, using an integrated image-analysis system (IBAS-2000, Kontron, Oberkochen, FRG), the area of the Sudan IV-positive surface was calculated.

Lipid Infiltration of the Aortic Wall and Measurement of Fatty Acid Composition in Cholesterol Esters in Plasma and Aorta
From the plasma samples taken at the end of the dietary period, plasma lipids were extracted according to a slightly modified version (Reference 39) of the method of Bligh and Dyer. Aortic samples (40–200 mg) were homogenized and analyzed for lipid content. In the delipidated extracts, DNA contents were measured as previously described.

For fatty acid analysis, plasma and aortic lipids were separated by thin-layer chromatography on Kieselgel 60 F254 plates (Merck, Darmstadt, FRG) activated at 110°C for 1 hour. The solvent system contained n-hexane/diethyl ether/acetic acid (60:40:1, vol/vol/vol). The spots were scraped off and the fatty acids transmethylated with 14% BF3 in methanol (Sigma Chemical Co., St. Louis, Mo.) as described earlier.

Blood Sample Collection and Isolation of Granulocytes
At the end of the dietary period, blood samples drawn from the animals from the five dietary groups were collected in tubes containing 15 mg EDTA (Terumo Europe, Leuven, Belgium), subsequently diluted 1:1 with phosphate-buffered saline (pH 7.4) in polypropylene tubes (Costar), and then layered on a cushion of sterile, pyrogen-free Lymphoprep (Nycomed AS, Torshov, Norway) to separate granulocytes and erythrocytes from mononuclear leukocytes and platelets. After centrifugation for 15 minutes at 800g at room temperature, the granulocyte-rich pellet was washed and erythrocytes were lysed at 4°C with 0.155 M NH4Cl in 0.01 M NaHCO3 buffer, pH 7.4, containing 0.1 mM EDTA. The cells were washed again and resuspended in Hanks’ balanced salt solution containing 5.5 mM glucose and kept on ice until use.

Superoxide Anion Assay
Superoxide production by granulocytes was determined by the superoxide dismutase–inhibited ferricytochrome c reduction method. As stimuli, 100 ng/mL phorbol myristate acetate (PMA), 1.25 mg/mL serum-treated zymosan (STZ), and 1 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP) were used. The rate of superoxide production was calculated for the linear part of the increase in absorption curve (maximal rate) by using a molar extinction coefficient for cytochrome c of 21.1 × 103 M⁻¹ cm⁻¹ and was expressed as nanomoles of superoxide per minute per 10⁶ granulocytes.

Intimal Thickening of Coronary Arteries
At the end of the dietary period, the hearts of the animals were excised and the coronary arteries dissected free. Every 10 mm, transverse sections were cut from the left anterior descending coronary artery, the left circumflex coronary artery, and the right coronary artery. The
sections were routinely stained with hematoxylin-azophloxine and resorcin-fuchsin after paraffin sections were made. Intimal proliferation was measured using a computer-assisted morphometric analysis technique.6,45

Statistical Analysis

Data are presented as mean±SEM. The Student’s t test was used for determining the statistical significance of the differences. Because the data on lipid content, sudanophilia of the aorta, and superoxide production by granulocytes were not always normally distributed, they have been expressed as the median and the range, and the nonparametric Mann-Whitney U test was used to determine statistical significance. Statistical significance was accepted for p<0.05 (two-tailed).

Results

Because five animals died suddenly after the endothelial denudation procedure (three in group II and two in group III_pREV) and one animal in group II died of pneumonia at 4 months, the numbers of animals for which data were obtained were 7, 14, 6, 9, and 9 in groups I_NORM, II_nd, II_LF, II_F0, and III_pREV, respectively. During the dietary period, animals in groups I_NORM, II_nd, and III_pREV (after 6 months), II_LF, and II_F0 (after 12 months) increased their weight to 96±2, 83±4, 96±5, 135±8, and 144±10 kg, respectively.

Blood Lipids

In group I_NORM animals, plasma levels of total cholesterol increased slightly (p<0.05) during the dietary period (Figure 2). In group II_nd, the addition of bile acids after 3 months led to an increase of plasma cholesterol to more than four times baseline level at 4 months, which remained at this level after 8 months. The increments of cholesterol in groups II_LF and II_F0, receiving the same diet, were generally the same. The increases in plasma cholesterol were partly due to changes in HDL cholesterol (not shown), which in group II Nd, increased from 1.02±0.12 to 2.26±0.22 mM (p<0.05) at the end of the induction period, with similar changes in the induction phase for groups II_LF and II_F0.

In the postinduction period in group II_pREV, total cholesterol decreased to 1.70±0.13 mM (p<0.05 versus baseline) within 1 month and increased thereafter to 2.50±0.14 mM (p<0.05 versus baseline) during the last 2 months (Figure 2). In group II_F0, total cholesterol was at baseline level during the entire postinduction period.

Fish oil ingestion during the induction period (group III_pREV) did not have a major effect on plasma cholesterol, as only at the end of the dietary period was plasma cholesterol lower in III_pREV than in group II Nd. The changes in HDL cholesterol were not affected by fish oil (not shown).

Plasma triglycerides remained unchanged in group I_NORM (baseline value of 0.37±0.05 mM), while in group II_nd the addition of bile acids resulted in a decrease in triglycerides (70% of the predietary value of 0.36±0.03 mM) at 4 months, which returned to baseline levels during the following months.

During the first 4 months of the induction phase in groups II_LF and II_F0, the decreases in plasma triglycerides were similar to those in group II Nd. During the postinduction period in group II_LF, triglycerides were at baseline level. At the end of the postinduction period, triglycerides were significantly lower in group II_F0 than in group II_LF. During the induction period, fish oil did not affect plasma triglycerides in group III_pREV.

Superoxide Production by Blood Granulocytes

Induction period with or without fish oil. When stimulated by fMLP, granulocytes from group II Nd animals showed a higher superoxide production rate in comparison with granulocytes from group I_NORM (p=0.038; Figure 3). Fish oil prevented this increase (p=0.003 for the difference between groups III_pREV and II Nd; Figure 3). During phagocytosis of STZ, however, no significant differences in the rates of superoxide production by granulocytes between groups I_NORM, II Nd, and III_pREV were found (Figure 3). After stimulation by PMA, granulocytes obtained from group III_pREV animals produced superoxide at a lower rate than granulocytes of group II Nd (p=0.039); between groups I_NORM and II Nd, however, no differences were demonstrated (Figure 3).

Postinduction period with or without fish oil. Four months of feeding a low-cholesterol diet with (group II_F0) fish oil or without (group II_LF) did not alter the superoxide production in response to fMLP, STZ, or PMA (Figure 3).

Lipid Infiltration of the Aortic Wall

There were no macroscopically visible lesions in the nonabraded ascending aorta in any group or in the abdominal aorta of group I_NORM animals.

Effect of endothelial denudation (group I_NORM) and high-cholesterol feeding (group II Nd). Assuming that in pigs there are no regional differences with respect to aortic lipids, the effect of abrasion of the aorta can be deduced from the differences in lipid infiltration in the nonabraded (ascending) and the abraded (abdominal)
parts of the aorta of the low-cholesterol-fed animals (group INORM; Table 1). In the abdominal aorta, free and total cholesterol and phospholipids were 40-46% higher (all \( p < 0.05 \)) than in the ascending aorta, indeed revealing the atherogenic effect of abrasion only.

The effect of high-cholesterol feeding on aortic atherosclerosis was demonstrated by the lipid infiltration in group IIIND in comparison with group INORM. In both the ascending and abdominal aortas, the high-cholesterol diet caused an increase in the levels of total and free cholesterol and cholesterol ester, while triglycerides and phospholipids were not affected (Table 1).

### Table 1. Effect of Dietary Fish Oil on Aortic Lipid Content of the Nonabraded (Ascending) and Abraded (Abdominal) Aorta in Pigs

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Ascending aorta (nonabraded)</th>
<th>Lower abdominal aorta (abraded)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INORM (n=7)</td>
<td>IIIND (n=8)</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>1.42 (1.17-1.61)*</td>
<td>1.79 (1.25-6.90)</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>0.00 (0.00-0.08)*</td>
<td>0.43 (0.09-1.37)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.21 (0.09-0.59)</td>
<td>0.30 (0.03-1.49)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.56 (1.30-1.82)</td>
<td>1.62 (0.82-2.19)</td>
</tr>
</tbody>
</table>

| Free cholesterol | 1.99 (1.70-2.28)* | 3.49 (2.20-4.33) | 5.59 (2.94-12.63)* | 2.67 (1.39-5.46)* | 3.04 (2.67-4.36)* |
| Cholesterol ester | 0.00 (0.00-0.18)* | 0.49 (0.00-2.29) | 1.36 (0.46-3.10)* | 0.53 (0.09-2.03)* | 1.53 (0.66-2.29)* |
| Triglycerides | 0.36 (0.21-1.15) | 0.33 (0.09-3.41) | 1.05 (0.40-2.24) | 0.74 (0.17-1.38)* | 0.58 (0.21-0.92) |
| Phospholipids | 2.28 (1.76-2.66)* | 2.55 (1.45-3.75)* | 3.91 (2.78-7.20)* | 2.34 (1.65-4.02)* | 2.31 (1.65-2.94)* |

Data are median (range) and are in micromoles per milligram of DNA.

\*\( p < 0.05 \) vs. IIIND; \( \dagger p < 0.05 \) vs. IIIF; \( \ddagger p < 0.05 \) vs. ascending aorta.
TABLE 2. Fatty Acid Composition of Cholesterol Esters and Total Phospholipid Fractions in Plasma, the Nonabraded Ascending Aorta, and the Abraded Abdominal Aorta of the Five Dietary Groups at the End of the Dietary Period

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>INORM</th>
<th>IIIND</th>
<th>II LF</th>
<th>II FO</th>
<th>III PREV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol ester</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>54.7±1.4§</td>
<td>72.6±1.4</td>
<td>53.8±1.0§</td>
<td>56.2±1.2§</td>
<td>58.4±0.8§</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>46.2±1.5§</td>
<td>26.9±1.5</td>
<td>45.6±1.2§</td>
<td>35.3±0.9</td>
<td></td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>0</td>
<td>0.8±0.2</td>
<td>0.8±0.2</td>
<td>8.5±0.5§</td>
<td>12.0±0.8§</td>
</tr>
<tr>
<td>Ascending aorta†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>...</td>
<td>90.0±1.3</td>
<td>67.7±1.4§</td>
<td>73.7±2.1§</td>
<td>67.5±1.6§</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>...</td>
<td>9.5±1.0</td>
<td>30.7±1.2§</td>
<td>23.3±2.0§</td>
<td>21.0±2.0§</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>...</td>
<td>0.4±0.4</td>
<td>1.5±1.3</td>
<td>0.3±0.2§</td>
<td>11.1±0.8§</td>
</tr>
<tr>
<td>Abdominal aorta‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>...</td>
<td>87.3±1.4</td>
<td>66.0±3.2§</td>
<td>75.0±2.7§</td>
<td>68.4±1.4§</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>...</td>
<td>12.7±1.3</td>
<td>33.3±3.2§</td>
<td>19.6±2.9§</td>
<td>20.7±1.0§</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>...</td>
<td>0</td>
<td>0.6±0.1§</td>
<td>5.4±0.8§</td>
<td>10.9±0.7§</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>63.5±0.5§</td>
<td>67.9±0.6</td>
<td>66.6±0.4</td>
<td>67.9±1.0</td>
<td>63.6±1.0§</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>32.0±0.48§</td>
<td>29.6±0.6</td>
<td>30.0±0.2</td>
<td>15.3±0.3§</td>
<td>16.7±0.3§</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>3.9±0.3§</td>
<td>2.6±0.1</td>
<td>3.5±0.3§</td>
<td>16.2±0.7§</td>
<td>19.7±1.0§</td>
</tr>
<tr>
<td>Ascending aorta†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>69.5±2.7</td>
<td>68.4±0.8</td>
<td>68.5±2.7</td>
<td>67.7±0.5</td>
<td>68.8±0.8</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>28.2±2.3</td>
<td>28.5±0.7</td>
<td>29.3±2.2</td>
<td>22.8±0.5</td>
<td>20.6±0.4§</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>2.3±0.4</td>
<td>2.2±0.1</td>
<td>2.2±0.5</td>
<td>9.5±0.1</td>
<td>10.4±0.4§</td>
</tr>
<tr>
<td>Abdominal aorta‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFAs+MUFAs</td>
<td>71.9±3.2</td>
<td>78.2±1.9</td>
<td>69.8±1.9§</td>
<td>78.0±3.3</td>
<td>69.5±1.1§</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>26.0±2.8</td>
<td>20.9±1.6</td>
<td>28.0±1.5§</td>
<td>15.5±1.5§</td>
<td>19.5±0.6</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>2.1±0.5</td>
<td>0.9±0.3</td>
<td>2.2±0.4</td>
<td>6.4±2.1</td>
<td>11.5±0.8§</td>
</tr>
</tbody>
</table>

Data are mean±SEM in percent. SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

* n=6 for group INORM, II LF, and III PREV; n=10 for group II IND; n=5 for group II FO.
†For cholesterol ester and phospholipid, n=4 for all groups except for cholesterol ester in group II IND (n=3) and phospholipids in group III PREV (n=7).
‡For cholesterol ester and phospholipids, n=4 for all groups except for cholesterol ester in group II IND (n=3) and cholesterol ester and phospholipids in group III PREV (n=6).
§ p<0.05 versus group INORM; || p<0.05 versus group II LF.

In the aortic tissue of the normolipidemic animals, the amount of cholesterol ester was too small to evaluate the fatty acid composition.

Effect of fish oil during the induction period (group II IND versus group INORM). The increase in free cholesterol content of the ascending aorta (group II IND versus INORM) was prevented by fish oil in group III PREV (Table 1), but the contents of cholesterol ester, triglycerides, and phospholipids in the ascending aorta of group III PREV animals were not significantly different from those in II IND. In the abdominal aorta, lipid infiltration was not affected by fish oil.

Fatty Acid Composition of Cholesterol Ester and Phospholipids in Plasma and the Aorta in the Five Dietary Groups

Effect of the high-cholesterol diet on cholesterol ester and phospholipid fatty acids in plasma (group INORM versus II IND and group II LF versus INORM). The high-cholesterol diet had no major effects on plasma phospholipids (group INORM versus II IND) but caused a marked increase in the sum of the saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in plasma cholesterol ester and decreases in n-6 polyunsaturated fatty acids (PUFAs) (Table 2). More specifically, oleic acid (18:1n-9) increased from 30.1±0.9% to 43.4±1.4%, while both arachidonic (20:4n-6) and linoleic (18:2 n-6) acids decreased from 3.8±0.2% to 1.4±0.2% and from 41.8±1.5% to 25.2±1.3%, respectively.

Four months of low-cholesterol feeding (group II LF) normalized the fatty acid composition of cholesterol ester and phospholipids (group II LF versus INORM), indicating the reversibility of the effects of a hypercholesterolemic diet on the fatty acid composition of plasma lipoproteins.

Effect of fish oil addition on cholesterol ester and phospholipid fatty acids in plasma. The most prominent result observed in plasma cholesterol ester after addition of fish oil to the diet during the postinduction period was the increase of the relative content of n-3 PUFAs from 0.8% (in group II LF) to more than 8% (in...
group II(F0) at the expense of n-6 PUFA s (Table 2). Similarly in plasma phospholipids, n-6 PUFA s were exchanged for n-3 PUFA s (group II LF versus II F0).

Addition of fish oil to the hypercholesterolemic diet (group III PREV versus II IND) caused the relative content of the total n-3 fatty acid pool of cholesterol ester to increase to 12%. The relative content of SFAs plus MUFAs in group III PREV returned to normal values (as observed in group II(NORM)). Again, in the phospholipids the incorporation of n-3 PUFA s was at the expense of n-6 PUFA s (group II IND versus III PREV).

Generally, the incorporation of n-3 PUFA s in cholesterol ester consisted almost entirely of 20:5n-3, while in phospholipids of group II F0, 22:5n-3 and 22:6n-3 were 2.0±0.2% and 3.4±0.2%, respectively. In group III PREV these respective values were 3.7±0.5% and 4.4±0.4% (not shown).

Effect of high-cholesterol feeding on the fatty acid composition of cholesterol ester and phospholipids of the aorta. In group II IND the relative content of n-6 PUFA s in plasma cholesterol ester was more than two times lower than in cholesterol ester in both parts of the aorta (Table 2).

The relative amount of n-6 PUFA s in phospholipids in plasma is higher than that in the plaque of the abraded abdominal aorta in group II IND. The fatty acid composition of phospholipids in the abraded and non-abraded parts of the aorta in group II IND is similar to that in group II(NORM), indicating that the changes in fatty acid composition caused by the hypercholesterolemic diet (compare abdominal aorta of groups II IND and II(NORM)) are reversible in the plaque of the abdominal aorta as well.

Effect of fish oil during the postinduction period on the fatty acid distribution of cholesterol ester and phospholipids in the aorta (group II(F0)). Feeding n-3 fatty acids during the postinduction period resulted in the appearance of n-3 fatty acids in cholesterol ester of the nonabraded aorta (group II IND). The relative content of the total n-3 pool was twice as large as in group II(F0) animals, consisting mainly of 20:5n-3 (not shown). In the plaques of group II(F0) animals, the total amount of n-3 fatty acids was higher than in the abraded aorta of group II LF (5.4% versus 0.6%), but incorporation of 20:5n-3 (2.3±0.3%) and 22:5n-3 (2.1±0.8%) was almost equal.

Effect of fish oil during the induction period (group III PREV) on the fatty acid distribution of cholesterol ester and phospholipids in the aorta. In contrast to group II(F0), in group III PREV the n-3 fatty acids were present during the formation of plaques and for a longer period of time (8 months versus 4 months), and there was a relatively higher intake (see “Methods” section). The increases in n-3 fatty acids in cholesterol ester in group III PREV are therefore more distinct (Table 2). The differences in n-3 fatty acid incorporation in phospholipids are less striking: only in the plaque is total n-3 fatty acid content almost twice as large in group III PREV (p<0.05) than in group II IND. In the abraded or nonabraded aorta, the incorporation of 20:5n-3 and 22:5n-3 in cholesterol ester and phospholipids was almost equal (not shown).

Sudanophilia of the Aortic Wall

None of the aortas of the normolipemic animals were stained with Sudan IV. In group II ND, coverage of the intimal surface with Sudan IV-positive lesions was 33% (4.2–72%), while in groups II LF and II IND sudanophilia was 8.7% (2.0–11.9%) and 6.6% (0.4–24%), respectively (both p<0.05 versus group II IND). In group III PREV this value was 35% (18–57%), which was not significantly different from that in group II IND.

Intimal Thickening of the Coronary Arteries

Effect of endothelial denudation and high-cholesterol feeding. In the normolipemic animals mean luminal encroachment of the abraded left anterior descending coronary artery (12.7±3.1%, Table 3) was significantly higher than in the nonabraded arteries, which indicates that in this model the most damaging incident for the coronary arteries is the abrasion of the vessel and not the lipid burden. This is supported by the luminal encroachment data for the coronary arteries of the high-cholesterol–fed animals in group II IND, which were similar to the group II(NORM) data.

Effect of fish oil during the postinduction period. Luminal encroachment of both the abraded left anterior descending coronary artery and the nonabraded left circumflex coronary artery in group II LF was not affected during the postinduction period (Table 3). Moreover, intimal proliferation in the right coronary artery was enhanced during this period, as luminal encroachment increased to 12.3±2.4% (p=0.011 versus group II IND). Fish oil prevented this progression of luminal encroachment in the right coronary artery (5.1±1.7%, p=0.025 versus group II LF). Development of atherosclerosis in the two other vessels was not significantly different in the two postinduction groups.

Effect of fish oil during the induction period. Luminal encroachment of the left anterior descending coronary artery in group III PREV was lower than in group II IND, but

### Table 3. Effect of Dietary Fish Oil on Luminal Encroachment of Porcine Nonabraded and Abraded Coronary Arteries

<table>
<thead>
<tr>
<th>Artery</th>
<th>Dietary group</th>
<th>Dietary group</th>
<th>Dietary group</th>
<th>Dietary group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I(NORM) (n=7)</td>
<td>II IND (n=14)</td>
<td>II LF (n=6)</td>
<td>II IND (n=9)</td>
</tr>
<tr>
<td>LADCA (abraded)</td>
<td>12.7±3.1</td>
<td>13.4±2.8</td>
<td>17.8±5.3</td>
<td>9.9±2.5</td>
</tr>
<tr>
<td>RCA (nonabraded)</td>
<td>1.5±0.3†</td>
<td>4.4±1.5†</td>
<td>12.3±2.4†</td>
<td>5.1±1.7†</td>
</tr>
<tr>
<td>LCXCA (nonabraded)</td>
<td>3.8±0.9†</td>
<td>3.1±0.8†</td>
<td>5.1±1.2†</td>
<td>5.9±3.6</td>
</tr>
</tbody>
</table>
| Values are mean±SEM in percent. LADCA, left anterior descending coronary artery; RCA, right coronary artery; LCXCA, left circumflex coronary artery. *p<0.05 versus abraded vessel (left anterior descending coronary artery) in the same group; †p<0.05 versus group II IND; §p<0.05 versus group II IND.
levels of significance were not reached \((p=0.075, \text{Table 3})\). Also in the nonabraded vessels, fish oil did not suppress atherogenesis.

**Discussion**

**Fish Oil and Regression of Atherosclerosis**

This is the fourth study reporting on the effects of fish oil on the regression of atherosclerosis. We previously presented results of a study in swine in which atherosclerosis was induced by endothelial denudation and high-cholesterol feeding for 4 months, after which time the animals were put on a low-cholesterol, fish oil (two doses)-containing diet for 3 months.\(^2\) Fish oil retarded the progression and caused regression of coronary atherosclerosis. In the present study, we added 0.5\% bile acids to the diet and prolonged the induction period to 8 months. Plasma cholesterol was indeed higher in the present study (about 9 mM compared with 4 mM in the previous study; Reference 25), but intimal thickening of the coronary arteries was still only 13.4\% in the induction group compared with 11\% in the previous study.\(^2\) However, during the subsequent postinduction period there was no regression of the severity of coronary atherosclerosis. The longer induction period in the present study may have contributed to the development of lesions, which became more resistant to fish oil treatment. In accordance with our previous study, fish oil prevented progression of established lesions. Fincham et al\(^F\) (24.5 months of induction and 20 months of postinduction) found that in the African green monkey fish oil did not cause regression of coronary atherosclerosis and, if anything, worsened aortic atherosclerosis as measured by an overall score including 16 variables, like the presence of foam cells, endothelial loss, and cholesterol clefts. On the other hand, Zhu et al\(^F\) (10 weeks of induction and 10 weeks of postinduction) demonstrated that fish oil significantly reduced sudanophilic lesions in the rabbit aorta and pulmonary artery. We also found a decrease in sudanophilic coverage in the two postinduction groups, but there was no difference between groups. However, aortic atherosclerosis as assessed by lipid content of the lesions was significantly lowered by fish oil, emphasizing the importance of the definition and criteria used for evaluating the severity of atherosclerosis.

**Fish Oil and Prevention of Atherosclerosis**

In the present study fish oil failed to prevent the incorporation of cholesterol into normal aortic tissue and plaques. This is in agreement with the studies of Hill et al,\(^F\) the only other studies in pigs that also described aortic lipid contents. However, they also found coronary and aortic atherosclerosis (based on grading techniques) to be decreased by fish oil in one of the studies.\(^F\) We found a lower level of coronary atherosclerosis, which, possibly because of the small number of animals, did not reach levels of significance. Using comparable doses of n-3 fatty acids, Weiner et al\(^F\) found a substantial reduction in the degree of coronary atherosclerosis in pigs after 8 months. Despite similar species, duration of study, dose of n-3 fatty acids, and definition and criteria of atherosclerosis, their control group exhibited a much higher luminal encroachment (up to 87\%) than in the present study, which could be due to the differences in diet (2\% bile acids, 29\% lard fat) resulting in higher levels of plasma cholesterol (to >17 mM) and a more vigorous endothelial denudation. The difference in the effect of fish oil could be due to the different types of fish oil, as we used ethyl esters, which are more purified than triglycerides. Little information is available on the metabolism of these ethyl esters, although we have shown that they easily enter the plasma and tissue lipids of pigs.

**Fish Oil and Fatty Acid Composition of Cholesterol Ester and Phospholipids in Plasma and Plate**

This is the first study reporting on the effects of fish oil, administered during the formation of atherosclerotic lesions, on the fatty acid composition of the developing plaque. During both induction and postinduction, a large incorporation of n-3 PUFAs occurred in the plasma and aortic cholesterol ester and phospholipids of groups II F0 and III PREv. The increases in the relative content of 22:5n-3 in both fish oil-fed groups II F0 and III PREv are absent (plasma cholesterol ester) or at least less pronounced (plasma phospholipids) than the increases in 20:5n-3, which was to be expected, as fatty acid chain elongation takes place predominantly in macrophages in the vessel walls.\(^2\) Indeed, the elongated product of 20:5 n-3 was shown to be present in the aortic lesion. Despite the presence (9\%) of 22:6n-3 in the diets, the increase in 22:6n-3 in phospholipids in the two fish oil-fed groups was only moderate compared with the effects on the incorporation of 20:5n-3 (13\% of the fatty acids in the fish oil diet). Moreover, in plasma cholesterol ester of group II F0 , it was not present and in group III PREv it was barely present. These findings support the study by Holub et al,\(^F\) who showed that the 22:6n-3/20:4n-6 ratio in plasma cholesterol ester was much lower than in plasma phosphatidylcholine, demonstrating docosahexaenoic acid's limited participation in the lecithin:cholesterol acyltransferase reaction. However, retroversion of 22:6n-3 to 20:5n-3 may have occurred in the liver.

After high-cholesterol feeding (group II IND), the relative amount of n-6 PUFAs in cholesterol ester in the aorta is higher than in plasma. This is analogous to the results of Rapp et al,\(^F\) who found a lower level of saturates and monounsaturates in human plaques derived from peripheral vascular surgery explants, with total n-6 PUFAs being higher.

We showed that during the postinduction period in the plaques of group II F0 , the total amount of n-3 PUFAs (5.4\%) was higher than in group II LF (0.6\%), indicating that n-3 fatty acids do enter an existing plaque, which again confirms the results by Rapp et al.\(^F\)

**Fish Oil and Granulocyte Superoxide Radical Production**

The present study shows that in diet-induced hypercholesterolemia, the rate of superoxide radical production by granulocytes after stimulation by PMA and STZ does not differ significantly from the normolipidemic controls. However, when stimulated by the membrane receptor–dependent stimulus fMLP, a significantly higher response was found. Granulocytes from fish oil-fed pigs (group III PREv) showed a significantly lower rate of superoxide production after stimulation by PMA and fMLP than did granulocytes from group II IND. Thus,
Fish oil prevented the increased response to fMLP in the induction phase. This observation supports the finding of Fletcher and Ziboh,\textsuperscript{37} who showed that fish oil treatment inhibited superoxide production after stimulation by fMLP and PMA. The fact that the response to fMLP in group IIHF was not affected cannot be readily explained.

The increased response to fMLP of granulocytes from hypercholesterolemic pigs could be relevant to the progression of aortic lesions found in this diet-induced disease. It has been demonstrated that N-formylated peptides are produced by mammalian mitochondria,\textsuperscript{47} which raises the possibility that the release of such peptides from damaged endothelial cells and smooth muscle cells provides a mechanism for recruiting blood granulocytes to those sites. Furthermore, fMLP increases the adherence of granulocytes to the endothelium\textsuperscript{48} and stimulates the respiratory burst.\textsuperscript{49} Superoxide is a growth promoter of vascular smooth muscle cells\textsuperscript{50} and releases Fe\textsuperscript{2+} from ferritin.\textsuperscript{51} Since Fe\textsuperscript{2+} can oxidize low density lipoprotein, this could also yield the atherogenic effects observed in the induction group. Thus, from the increase in fMLP-stimulated superoxide production by granulocytes from group IIHF and the postinduction groups IIHF and IIIHF in comparison with groups I NORM and III REV, a higher luminal encroachment in the nonabraded coronary arteries of the three former groups could be anticipated. However, luminal encroachment of the nonabraded coronary arteries was too small to draw a firm conclusion regarding a possible correlation between atherogenesis and granulocyte function.

Acknowledgments

We thank Regina Krak-Slee and Jacqueline Boonman for expert technical assistance.

References

37. Fletcher MP, Ziboh VA: Effects of dietary supplementation with eicosapentaenoic acid or gamma-linolenic acid on neutrophil phospholipid fatty acid composition and activation responses. *Inflammation* 1990;14:585-597
43. Lamers JMJ, Dekkers DHW, De Jong N, Meijs JTA: Modification of fatty acid composition of the phospholipids of cultured rat ventricular myocytes and the rate of phosphatidylinositol-4,5-biphosphate hydrolysis. *J Mol Cell Cardiol* 1992;24:605-618
Development and regression of atherosclerosis in pigs. Effects of n-3 fatty acids, their incorporation into plasma and aortic plaque lipids, and granulocyte function.
L M Sassen, J M Lamers, W Sluiter, J M Hartog, D H Dekkers, A Hogendoorn and P D Verdouw

doi: 10.1161/01.ATV.13.5.651

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/5/651