The Effects of Dietary ω3 Fatty Acids on Platelet Composition and Function in Man: A Prospective, Controlled Study

By Scott H. Goodnight, Jr., William S. Harris, and William E. Connor

The rarity of atherosclerotic vascular disease and a mild bruising tendency in Greenland Eskimos has been linked to their ingestion of ω3 fatty acids contained in foods obtained from the sea. Previous studies have shown that feeding salmon oil to normal volunteers resulted in reductions of plasma cholesterol and triglycerides. We wished to learn whether salmon oil feeding would result in the incorporation of ω3 fatty acids into platelets and whether platelet function or platelet-vascular interactions would be altered. Diets containing salmon oils led to the incorporation of eicosapentaenoic acid (C20:5 ω3) into platelets (6.1%) with a reduction in arachidonic acid (C20:4 ω6). The ratio of C20:5/C20:4 increased from 0.0045 on the control diet to 0.3 on the salmon diet. Bleeding times were prolonged (from 6.75 to 10 min, p < 0.006), platelet retention on glass beads was mildly reduced (from 89% to 78%, p < 0.005), and platelet aggregation in response to dilute concentrations of ADP was inhibited in the subjects ingesting the salmon oil. We conclude that in normal subjects dietary ω3 fatty acids derived from salmon oil are incorporated into platelet phospholipids and that these changes are accompanied by alterations in bleeding time and platelet function.

The relationships of diet, vascular disease, platelets and thrombosis have intrigued clinical investigators for several generations. Recently, Danish workers have focused attention on Eskimos from the West Coast of Greenland who have a low incidence of myocardial infarction and stroke as well as a mild tendency to bruise. Their dietary fat is derived largely from whale, fish, and seal. Compared to Danes, the Eskimos had lower levels of plasma cholesterol and triglyceride, increased bleeding times, and increased plasma concentrations of the distinctive ω3 very long chain and highly polyunsaturated fatty acids (i.e., eicosapentaenoic and docosahexaenoic acids) characteristic of fish and other marine animals.

Dietary fatty acids may be classified into families by the location of the first (ω) double bond from the methyl end of the molecule.* For example, arachidonic acid (C20:4 ω6) is the familiar ω6 series fatty acid precursor for prostaglandin synthesis in platelets, endothelial cells, and other tissues, whereas the ω3 family is composed of fatty acids such as eicosapentaenoic acid (C20:5 ω3) found principally in nature in fish, shellfish, phytoplankton, and in animals that ingest marine life for food.

In platelets, arachidonic acid is converted by the enzyme cyclooxygenase to cyclic endoperoxides, which are rapidly transformed by thromboxane synthetase to thromboxane A2, an extremely potent vasoconstrictor and platelet aggregating substance. In contrast, the vascular endothelial cell converts arachidonic acid via the same endoperoxides to PG1 (prostacyclin), which is a vasodilator and inhibits platelet aggregation. To explain the prolonged bleeding times and lack of vascular disease in the Greenland Eskimos, it has been suggested that eicosapentaenoic acid (C20:5 ω3) may compete with arachidonic acid for cyclooxygenase and may thereby alter platelet–vessel interactions.

With this background, we performed a prospective, controlled trial in which normal subjects were fed diets containing large amounts of ω3 fatty acids in the form of Pacific Northwest Chinook salmon and salmon oil. Initial studies indicated that these dietary changes led to the appearance of ω3 fatty acids in the plasma and to a significant reduction of plasma cholesterol and triglyceride. In the present study, we wished to learn whether salmon oil feeding would result in the uptake of ω3 fatty acids into platelets and whether platelet function would be inhibited or bleeding times prolonged. If so, the combination of reduced blood

*The ω (omega) nomenclature is a system of fatty acid classification that designates fatty acids by the position of the first double bond counting from the terminal methyl group. Thus, ω3 fatty acids all have their first double bond between the third and fourth carbons from the methyl end. Common ω3 fatty acids are linolenic (C18:3) and the two fatty acids found in the salmon oil used in this study, C20:5 and C22:6. Other common fatty acid families are the ω6 family that contains arachidonic acid (C20:4) and linoleic acid (C18:2) and the ω9 family, of which oleic acid (C18:1) is a member.

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lipids and inhibition of platelet–vessel interactions induced by salmon oil feeding might prove to be beneficial in the prevention or therapy of vascular disease in man.

MATERIALS AND METHODS

Subjects

Informed consent was obtained from 6 normal healthy male and 5 female nonsmoking volunteers who were taking no medications that could influence platelet function. The mean age of the study subjects was 39 yr.

Study Design

The subjects were outpatients in a clinical research center (CRC) environment and ate all meals under the supervision of the dietary staff. Each subject was randomly assigned to either the control or salmon diet for 4 wk. After a 3-wk rest period during which the subjects usual home diet was eaten, the alternate diet was eaten for an additional 4 wk. Plasma and platelet lipid analyses, platelet function tests, and bleeding times were performed at least twice during the last 2 wk of each dietary period.

Diets

Diets for all subjects were prepared daily by CRC dieticians. The control diet approximated the "typical American diet," was isocaloric and contained 45% of calories as fat, 15% as protein, and 40% as carbohydrate. The fat was largely saturated (36%) and monounsaturated (46%) and was derived from egg yolk, cocoa butter, and peanut oil. Virtually no w3 fatty acids were present. The dietary cholesterol was 500 mg/day. The diet consisted of fruits, vegetables, bread, and other cereal products plus a blended formula that contained the cholesterol and most of the fats. Vitamin E (50 mg/day) was further added to the diet.

The salmon diet was essentially identical in composition, calories, cholesterol, and percent fat, protein and carbohydrate except that almost all of the dietary fat and all of the dietary cholesterol were derived from salmon steaks (about 1 lb/day) and salmon oil (60–90 ml/day). Each subject ingested approximately 10 g of w3 fatty acids, which totaled 15% of fat calories. Since fish oils contain relatively smaller amounts of linoleic acid, the salmon diet contained 4% of fat calories as linoleic acid compared to 18% in the control diet.

Both diets were well tolerated and body weights did not change throughout the study.

Test Methods and Procedures

Platelets were enumerated and sized with electronic counting equipment (Coulter Instruments, Hialeah, Fla.) and results verified by phase microscopy. Wright’s stained blood smears were examined by light microscopy.

Platelet Lipids

Platelet-rich plasma (PRP) was prepared from EDTA-treated blood and the platelet count determined. Platelets were then sedimented at 7000 g for 20 min and the lipids extracted according to Bligh and Dyer. A recovery standard 4-14C-cholesterol was added for the assay of platelet cholesterol. The extracted lipids were separated into individual lipid classes according to previously described methods. After separation, methyl heptadecanoate was added to the phospholipid band as a recovery marker and an internal standard for gas liquid chromatography. Cholesterol was quantified as previously described and fatty acids were measured using a HP 5830A chromatograph (Hewlett-Packard, Avondale, Pa.) with a SP 2330 column (Supelco, Inc. Bellefonte, Pa.). Column conditions were oven 180°C for 10 min, then rising at 5°C/min to 200°C for 20 min; injection port 250°C; flame ionization detector, 250°C; N2 flow, 44 ml/min. Both the relative proportions of fatty acids in platelet phospholipids and the absolute amount of each acid were determined by comparison to the internal standard.

Platelet Protein

Washed platelets were analyzed for total protein by the Hartree modification of the Lowry method.

Ivy Bleeding Time

Ivy bleeding times were performed using a disposable Simplate II device (General Diagnostics, Warner-Lambert Co, Morris Plains, N.J.). In brief, a standard sphygmomanometer cuff was applied to the upper arm and inflated to 40 mm Hg. The upper lateral aspect of the volar surface of the forearm was shaved, lightly cleaned with an alcohol sponge and allowed to dry. After horizontal bleeding time incisions were made, blood was absorbed by capillary attraction every 30 sec using the edge of torn strips of filter paper, with care taken not to disturb the edges of the wound. The time was recorded when blood completely stopped flowing from each of the sites and the times were averaged. A group of 35 normal control subjects (average age 30 yr) taking no medications had a mean bleeding time of 6.8 ± 2.64 min with this method.

Platelet Retention

Platelet retention on glass beads was performed immediately after venipuncture on freshly drawn nonanticoagulated blood using glass bead columns (Cutter Laboratories, Berkeley, Calif.) and a syringe pump set to flow at 5.8 ml/min. Normal values range from 75%–100%.

Platelet Aggregation

Platelet aggregations were carried out on citrated platelet-rich plasma (200,000/cu mm) using a platelet aggregometer (Chronolog Corp., Havertown, Pa.) and paper strip recorder. Aggregating agents included ADP (Sigma, St. Louis, Mo.) collagen (Bio/Data Corp., Willow Grove, Pa.), epinephrine (Parke Davis, Morris Plains, N.J.), thrombin (Parke Davis), arachidonic acid (Nu Chec Prep, Inc., Elysian, Minn.), and ristocetin (Cutter Laboratories). Various concentrations of aggregating agents were added to PRP and height of aggregation recorded at 4 min. In addition, the concentrations of ADP needed to induce 90%, and 50% maximal aggregation was recorded. All aggregations were carried out at 37°C, stable pH, stirred at 1000 rpm and within 90 min of venipuncture. Serotonin release was measured with the use of 14C-serotonin (New England Nuclear, Boston, Mass.).

Malondialdehyde (MDA) production by platelets after stimulation by N-ethylmaleimide was determined with a thiobarbituric colorimetric reaction.

Statistical analysis was performed using the Student’s t test for paired observations; p < 0.05 was considered significant.

RESULTS

Platelet Composition (Table 1)

The cholesterol, protein, and fatty acid contents of the phospholipid fraction of platelets were measured during the control and salmon dietary periods in 5 of
Table 1. Platelet Composition

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Salmon Diet</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>µg/10⁹ platelets</td>
<td>56 ± 13.8</td>
<td>68 ± 7.5</td>
<td>p &lt; 0.025</td>
</tr>
<tr>
<td>µg/mg platelet protein</td>
<td>45.8 ± 4.9</td>
<td>48.8 ± 9.7</td>
<td>NS</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/10⁹ platelets</td>
<td>1.10 ± 0.3</td>
<td>1.24 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Fatty acids (phospholipid fraction)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0 + 18:0</td>
<td>34.6 ± 1.9</td>
<td>34.6 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>C18:1 ω 9</td>
<td>16.1 ± 0.9</td>
<td>18.8 ± 0.3</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>C18:2 ω 6</td>
<td>7.5 ± 1.5</td>
<td>4.0 ± 1.2</td>
<td>p &lt; 0.025</td>
</tr>
<tr>
<td>C20:4 ω 6</td>
<td>27.6 ± 3.0</td>
<td>20.2 ± 2.5</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>C20:5 ω 3</td>
<td>0.1 ± 0.2</td>
<td>6.1 ± 1.4</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>C22:6 ω 3</td>
<td>1.1 ± 0.7</td>
<td>3.7 ± 0.7</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Others</td>
<td>12.9 ± 4.2</td>
<td>12.5 ± 4.6</td>
<td>NS</td>
</tr>
<tr>
<td>µg Fatty acid/10⁹ platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:5 (ω 3)</td>
<td>0.28 ± 0.29</td>
<td>7.42 ± 3.46</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>C20:4 (ω 6)</td>
<td>31.07 ± 10.4</td>
<td>18.97 ± 3.6</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.0045</td>
<td>0.30</td>
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the 11 study subjects. Platelet phospholipid fatty acids as measured by gas liquid chromatography changed significantly after the subjects ingested the salmon diet. Both ω3 fatty acids (C20:5, C22:6) rose and ω6 fatty acids C20:4 and C18:2 fell during the experimental period. The most dramatic change was the rise in C20:5 from 0.1% of total fatty acids to 6.1%. Moreover, the ratio of C20:5/C20:4 greatly increased during salmon feeding from 0.0045 to 0.30. There were no significant changes in the levels of other platelet fatty acids.

Although dietary cholesterol intake was the same during the 2 dietary periods, and the plasma cholesterol was lower during the salmon period, the platelet cholesterol rose from a mean of 56 to 68 µg/10⁹ platelets (p < 0.025). Since platelet protein increased from 1.11 ± 0.3 mg/10⁹ platelets on the control diet to 1.24 ± 0.4 mg/10⁹ platelets on the salmon diet, the calculated platelet cholesterol/mg of protein also rose slightly from 45.8 to 48.8 µg platelet cholesterol/mg platelet protein.

Platelet Function (Fig. 1)

Platelet function was assessed by measurements of platelet retention on glass beads, platelet aggregation, ¹⁴C-serotonin release, and an estimate of platelet prostaglandin production, the formation of malondialdehyde. Platelet–vessel wall interactions were studied using the standardized Ivy bleeding time.

In 11 subjects receiving the salmon diet, the bleeding time was prolonged from 6.75 to 10 min (p < 0.005). The bleeding time in the subject with a platelet count of 90,000/cu mm was 9 min 45 sec. Platelet retention on glass beads using freshly drawn nonanticoagulated blood was reduced from 89% to 78% (p < 0.0005) during the salmon period.

Platelet aggregation testing was carried out using a wide variety of aggregating agents including collagen, ADP, epinephrine, thrombin, and arachidonic acid (Table 2). The magnitude of platelet aggregation to ADP decreased significantly when subjects were ingesting the salmon diet. These findings were seen only at low dilutions of ADP (e.g., a final concentration of 1.4 or 2 µM). The concentration of ADP needed to induce 90% or 50% maximal aggregation is greater during the salmon phase, indicating less responsivity of the platelets to the aggregating agent. However, these findings were not significantly different at a p value of <0.05. Using relatively high concentrations of
Fig. 1. Tests of platelet number and function during control and salmon diets in 11 normal study subjects.

Platelet production of malondialdehyde was assessed following stimulation of the platelets with the sulfhydryl blocking agent n-ethylmaleimide. No reduction, and in fact, an increase of malondialdehyde, occurred in platelets from subjects consuming the salmon diet.

DISCUSSION

We prospectively studied platelet lipid composition and function in 11 normal individuals ingesting a diet rich in salmon oil under carefully defined conditions and compared the findings to those obtained during a control (typical American) diet. Distinctive ω3 fatty acids (e.g., C20:5, C22:6) increased greatly in the plasma and in the platelets of the study subjects during the salmon phase. In recent reports, these fatty acids were also found in the plasma and platelets of Eskimos eating a seal and whale diet and in German subjects fed large quantities of mackerel for 6 days.

The platelet content of cholesterol was measured since plasma cholesterol in our subjects decreased by 17% and alterations in platelet reactivity have been...
linked to changes in platelet cholesterol.\textsuperscript{21} In our study, platelet cholesterol did not decrease, and therefore, reduced platelet reactivity induced by lowered membrane cholesterol levels cannot explain the prolongation of bleeding times observed in the test subjects.

A mild to moderate fall in the platelet count (but not WBC or RBC) occurred in all but one of the subjects, and platelet numbers fell to less than 150,000/cu mm in three subjects. In these instances the platelet count rose to normal in about 1 wk after salmon feeding was discontinued. Whether the diminished circulating platelet count was due to decreased or altered marrow production, splenic sequestration or accelerated consumption is not known, but the presence of megathrombocytes may indicate shortened platelet survival and consequent increased marrow production of platelets or perhaps a direct effect of the lipid changes on the megakaryocyte. The reductions in platelet count cannot explain the prolonged bleeding times since levels of less than 100,000/cu mm are necessary to significantly prolong the bleeding time in normal individuals.\textsuperscript{22} No subject in our study had any bleeding phenomenon. The falls in platelet count are consonant with the lower levels of platelets seen in Eskimos compared to Danish controls (171,000/cu mm versus 232,000/cu mm).\textsuperscript{3}

The increase in Ivy bleeding times from the salmon diet was similar to the longer bleeding times found in Greenland Eskimos when compared to a Danish control population.\textsuperscript{3} Studies of platelet aggregation in response to a variety of aggregating agents showed no dramatic alterations but did show a significant diminution of aggregation to dilute concentrations of ADP. These findings are similar to those reported by Dyerberg and Bang\textsuperscript{4} in the Eskimo population. At a time when our study was largely complete, Siess et al.\textsuperscript{20} reported that subjects ingesting a mackerel diet had decreased platelet aggregation to very low concentrations of collagen. We used higher concentrations of collagen and found no difference in aggregation, although \textsuperscript{14}C-serotonin release was reduced by 20%.

We did observe a small but significant reduction in platelet retention to glass beads when freshly drawn, nonanticoagulated, and immediately processed blood was used for the assay. Plasma factors known to influence platelet adhesiveness such as fibrinogen or von Willebrands factor (VII\textsubscript{VW}) did not change during the dietary periods. Whether another plasma factor such as PGI\textsubscript{2} or PGI\textsubscript{1} generated during venipuncture may have had an effect is not known.

The \textit{w3} fatty acid, eicosapentaenoic acid (C20:5) from fish oil has been shown to participate in prostaglandin metabolism in vitro. Moncada postulated that C20:5 would displace C20:4 (arachidonic acid) in the prostaglandin pathway and produce thromboxane A\textsubscript{3}, which was originally thought to be inactive, rather than the active thromboxane A\textsubscript{2}. In contrast, PGI\textsubscript{2} generated from endothelial cells inhibited platelet aggregation.\textsuperscript{8} More recently, however, Needleman et al.\textsuperscript{23,24} have suggested that C20:5 competitively inhibits platelet cyclooxygenase and is itself a poor substrate for thromboxane synthetase leading to diminished production of both thromboxane A\textsubscript{2} and thromboxane A\textsubscript{3}.

Since assays for thromboxane B\textsubscript{2} were not available to us at the time of our study, the platelet production of malondialdehyde was chosen as an estimate of prostaglandin synthesis in an effort to determine if \textit{w3} fatty acids from fish oil might inhibit prostaglandin synthesis. For example, aspirin in low doses will virtually eliminate malondialdehyde production by platelets in humans through inhibition of cyclooxygenase\textsuperscript{25} and has been used to estimate thromboxane synthesis in clinical studies. In 10 of 11 subjects, the production of malondialdehyde by platelets failed to decrease and, in fact, increased during the salmon phase of the study. The explanation for this finding is, as yet, unknown and differs from the "in vitro" work previously cited\textsuperscript{23,24} and the short-term mackerel feed-

<table>
<thead>
<tr>
<th>ADP Aggregation</th>
<th>Collagen Aggregation and Release</th>
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<tr>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>5 \mu m*</td>
<td>2 \mu m</td>
</tr>
<tr>
<td>Control Diet</td>
<td>61.75  48.10  39.70</td>
</tr>
<tr>
<td>Salmon Diet</td>
<td>57.30  36.55  28.14</td>
</tr>
<tr>
<td>Difference</td>
<td>-3.45  -11.55  -12.93</td>
</tr>
<tr>
<td>(± 1SD)</td>
<td>(± 8.8) (± 18.6) (± 13.2)</td>
</tr>
<tr>
<td>Significance</td>
<td>NS     p &lt; 0.05 p &lt; 0.05</td>
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</table>

\*Final concentrations of aggregating agent in platelet-rich plasma.  
†Units of aggregation. Total possible units equal 80.  
‡\mu ADP required to reduce maximal platelet aggregation to 90% or 50%, respectively.  
§Percent serotonin released after aggregation induced by 0.2 mg/ml collagen.
ing study where levels of thromboxane B2 were shown to diminish.20 The agent used to stimulate prostaglandin generation in our study was n-ethylmaleimide, which may act differently in platelet lipid peroxidation than low doses of other aggregating agents such as collagen.

These relatively small changes in dilute ADP and collagen aggregation, platelet retention on glass beads, and thromboxane generation may be sufficient to explain the lengthened bleeding times, since the local concentration of aggregating agents at the skin incision site is unknown and could be equivalent to the concentrations used in the aggregation assays. However, alternative explanations such as increased vascular production of PGI2 or platelet production of PGD2 must also be considered.

Although this and other studies have shown that fish oil feeding may result in lowered plasma lipids and altered platelet-vessel interactions, it is far too early to advocate a change in diet for normal individuals or for those with vascular disease. Additional studies are clearly needed to document any possible long-term toxicity, to determine the mechanisms of the lipid and platelet changes, and to provide direct evidence that such treatment would be efficacious in the prevention or therapy of atherosclerotic vascular disease.

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The effects of dietary omega 3 fatty acids on platelet composition and function in man: a prospective, controlled study

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