Variable Hypocoagulant Effect of Fish Oil Intake in Humans

Modulation of Fibrinogen Level and Thrombin Generation

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Objective—The beneficial effect of dietary fish oil, rich in omega-3 polyunsaturated fatty acids (PUFAs), on cardiovascular disease is multifactorial and may partly rely on their anticoagulant action. We studied how fish oil intake influenced thrombin generation in plasma and which factors were involved herein.

Methods and Results—Twenty-five healthy males with borderline overweight received 3.0 g omega-3 PUFAs daily for 4 weeks. Fish oil intake reduced plasma triglycerides and lowered platelet integrin activation, as well as plasma levels of fibrinogen and factor V, but had no effect on vitamin K-dependent coagulation factors. Before fish oil intake, thrombin generation (reflecting the coagulant potential) considerably varied between plasmas from individual subjects, which were partly explained by variation in prothrombin, antithrombin, fibrinogen, and factor V levels. Fish oil intake reduced thrombin generation in the presence and absence of platelets. This reduction correlated with the fish oil effect on fibrinogen and factor V levels. Interestingly, the lowering effect of fish oil on thrombin generation and fibrinogen clustered around subjects with high fibrinogen carrying a structural fibrinogen α-chain polymorphism.

Conclusions—Dietary omega-3 PUFAs provoke a hypocoagulant, vitamin K-independent effect in humans, the degree of which may depend on fibrinogen level. (Arterioscler Thromb Vasc Biol. 2004;24:1734-1740.)

Key Words: coagulation • factor V • fibrinogen • fish oil • thrombin generation

Since the 1970s, fish oil has been studied as a nutritional component with antithrombotic potential.1 The protective effect on thrombosis has been attributed to the omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid, and docosahexaenoic acid, which are abundantly present in fish oil. Early epidemiological and intervention studies pointed to a strong association between consumption of omega-3 PUFAs and a reduced risk of coronary heart disease, even with only 2 fish dishes per week.2,3 Currently, a daily intake of 0.3 g of omega-3 PUFAs is recommended for healthy adults, and a daily dose up to 1 to 3 g is recommended for patients with coronary heart disease or hypertriglyceridemia.4

Despite 30 years of study, the precise mechanisms of action of omega-3 PUFAs are still a matter of debate.5 Established effects include an altered heart and vessel function, a decreased risk for arrhythmias, and lowering of blood pressure. Many reports also describe effects on plasma hemostatic variables, but usually with high interstudy variation. Best documented is that omega-3 PUFA intake reduces plasma triglycerides levels, whereas plasma cholesterol is decreased in only few studies.6–8 Part of published studies show, often mild, lowering effects of omega-3 PUFA on platelet activation and bleeding time.9

With respect to coagulation, some trials point to a moderate reduction by fish oil of the plasma levels of fibrinogen and coagulation factors V, VII, and X,10–12 whereas other studies fail to detect this.5,9 Because some of these factors require vitamin K-dependent carboxylation for coagulant activity, it was suggested that fish oil interferes with the vitamin K action. In rat, we and others have found that high amounts of dietary omega-3 PUFAs reduce the levels of the vitamin K-dependent factors X and prothrombin.13,14 By continuous measurement of thrombin generation, which provides a highly sensitive method of measuring the coagulant potential of plasma,15,16 we established that this lowering of coagulation factor levels resulted in hypocoagulant activity.13 However, because this hypocoagulant effect in rat was not enlarged by vitamin K depletion and was accompanied by a reduction in (vitamin K-independent) factor V, the hypocoagulant effect of fish oil has at least a partially vitamin K-independent cause.17 How intake of omega-3 PUFAs influences thrombin generation in humans is still unknown.
Genetic variation in coagulation factors and adhesive platelet glycoprotein is likely to contribute to the risk for arterial and venous thrombosis.\(^{18,19}\) There is limited evidence that genetic variety may also contribute to the antithrombotic response to nutrition. For instance, the reducing effect of dietary omega-6 fatty acids on lipoprotein levels has been found to differ between carriers of polymorphisms of several genes encoding for apolipoproteins.\(^{20}\) It is therefore possible that the hypocoagulant effect of omega-3 PUFAs is also sensitive to gene–environmental interactions which, in turn, contribute to the variable outcome of fish oil intervention studies.

In the present study, we investigated the effects of fish oil-derived omega-3 PUFAs on thrombin generation (reflecting the coagulant potential) in a group of subjects with borderline overweight and therefore slightly increased thrombotic risk. Because thrombin generation in plasma is critically dependent on the presence of procoagulant phospholipids,\(^ {21}\) this process was measured in the presence of either phospholipid vesicles or autologous platelets. It appeared that fish oil intervention decreased thrombin generation even in the absence of platelets, along with coagulation factors fibrinogen and factor V. Intriguingly, this hypocoagulant effect was clustered in a subgroup with relatively high baseline levels of fibrinogen who were carrying the 312A polymorphism in the fibrinogen-\(\alpha\) gene.

### Methods

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### Results

#### Effects of Fish Oil Intervention on Coagulation Factor Levels and on Thrombin Generation in the Presence and Absence of Platelets

Twenty-five healthy male subjects, aged 48.5±9.8 years (mean±SD), with borderline overweight (body mass index 29.0±2.5 kg/m\(^2\)) participated in the fish oil study. The intervention consisted of intake of capsules with 3.0 g omega-3 PUFAs per day for 4 weeks, which was equivalent to, on average, 32.8 mg omega-3 PUFAs per kg body weight daily. Blood samples were taken 4 weeks before, immediately before, and 4 weeks after fish oil treatment. In baseline blood samples, all 25 subjects had normal counts of platelets (215±9×10\(^3\)/L), erythrocytes (5.2±0.1×10\(^12\)/L), and leukocytes (6.6±0.4×10\(^9\)/L). Fish oil intake did not affect these parameters. The intervention resulted in significantly lower levels of plasma triglycerides, which is a common effect of fish oil. In contrast, cholesterol in low-density lipoprotein tended to be increased (borderline significant; Table 1).

After earlier diet studies with rats,\(^ {13,17}\) vitamin K-(in)dependent coagulation factors were measured. In the group of 25 volunteers, fish oil reduced plasma levels of the vitamin K-independent coagulation factor V (significant) and of fibrinogen (borderline significant), but not of the vitamin K-dependent factors, prothrombin, factor VII, and factor X (Table 1). Fibrinogen antigen level was significantly reduced after fish oil by \(-4.1±3.1\%\) (mean±SE; \(P=0.03\)) when an EIA-based test was used instead of the activity-based Clauss test. Anticoagulant proteins antithrombin and protein C were not altered (Table 1).

To measure platelet activation, platelets in platelet-rich plasma (PRP) were stimulated with the PAR1 thrombin-receptor agonist, SFLLRN. Flow cytometric analysis indicated that fish oil intake significantly lowered SFLLRN-induced activation of \(\alpha\)IIb\(\beta\)3 integrin (\(-18.4\%\)), but not SFLLRN-provoked exposure of P-selectin, when compared with effects of SFLLRN in pre-intervention PRP samples (Table 2).

As a sensitive way to monitor the coagulant potential under hypocoagulant conditions, we measured thrombin generation in tissue factor-triggered plasma after the thrombogram method.\(^ {15}\) To provide procoagulant phospholipids, plasma was
supplied with platelets (PRP of standardized platelet count) or with a nonlimiting concentration of 4 μmol/L phospholipids (platelet-free plasma [PFP]/phospholipids). Coagulation in PRP was initiated with a low concentration tissue factor (0.5 pmol/L) sufficient to start intrinsic coagulation and to detect platelet-dependent effects. Coagulation in PFP/phospholipids was triggered with optimal tissue factor (5 pmol/L). The thrombograms were analyzed on the following parameters: time to thrombin peak; peak height (indicative of maximal rate of thrombin formation); and area-under-the-curve or time-to-peak and had a smaller (borderline significant) effect on peak height and ETP of ∼3%. Typically, in some, but not in all, subjects, fish oil intake resulted in a decreased thrombin generation (Figure 2). The intervention did not significantly influence nAPCsr, indicating that factor Va inactivation remained unchanged. These data thus indicate that the reducing effect of fish oil on coagulant potential in the presence and absence of platelets is caused by reduced thrombin formation rather than by increased APC-dependent factor Va inactivation or increased thrombin inhibition.

**Contribution of Fibrinogen and Factor V to Fish Oil Effect on Thrombin Generation**

By multivariate regression analysis, the contribution of plasma (anti)coagulant factors to the large intersubject variation in thrombin generation was evaluated. Comparison of pre-intervention values of the 25 subjects (Table 2) showed that levels of prothrombin and antithrombin were main determinants of ETP with PFP/phospholipids (R=0.26, P=0.042). This is in agreement with published kinetic data. The levels of only fibrinogen and factor V further contributed to the ETP variation. Together, these 4 factors explained ∼30% of the variation of peak height and ETP (R=0.31, P=0.036). The vitamin K-dependent factor VII, factor X, and protein C, which were covariants (P<0.03), only determined components. The results were expressed as normalized APC sensitivity ratio (nAPCsr), which by definition has a value of 1.0 in normal pooled plasma. Before intervention, plasmas from the 25 subjects displayed variable nAPCsr values, with 2 subjects showing a nAPCsr >2.5 (Figure 1C).

When averaged for all 25 subjects, thrombogram parameters of PRP were significantly altered after 4 weeks of fish oil intake. The intervention significantly prolonged the time-to-peak and decreased the peak height and the ETP by 30% of the variation of peak height and ETP (∆APC). Together, these 4 factors explained 30% of the variation of peak height and ETP (∆ETP). The vitamin K-dependent factor VII, factor X, and protein C, which were covariants (P<0.03), only determined components. The results were expressed as normalized APC sensitivity ratio (nAPCsr), which by definition has a value of 1.0 in normal pooled plasma. Before intervention, plasmas from the 25 subjects displayed variable nAPCsr values, with 2 subjects showing a nAPCsr >2.5 (Figure 1C).

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Hypocoagulant Fish Oil Effect

Given the high variation in thrombograms between subjects, we explored the possibility that genetic variation in fibrinogen and factor V contributed to the variable effects of fish oil intervention on the coagulant potential. As a start, common polymorphisms in fibrinogen-αβ and factor V genes were determined that have been associated with an increased risk of thrombosis. With respect to the fibrinogen-β gene, 11 subjects were carrying the less common allele of all the G-854A, G-455A, and C-148T polymorphisms (1 homozygous), which are known to be in linkage disequilibrium. For these polymorphisms, we did not find associations with ETP or fish oil effects on ETP. In contrast, 10 subjects carrying the 312Ala allele of the fibrinogen-α T312A polymorphism,25 had higher baseline levels of fibrinogen and a greater reduction in fibrinogen on fish oil consumption (P=0.008 and 0.021, respectively) in comparison to the 15 noncarriers (Figure 4A). Fibrinogen reduction in the group of carriers correlated with the decrease in thrombin generation after fish oil (P=0.048). Analysis of data from all subjects indicated that the 312Ala carriers were responsible for the fish oil effect on fibrinogen and thrombin generation. In the heterozygote TA group, the average decrease in fibrinogen was 8%, whereas in the homozygote TT group, fibrinogen increased 0.5% (Figure 4B).

Five of 25 subjects carried the less common 1299Arg allele of the A4070G polymorphism of factor V, which is invariably associated with the HR2 haplotype.26,27 These carriers had lower factor V levels than noncarriers both before and after the intervention period (P=0.032 and 0.035, respectively). They had a tendency to greater factor V reduction with fish oil (P=0.06) in comparison to noncarriers, which correlated with the reduced thrombin generation (Figure 4C and 4D). Two subjects carried the Gln506 (factor VLeiden) allele of the G1691A factor V polymorphism. They were responsible for the high nAPCsr levels >2.5 (Figure 1C), which is in

**Figure 1.** Intersubject variation in thrombin generation. A, Tissue factor-induced thrombin generation in plasma from 2 representative subjects with either 100×10⁴ platelets/L (PRP) or 4 μmol/L phospholipids (PFP+PL). Note the high curves for subject A in comparison to subject B. B, Correlation between ETP levels determined in plasma from all 25 subjects in the presence of platelets and with phospholipids (95% CI is shown, R=0.48, P=0.001). C, Normalized APC sensitivity ratio (nAPCsr) determined in plasma from the 25 subjects. *Heterozygous factor V<sub>312Ala</sub> carriers.

the time-to-peak (P<0.046) but did not further contribute to peak height and ETP level.

Multiregression analysis of data from all subjects was also used to determine whether the fish oil effect on coagulation factor levels could explain its effect on thrombin generation. For PFP, the fish oil-evoked reduction in ETP significantly correlated with the reduction in fibrinogen or factor V level of R=0.48 (P=0.015) or R=0.41 (P=0.045), respectively. The variable decrease in thrombin peak height also correlated with the reduction in factor V (P=0.041), whereas the increase in lag-time of thrombin formation correlated with the reduction in fibrinogen (P=0.021). Also in PRP, the intervention effects on ETP and factor V level were correlated (P=0.016), but not those on ETP and fibrinogen level (P=0.77). An indication that variable fibrinogen did contribute to thrombin generation in PRP came from reanalysis of calibrated curves; fish oil effects on thrombogram peak height were correlated with fibrinogen level (P=0.048, n=11). However, there was no correlation between the fish oil effect on αIIbβ3 integrin activation and on thrombin generation in PRP (Table 2).

Control experiments were performed to determine whether changes in fibrinogen and factor V could influence thrombogram characteristics. Figure 3 shows that addition of 10% human fibrinogen to normal pooled PFP/phospholipids resulted in increased thrombin generation, as indicated by higher thrombin peak and ETP levels. Partial depletion of fibrinogen but not factor V led to decreased thrombin generation. Complete depletion of factor V but not of fibrinogen abolished thrombin generation.
agreement with expected APC resistance, but they did not show particular responses to fish oil.

With respect to platelet receptors, 12 subjects carried the high-risk 807T variant of integrin-β2, and 5 subjects carried the Leu33 (Pl A2) variant of the C1565T integrin-β3 polymorphism. Thrombograms of PRP with or without fish oil were not different between carriers and noncarriers (P > 0.073).

Discussion

The present data are a first report in humans on reduced thrombin generation in response to fish oil intake. This effect was achieved in healthy male volunteers with borderline overweight, consuming 3.0 g fish oil-derived omega-3 PUFAs daily for 4 weeks, and it was accompanied by a reduction in plasma levels of mainly fibrinogen and factor V. In agreement with other studies, fish oil intake lowered the plasma triglyceride concentrations. This latter effect pointed to efficacy of the omega-3 PUFA intervention. The intervention led to a small increase in low-density lipoprotein cholesterol, an effect that is also not uncommon after fish oil intervention in normal and hyperlipoproteinemic subjects.

The data suggest that the reducing effects of fish oil on tissue factor-induced thrombin generation and on fibrinogen level are causally related. First, variability analysis shows that fibrinogen, in addition to prothrombin, antithrombin, and factor V, is a main coagulation factor contributing to the intersubject variation in thrombogram parameters (peak height and ETP). The enhancing effect of fibrinogen on thrombin generation in situ has earlier been described by Hemker et al. Second, the intervention effect on thrombin generation in individuals significantly correlates with the effects on fibrinogen and factor V level. This holds for thrombograms obtained in the presence and absence of platelets. Third, in vitro modulation of fibrinogen in normal pooled plasma causes similar changes in thrombin generation as the fish oil intervention. Because artificial addition or depletion of factor V in normal plasma did not influence thrombin generation, it is likely that factor V, in the thrombogram variation, is a confounder for another related plasma factor that influences thrombin formation or inactivation. The lack of fish oil effect on nAPCsr suggests that this concerns a coagulant rather than anticoagulant factor.

Earlier studies often failed to measure anticoagulant effects of fish oil consumption, most probably because less sensitive coagulation assays like the prothrombin time were used. However, in some but not all human studies, a reduction in plasma fibrinogen and/or factor V activity was observed in response to fish oil. In addition, in rats, feeding of low doses of omega-3 PUFAs lowered factor V levels. With respect to fibrinogen, which is likely an independent cardiovascular risk factor when increased, fish oil has been shown to decrease this factor mainly in subjects with elevated baseline levels. The present data agree with this.

At the applied dose of 3.0 g omega-3 PUFAs/d, we did not find an intervention effect on vitamin K-dependent coagulation factors, prothrombin, factor VII, factor X, and protein C. This contradicts to the idea that fish oil can interfere with vitamin K action, as proposed from other studies in which factor VII and factor X were moderately reduced by fish oil. In rat plasma, we and others have measured reduced levels of prothrombin and factor X, an observation that was compatible with vitamin K antagonistic activity. However, this was seen after relatively high doses of fish oil (≥3 energy%), and the hypocoagulant effect was not further affected by vitamin K depletion. This strongly suggests that the main fish oil effect, especially at lower doses, is independent of vitamin K.

Quantitatively, the fish oil-induced decline in thrombin generation (peak level) was greater in the presence (−15%) than in the absence (−3%) of platelets. It is noted that
relations between thrombin generation with platelets and, eg, fibrinogen levels are more difficult to establish because of the high intrasubject variation in the assay with PRP. Fish oil reduced platelet integrin αIIbβ3 activation in response to thrombin-receptor stimulation. This is compatible with the notion that thrombin generation depends on the mutually stimulatory interactions of platelet activation and coagula-

tion, and that integrin αIIbβ3 activation significantly contributes to platelet-dependent thrombin generation. Thus, as proposed earlier, moderate antiplatelet and hypocoagulant effects of fish oil may add in lowering the thrombogram curve. From the present results, we can conclude that the fish oil effect on thrombin generation is enhanced by the presence of platelets, but that the contribution of integrin activation is still unclear.

As considerable intersubject variation was observed in coagulation factor levels and size of the thrombogram, the 25 volunteers were evaluated on the presence of frequent polymorphisms in fibrinogen and factor V genes with reported increased thrombotic risk. Typically, carriers of fibrinogen α-chain 312Ala variant (n = 10 heterozygotes/25) also had higher baseline levels than noncarriers (3.70 ± 0.20 versus 2.57 ± 0.19 g/L), and showed a greater reduction after fish oil (P = 0.016). *P < 0.05 (Mann–Whitney).

Figure 3. Effect of fibrinogen and factor V on thrombin generation. Normal pooled plasma was prepared with variable concentrations of human fibrinogen or factor V. Tissue factor-induced thrombin generation was measured with 4 μmol/L phospholipids at the following conditions: (A) 0% to 110% fibrinogen and 100% factor V; (B) 0% to 110% factor V and 100% fibrinogen. Curves of nanomolar thrombin concentrations were obtained by using separate calibrator traces for each different sample to eliminate differences in light scatter. Indicated factor levels (compared with normal pooled plasma) were always checked in the reconstituted plasma samples. Data are representative for 3 or more experiments. Thrombin generation curves with plasmas from carriers of 1299Arg and His1299 factor V gave the same factor V dependency.

Figure 4. Effect of fish oil on fibrinogen and factor V levels and thrombin generation in plasma/phospholipids. A and B, Carriers of fibrinogen α 312Ala allele (n = 10 heterozygotes/25). C and D, Carriers of factor V 1299Arg allele (n = 5 heterozygotes/25). Data (mean ± SE) were obtained as described for Tables 1 and 2. When fibrinogen was measured as antigen level, 312Ala carriers also had higher baseline levels than noncarriers (3.70 ± 0.20 versus 2.57 ± 0.19 g/L), and showed a greater reduction after fish oil (P = 0.016). *P < 0.05 (Mann–Whitney).

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In general, limitation, and strength, of this study is that the effect evaluation was analyzed in samples from a limited number of 25 individuals with borderline increased body mass index. This limits the statistical power and precludes the finding of small effects but, when effects are found, these are likely to be biologically and medically significant. The small numbers make it difficult to draw strong conclusions on differences between the polymorphisms. Yet, this report provides a first indication that genetic variation can contribute to a variable hypocoagulant (thromboprotective) fish oil effect.

Considering that the hypocoagulant effect of in fish oil is vitamin K-independent and has a genetic component, it is of interest to speculate on the mechanism of action. In mice, omega-3 PUFAs can downregulate the hepatic expression of the sterol regulatory element-binding protein-1 and of the peroxisome proliferator-activated receptor-α system. Further, peroxisome proliferator-activated receptor-α controls fibrinogen levels in humans. One possibility, therefore, is that omega-3 PUFAs provoke hypocoagulant conditions by influencing transcription systems, in which genetic variation may play a modulatory role. However, fish oil may also act on the translation or posttranslation level, eg, altering hepatic secretion quantitatively or qualitatively.

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References

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Materials and Methods

Subjects and Study Design

After full informed consent, 27 healthy males aged 48.5±9.8 year (mean±SD, median 51 year) enrolled in the study. The volunteers had a body weight of 92.4±9.9 kg and body-mass index (BMI) of 29.0±2.5 kg/m². They did not suffer from clinical cardiovascular diseases, diabetes mellitus or hypertension, and they did not use cholesterol-lowering, antiplatelet or anticoagulant medication. During the trial, one of the subjects withdrew due to gastrointestinal discomfort, and one other for unknown reasons. Data from these two subjects were not used for analysis. Four subjects were smoking.

Volunteers completed a short dietary questionnaire, indicating that they maintained their normal nutritional, smoking and alcohol consumption pattern, and had not consumed fish or marine products before or during the intervention period. During 4 weeks, the subjects took, in addition to their normal diet, nine soft gelatin capsules per day. Each capsule contained 500 mg fish oil, of which 475 mg was free fatty acids (35% EPA, 25% DHA and 10% other omega-3 PUFAs). Total omega-3 PUFA intake was 3.0 g daily. At three times, blood was drawn after overnight fasting: at 4 weeks and just before start of fish-oil intake (no treatment values), and after 4-weeks of fish-oil intervention. The study protocol was in agreement with the Helsinki declaration and was approved by the local medical ethics committee.

Materials

The fish oil supplement was a kind gift of Pharma Nord (Vejle, Denmark). Fluorescein isothiocyanate (FITC)-labeled monoclonal PAC-1 antibody came from Becton-Dickinson (San José, CA); FITC-labeled AK6 antibody against P-selectin (anti-CD62P-FITC) was from CLB (Amsterdam, The Netherlands). SFLLRN and benzyloxycarbonyl-Gly-Gly-Arg 7-amido-4-methyl coumarin (Z-GGR-AMC) were purchased from Bachem (Bubendorf, Switzerland). Recombinant human tissue factor was from Dade (Miami, FL). Human thrombin calibrator and thrombogram software were supplied by
Synapse (Maastricht, The Netherlands). Coagulation factor-deficient plasmas and kits to determine coagulant proteins were from sources described before.\textsuperscript{1,2} Test kits for determination of antithrombin and protein C came from Chromogenix (Möln达尔, Sweden); kits to determine fibrinogen activity and free plasma glycerol were from Roche Diagnostics (Mannheim, Germany), as was high-pure kit for isolation of genomic DNA. An EIA-based kit to determine fibrinogen antigen levels was obtained from Gaubius-TNO (Leiden, The Netherlands). Reagents to determine plasma total cholesterol and high density lipoproteins were from ABX Diagnostics (Montpellier, France), while Sigma (St. Louis, MO) supplied a kit for measurement of total glycerol. DNA primers were synthesized by Eurogentec (Liège, Belgium). Taq DNA Polymerase, buffer and MgCl\textsubscript{2} for polymerase chain reaction (PCR) were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Restriction enzymes with buffers were from the following sources: Hae III and Mnl I from New-England Biolabs (Beverly, MA); EcoR I from Gibco BRL (Breda, The Netherlands); Hind III and dNTP from Fermentas (St. Leon-Rot, Germany); Rsa I from Promega (Leiden, The Netherlands).

**Blood Collection and Preparation**

Blood was collected into open tubes with 129 mmol/L trisodium citrate (10 vol\%) and in K\textsubscript{2}EDTA tubes (Vacutainer Systems, Plymouth, UK). Platelet-rich plasma (PRP) and buffy coats were immediately prepared from citrated blood by centrifugation at 260 g for 15 minutes. Platelet-free plasma (PFP) was obtained by centrifuging PRP at 870 g for 10 minutes, followed by two centrifugation steps at 18,000 g for 10 minutes. Platelet concentration in PRP was determined with a Beckman MicroDiff\textsuperscript{18} counter (Coulter Electronics, Luton, UK) and normalized to 30 or 150×10\textsuperscript{9} platelets/L with autologous PFP. The PRP was used within 90 minutes. Buffy coats and PFP were snap-frozen in liquid nitrogen and stored at -80°C for later coagulation measurements.

Standard hematological parameters were determined in EDTA-anticoagulated blood; remaining EDTA-anticoagulated blood was centrifuged twice at 870 g for 10 minutes. Collected plasma was stored at -80°C for lipid analysis.
**Plasma Lipids and Coagulation Factors**

In EDTA-anticoagulated plasma, total and free glycerol as well as total cholesterol and cholesterol in high density lipoproteins were measured using commercially available test kits. Triglycerides were determined by subtracting levels of free glycerol from total glycerol. Plasma levels of coagulation factors II and VII\(^1\) and of factors V and X\(^2\) were measured at two dilutions using factor-deficient plasmas, as described. Levels of protein C and antithrombin were determined with test kits from Chromogenix, following the manufacturer’s instructions. Factor levels were expressed as percentages of activity compared to a ‘normal’ plasma pool of 40 healthy subjects (males and females). Basal levels of antithrombin in the subjects participating in the intervention study were below ‘normal’, probably because of their sex, age, and high BMI.\(^3\) Fibrinogen activity in plasma was determined according to Claus (test variability about 3%).\(^4\) In addition, total fibrinogen concentrations in plasma were measured with an in-house EIA that uses a pool of rabbit anti-human fibrinogen IgGs as catching antibodies,\(^5\) and a peroxidase-conjugated monoclonal antibody specific for human fibrinogen, fibrinopeptide A-containing fragments (Y18) as tagging antibody.\(^6\)

Plasma partially deficient in fibrinogen or factor V was prepared by mixing normal pooled plasma with the respective factor deficient plasma. Plasma enriched in fibrinogen or factor V was obtained by addition of respective purified human factor to normal pooled plasma. Levels of fibrinogen or factor V were checked after reconstitution of these plasma samples.

**Flow Cytometry**

Activated integrin \(\alpha\)IIb\(\beta\)3 and surface exposure of P-selectin on platelets was determined in duplicate using flow cytometry on an Epics-XL flow cytometer from Coulter Electronics (Luton, UK). For integrin activation, PRP containing 30×10\(^9\) platelets/L was activated with sub-maximal SFLLRN (5 \(\mu\)M, 10 min) in the presence of FITC-labeled PAC-1 antibody against activated \(\alpha\)IIb\(\beta\)3. To measure P-selectin exposure, PRP was stimulated with SFLLRN, diluted 30-times with phosphate-buffered saline, and incubated with FITC-labeled anti-CD62P antibody. Fractions of platelets expressing activated \(\alpha\)IIb\(\beta\)3 or P-selectin were determined from at least 10,000 events.
Thrombin Generation Measurements

Thrombin generation (thrombogram method) was measured in PRP and PFP, basically following described procedures. Contact activation of plasma was minimized by using hydrophobic materials. Briefly, triplicate samples of 80 µL freshly isolated PRP (150×10⁹ platelets/L) were pipetted into a 96-wells plate, containing 20 µL recombinant tissue factor (3 pmol/L) in buffer A (20 mmol/L Hepes, 140 mmol/L NaCl, 5 mg/mL bovine serum albumin, pH 7.35). Polystyrene well plates (Immulon 2HB, Dynex Technologies, Chantilly, VA) were used, which were selected for minimal contact activation. The plate was inserted into a Fluoroskan Ascent well-plate reader (Thermo Labsystems, Helsinki, Finland), and pre-heated to 37°C. Coagulation was started by automated addition of 20 µL fluorescent thrombin substrate (Z-GGR-AMC, 2.5 mmol/L), suspended in buffer B (20 mmol/L Hepes, 0.1 mol/L CaCl₂, 60 mg/mL bovine serum albumin, pH 7.35) under shaking. Final tissue factor concentration in experiments with PRP was 0.5 pmol/L. When using PFP instead of PRP, 4 µmol/L phospholipids (phosphatidylserine : phosphatidylcholine : phosphatidylethanolamine, 1:3:1) were present, and coagulation was triggered with 5 pmol/L tissue factor (f.c.). In the absence of tissue factor, the lag-time of onset of thrombin generation was >50 min. Addition of corn trypsin inhibitor (50 µg/mL) to block the intrinsic coagulation system did not significantly alter the thrombin generation curves (data not shown). Thrombin generation curves with PRP had a long lag-time, which depended on the slow rate of phosphatidylserine exposure of platelets under these conditions.

Fluorescence accumulation was semi-continuously measured in time at excitation and emission wavelengths of 390 and 460 nm, respectively (37°C). First-derivative curves of accumulation of fluorescence intensity were converted into curves of nanomolar of thrombin using human thrombin calibrator and thrombinscope software to correct for non-linearity of fluorescence with AMC concentration, depletion of fluorescent substrate, and fluorescence accumulation due to non-coagulant α₂-macroglobulin-bound thrombin as described. The calibration samples contained the same plasma, to which a fixed amount of stable thrombin calibrator was added. In each analytical sample, deviation of fluorescence accumulation from linearity was corrected in one single step by comparing the fluorescence curve of the sample with the corresponding calibrator curve. Thrombograms were always...
run in triplicate, and analyzed on time to thrombin peak, peak height (indicative of maximal rate of thrombin formation), and area-under-the-curve or endogenous thrombin potential (ETP), reflecting the activity of thrombin during the total coagulation process. The assay variability of obtained thrombogram parameters, when measured repeatedly with the same plasma sample, was <3%. Described intra-individual coefficients of variation of this assay with PFP/phospholipids were about 4.5% for time-to-peak, peak height and ETP. Variation coefficients of the assay with PRP were 5% for time-to-peak and 8-9% for peak height and ETP.\(^8\)

Activated protein C (APC) resistance was measured with a modified thrombin generation test. A normalized APC sensitivity ratio (nAPCsr), indicative of APC resistance, was determined as the ratio of the amounts of \(\alpha_2\)-macroglobulin-thrombin complex present in plasma activated in the presence and absence of APC, divided by the same ratio determined in normal pooled plasma.\(^9\)

**Isolation of DNA and Determination of Genetic Polymorphisms**

Leukocyte DNA was isolated from blood buffy coats using a high-pure kit according to instructions of the manufacturer (Roche). Polymorphisms were determined using PCR primers suitable for analysis by RFLP as described: factor V A4070G (R\(_2\)FV)\(^{10}\) and G1691A (FV\(_{\text{Leiden}}\));\(^{11}\) fibrinogen-\(\alpha\) T312A;\(^{12}\) fibrinogen-\(\beta\) C-148T and G-854A\(^{13}\); fibrinogen-\(\beta\) G-455A.\(^{14}\) PCR was for 30-35 cycles (temperature conditions according to characteristics of the primers) with 50 ng isolated DNA, dNTPs (2 mmol/L of each), 1.5 mmol/L MgCl\(_2\), forward and reverse primers (10 \(\mu\)mol/L), Taq polymerase (5 U/\(\mu\)L) and suitable PCR buffer. Housekeeping \(\beta\)-actin gene was co-amplified to control for quality of the isolated DNA. Generated PCR fragments and endonuclease digestion products were controlled for purity and fragment size by electrophoresis on ethidium bromide-stained agarose gels.

**Statistics**

To determine the effect of fish oil, per individual the values from two baseline blood samples were averaged and then subtracted from the value obtained after intake of fish oil. For statistical analysis, non-parametric tests were used, because some of the parameters were not distributed in a normal way.
Per parameter, the differences between baseline samples were statistically compared to the effect of fish-oil intake with the Wilcoxon signed rank-test. Correlation between individual parameters was determined with a Spearman test. Contribution of various coagulation factors to thrombin generation was evaluated by multivariate regression analysis. Mann-Whitney U test was used to compare genotypic groups. Tests were carried out with the statistical package for the social sciences (SPSS, Chicago, IL).

References


