For a period of 6 weeks, 76 healthy male volunteers consumed during their daily main meal the contents of one tin (~135 g) of either fish (mackerel) paste or meat paste. Fibrinolytic parameters were determined in plasma samples obtained at the beginning and at the end of the experimental period. No changes were found in plasminogen, α₂-antiplasmin, tissue-type plasminogen activator (t-PA) antigen, and euglobulin t-PA activity. In the control group (n = 39), plasminogen activator inhibitor activity did not change. In the fish group (n = 37), however, total plasma PA inhibitor (PAI) activity increased by 45%, due to a 71% increase in PA inhibitor type-I. This increase could not be ascribed to a diet-induced acute phase-type reaction and could not be explained by changes in serum triglycerides or insulin.

Consumption of fish may be of benefit in prevention of ischemic cardiovascular disease. Kromhout et al recently demonstrated an average daily intake of 30 g fish to be associated with a reduced risk of coronary heart disease. Similar associations have been obtained by other, but not all, investigators. These epidemiologic data suggest that consumption of fish may protect against ischemic heart disease. The mechanisms involved have not yet been fully elucidated, but may include changes in blood lipids and lipoproteins and reduction of the interaction between blood platelets and the vessel wall.

Hamsten et al recently reported that in myocardial infarction patients the risk of reinfarction is positively related with the plasma level of plasminogen activator inhibitor (PAI) activity, a risk which may be associated with a reduced blood fibrinolytic activity. Because the plasmatic PAI activity repeatedly correlated with triglyceride concentration in the plasma, and since dietary fish and fish oil very consistently lower the plasma triglyceride level, consumption of a fish-enriched diet might result in a decreased level of PAI and thus improve fibrinolysis. Such an effect would provide an additional explanation for the link between fish (oil) consumption and the reduced incidence of cardiovascular disease. We now report, however, that a diet moderately enriched in fish actually increased plasma PAI activity by ~45% due to a 70% increase in the plasma level of PAI type-I.

Materials and Methods

Experimental design. The study design has been described in detail elsewhere. In three different centers, healthy, nonsmoking male volunteers (aged 20 to 45 years and with normal dietary habits) were assigned to either a control or an experimental group by stratified randomization, on the basis of serum triglyceride (TG) content and systolic blood pressure (SBP). In all, 84 volunteers entered the study, 42 in each group. During a 6-week experimental period, the volunteers consumed the contents of one tin (~135 g) of mackerel paste (fish group) or meat paste (control group) each day. The experimental period was preceded by a 2-week run-in period during which all volunteers consumed one tin of meat each day. The dietary supplement had to replace the fish, meat, cheese or eggs normally consumed during the main meal. The volunteers were requested not to change their dietary habits for the other meals.

Compliance was checked by measuring the urinary excretion of lithium, added to the dietary supplements as a tracer. During the entire 8-week period no use of aspirinlike drugs was allowed. Each volunteer was requested to keep a diary and record any changes in physical condition, abnormal events, or failure to comply. No major changes were noted in these respects, and the volunteers did not change their dietary habits. From 84 volunteers who entered the study, 76 complete sets of plasma were available for the analysis of fibrinolytic components (37 in the fish group and 39 in the meat group). Of the eight missing sets of plasma, six were incomplete due to a technical failure during plasma preparation. One volunteer dropped out and one volunteer had to be removed from the study because of low compliance. The study protocol was approved by the Medical Ethical Committee of the University of Limburg, Maastricht, The Netherlands, and written informed consent was obtained from all volunteers.

Composition of dietary supplements. The dietary supplements were prepared by the Institute of Fishery Products TNO, Ijmuiden, The Netherlands (mackerel paste) and TNO-CIVO Toxicology and Nutrition Institute, Zeist, The Netherlands (meat paste). The dietary supplements have been described previously. The fish paste provided 1.7 g timnodonic acid (20:5 w3, eicosapentaenoic acid) and 3.0 g cervonic acid (22:6 w3, docosahexaenoic acid) per tin.

Blood sampling and plasma preparation. Blood sampling was performed between 8:00 AM and noon after an overnight fast and a 24-hour period of abstinence from alcohol. Blood samples were obtained after a ten-minute rest period in a recumbent position under minimal stasis from a cannula in a forearm vein. After a sample was obtained for basic hematologic measurements, 4.5 mL freely dripping blood was collected from the cannula into a precooled plastic tube containing 0.5 mL ice-cold sodium citrate (109 mmol/L, adjusted to 7.2 to 7.4 pH with citric acid, 10% wt/vol), and then placed back into melting ice. Platelet-free plasma was prepared (4°C, 3,000 g for 15 minutes) and quick-frozen at ~20°C. Blood samples were obtained at the end of the run in period (week 0) and at the end of the experimental period (week 6). All assays were performed.
performed on citrated plasma; TG concentrations were determined in serum.

**PAI assays.** Total PAI activity of plasma was determined spectrophotometrically by titrating a plasma sample (10 μL) with melanoma-derived tissue-type plasminogen activator (t-PA) and measuring the residual t-PA activity.\(^{18}\) PAI activity was then determined by graphic extrapolation. The thermostable PAI activity was determined by the same procedure, using plasma that had been incubated for 18 hours at 37°C. To determine the contribution of PAI type-1 (PAI-1) to total PAI activity, plasma samples (10 μL) were preincubated with a quenching mouse monoclonal anti-PAI-1 IgG, (Monozyme, Charlottenlund, Denmark)\(^{19}\) performed at a concentration of 20 μg/mL in the assay buffer at 37°C for 15 minutes before the assay procedure was started by addition of t-PA, plasminogen, and the substrate S-2251. Preliminary experiments had shown that this amount of antibody gave maximum inhibition of plasmas containing as much activity as the sample with the highest PAI activity from this series (21 IU/mL).

Controls without added antibodies were run in parallel. PAI-1 activity was then calculated by subtraction. PAI and t-PA activities are expressed as international units (IU), as defined by the international t-PA standard (batch 83/517). The intra- and interassay coefficient of variation (CV) of the PAI assay was 11% and 12%, respectively, for pooled normal plasma, and the interassay CV was 8% for samples with values >200% of pooled plasma.

**Plasminogen.** A spectrophotometrical assay with Titertek equipment was used after activation of plasma with streptokinase.\(^{20}\) Procedural details were provided by Dr L. Engesser of the Gaubius Institute. Plasma (10 μL) was incubated with 250 U streptokinase (KabiVitrum, Stockholm) in 0.1 mol/L Tris/HCl buffer, pH 7.5, containing 0.1% (vol/vol) Tween 80 (final volume 125 μL) for nine minutes at 37°C. Then 125 μL Tris/Tween buffer was added, and 10 μL was transferred to microtiterplate cups containing 170 μL buffer and 30 μL S-2251 (3.5 mmol/L). The plates were incubated at 25°C, and absorbance at 405 nm was measured at 15-minute intervals for 60 minutes.

**α1-Antiplasmin.** Activity of α1-antiplasmin was determined spectrophotometrically\(^{23,24}\) on Titertek equipment. To microtiterplate cups containing 120 μL buffer (0.05 mol/L Tris/HCl, 0.11 mol/L NaCl, 0.01% Tween 80, Carbowax 1.4 mg/mL, pH 7.4) 40 mL S-2251 (3.5 mmol/L) and 10 μL plasma (diluted 1:20 and 1:40) were added. After equilibration for five minutes at room temperature, 40 μL plasmin (casein units 0.15/μL; KabiVitrum) was added, and absorbance at 405 nm was determined at five-minute intervals for 15 minutes. During this time span, the change in absorbance was linear with time. Plasmin controls were run without plasma. With this assay procedure, no antiplasmin activity was detected in the plasma from an α1-antiplasmin-deficient patient.

**Plasma t-PA activity.** The t-PA activity in the plasma euglobulin fraction (dilution 1:10, pH 5.9) was determined by the method of Verheijen et al\(^{25}\) with a 25 μg euglobulin sample, with and without 10 μL rabbit anti-human t-PA IgG (Cappel-Organon Teknika, Turnhout, Belgium).

**Plasma t-PA antigen.** A commercial enzyme-linked immuno- sorbent assay (ELISA) test kit (Imulysé; Biopool, Umed, Sweden) was used according to the manufacturer's instructions. Disodium-EDTA (10 mmol/L) had been added to the plasma samples.\(^{26}\) In four sets of plasma (two from the fish group and two from the control group) t-PA antigen could not be determined due to intractable high background absorbance.\(^{27}\)

**Fibrinogen.** The method of Clauss\(^{24}\) was used.

**C-reactive protein (CRP).** A radial immunodiffusion procedure was used according to the manufacturer's instructions (Behring-werke, Marburg, FRG) with citrated plasma. The lower limit of sensitivity of the assay was 1.1 μg/mL.

**Fasting serum TG.** TG was determined by a two-component kinetic method, using a Vitatron PA 800 automatic analyzer and commercial kits (Boehringer Mannheim, FRG. cat. no. 644200).

**Insulin.** Plasma concentrations were determined by radioimmunoassay (RIA), using dextran-coated charcoal to separate bound from free insulin.\(^{23}\)

**Statistics.** Differences between the values obtained at week 0 and week 6 were evaluated with Student's paired t test or Wilcoxon's two-sample rank test, as indicated. A difference was considered significant if \(P < .05\).

**RESULTS**

**Fibrinolytic parameters.** In the plasma samples obtained at week 0 and week 6 we determined plasminogen, α1-antiplasmin, t-PA antigen, and t-PA activity. At week 0, we observed no significant differences between the control and the fish group for any of the fibrinolytic parameters measured (Table 1), showing that the two groups were adequately matched in these respects. Occasionally, significant changes occurred during the 6-week experimental period. For none of these changes, however, did any differences between the two groups appear to be significant. Consequently, the fish supplement did not have a specific effect on these fibrinolytic parameters (Table 1).

**PAI activities.** Preincubation of plasma with a monoclonal antibody quenching the activity of PAI-1 allowed subdivision of total PAI activity into two portions: a portion quenched by the antibody (designated as PAI-1 activity) and a residual portion (designated as non–PAI-1–related activity).

At week 0, none of the PAI activities proved different for the control and the fish groups (Table 2). In the control group, total PAI activity and PAI-1 activity did not change significantly during the 6-week experimental period (Table 2). In the fish group, however, total PAI activity as well as PAI-1 activity increased significantly, + 2.3 IU/mL (+ 45%) for total PAI activity (\(P < .01\)), and + 2.4 IU/mL (+ 71%) for PAI-1 activity (\(P < .002\)) (Table 2).

Because PAI-1-unrelated activity did not change significantly in this group, the increase in PAI activity during the experimental period can be ascribed to an increase in PAI-1. At week 0, PAI-1 activity accounted for ~64% of all PAI activity (63% in the control group, 66% in the fish group). Because PAI-1 activity is unstable during in vitro incubation at 37°C\(^{28}\) (half-life two to three hours), plasma samples were also assayed after overnight incubation at 37°C. After this treatment, 67% (66% in the control group and 68% in the fish group) of PAI activity had disappeared in week-0 samples, in agreement with the percentage quenched by the antibody (64%). In week-6 samples, the percentages of the antibody- and temperature-sensitive PAI activities were 77% and 78%, respectively, in the fish group and 66% and 65% in the control group.

In the control group, individual PAI-1 activities at week 0 and week 6 showed a strong correlation (\(r = .850, n = 39, P < .001\)), confirming the observation\(^{29}\) that plasma PAI activity normally shows little intra-individual variability. Even in the fish group, this correlation persisted (\(r = .518, n = 37, P < .001\)) despite a 71% increase in mean PAI-1 activity.
Acute-phase proteins. To decide whether the increase in PAI-1 activity in the fish group might have been caused by an acute-phase reaction, the rapidly reacting acute-phase protein CRP and the more slowly reacting acute-phase protein fibrinogen were measured. Only minor, nonsignificant changes in CRP (Table 3) and fibrinogen (Table 1) were noted in the two groups. In volunteers in whom a change in CRP was observed, no significant correlation was observed with change in PAI-1 (fish group: \( r = -0.082, n = 10, \text{NS} \); control group: \( r = 0.132, n = 11, \text{NS} \)).

TG, body mass index, and insulin. A highly significant positive correlation was observed between fasting TG and PAI-1 levels at week 0 for all volunteers (\( r = 0.382, n = 75, P < .001 \)). In the control group, mean TG level did not change during the 6-week period (\( \Delta \text{TG} = 0.03 \pm 0.27 \text{mmol/L} (\text{mean} \pm \text{SD}, n = 39) \)), whereas individual changes in TG and PAI-1 values showed a positive correlation (\( r = 0.497, n = 39, P < .001 \)). In the fish group, mean TG level decreased (\( \Delta \text{TG} = -0.30 \pm 0.34 \)), but the individual changes in TG and PAI-1 levels were not significantly related to each other, although there was a tendency toward a negative correlation (\( r = -0.235, n = 36, P = .163 \)). We also observed a significant correlation for all volunteers, between PAI-1 and body mass index (\( r = 0.323, n = 76, P = .004 \)) at week 0.

At week 0, a significant correlation was noted between PAI-1 and insulin (\( r = 0.252, n = 76, P = .028 \)). Insulin levels did not change significantly during the 6-week period in either group, and neither were individual changes in insulin and PAI-1 significantly correlated (control group: \( r = -0.049, n = 38, P = .77 \); fish group: \( r = 0.224, n = 37, P = .184 \)).

### DISCUSSION

The fibrinolytic capacity of plasma is largely determined by the balance between profibrinolytic factors such as plasminogen and fibrinogen (pro)activators on the one hand, and antifibrinolytic factors (eg, antiplasmins, PAIs) on the other. We report that a fish-enriched diet influenced this balance by causing a 71% increase of the plasma level of PAI-1, a fast-acting inhibitor of t-PA and of urokinase.27 No such change was noted in the (meat-consuming) control group. The other fibrinolytic components showed either no significant change during the 6-week experimental period (\( \alpha_2 \)-antiplasmin, t-PA activity) or a similar small change in both the fish and the control groups (plasminogen, t-PA antigen).

Of the total PAI activity in plasma at week 0, about two thirds was due to PAI-1, as determined by antibody quenching and temperature stability experiments. The remaining one third was presumably due to the (still poorly defined) PA binding protein described by Kluit et al,28 as other known specific PA inhibitors (PAI-2; protease nexin-1) are not

### Table 1. Fibrinolytic Parameters at Weeks 0 and 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Week 0</th>
<th>Week 6</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen activity *</td>
<td>7.41 ± 0.15</td>
<td>7.71 ± 0.15</td>
<td>+0.30 ± 0.12 †</td>
</tr>
<tr>
<td>Fish group (n = 37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n = 39)</td>
<td>7.49 ± 0.12</td>
<td>7.74 ± 0.13</td>
<td>+0.25 ± 0.11 †</td>
</tr>
<tr>
<td>α2-Antiplasmin activity ‡</td>
<td>7.76 ± 0.23</td>
<td>7.92 ± 0.25</td>
<td>+0.16 ± 0.20</td>
</tr>
<tr>
<td>Fish group (n = 37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n = 39)</td>
<td>8.28 ± 0.24</td>
<td>8.58 ± 0.24</td>
<td>+0.31 ± 0.23</td>
</tr>
<tr>
<td>t-PA antigen (ng/mL)</td>
<td>7.5 ± 0.8</td>
<td>8.1 ± 0.5</td>
<td>+0.6 ± 0.4</td>
</tr>
<tr>
<td>Fish group (n = 37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n = 39)</td>
<td>6.6 ± 0.6</td>
<td>7.4 ± 0.6</td>
<td>+0.8 ± 0.3 †</td>
</tr>
<tr>
<td>t-PA activity (mIU/mL) §</td>
<td>59 (16, 0-695)</td>
<td>39 (7, 0-263)</td>
<td>-20 (0, -622+84)</td>
</tr>
<tr>
<td>Fish group (n = 37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (n = 39)</td>
<td>69 (16, 0-541)</td>
<td>57 (17, 0-338)</td>
<td>-12 (0, -209+214)</td>
</tr>
</tbody>
</table>

All data are mean ± SEM, except for t-PA activity which is presented as median (range).

*Plasminogen activity is defined as (ΔA₉₀₉/min) · 10³ after activation of acidified plasma by streptokinase; experimental details are in the Materials and Methods section.

†Significant increase from week 0 to week 6 (paired t test, \( P < .025 \)). No significant differences between fish group and control group (t test).

‡α2-Antiplasmin activity is defined as the decrease in plasmin activity, expressed as (ΔA₉₀₉/min) · 10³, in the presence of 0.5 μL plasma; experimental details are described in the Materials and Methods section.

§No significant changes in either group by Wilcoxon’s two-sample rank test.

### Table 2. PAI Activities (mIU/mL) at Weeks 0 and 6

<table>
<thead>
<tr>
<th>Activity</th>
<th>Week 0</th>
<th>Week 6</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish group (n = 37)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>3.4 ± 0.6*</td>
<td>5.8 ± 0.8</td>
<td>+2.4 ± 0.7*</td>
</tr>
<tr>
<td>Non-PAI-1</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>-0.1 ± 0.1</td>
</tr>
<tr>
<td>Total PAI</td>
<td>5.1 ± 0.6</td>
<td>7.5 ± 0.8</td>
<td>+2.3 ± 0.7†</td>
</tr>
<tr>
<td>Control group (n = 39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>3.1 ± 0.6</td>
<td>2.9 ± 0.4</td>
<td>-0.2 ± 0.3</td>
</tr>
<tr>
<td>Non-PAI-1</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>-0.5 ± 0.1†</td>
</tr>
<tr>
<td>Total PAI</td>
<td>4.9 ± 0.6</td>
<td>4.3 ± 0.4</td>
<td>-0.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

†Significant increase from week 0 to week 6 (paired t test, \( P < .002 \)), and significantly different from control group (t test, \( P < .002 \)).

‡Significant increase from week 0 to week 6 (paired t test, \( P < .01 \)), and significantly different from control group \( (P < .001) \).

§Significant decrease from week 0 to week 6 (paired t test, \( P < .001 \)), and significantly different from fish group \( (P < .02) \).
showed a small (−27%) but significant decrease in the control group only.

In the fish group, despite the increase of PAI-1 (Table 2) and the absence of a change in t-PA antigen (Table 1), the euglobulin t-PA activity did not change. This suggests that the free t-PA concentration in the plasma did not change and that any eventual coprecipitation of PAI-1 with t-PA during the euglobulin fractionation procedure was of no quantitative importance in these samples.

Individual levels of PAI-1 activity appear to be tightly regulated, as we also showed by the excellent correlation between PAI-1 values at weeks 0 and 6. This would also apply to PAI-1 antigen levels, since PAI-1 antigen and activity are strongly correlated. Yet little is known about factors involved in regulation of PAI-1. Neither do we know which cell type(s) synthesizes PAI-1 in vivo. Plasma levels of PAI-1 increase rapidly after surgery and trauma and during septicemia. No such acute phase-type reaction appeared to be involved in the PAI-1 increase induced by the fish diet, since the acute-phase reactants CRP and fibrinogen showed no significant changes, and the small changes observed were not correlated with changes in PAI-1.

Under physiologic conditions, plasma PAI activity has been reported to be positively correlated with total serum TG,6,10,13,14 VLDL and LDL triglycerides,6 oral glucose intolerance,6 body mass index,6,11,13,14 and insulin.11,14 In this study, a highly significant correlation was also shown between fasting TG and PAI-1 levels at week 0 for all volunteers and (in the control group) between individual changes in TG and in PAI-1 levels during the experimental period. These positive correlations make the increase in PAI-1 for the fish group, in which TG levels decreased by 40%, even more surprising. Our data also confirm the previously reported data on the correlation between total PAI activity and body mass index,6,11,13,14 as well as between total PAI activity and insulin. Yet the increase in PAI-1 activity induced by the fish diet cannot be explained by changes in serum TG and insulin in the fish group during the experimental period.

In contrast to our data, Barcelli et al. reported that consumption of 15 capsules of Max-EPA/day (providing ω-3 fatty acid content of one tin of fish) for 2 weeks decreased (in nine volunteers) plasma PAI activity by −15%. However, because no control group was included, the data cannot be interpreted correctly. Moreover, direct comparison of their data and ours is difficult, since the assay procedures used show essential differences. The overnight procedure used by Barcelli et al. for measuring inhibition probably measured slow-acting high-affinity PA inhibitors such as α2-antiplasmin, α2-macroglobulin, and C1-inhibitor as well, and is not necessarily specific for presumably physiologically more relevant fast-acting high-affinity PAIs such as PAI-1. Neither did we detect the reported changes in α2-antiplasmin and t-PA activity. Another explanation for the different results in this study and the study by Barcelli et al. may be that effects induced by whole fish and by a specially processed fish oil preparation are not identical. On the other hand, Fröschl et al. also reported (as we observed for fish) an increase in PAI activity in diabetic patients consuming fish oil.

Recently, Andersen et al. reported that dietary intervention (reduction in total fat and carbohydrate intake, and substitution of polyunsaturated-to-saturated fats by, among other factors, increasing the intake of fat fish) resulted in good responders (defined as a decrease of ≥20% in their plasma TG) in a normalization of the fibrinolytic response to venous occlusion or desmopressin. Because a defective fibrinolytic response is often due to an increased PAI level, dietary intervention may have decreased PAI levels. Unfortunately, PAI was not specifically measured by Andersen et al.

Increased PAI activity has been reported in patients with idiopathic deep vein thrombosis and in young and older postmyocardial infarction patients and is believed to be a risk factor for occurrence of reinfarction and development of postoperative deep vein thrombosis. Our observation that a fish-enriched diet increased PAI-1 suggests caution in the use of fish-enriched diets in such conditions.

ACKNOWLEDGMENT

Fibrinogen measurements were performed by A.D. Muller, Department of Biochemistry, Limburg University, Maastricht, The Netherlands. TG determinations were performed by Dr H. Zevenbergen, Unilever Research, Vlaardingen, The Netherlands. Insulin determinations were performed in the Central Laboratory for Clinical Chemistry (Dr M. Fröhlich) of the University Hospital Leiden, with the skillful technical assistance of M. van Dijk-Besting. The mackerel used in this study were provided by the International Association of Fish Meal Manufacturers, Potters Bar, Herts, England.

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<table>
<thead>
<tr>
<th>Table 3. CRP at Weeks 0 and 6</th>
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<tr>
<td><strong>Fish Group (n = 37)</strong></td>
</tr>
<tr>
<td>Week</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

No significant difference between the fish group and the control group in change in CRP from week 0 to week 6 (Wilcoxon's two-sample rank test).

*Mean ± SEM (number of volunteers).
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A moderate fish intake increases plasminogen activator inhibitor type-1 in human volunteers

JJ Emeis, AC van Houwelingen, CM van den Hoogen and G Hornstra