

Effect of a Moderate Fish Intake on Platelet Aggregation in Human Platelet-Rich Plasma

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Key words

Fish – Diet – Compliance – Platelet aggregation – Platelet number – PRP – Collagen – Thrombin

Summary

This paper describes the results of an international study to investigate the effect of a reasonable amount of dietary fish on platelet aggregation in platelet-rich plasma (PRP) induced by collagen and thrombin. In Maastricht, Tromsø, and Zeist two groups of healthy male volunteers were given a daily dietary supplement consisting of 135 g of canned mackerel paste (experimental group, $n = 40$) or meat paste (control group, $n = 42$) for a 6-week period. Compliance, calculated on the basis of the urinary excretion of lithium, added to the supplements, was about 80%. Platelet number in PRP decreased significantly in the fish group. Collagen-induced platelet aggregation in PRP differed widely between the three centres despite the attempt to use exactly the same conditions. Nonetheless, aggregation decreased significantly in the fish group. The mackerel effect on thrombin-induced aggregation was inconsistent.

Introduction

Blood platelets play a major role in the development of ischaemic cardiovascular disease because of their ability to adhere to damaged vascular tissue and to aggregate with each other. It has been suggested that the consumption of polyunsaturated fatty acids of the (n-3) family, present in fish and fish products, diminishes the interaction between platelets and vessel wall by causing a shift in the balance between pro- and anti-aggregatory prostanooids formed by the platelets and the vessel wall, respectively (1). However, previous studies on the effects of additional dietary (n-3) PUFAs on platelet aggregation generated conflicting results. Although a large number of human studies have been performed, only eight included a proper control group (2), whereas methods used to monitor compliance with the treatment were often absent. Moreover, there was a large variability in quantities and types of dietary supplementation which, frequently, were not realistic at all.

We, therefore, conducted a well-controlled study to investigate the effect of a reasonable amount of fish in the diet on collagen- and thrombin-induced platelet aggregation in platelet-rich plasma (PRP). Thrombin was chosen to activate the platelets because it is more and more recognized as an important physiological platelet activator (3, 4). Moreover, collagen was used, not only because it

is another physiological platelet activator (5, 6), but also because it has been widely used in previous fish(oil) studies (7–32).

Materials and Methods

The study was part of a trial conducted by the International Working Party "Fish against thrombosis?". The design of the study was published in detail before (33) and is briefly reiterated below. Experimental details will be given only as far as they are related to the results to be presented in this paper.

In Tromsø (Norway) and in Maastricht and Zeist (The Netherlands) 84 healthy male volunteers (20–45 years of age, see Table 1) participated in the study. The experiment lasted 6 weeks, and was preceded by a run-in period of 2 weeks. During this run-in period all volunteers were requested to consume one tin of meat paste (135 g) per day. Subsequently, they were randomized into a mackerel and a meat (control) group. During the 6 experimental weeks the volunteers of the mackerel group consumed one tin of mackerel paste (135 g) and the controls continued the consumption of meat paste. The dietary supplements were given as a replacement of the fish, meat, cheese and eggs normally consumed during the main meal. The mackerel paste provided 1.7 g timnodonic acid (EPA, 20:5 n-3) and 3.0 g cervonic acid (DHA, 22:6 n-3) per day.

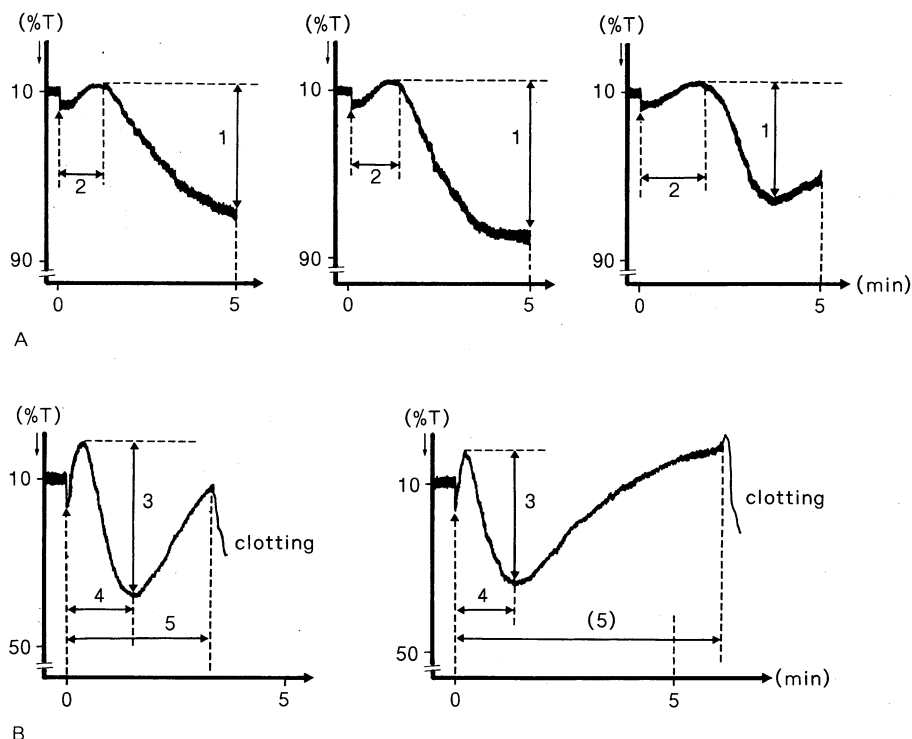
Compliance was calculated on the basis of the urinary excretion (%) of a standard amount of lithium added to the supplements. The maximum lithium intake per day was about 100 times the amount naturally available in the diet and yet represented only 1% of the therapeutic dose (34). Compliance was also calculated on the basis of urinary lithium per urinary creatinine ($\mu\text{mol}/\text{mmol}$). To correct for a possible influence of inaccuracies during urine collection and to minimize the influence of differences in body mass and sudden changes in exercise level, both compliance indices were multiplied, resulting in a variable (the compliance index) which was used to investigate possible relationships between dietary adherence and diet-induced changes in platelet aggregation.

At week 0 (the end of the run-in period) and week 6, blood was drawn under fasting conditions after the volunteers had been resting for 20 min in a supine position. Under minimum stasis a forearm vein was punctured using a 19 G butterfly-venisystem (No. 4590, Luer, or No. 8488, Luer and Record; Abbott Ireland Ltd., Sligo, Republic of Ireland).

Platelet Aggregation in PRP

Eighteen ml of blood were taken slowly into a 20 ml syringe, prefilled with 2 ml citrate solution (109 mmol/l, pH 7.2–7.4). The anticoagulated blood was mixed gently and PRP was prepared by centrifugation for 10 min at $120\text{--}160 \times g$. After removing the PRP, the residue was centrifuged again for 10 min at $1500 \times g$. The platelet-poor plasma (PPP) was carefully removed. Platelet concentrations in PRP were measured with a Thrombocounter® (Coulter Electronics, Luton, Beds. England). PRP was normalized to contain 220×10^9 platelets/l by adding autologous PPP. Aggregation was measured with a Chrono-log® aggregometer (model 430, Chrono-log Corporation, Havertown, PA, USA). For each aggregation measurement 450 μl PRP was brought into a prewarmed (37.5° C), siliconized (Prosil®, No. 11975-0, Ventron, Alfa Produkte, Karlsruhe, FRG) glass cuvette (No. 312, AHS, Maarssenbroek, The Netherlands) and stirred at 1000 rpm. Exactly 3 min later 50 μl of the

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Figs. 1A, B Schematic representation of aggregation curves in PRP induced by collagen (A), and thrombin (B). 1: Maximum aggregation at or within 5 min after collagen addition (change in percentage of light transmission: %T). 2: Aggregation induction time (tai, sec). 3: Maximum aggregation (%T) occurring within 5 min after thrombin administration. 4: Time to maximum aggregation (t_{max} , sec). 5: Clotting time (t_c , sec). If clotting did not occur within 300 sec after thrombin addition, this parameter was set to 301

activator (collagen or thrombin) was added and the change in percentage of transmitted light (%T, see Fig. 1) was monitored continuously for 5 min or until clot formation occurred (thrombin only). In each case collagen (Collagen Horm®, Hormon Chemie, Munich, FRG) was used as the first agonist. At week 0 a range of collagen concentrations was tested, two of which were selected giving a difference in %T within 5 min of about 30% (low dose) or 60% (high dose), respectively. These doses were used at week 6 again in exactly the same order as at week 0. A similar dose-finding procedure was followed for thrombin. Thrombin (T 9010, lyophilized, Sigma, St. Louis, MO, USA) was dissolved in complete Tyrode solution to a stock solution containing 10 U/ml (NIH units). This stock solution was stored in 0.15 ml portions in plastic tubes at -15°C for a maximum period of 2 weeks. The working solutions were prepared fresh daily, using Tyrode solution as a diluent. At week 0, doses were used which gave a maximum aggregation response (%T) within 5 min of about 10% (low dose) or 30% (high dose) respectively. At week 6 these doses were used in exactly the same order.

Table 1 Age (years) of volunteers and initial values for platelet counts in PRP ($\times 10^9/\text{l}$)

Centre	Group	Age		Platelet count in PRP	
		n	Mean (range)	n	Mean \pm SEM
Maastricht	mackerel	19	32.4 ¹ (21–45)	19	314 \pm 14.6
	control	20	32.6 ¹ (22–44)	20	316 \pm 12.3
Thromsø	mackerel	11	25.0 (22–30)	9	370 \pm 34.8
	control	12	25.1 (20–29)	10	424 ² \pm 27.6
Zeist	mackerel	10	23.9 (21–27)	10	365 \pm 19.6
	control	10	23.0 (21–26)	10	382 \pm 28.4
Total	mackerel	40	28.3 (21–45)	38	341 \pm 12.8
	control	42	28.2 (20–44)	40	359 \pm 13.5

¹ Maastricht differed significantly from Tromsø and Zeist ($p < 0.0001$, Bonferroni inequality test)

² Significantly different from Maastricht controls ($p < 0.01$, Bonferroni inequality test)

Aggregation was quantified as shown by the diagrams given in Fig. 1. Transmitted light was adjusted at 90% for PPP and 10% for PRP. When thrombin is used to activate the platelets, aggregation is followed by clotting. To prevent interference of this latter process with the aggregation recordings, only those aggregation results were accepted, showing a time lapse of at least 30 sec between the moment of maximum aggregation and the occurrence of clotting. This interval was chosen on the basis of a series of measurements in PPP which revealed that the time between fibrin formation (decrease in light transmission) and clotting (increase in light transmission) was never more than 10 sec.

All measurements were done in duplicate between 60 and 120 min after blood collection. All working solutions were stored in melting ice and all platelet handling was carried out in plastic material and at room temperature.

Statistical Evaluation

Frequency distributions of all data were checked and when necessary, transformation of the data was applied to normalize the distribution. Subsequently significant outliers were omitted (35).

Student's t-test for paired data was used to evaluate the changes within the mackerel and the control groups after 6 weeks.

Differences between the mackerel and control groups were evaluated using Student's 2-sample t-test. To compare these differences for the three experimental centres, combined analysis of variance (ANOVA) was used.

Occasionally, other statistical methods were used which will be outlined when describing the results.

Differences were considered significant when the two-sided P value (p) was equal to, or smaller than 0.050.

Results

As described before (33) average dietary adherence was 85% for the control group and 78% for the mackerel group. One volunteer had to be omitted because of a very poor compliance. No side effects of the dietary supplements were observed during the study (33).

Transformation of the data was required for time-related parameters only. For the sake of clarity, non-transformed values will be given only.

Platelet Concentration in PRP

Initial values for the platelet counts in PRP are given in Table 1. No significant differences were observed between the mackerel and control groups (ANOVA). Moreover, there were no significant differences between the three centres, except for the control group in Tromsø, which showed a significantly higher platelet count than the control group in Maastricht ($p < 0.05$, Bonferroni inequality test; ref. 36). In each centre, the number of platelets in PRP tended to decrease in the mackerel group (Fig. 2). This decrease was significant for Zeist ($p < 0.01$) as well as for all centres combined ($p < 0.05$). In the control groups the changes in platelet count were very inconsistent, showing a significant increase in Maastricht ($p < 0.01$) and no significant changes in the other experimental centres. Taking all data together it appeared that, as compared to the control group, platelet concentration in PRP decreased significantly in the mackerel group ($p < 0.01$). Analysis of covariance demonstrated that this fish effect was not influenced by the initial difference in the platelet count of the Tromsø-control group. As was also observed for whole blood (37), this lowering effect of the fish supplement on platelet number was already maximal after 3 weeks (data not shown). The fish-induced decrease in platelet number did not correlate significantly with the dietary adherence as reflected by the compliance index.

Collagen-Induced Aggregation in PRP

In the three different centres the aggregation response to the same amount of collagen varied widely. In Table 2 the aggregation response to the dose of $0.8 \mu\text{g}$ collagen/ml final concentration (f.c.) is shown. This collagen dose was used simultaneously but independently from the present aggregation study and was meant to investigate the effect of the dietary supplements on the formation of cyclo-oxygenase and lipoxygenase products by collagen-activated platelets, to be published elsewhere. In Maastricht the aggregation response was much higher than in Tromsø and Zeist ($p < 0.01$, Bonferroni inequality test). In all three centres collagen of the same batch was used as well as identical aggregometers.

In Figs. 3A and 3B the changes in maximum aggregation after 6 experimental weeks are shown for the measurements giving an initial response (max. %T) of approximately 60% and 30%, respectively. Values obtained at week 0 are given in Table 3. For the high doses (initial aggregation response about 60%) the changes in aggregation during the study were significantly different from zero, for the mackerel as well as for the control groups (Tromsø excluded). The decrease in the fish group always tended to be more pronounced than that in the control group. This difference reached statistical significance in the total group (Student's t -test $p = 0.04$; analysis of variance $p < 0.02$, Fig. 3A).

At the lower doses (response about 30%) the change in maximum aggregation in the fish group was significantly different from zero in Maastricht and Zeist. Compared with the control group the aggregation in the mackerel group tended to be more decreased, but these differences were not significant. Again in Tromsø, different results were obtained: during the experimental period aggregation increased. This increase was significantly less for the mackerel group than for the control group ($p < 0.04$, see Fig. 3B). Using analysis of variance it appeared that, as compared with the control group, the mackerel supplement decreased platelet aggregation significantly ($p < 0.02$).

Due to inter-individual differences in sensitivity of platelets towards collagen, the collagen dose-range used for the various individuals differed substantially. However, in each centre there was always one particular dose which was used most frequently.

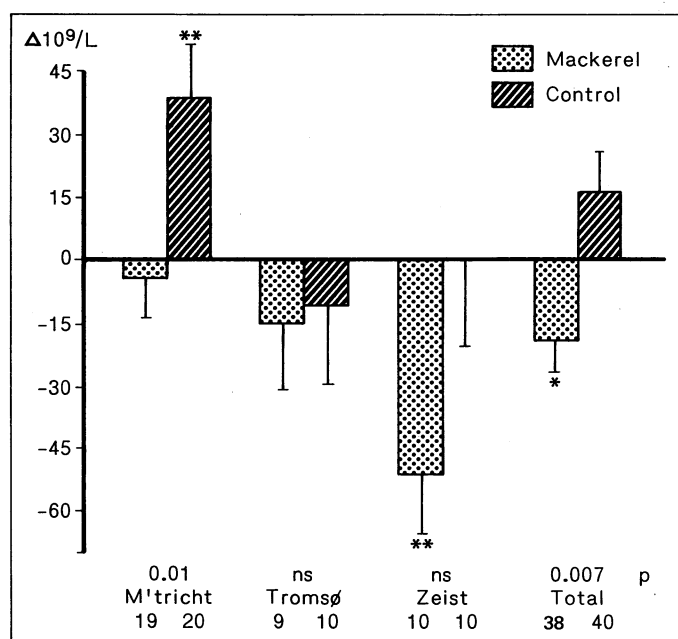


Fig. 2 Effect of dietary supplements on the change in platelet count in PRP ($\times 10^9/\text{l}$) after 6 experimental weeks (mean and sem). For initial values: see Table 1. p: significance of differences between mackerel and control groups. ns: not significant. Values significantly different from zero: * $p < 0.05$, ** $p < 0.01$

Table 2 Platelet aggregation in PRP (%T) in response to the same dose of collagen ($0.8 \mu\text{g}/\text{ml}$, f.c.) in the three different centres

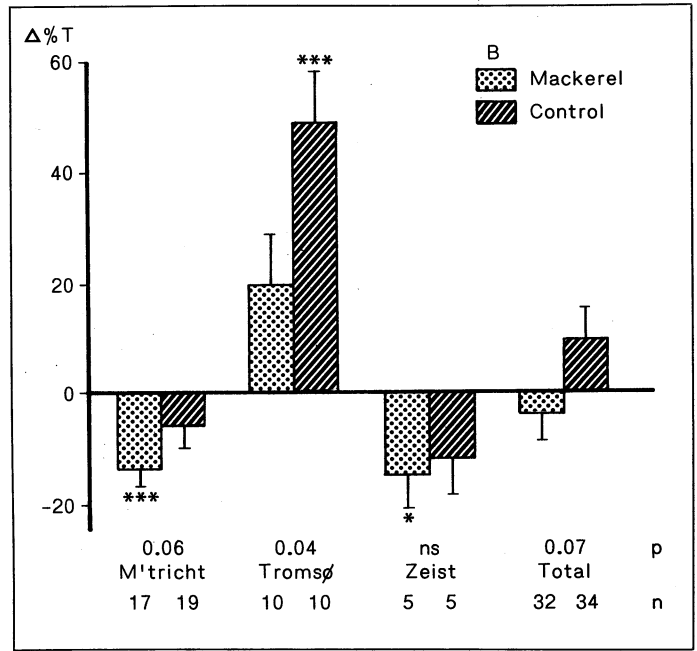
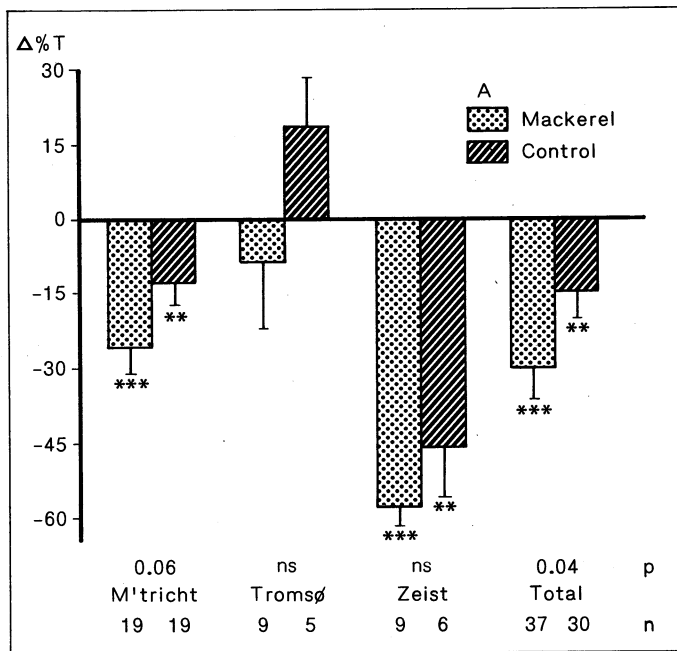
Centre	n	Mean	SEM
Maastricht	36	62.7 ¹	2.07
Tromsø	23	13.1	4.29
Zeist	20	23.4	4.94

¹ Significantly different from other centres ($p < 0.01$, Bonferroni inequality test)

In Maastricht this collagen dose was $0.5 \mu\text{g}/\text{ml}$ (f.c.) and the aggregation-inhibiting effect of the mackerel supplement after 6 weeks as measured with this amount of collagen was significantly more pronounced than that of the meat supplement ($p = 0.004$, Fig. 4). In Tromsø the most frequently used collagen dose was $1.0 \mu\text{g}/\text{ml}$ (f.c.); no significant effect of the mackerel paste was observed. In Zeist the collagen dose used most often at week zero ($0.8 \mu\text{g}/\text{ml}$, f.c.) scarcely gave a response at week 6: in 15 out of 16 cases no aggregation occurred at all. Therefore these measurements were disregarded. When the results of Maastricht and Tromsø were combined the inhibition of maximum aggregation by the mackerel group was significant as compared with the change in the control group (ANOVA, $p = 0.04$).

The effect of the dietary supplements on the aggregation induction time was analysed in a similar way as described above for the maximum aggregation. Similar results were obtained (data not shown) which was to be expected because of the strong correlation between both aggregation variables at week 0 ($r = -0.74$, $p < 0.001$, $n = 65$).

Taking all these results together it can be concluded that the fish supplement lowered collagen-induced platelet aggregation in PRP slightly but significantly. No significant regression was observed between the dietary adherence and the extent to which



Figs. 3A, B Effect of dietary supplements on the change in collagen-induced aggregation in PRP (%T and sem) of the measurements which gave a maximum aggregation response of about 60% (but always >35%, A) and about 30% (always <35%, B). See Table 3 for initial values. p: significance of differences between mackerel and control groups. ns: not significant. Values significantly different from zero: *p < 0.05, **p < 0.01 and ***p < 0.005. Using analysis of variance the overall fish effect was significant (p = 0.02) for the high as well as the low dose of collagen

the fish supplement inhibited the collagen-induced platelet aggregation. Moreover, within the experimental group the mackerel effect on platelet aggregation was similar in those 50% of the volunteers having the highest compliance as compared with the other 50% having the lowest compliance. Hence, the fish-induced changes in platelet aggregation were not significantly influenced by differences in dietary adherence.

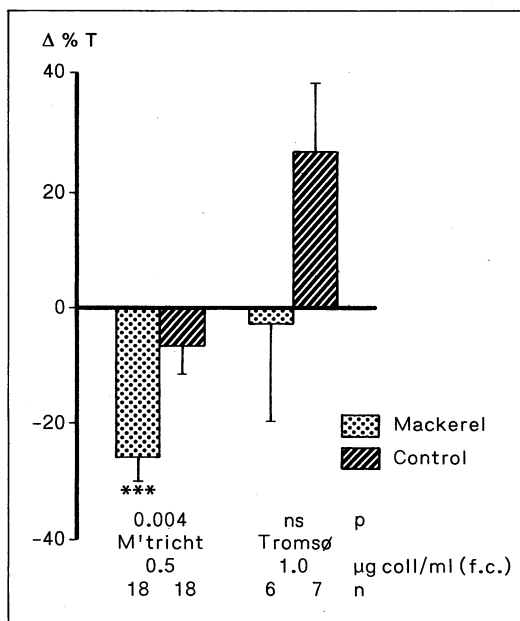


Fig. 4 Effect of dietary supplements on the change in maximum aggregation in PRP (%T and sem) due to a specific dose of collagen (Maastricht and Tromsø). p: significance of differences between mackerel and control groups. ns: not significant. Values significantly different from zero: ***p < 0.005. Using analysis of variance the fish effect was significant (p < 0.04)

Thrombin-Induced Platelet Aggregation in PRP

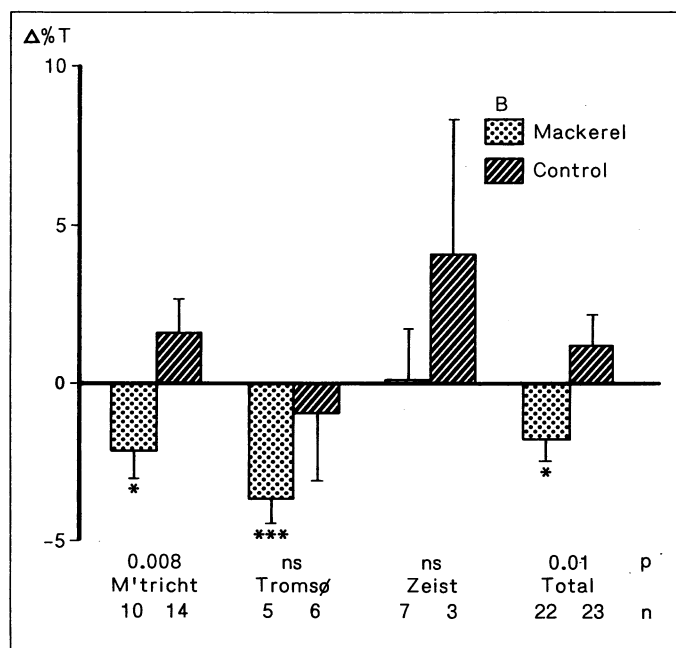
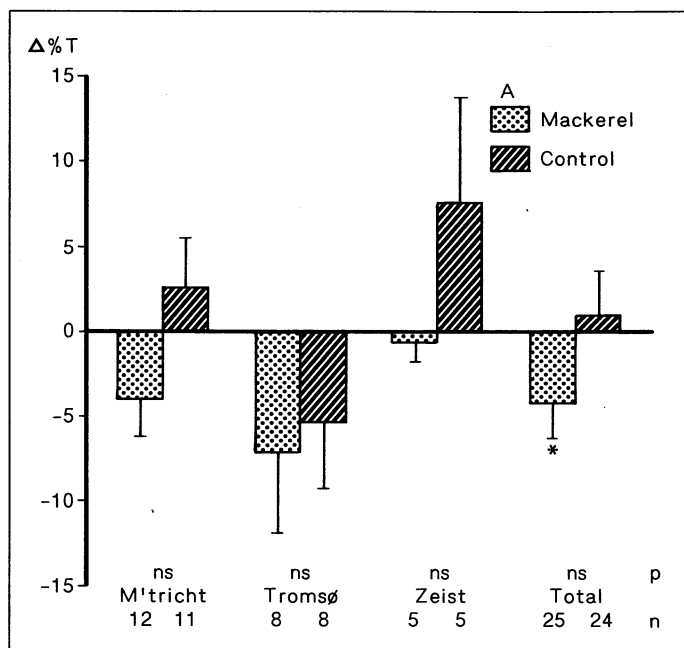
In the three experimental centres, the inter-individual variation in aggregation response was far less for thrombin than for collagen as a platelet activator. However, the limited supply of PRP did not always enable us to obtain the required aggregation responses of 10 and 30% for each volunteer. Because of this the number of observations in each centre was often too small to warrant reliable results. At the higher doses of thrombin (giving a %T response of about 30% at week zero, see Table 4), in none of the centres significant changes in aggregation occurred during the 6 experimental weeks. When the results from the three centres were taken together, a small decrease in aggregation was evident in the mackerel group which was significantly different from zero (p < 0.05) but which was not significantly different from the (non significant) change in aggregation, observed in the control group (Fig. 5A).

Results for the lower doses of thrombin (initial aggregation response about 10%, see Table 5) are given in Fig. 5B.

In the mackerel groups a significant decrease in aggregation was observed (Zeist excepted) whereas the changes in the control groups were not significantly different from zero. For Maastricht as well as for the three centres combined this resulted in a significant mackerel effect as compared with the control groups. Using analysis of variance, however, no significant mackerel effect could be observed for the three centres combined although a tendency was obvious (p = 0.09).

Results obtained with the dose of thrombin used most frequently, were analysed in a similar way; no significant differences were found between the mackerel and the control groups, neither for each centre separately, nor for the three centres combined.

The effect of the mackerel supplement on the time to maximum aggregation was analysed in an identical way as described for the maximum aggregation. In general, results were similar (data not shown) which can be explained by the correla-



Figs. 5A, B Effect of dietary supplements on the change in thrombin-induced aggregation in PRP (%T and sem) of the measurements which gave a maximum aggregation response of about 30% (A) and of about 10% (B). See Table 4 for initial values. p: significance of differences between mackerel and control groups. ns: not significant. Values significantly different from zero: * $p < 0.05$, *** $p < 0.005$

tion between both aggregation variables at week 0 ($r = 0.64$, $p < 0.001$, $n = 40$).

Clotting times (low thrombin dose: 200 ± 11 sec, $n = 45$; high dose: 144 ± 10 sec, $n = 49$) were not affected by the dietary supplements (data not shown).

In general, the results did not change when corrections were made for dietary adherence. Therefore, it can be concluded that thrombin-induced platelet aggregation in PRP is hardly affected by the mackerel supplement.

Discussion

The study reported in this paper was part of a trial conducted by the International Working Party "Fish against thrombosis?". The general outline of this trial has been published in detail before (33). In the present paper the effects of the daily administration of mackerel for 6 weeks on platelet aggregation in PRP are described.

The aggregation measurements were performed in three different experimental centres. Maximum care was taken to perform the measurements under identical conditions in each centre. Detailed instructions were given to the research teams and identical equipment and reagents were made available. In spite of this, the response to the same amount of collagen was much higher in Maastricht than in Tromsø and Zeist (Table 2), although all volunteers consumed the meat supplement during the 2 weeks "run-in period", which was intended to reduce the possible differences in starting conditions between the three centres. A difference in platelet sensitivity was not seen for thrombin-induced aggregation.

It should be pointed out here that the Maastricht volunteers were recruited from the general population, and were, on average, significantly older (Table 1) than the participants in Tromsø and Zeist, who were all university students.

Most probably, the difference in collagen-sensitivity of the platelets in the three centres did not interfere with our experimental results, because the effects of the diet were calculated in

Table 3 Initial values for maximum aggregation in PRP induced by collagen (%T \pm SEM)

	Response ca. 60%		Response ca. 30%		Most frequently used collagen dose (μ g/ml, f.c.) ¹	
	n	%T	n	%T	f.c.	%T
Maastricht					0.5	
Mackerel	19	59 ± 1.9	17	23 ± 2.1	18	44 ± 4.7
Control	19	59 ± 1.5	19	22 ± 2.0	18	33 ± 4.1
Tromsø					1.0	
Mackerel	9	60 ± 5.8	10	22 ± 3.0	6	52 ± 10.9
Control	5	58 ± 4.3	10	20 ± 2.6	7	25 ± 9.9
Zeist					0.8	
Mackerel	9	65 ± 2.4	5	17 ± 5.5	8	29 ± 9.3
Control	6	61 ± 4.1	5	17 ± 4.8	8	32 ± 10.0

¹ f.c.: final concentration

Table 4 Initial values for maximum aggregation in PRP, induced by thrombin (%T \pm SEM)

	Response ca. 30%		Response ca. 10%		Most frequently used thrombin dose (U/ml, f.c.) ¹	
	n	%T	n	%T	f.c.	%T
Maastricht					0.15	
Mackerel	12	25 ± 1.9	10	11 ± 0.6	15	16 ± 2.6
Control	11	21 ± 1.3	14	11 ± 0.5	16	14 ± 1.8
Tromsø					0.15	
Mackerel	8	23 ± 2.7	5	12 ± 0.9	5	14 ± 1.4
Control	8	28 ± 2.4	6	11 ± 1.4	4	25 ± 9.3
Zeist					0.16	
Mackerel	5	23 ± 2.6	7	12 ± 0.8	7	15 ± 3.7
Control	5	20 ± 1.2	3	9 ± 0.9	5	14 ± 2.9

¹ f.c.: final concentration

relation to a given, pre-determined response level of the platelets at week zero, irrespective of the collagen doses. Since this approach is quite uncommon, we also analysed a series of measurements obtained with one dose of collagen for each centre. The results of both approaches were almost identical.

Our study indicates that the fish supplement had a slight but significant reducing effect on collagen-induced platelet aggregation in PRP, which might be due to a potency of activated platelets to produce thromboxane A₂ (25). Results with respect to collagen-induced aggregation, as reported in the literature, are not unanimous at all, which may be due to methodological differences between the various studies. Although most investigators used the same type and source of collagen we did (Hormon Chemie), the collagen doses applied to stimulate the platelets varied widely (range 0.1–70 µg/ml, f.c.). Moreover, the concentration of platelets in the PRP varied considerably (between 200–400 × 10⁹/l). The amounts and types of fish-(products) investigated were by no means similar; daily doses of timnodonic acid varied between 0.15 to 6 g and the duration of supplementation ranged from one meal to 20 weeks daily. However, the equivocal results of the studies are unlikely to be explained by this wide dose range. Finally, the populations under investigation were quite different, comprising healthy young individuals, elderly diabetics, hyperlipidaemic patients, etc.

From our study it is evident that a proper control group is essential in this type of nutritional study. Unfortunately, of the 26 collagen-aggregation studies reported so far only 8 included a proper control group (7–14). In 5 (62%) of these latter studies, fish(oil) administration was shown to reduce collagen-induced aggregation in PRP in a significant way. This percentage was higher (72%) in the poorly- and non-controlled studies (15–32) but it can never be excluded that the positive effect in these studies is (partly) due to an artefact.

Thrombin-induced platelet aggregation in PRP was scarcely affected by the fish supplement. This might be due to the fact that thrombin-induced aggregation is very little affected by TxA₂ formation (38) which may be diminished as a consequence of dietary fish (25). Our results compare quite well with two recently published well-controlled studies (8, 10) and with one poorly controlled investigation (21). In another poorly designed experiment, thrombin-induced platelet aggregation appeared to be inhibited by the administration of cod-liver oil (39).

The importance of platelet aggregation as a risk factor for cardiovascular disease is poorly documented. Studies performed after myocardial infarction or in angina pectoris patients demonstrate that platelet aggregation may be enhanced or not altered (40, 41). So far, prospective studies to support the concept that increased platelet aggregability is a risk factor for the development of ischaemic cardiovascular disease have not been reported. This implies that a decrease in platelet aggregation can, in fact, not be interpreted in terms of myocardial risk. Therefore, to investigate the potential of dietary fish for prevention and therapy of cardiovascular ischaemia, a long term, prospective study is required which should focus on incidence and mortality of atherosclerotic diseases.

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