Effects of dietary *n*-3 polyunsaturated fatty acids from plant and marine origin on platelet aggregation in healthy elderly subjects

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In the present intervention study we compared the effects of α-linolenic acid with those of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on platelet aggregation *in vitro* and *ex vivo* in healthy non-institutionalized elderly subjects. We also compared the effects of α-linolenic acid on platelet aggregation in elderly subjects with those in younger volunteers. During a run-in period of 3 weeks all subjects (thirty-eight elderly (> 60 years) and twelve younger volunteers (< 35 years)) received a diet rich in oleic acid. For the next 6 weeks the elderly subjects received a diet rich in oleic acid (*n* 11), α-linolenic acid (*n* 14) (6·8 g/d) or EPA/DHA (*n* 13) (1·05 g EPA plus 0·55 g DHA). The younger subjects were given a diet rich in α-linolenic acid. The diets did not affect ADP- or collagen-induced platelet aggregation *in vitro* in either platelet-rich plasma or whole blood. The *ex vivo* platelet aggregation as measured with filtragometry was significantly decreased in the elderly group that received EPA/DHA compared with the α-linolenic acid ($P = 0{\cdot}006$) and the oleic acid ($P = 0{\cdot}005$) diet groups. Effects of α-linolenic acid were not age-dependent. Our results suggest that α-linolenic acid and EPA/DHA do not change *in vitro* platelet aggregation. Compared with oleic acid, EPA/DHA, but not α-linolenic acid, favourably affects *ex vivo* platelet aggregation in healthy elderly subjects.

α-Linolenic acid: Eicosapentaenoic acid: Docosahexaenoic acid: Platelet aggregation

A major part of the daily intake of α-linolenic acid, an essential fatty acid that belongs to the *n*-3 family, is provided by vegetable oils like rapeseed and soyabean oils and products derived from these oils (Voskuil *et al*. 1996). However, other food sources such as walnuts, dairy products and green vegetables (Hepburn *et al*. 1986) also contribute to α-linolenic acid intake.

In the body, α-linolenic acid can be converted into longer-chain *n*-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Emken *et al*. 1988). In the diet these longer-chain *n*-3 fatty acids are mainly found in fatty fish and fish-oil concentrates. Although the estimated habitual daily intake of EPA plus DHA (0·2–0·5 g) is much less than that of α-linolenic acid (1·5–2·0 g; Hunter, 1990), most studies have focused on the health effects of EPA/DHA. In particular, consumption of fatty fish is thought to decrease platelet aggregation (Kinsella *et al*. 1990*b*).

Human studies comparing the effects of α-linolenic acid with those of EPA/DHA on platelet aggregation are scarce. Freese & Mutanen (1997) reported that EPA/DHA increased the sensitivity of platelets to ADP-induced aggregation compared with α-linolenic acid. However, no differences between α-linolenic acid and EPA/DHA were observed for collagen-induced aggregation.

The capacity of human beings to convert α-linolenic acid into EPA and DHA is rather low (Emken *et al*. 1988), and may decrease with ageing (Debry & Pelletier, 1991). Elderly people are therefore a relevant study population. Until now, however, no studies on the effects of α-linolenic acid on platelet aggregation in elderly subjects have been carried out. Therefore, in the present intervention study we compared the effects of α-linolenic acid with those of EPA/DHA on platelet aggregation in healthy elderly subjects (60 years and older). A group of subjects younger than 35 years was also included. Since they were given α-linolenic acid, we could also examine if the potential effects of α-linolenic acid on platelet aggregation are age-dependent.

Materials and methods

Subjects

Subjects were recruited via advertisements in local newspapers, in a university newspaper and via posters in public buildings. Sixty-seven subjects, nineteen aged younger than

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PRP, platelet-rich plasma; Tx, thromboxane.

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35 years and forty-eight older than 60 years, underwent the selection procedure. Nineteen young adults and forty-one non-institutionalized elderly volunteers met all our eligibility criteria: serum total cholesterol concentration $< 8{\cdot}0$ mmol/l, serum triacylglycerol concentration $< 3{\cdot}0$ mmol/l, not taking a prescribed or weight-reducing diet, and not taking any medication known to affect the variables measured. In addition, all sixty volunteers were apparently healthy, as indicated by a medical questionnaire and by absence of protein and glucose in the urine.

The subjects were informed about the nature of the study and gave their written consent. The study protocol was reviewed and approved by the Medical Ethical Committee of the university.

Ten subjects did not complete the study due to job or study commitments (four younger subjects), use of non-steroidal anti-inflammatory drugs (two elderly subjects and one younger subject), family circumstances (one elderly subject), illness (one younger subject) or blood donation (one younger subject). Thus, twelve young adults (three men and nine women) and thirty-eight elderly subjects (fourteen men and twenty-four women) completed the study. The young adults were between 16–33 years old (mean 24 years), weighed 49–80 kg (mean 66 kg), were between 1·58 and 1·85 m in height (mean 1·70 m) and had BMI from 19 to 27 kg/m^2 (mean 23 kg/m^2). The elderly subjects were between 60 and 78 years old (mean 65 years), weighed 51–99 kg (mean 73 kg), were between 1·48 and 1·79 m in height (mean 1·67 m) and had BMI from 20 to 32 kg/m^2 (mean 26 kg/m^2). Physical characteristics did not differ between the persons who withdrew and the persons who completed the study.

All nine young women used oral contraceptives, while the elderly women were all post-menopausal. In the younger group, one person smoked, in the elderly group six subjects smoked. Subjects were asked to maintain their usual pattern of activity and not to change their smoking habits or use of oral contraceptives.

Experimental design and statistical analyses

The experiment had a parallel design and consisted of a run-in period of 3 weeks, immediately followed by an experimental period of 6 weeks. The experiment was carried out during two separate periods. Eight younger and twenty-one elderly subjects were studied in the first period, while the remaining subjects (four younger and seventeen elderly subjects) participated in the second period.

During the run-in period, all subjects received a diet enriched with oleic acid. After this period, all the younger volunteers received during the experimental period a diet enriched with α-linolenic acid. The elderly volunteers, however, were first categorized according to sex. As there were more women than men, we first divided the elderly men at random over the three groups and added, if participating, the women they were married to in the same group. Then, we randomly divided the remaining women over the three groups. These three groups received a diet enriched with oleic acid, α-linolenic acid, or EPA/DHA. The numbers of subjects in each experimental group were balanced over the two study periods.

Effects of the three diets in the elderly group were examined by a one-way factor analysis of covariance including the end of the experimental period as the dependent variable, the end of the run-in period as covariate and diet as factor. When the analyses indicated a significant diet effect ($P < 0{\cdot}05$), the Bonferroni method was used to compare the diets pairwise. As this involved three simultaneous comparisons, the upper limit of statistical significance was set at one-third of the customary 0·05, i.e. 0·016. Differences in responses between the younger and elderly volunteers on the α-linolenic acid diets were analysed similarly but with age group as factor instead of diet. A two-tailed P-value $< 0{\cdot}05$ was considered statistically significant. Pearson correlation coefficients were calculated between the various platelet function tests (Kleinbaum *et al*. 1988). All values are expressed as means with their standard errors.

Diets

During the study, subjects were provided with products in which a part of the habitual fat was replaced by experimental shortenings. The high-oleic acid shortening was a mixture of sunflower oil high in oleic acid (617 g/kg), an oleic-acid rich fraction of palm oil (150 g/kg), fully hydrogenated palm oil (48 g/kg), and palm oil (185 g/kg). The shortening high in α-linolenic acid consisted of palm oil (151 g/kg), fully hydrogenated palm oil (60 g/kg), linseed oil (429 g/kg), and Trisun-80 (360 g/kg). The fat high in EPA/DHA consisted of palm oil (66 g/kg), fully hydrogenated palm oil (60 g/kg), menhaden oil (306 g/kg), Trisun-80 (530 g/kg), and high-linoleic safflower oil (38 g/kg). The fatty acid compositions of the three experimental shortenings, which were prepared by Unilever Research Vlaardingen, The Netherlands, are given in Table 1. The experimental shortenings high in oleic acid, α-linolenic acid and EPA/DHA contained 724, 1090 and 808 mg tocopherols/kg and 118, 85 and 0 mg tocotrienols/kg respectively.

Table 1. Fatty acid composition (g/100 g total fatty acids identified) of the experimental shortenings

	Type of shortening*		
Fatty acid	Oleic acid	α-Linolenic acid	EPA/DHA
ΣSAFA	15·7	20·7	24·2
14:0	0·6	0·3	3·3
16:0	8·2	13·3	13·8
18:0	6·9	7·1	7·1
ΣMUFA	62·1	44·0	54·7
16:1*n*-9	0·1	0·1	3·8
18:1*n*-9	62·0	43·9	50·9
ΣPUFA	9·3	32·4	14·4
Σ*n*-6 PUFA	9·2	10·9	8·7
18:2*n*-6	9·2	10·9	8·7
Σ*n*-3 PUFA	0·1	21·5	5·7
18:3*n*-3	0·1	21·5	0·5
20:5*n*-3	0·0	0·0	3·4
22:6*n*-3	0·0	0·0	1·8
PUFA : SAFA	2·8	3·7	2·9

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

* For details of shortenings, see p. 184.

The shortenings were provided as spreads or were incorporated into pies, cake and chocolate paste. No heating was used for the preparation of the experimental products to prevent oxidation of the highly unsaturated fatty acids. Subjects were not allowed to use the spreads for baking or frying, but had to consume 30 g of the experimental shortenings daily. This resulted in an additional daily consumption of 6·8 g α-linolenic acid and 1·05 g EPA plus 0·55 g DHA. The oleic acid and α-linolenic acid groups were not allowed to consume any fatty fish.

Volunteers came to the department at least once weekly to receive a new supply of products. Body weight was recorded every week and, if necessary, subjects were advised to adjust energy intake to avoid weight changes. In addition, they were asked to record in diaries any signs of illness, medication used, alcoholic drinks, menstrual phase, changes in physical activity, and any deviations from the diet or the study protocol.

Blood sampling

Venous blood was sampled from an antecubital vein under minimal stasis with the volunteer in a recumbent position, after resting for 15 min, and after an overnight fast. Subjects were instructed to abstain from drinking alcohol on the preceding day and to abstain from smoking on the morning before blood sampling. All venepunctures were performed at the same location and for each subject generally at the same time of the day. Before the study began, all the subjects were assigned a random number that was used for labelling their tubes. In this way, the technicians who sampled the blood and performed the analyses were blinded as to the subjects' diets.

Blood was collected on day 21 (end of run-in period) and on day 63 (end of experimental period) with an infusion needle (1·0 mm/G19; Microflex, Écouen, France). The first 2–3 ml blood was used for analysis of haematological variables (numbers of platelets, erythrocytes and leucocytes, packed cell volume and haemoglobin concentration) on a Coulter® MD 18 (Coulter Corporation, Miami, FL, USA). The following 13–14 ml was collected for aggregation measurements *in vitro*, followed by 30 ml for additional measurements to be presented elsewhere. Finally, the infusion needle was connected to the filtragometer for the measurement of platelet aggregation *ex vivo*.

Platelet aggregation in vitro

In vitro platelet aggregation in platelet rich plasma (PRP) and in whole blood was measured with a dual sample aggregometer (Model 540; Chrono-Log® Corporation, Havertown, PA, USA).

For platelet aggregation in PRP, 10·8 ml blood was drawn slowly into a plastic tube containing 1·2 ml sodium citrate solution (32 g/l). The blood was gently mixed and immediately centrifuged at 200 ***g*** for 12 min at room temperature to obtain PRP. Platelet-poor plasma was obtained after centrifugation of a part of the PRP at 1500 ***g*** for 5 min at room temperature. Platelet concentrations in PRP were measured with a Coulter® MD 18 (Coulter Corporation) and platelet counts adjusted to 185×10^3 platelets/μl by adding autologus platelet-poor plasma.

For each measurement, 400 μl PRP was brought into a prewarmed (37°) siliconized glass cuvette with a plastic pipette and stirred at 1000 rev./min. Exactly 3 min later, aggregation was induced in both channels with either 10 μl ADP (ADP, A6521; Sigma Chemical, St Louis, MO, USA: final concentration 1·5 μmol/l) or 10 μl collagen (Collagen Horm®; München, Germany: final concentration 1·0 μg/ml). The change in percentage of transmitted light was monitored continuously by a computerized system for 7 min. Transmission was set at 0 % with PRP and at 100 % with platelet-poor plasma and the change in transmission was expressed as a percentage of 100 % light transmission.

All measurements were completed between 30 and 60 min after blood collection, and all platelet handling was carried out at room temperature.

The aggregations in PRP were quantified by measuring the aggregation velocity (Va, the steepest slope of the aggregation curve in %/min), and the maximum aggregation (Imax, the total increase in optical density in %).

For platelet aggregation in whole blood, 2·7 ml blood was drawn slowly into a plastic tube containing 0·3 ml recombinant desulfatohirudin variant 1 (Revasc™; a kind gift from Novartis Pharmaceuticals, Horsham, West Sussex, UK), (final concentration 0·07 mg/ml). The blood was gently mixed and 1 ml anticoagulated blood was immediately transferred into a prewarmed (37°) plastic cuvette with a stir bar. Electrodes were placed in the cuvette and the sample was equilibrated for 5 min at 37° at 1000 rev./min. After 5 min pre-incubation and calibration, aggregation was induced by addition of 10 μl ADP (final concentration 1·5 μmol/l) or 10 μl collagen (final concentration 0·05 μg/ml), and the change in impedance was monitored continuously by a computerized system for 15 min.

Whole-blood platelet aggregation was quantified by measuring the aggregation velocity (Va) and the maximum aggregation (Imax).

For ADP-induced aggregations in PRP or whole blood, aggregation variables of the first wave only were used as outcome variables. If present, the second wave was neglected.

At 15 min after induction of aggregation in whole blood, the activated blood samples were centrifuged for 5 min at 9700 ***g*** in an Eppendorf centrifuge. Supernatant fractions were collected, immediately frozen in liquid N_2, and stored at –80° until determination of the thromboxane (Tx) B_2 concentration. The TxB_2 concentration was determined with an enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions.

Platelet aggregation ex vivo: *filtragometry*

Platelet aggregation *ex vivo* was measured with the filtragometer. The filtragometer is a device for measuring platelet aggregation in flowing venous blood in man. The principle of the method is based on the continuous measurement and registration of the pressure difference (ΔP) across a filter with pores of 20 μm diameter through which blood from a forearm vein is drawn. Platelet aggregates, obstructing the filter, cause a change in the pressure difference (Hornstra & ten Hoor, 1975), which is proportional to the mass of the platelet aggregates that obstruct the filter.

Blood was drawn through the filtragometer by a motor-driven syringe at a flow rate of 2 ml/min. The filter and connecting tubing, which were siliconized, were maintained at 37°. Heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands; final concentration 5 IU/ml), was infused at the beginning of the filtragometer tubing system, before a mixing chamber, to prevent coagulation in the apparatus. All solutions used in the filtragometer had been filtered and degassed by sonication in order to remove small particles and air bubbles, which might occlude the filter.

The pressure difference across the filter, measured by two pressure transducers on either side, is a measure of filter occlusion. A 5 mmHg pressure difference corresponds to 25 % filter pore occlusion. When ΔP reaches 5 mmHg, the heparin is switched off and a citrate infusion (final concentration 4·04 g/l) is started (Hornstra & ten Hoor, 1975). The citrate infusion may partially reverse the occlusion of the filter with a transient decrease in ΔP.

The following parameters were calculated from the ΔP curve: aggregation time (tA, the time (s) necessary to reach a ΔP-value of 5 mmHg), aggregation slope (Ts, the slope of the tangent to the curve at ΔP = 5 mmHg), maximum aggregation (Amax, the maximum height of the aggregation curve in mmHg) and desaggregation induction time (tDI, the time (s) between termination of the heparin infusion and the beginning of desaggregation).

If the 5 mmHg pressure difference was not reached within 600 s, the filtragometer was switched off and the aggregation time set at 600 s. In those cases none of the other parameters could be calculated.

Due to the asymmetric distribution of the filtragometry data, logarithmic transformation was performed.

Fatty acid composition of erythrocyte neutral phospholipids

The fatty acid composition of erythrocyte neutral phospholipids was determined in EDTA-treated blood as a measure of compliance with the diet. After centrifugation, the plasma was collected and used for other analyses. The remaining blood cells were washed twice with an EDTA–NaCl solution (28·64 g EDTA, 7·0 g NaCl, 1 litre water) and the erythrocytes were stored under N_2 at −80°, until lipid extraction. Lipids in the erythrocytes were extracted within 48 h after collection, according to the method of Bligh & Dyer (1959). The neutral phospholipid fraction was separated from the lipid extract by aminopropyl bonded silica columns (Varian®, Harbor City, CA, USA; Kaluzny *et al*. 1985). The neutral phospholipids were hydrolysed and the resulting fatty acids transmethylated to their corresponding methyl esters by reaction with BF_3 (100 g/l) in methanol at 100° for 1 h (Morrison & Smith, 1964). The fatty acid methyl esters were analysed using an Autosystem (Perkin Elmer, Gouda, The Netherlands) GC, fitted with a 50 m CP Sil 88 polar capillary column with 0·25 mm i.d., film thickness 0·20 μm (Chrompack®, Middelburg, The Netherlands) using a split ratio of 1 : 20. The injection and detection temperatures were both 300°, and He was used as carrier gas (130 kPa). The starting temperature of the column was 160°. At 10 min after injection, the temperature was increased to 190° at a rate of 3·2°/min; after 15 min at 190°, the temperature was increased to 230° at a rate of 5°/min. The final temperature of 230° was maintained for 7 min.

A standard mixture of fatty acid methyl esters was used to identify the fatty acid methyl esters in the samples by means of retention times. Using computer-assisted analysis (Turbochrome ll, Perkin-Elmer), the fatty acid profiles were corrected for blank runs. The results are expressed in relative amounts (g/100 g total fatty acids identified).

Results

At the end of the run-in period, the fatty acid compositions of the erythrocyte neutral phospholipids were comparable for the elderly and the younger volunteers. Only the proportion of 18:3*n*-3 in the future oleic acid diet group was significantly higher than that in the elderly volunteers randomized into the α-linolenic acid diet group ($P = 0·009$).

The proportion of 18:3*n*-3 in the α-linolenic acid diet group increased significantly more than in the oleic acid ($P < 0·001$) and the EPA/DHA diet ($P < 0·001$) groups. In the EPA/DHA diet group dietary compliance was confirmed by a rise of 1·3 % in the proportion of 20:5*n*-3 and of 0·8 % in 22:6*n*-3 at the expense of a decrease of 2·2 % in the proportion of 18:2*n*-6. All these changes were significantly different from those in the oleic acid and the α-linolenic acid diet groups (Table 2).

The increase in the ratio Σ(*n*-3) : Σ(*n*-6) of 0·14 (SEM 0·03) in the EPA/DHA group was significantly higher than the slight increases of 0·02 (SEM 0·02) in the oleic acid ($P < 0·001$) and of 0·05 (SEM 0·01) in the α-linolenic acid diet groups ($P < 0·001$).

Effects of α-linolenic acid supplementation on the fatty acid composition of erythrocyte neutral phospholipids were similar for the younger and elderly subjects ($P = 0·056$; Table 2).

No significant diet effects were found on either ADP- or collagen-induced aggregation velocity and maximum aggregation in PRP or whole blood (Tables 3 and 4). Occasionally, ADP-induced aggregation was accompanied by a second wave. At the end of the run-in period this was observed in two (8 %) younger and six (8 %) elderly subjects. At the end of the experimental period this was observed for seven (29 %) of the younger subjects and seven of the elderly subjects, one (5 %) on the oleic acid diet, two (8 %) on the α-linolenic acid diet and four (14 %) on the EPA/DHA diet. At the end of the run-in period collagen-induced platelet aggregation velocity (Va) in whole blood was significantly lower in the older compared with the younger adults (4·20 (SEM 0·39) *v*. 5·26 (SEM 0·47) ohm/min, $P = 0·031$).

Changes in the TxB_2 concentrations in plasma, obtained after ADP- or collagen-induced platelet aggregation in whole blood did not differ significantly between the three groups of elderly subjects or between the two α-linolenic acid diet groups (Table 4). TxB_2 production was significantly related to the Va of collagen-induced platelet aggregation in whole blood at the run-in period (r 0·387, $P = 0·009$), but not to the ADP-induced platelet aggregation (r 0·087, $P = 0·587$) at that period.

Table 2. Effects of diets enriched in oleic acid, α-linolenic acid or eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA) on the fatty acid composition (g/100 g total fatty acids identified) of erythrocyte neutral phospholipids in elderly subjects and young adult subjects‡

(Mean values with their standard errors)

	Elderly subjects (*n* 38)												Young adults (*n* 12)			
	Oleic acid (*n* 11)				α-Linolenic acid (*n* 13)				EPA/DHA (*n* 14)				α-Linolenic acid (*n* 12)			
	Run-in		Change		Run-in		Change		Run-in		Change		Run-in		Change	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
ΣSAFA	45·3	0·6	0·5	0·5	45·8	0·8	0·2	0·6	45·8	0·6	0·2	0·5	45·3	1·0	–0·6	1·0
ΣMUFA	20·2	0·4	0·2	0·3	19·3	0·4	0·0	0·3	19·4	0·7	0·5	0·6	19·9	0·5	–0·5	0·4
18:1*n*-9	13·2	0·3	0·4	0·2	12·7	0·3	0·3	0·3	13·0	0·4	0·5	0·5	13·2	0·3	–0·7	0·3
ΣPUFA	33·6	0·5	–0·6	0·3	34·0	0·6	0·0	0·6	34·2	0·6	–0·7	0·6	33·9	0·8	0·4	0·5
Σ*n*-6 PUFA	28·1	0·5	–1·0	0·5	28·3	0·5	–0·9	0·5	28·0	0·6	–3·5**	0·7	28·2	0·8	–0·4	0·5
18:2*n*-6	13·3	0·5	–0·7	0·3	12·8	0·3	–0·1	0·2	13·4	0·9	–2·2***††	0·9	13·2	0·5	–0·6	0·6
20:4*n*-6	9·9	0·3	–0·2	0·3	10·5	0·5	–0·7	0·2	10·1	0·4	–0·8	0·3	9·6	0·3	0·9	0·3
Σ*n*-3 PUFA	5·5	0·3	0·4	0·3	5·6	0·2	1·1	0·3	6·0	0·5	2·8†	0·7	5·3	0·5	1·1	0·6
18:3*n*-3	0·1	0·0	0·0	0·0	0·0	0·0	0·4†††	0·0	0·0	0·0	0·0***	0·0	0·1	0·0	0·2	0·1
20:5*n*-3	0·6	0·1	0·0	0·1	0·6	0·1	0·4	0·1	1·0	0·3	1·3***†††	0·4	0·5	0·2	0·2	0·2
22:6*n*-3	3·2	0·3	0·1	0·2	3·5	0·2	–0·3	0·1	3·5	0·2	0·8***†††	0·2	3·5	0·3	–0·1	0·3
Σ(*n*-3) : Σ(*n*-6)	0·20	0·01	0·02	0·02	0·20	0·00	0·05	0·01	0·22	0·02	0·14***†††	0·03	0·20	0·02	0·04	0·02

SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Mean values were significantly different from those for α-linolenic acid in the elderly: ** $P < 0·01$, *** $P < 0·001$ (analysis of covariance).
Mean values were significantly different from those for the oleic acid group: †† $P < 0·01$, ††† $P < 0·001$ (analysis of covariance).
‡ For details of diets and procedures, see pp. 183–186.

Table 3. Effects of diets enriched in oleic acid, α-linolenic acid or eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA) on *in vitro* platelet aggregation in platelet-rich plasma induced with ADP or collagen*†

(Mean values with their standard errors)

	ADP				Collagen			
	Run-in		Change		Run-in		Change	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Va (%)								
Elderly (*n* 38)								
Oleic acid (*n* 11)	50·7	5·8	–0·6	8·9	42·8	5·8	9·9	11·0
α-Linolenic acid (*n* 13)	52·9	4·2	–2·5	6·1	40·2	6·1	6·1	5·4
EPA/DHA (*n* 14)	48·2	2·8	6·1	7·8	46·5	2·4	3·7	8·6
Young adults (*n* 12)								
α-Linolenic acid (*n* 12)	46·6	6·4	–4·2	8·3	47·8	4·2	5·1	7·6
Imax (%)								
Elderly (*n* 38)								
Oleic acid (*n* 11)	68·5	9·4	7·0	12·1	63·9	6·0	–2·9	9·2
α-Linolenic acid (*n* 13)	73·3	7·3	–8·6	11·1	50·2	6·9	7·5	4·2
EPA/DHA (*n* 14)	69·6	5·1	9·2	5·8	65·7	3·0	–0·1	7·4
Young adults (*n* 12)								
α-Linolenic acid (*n* 12)	62·1	7·6	–0·5	11·1	61·5	5·1	4·1	7·3

Va, platelet aggregation velocity; Imax, maximum aggregation.
*For details of diets and procedures, see pp. 183–186.
† The final ADP concentration was 1·5 μmol/l and the final collagen concentration was 1·0 μg/ml.

The *ex vivo* platelet aggregation, as measured with the filtragometer, was differently affected by the experimental diets. The aggregation time (tA) was significantly prolonged in the elderly group that received EPA/DHA compared with the α-linolenic acid ($P = 0·006$) and the oleic acid ($P = 0·004$) diet groups (Fig. 1).

The α-linolenic acid diet group showed a tendency to lower Ts values compared with the oleic acid diet group

Table 4. Effects of diets enriched in oleic acid, α-linolenic acid or eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA) on *in vitro* platelet aggregation in whole blood induced with either ADP or collagen and on thromboxane B_2 production in plasma after ADP- or collagen-induced aggregation in whole blood*†

(Mean values with their standard errors)

	ADP				Collagen			
	Run-in		Change		Run-in		Change	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Va (ohm/min)								
Elderly (*n* 38)								
Oleic acid (*n* 11)	3·70	0·68	0·76	0·58	4·21	0·39	0·19	0·46
α-Linolenic acid (*n* 13)	3·97	0·51	0·19	0·53	3·82	0·41	0·34	0·53
EPA/DHA (*n* 14)	3·92	0·52	–0·74	0·46	4·56	0·38	–0·99	0·43
Young adults (*n* 12)								
α-Linolenic acid (*n* 12)	4·32	0·70	0·13	0·63	5·26	0·47	–0·55	0·69
Imax (ohm)								
Elderly (*n* 38)								
Oleic acid (*n* 11)	10·6	1·9	0·5	2·3	19·3	1·2	–0·9	1·5
α-Linolenic acid (*n* 13)	11·1	1·8	–0·9	1·2	15·4	1·5	2·5	1·8
EPA/DHA (*n* 14)	12·9	1·7	–2·0	2·1	20·0	1·4	0·1	1·4
Young adults (*n* 12)								
α-Linolenic acid (*n* 12)	10·2	2·0	0·9	1·8	21·3	2·3	–0·5	2·6
Thromboxane B_2 (ng/ml)								
Elderly (*n* 38)								
Oleic acid (*n* 11)	4·1	1·5	0·2	1·4	53·9	8·6	–9·5	8·8
α-Linolenic acid (*n* 13)	4·0	1·3	–1·8	1·4	48·5	8·9	–12·7	5·1
EPA/DHA (*n* 14)	4·6	0·9	–1·7	0·5	46·1	4·6	–10·7	3·5
Young adults (*n* 12)								
α-Linolenic acid (*n* 12)	2·8	0·8	0·0	0·7	47·2	5·9	–6·8	4·4

Va, platelet aggregation velocity; Imax, maximum aggregation.
*For details of diets and procedures, see pp. 183–186.
† The final ADP concentration was 1·5 μmol/l and the final collagen concentration was 0·05 μg/ml.

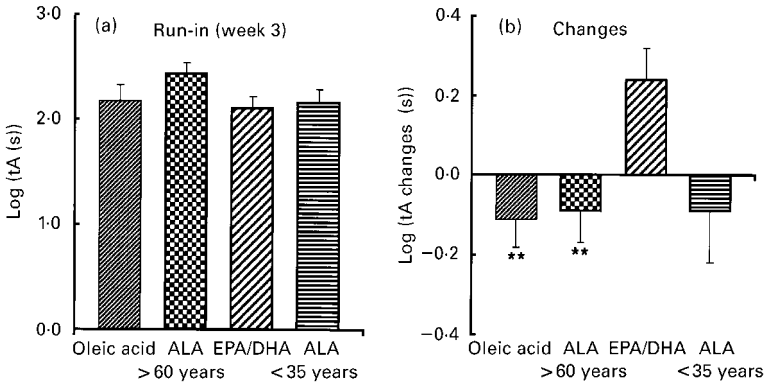

Fig. 1. (a) Aggregation times (tA) of platelets measured *ex vivo* by filtragometry in four groups of subjects during a run-in period before dietary intervention with different fats. (b) Changes in tA as a result of a 6-week dietary intervention with different fats. Dietary groups were: oleic acid (▨), elderly subjects (*n* 11); α-linoleic acid (ALA; ▩), elderly subjects (*n* 13); eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA; ▨), elderly subjects (*n* 14); ALA (▤), younger subjects (*n* 12). Values are means with their standard errors indicated by vertical bars. Mean values were significantly different from that for the EPA/DHA group: ** $P < 0{\cdot}01$.

($P = 0{\cdot}038$). No significant differences in effects were found between the three elderly groups for the changes in maximum aggregation (Amax) or desaggregation induction time (tDI) (results not shown). Also, effects of α-linolenic acid were not age-dependent.

At the end of the run-in period, a longer tA correlated with a lower platelet aggregation *in vitro*, as shown by the negative correlation between *ex vivo* platelet aggregation (tA) and collagen-induced *in vitro* platelet aggregation in PRP (Va) (r −0·50, $P = 0{\cdot}023$) and in whole blood (Va) (r −0·63, $P = 0{\cdot}001$) in the elderly group. For the younger group, these correlations were not significant.

For all subjects, the number of platelets changed during the study by 1·3 (SEM 2·4) %, the number of erythrocytes by −3·4 (SEM 1·0) %, and the number of leucocytes by 3·1 (SEM 3·1) %. Changes were similar for the three groups of elderly, and effects of α-linolenic acid were not age-dependent. The other haematological variables also did not change during the study (results not shown).

Discussion

In the present study we compared in healthy elderly subjects the effects of α-linolenic acid and EPA/DHA with those of oleic acid on *in vitro* and *ex vivo* platelet aggregation. Our results suggest that α-linolenic acid and EPA/DHA do not change *in vitro* platelet aggregation in whole blood or PRP, and that EPA/DHA, but not α-linolenic acid, favourably affects *ex vivo* platelet aggregation compared with oleic acid. We also compared the effects of α-linolenic acid on platelet aggregation in elderly subjects with those in younger volunteers. Our results also suggest that effects of α-linolenic acid are not age-dependent. Differences in the fatty acid composition of the diets were reflected by the fatty acid composition of erythrocyte neutral phospholipids, indicating good dietary compliance.

Only a few studies have compared the effects of α-linolenic acid with those of EPA/DHA on *in vitro* platelet aggregation. Freese & Mutanen (1997) supplemented the diets of healthy middle-aged women and men for 4 weeks either with encapsulated linseed oil (5·9 g α-linolenic acid/d) or encapsulated fish oil (2·9 g EPA plus 2·3 g DHA/d). Compared with baseline values, these treatments did not affect collagen-induced platelet aggregation in PRP, while ADP-induced platelet aggregation was increased after fish-oil supplementation but not after supplementation with linseed oil. Results, however, may have been confounded by aspecific drifts with time, as no control group was used, and no comparison was made between the differences in changes of the two diet groups.

A large number of human studies have examined the effects of dietary fish, fish oil or fish-oil concentrates on *in vitro* platelet aggregation. Only a few of these studies have used a proper control group and the results are equivocal (Hornstra, 1989). It has been postulated that effects, if any, might be mediated by a decreased production of TxA_2, a potent proaggregatory eicosanoid (Kinsella *et al.* 1990*a*). However, we found no effects of the diets on the concentration of TxB_2, a stable metabolite of TxA_2, after collagen- or ADP-induced aggregation of whole blood, although a positive correlation was found between the aggregation velocity (Va) of collagen-induced platelet aggregation *in vitro* in whole blood and TxB_2 production.

The effects of α-linolenic acid on platelet aggregation *in vitro* have been less frequently studied than those of fish oil. In a recent study (Allman *et al.* 1995), a low-fat diet supplemented with 40 g flaxseed oil (23 g α-linolenic acid/d) decreased collagen-induced platelet aggregation in whole blood compared with a diet supplemented with 40 g sunflowerseed oil (25 g linoleic acid/d). No changes were seen in ADP-induced aggregation. Freese *et al.* (1994) found no effects on platelet aggregation in PRP after a rapeseed-oil diet (1·6 g α-linolenic acid/d) compared with the habitual baseline diet. In contrast, Mutanen *et al.* (1992) reported that

platelet aggregation in PRP, induced by ADP and collagen, was significantly increased after rapeseed oil supplementation (5·8 g α-linolenic acid/d) and sunflowerseed oil as compared with a milk-fat diet. Compared with oleic acid and EPA/DHA, we found no effects of α-linolenic acid on platelet aggregation *in vitro* induced by either ADP or collagen. Thus, as for EPA/DHA, results on the effects of α-linolenic acid on platelet aggregation are not consistent.

Studies on the effects of diets on platelet aggregation *in vitro* are difficult to compare, because many different techniques have been used. We, therefore, decided not only to measure platelet aggregation *in vitro*, but also *ex vivo* with the filtragometer, because this may reflect *in vivo* platelet aggregation better than the conventional *in vitro* techniques. Validation studies have shown that filter occlusion is mainly due to platelet aggregates and that acetylsalicylic acid prolongs filtragometry readings (tA), (Hornstra & ten Hoor, 1975), which indicates a lower aggregability *in vivo*. We now report that compared with both oleic acid and α-linolenic acid, a prolonged tA was found after 6 weeks of dietary EPA/DHA enrichment. This may suggest a positive effect of the longer *n*-3 polyunsaturated fatty acids on cardiovascular risk.

Earlier studies (Hornstra *et al*. 1973; Fleischman *et al*. 1975) with the filtragometer reported decreased platelet aggregation *in vivo* after increased linoleic acid intake. Hornstra *et al*. (1973) have shown that increasing the dietary polyunsaturated : saturated fatty acid ratio significantly decreased platelet aggregation *ex vivo*. It was suggested that this altered platelet behaviour may have contributed to the reported effect of this high polyunsaturated : saturated fatty acid diet in the lower incidence of IHD in the Helsinki trial (Miettinen *et al*. 1972). In a study by Fleischman *et al*. (1975), an increased intake of linoleic acid for 2 weeks, at the expense of saturated and monounsaturated fatty acids, was also associated with decreased platelet aggregation. However, O'Brien *et al*. (1976) found no differences in platelet aggregation after 5 weeks of increased linoleic acid intake. The fact that replacement of saturated fatty acids by linoleic acid was not strictly controlled might be a reason for this discrepancy.

During the run-in period, collagen-induced platelet aggregation in whole blood was significantly lower in the older compared with the younger volunteers. ADP-induced aggregation in whole blood and aggregation in PRP were not different. Studies on the effects of age on platelet aggregation have not given consistent results so far. Some investigators found that aggregation increased with ageing (Meade *et al*. 1985; Vericel *et al*. 1988; Vilén *et al*. 1989), while others found no effects of age on platelet aggregation (Abbate *et al*. 1986). These studies were carried out in whole blood activated with collagen or ADP (Abbate *et al*. 1986; Vericel *et al*. 1988) or in PRP activated with collagen (Meade *et al*. 1985; Vericel *et al*. 1988) or ADP (Meade *et al*. 1985; Abbate *et al*. 1986; Vericel *et al*. 1988; Vilén *et al*. 1989).

In conclusion, our findings suggest that effects of *n*-3 polyunsaturated fatty acids from vegetable and marine sources on platelet aggregability are not similar. Compared with oleic acid, a mixture of EPA and DHA significantly reduced platelet aggregation *ex vivo* (as measured by filtragometry) in elderly subjects. No such effect was found for α-linolenic acid. The postulated decreased conversion of α-linolenic acid into longer-chain polyenes in elderly subjects does not explain these differential results, as effects of α-linolenic acid were not age-dependent.

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