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FLOW-MEDIATED VASODILATION AND DIETARY INTAKE OF N-3 POLYUNSATURATED ACIDS IN HEALTHY SUBJECTS

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Abstract – Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two major marine n-3 polyunsaturated fatty acids (PUFA), have been proposed to decrease the risk of atherosclerosis and coronary heart disease. An early event during atherogenesis is endothelial dysfunction. We studied the correlation between fish consumption, serum phospholipid (sPL) levels of DHA and EPA and flow-mediated vasodilation (FMD), a measure of endothelial function. Furthermore, subjects were classified according to whether they did (Fish+, n = 19) or did not (Fish-, n = 21) follow the Danish recommendations, consuming at least 300 g fish/week. Neither the fish intake, sPL EPA nor sPL DHA significantly correlated with FMD, -0.20 (p = 0.23), -0.23 (p = 0.15) and -0.06 (p = 0.72), respectively. Also, when comparing the Fish+ and the Fish- group we did not find any significant differences in FMD (p = 0.33). In conclusion, our results did not show any correlation between intake and sPL levels of marine n-3 PUFA and FMD in healthy subjects.

Key words: Docosahexaenoic acid, eicosapentaenoic acid, endothelial function, fish consumption, flow-mediated vasodilation, marine n-3 polyunsaturated fatty acids.

INTRODUCTION

Consumption of marine n-3 polyunsaturated fatty acids (PUFA) has been associated with a reduction in cardiovascular risk factors, cardiovascular events, and mortality (1,6,13,27,28). The exact mechanisms of action are unknown, but it is likely that it is due to a combination of beneficial effects including effects on lipids, platelet aggregability, arrhythmias, blood pressure, and anti-thrombotic and anti-inflammatory effects (17,28,29). One of the earliest steps in atherogenesis is believed to be endothelial dysfunction, characterized by, among others, a reduced bioavailability of vasodilators, such as nitric oxide, and an increased bioavailability of endothelium-derived contracting factors (2,5). In recent years, ultrasound measurement of flow-mediated vasodilation (FMD) has gained popularity as a measure of endothelial function since it is a non-invasive procedure. One drawback of FMD has been its lack of standardization together with observer variability issues.

There is some evidence that marine n-3 PUFA may increase FMD (12,16,26,30), although other studies have failed to reproduce these findings (11,18). The present study investigates whether dietary intake of marine n-3 PUFA and serum phospholipid (sPL) levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was related to FMD in healthy subjects. Furthermore, we studied whether participants adhering to the recommendations for fish intake had a higher FMD than those who ate less fish than recommended.

Abbreviations: **DHA,** docosahexaenoic acid; **EDV,** Endothelial dependent vasodilation; **EIDV,** Endothelial independent vasodilation; **eNOS,** endothelial nitric oxide synthase; **EPA,** eicosapentaenoic acid; **FMD,** flow-mediated dilation; **NO,** nitric oxide; **PUFA,** polyunsaturated fatty acids; **SLE,** systemic lupus erythematosus; **sPL,** serum phospholipid(s); **TFA,** trans fatty acids

MATERIAL AND METHODS

Subjects

A total of 40 healthy volunteers (20 men and 20 women) were recruited (Table 1). Except for birth control pills none were taking any medication, and pre- and

postmenopausal women were equally represented. All subjects gave their written consent and the protocol was approved by the local Ethics Committee.

Methods

Examinations were conducted on two separate occasions, four weeks apart. Premenopausal women were examined in the same period of their menstrual cycle in order to reduce hormonal influence on FMD (34). Results are given as the mean of the two examinations.

Serum phospholipids

Blood samples were collected in the morning after at least ten hours of fasting (including caffeine and tobacco) and 10 minutes' rest in the supine position. Samples were left to coagulate for at least one hour before centrifugation at 3000 x g for 20 minutes, and vials containing serum aliquots were filled with nitrogen to avoid oxidation and frozen at –80°C until analysis. Samples from each individual were analyzed in the same analytical run to avoid inter-assay variability.

sPL were analyzed as described earlier (31). In brief, total lipids were extracted from serum using chloroform ($CHCl_3$) and methanol containing butylated hydroxytoluene as antioxidant. Organic $CHCl_3$ phase was transferred to preconditioned Sep Pak NH_2 columns (Waters Corporation, Massachusetts, USA) and phospholipids were separated from other lipid classes. The extracted phospholipids were dried under nitrogen, redissolved in heptane, and transesterified using 0.5 M sodium methoxide and acetic acid. The fatty acid composition was analyzed by gas chromatography using a Chrompack CP-9002 gas chromatograph (Varian, Middleburg, Netherlands), and a CP-sil 88 capillary column, fatty acid methyl esters with 14 to 24 carbon atoms and separation of several trans fatty acids were quantified. Inter-assay coefficients of variation were 3.5% and 2.8% for EPA and DHA, respectively.

Blood pressure

Blood pressure was assessed once before each examination using an automatic blood pressure apparatus, OMRON M6 (OMRON HEALTHCARE Co., Ltd., Kyoto, Japan). The measurement was conducted on the left arm after subjects had rested for at least 15 minutes in the supine position (Table 1).

Table 1. Baseline characteristics of the subjects.

	Minimum	1st Quartile	Median	3rd Quartile	Maximum
Age (years)	24.0	38.0	51.0	58.3	66.0
Systolic blood pressure (mmHg)	91.0	107.0	112.0	119.3	159.0
Diastolic blood pressure (mmHg)	53.0	68.0	72.5	76,3	91.0
BMI (kg/m^2)	19.0	22.0	24.0	26.0	32.0
Current smoker (n)	4 (10 %)				
Former smoker (n)	9 (22.5 %)				

Table 2. Structure of the Food Frequency Questionnaire.

			Points								Score
			1	2	3	4	5	6	7	8	
Type of fish	Fat	Cold	<½ slice /month†	½ slice /month	2-3 slices /month	½ slice /week	2-3 slices /week	4-6 slices /week	½ slice /day	2-3 slices /day	A (1 – 8)
		Warm	< 1 /month	1 /month	2-3 /month	1/week	2-3 /week	4-6 / week	daily	2 /day	B (1 – 8)
	Lean	Cold	<½ slice /month	½ slice /month	2-3 slices /month	½ slice /week	2-3 slices /week	4-6 slices /week	½ slice /day	2-3 slices /day	C (1 – 8)
		Warm	< 1 /month	1 /month	2-3 /month	1/week	2-3 /week	4-6 / week	daily	2 /day	D (1 – 8)
										Fish score	A + B + C + D

† Intake of cold fish was assessed by asking how many slices of bread with fish subjects consumed. It was calculated that one warm portion equalled 125-150 g and that one slice of bread reflected a serving of 30-40 g of fish.

Food frequency questionnaire

The intake of fish, both as warm and cold servings and regarding type of fish (fat vs. lean), was assessed by a food frequency questionnaire (Table 2). The food frequency questionnaires were filled out on the first examination and subjects were classified according to whether they followed the Danish recommendations of a fish intake of at least 300 g/week (Fish+, n = 19) or not (Fish-, n = 21). Furthermore, a fish score was conducted ranging between 4 and 32, with score 32 reflecting the highest possible fish intake (Table 2).

Endothelial function

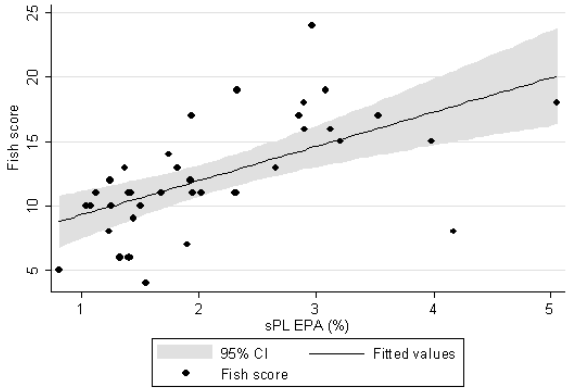
FMD was measured using a Siemens Sonoline G50 ultrasonography apparatus (Siemens Medical Solutions USA, Inc., Japan). This was fitted with a L10-5 6.5-10 MHz transducer, with measurements made with a 10 MHz probe. Before the examination, subjects had at least 15 minutes of supine rest in a quiet and tempered room. Measurements were made on the right brachial artery 3-5 cm distal to the ante-cubital fossa, and when the clearest B-mode image was obtained, the ultrasound probe was fixed with a stereotactic clamp. Baseline images were recorded as three clips, each of 4 sec. duration. Subsequently, a blood pressure cuff was placed on the upper arm and inflated to 300 mmHg for 5 minutes. The cuff was then rapidly deflated, a new series of clips was made, and FMD was determined 60 sec. after cuff deflation. Measurements were made from media to media on a longitudinal section of the artery in end-diastole at the beginning of the R-wave of the ECG and conducted in 4 consecutive heart cycles. The mean diameter was determined. The between-operator and within-subject standard deviations were 4.0 % and 2.9 %, respectively (24). Finally, FMD was calculated as a percentage increase in diameter from baseline to one minute after cuff deflation. Afterwards, to ensure that the capacity for vasodilation was present, endothelial independent dilation was measured four minutes after sublingual admission of 0.4 mg glyceryl trinitrate.

Statistics

All statistical analyses were performed using R (Version 2.0.1). Data from the two examinations were used to calculate mean FMD, sPL DHA and sPL EPA. These mean values were then used in the later analysis. A Q-Q plot was used to test if FMD was normally distributed.
Pearson's coefficient of correlation was used to describe the associations. A p-value < 0.05 (two-tailed) was considered significant.

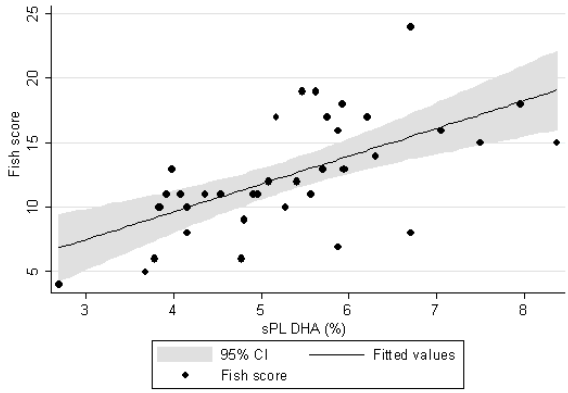
RESULTS

Our analysis revealed a mean sPL content of 2.2% (95% CI: [1.9; 2.5]) and 5.32% (95% CI: [4.9; 5.7]) for EPA and DHA, respectively. The median of the fish score and FMD was 11.0 (1st quartile = 10.0; 3rd quartile = 15.8) and 9.9% (1st quartile = 7%; 3rd quartile = 11.6%), respectively. There were significant and positive correlations between the amount of EPA and DHA in phospholipids and the fish score with correlations of r = 0.60 (p < 0.001) and r = 0.62 (p < 0.001), respectively (Fig. 1-2).



Correlation: r = 0.60 (p < 0.001)

Figure 1. Fish score vs. the content of EPA in serum phospholipids

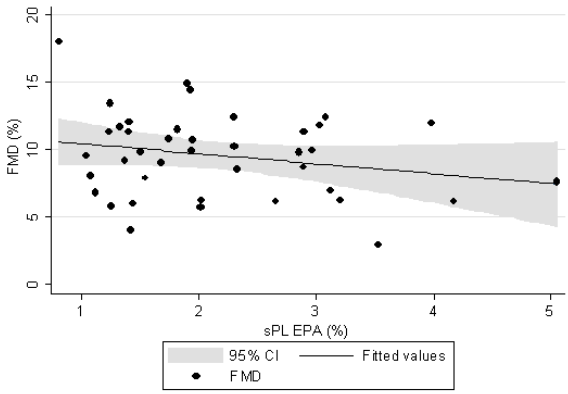


Correlation: r = 0.62 (p < 0.001)

Figure 2. Fish score vs. the content of DHA in serum phospholipids

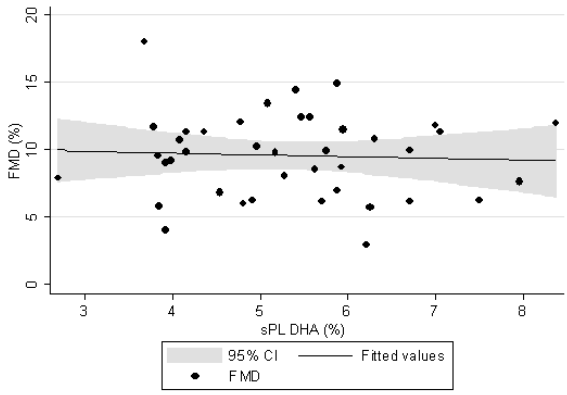
Serum phospholipids and FMD

Neither the content of sPL EPA nor of sPL DHA was significantly correlated to FMD (Fig. 3-4). FMD vs. EPA: r = -0.23 (p = 0.15) and FMD vs. DHA: r = -0.06 (p = 0.72).



Correlation: r = -0.23 (p = 0.15)

Figure 3. FMD vs. the content of EPA in serum phospholipids

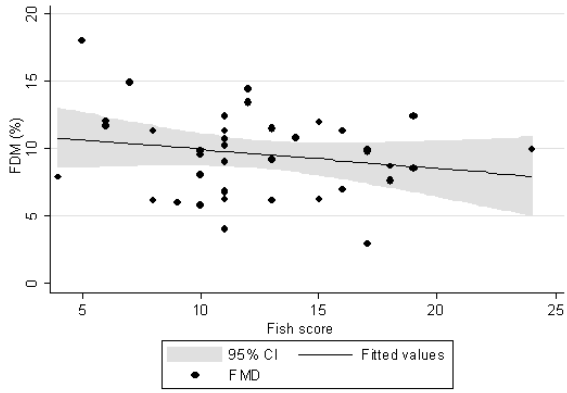


Correlation: r = -0.06 (p = 0.72)

Figure 4. FMD vs. the content of DHA in plasma phospholipids

Fish score and FMD

The fish score was not significantly correlated with FMD, r = -0.20 (p =0.23) (Fig. 5). In the Fish+ (>300 g/week) and Fish– groups (<300 g/week), sample mean FMD was 9.1% (95% CI: [7.7; 10.5]) and 10.1% (95% CI: [8.6; 11.6]), respectively (p = 0.33).



Correlation: r = -0.20 (p = 0.23)

Figure 5. FMD vs. Fish score.

DISCUSSION

The results of this study demonstrate that sPL concentrations of DHA and EPA were not significantly correlated to FMD in healthy subjects. Furthermore, the fish score was not significantly correlated with FMD (p = 0.23). We found that the fish score was positively and significantly correlated with the sPL concentration of both DHA and EPA (p < 0.001). However, based on the official Danish recommendations for fish intake (>300 g/week) the Fish- and the Fish+ groups were compared, but this did not show any significant difference regarding FMD (p = 0.33).

Takase et al. (32) have reported a positive and significant correlation between adenosine triphosphate disodium-induced vasodilation in coronary arteries and FMD in the brachial artery in patients with suspected coronary artery disease. Other studies have also reported that assessment of peripheral endothelial function, to some extent, can be used as a prognostic predictor of future cardiovascular events (14,23). Criticism has, however, been raised towards the use of FMD since many factors can affect the accuracy of this parameter and because of the lack of standardization between research laboratories and observer and patient variability (3,8,10). In the present study only experienced operators undertook the examination and by selecting the mean of two determinations of FMD, variability was further reduced. Also, our results regarding variability have previously been reported as acceptable (24).

In accordance with guidelines by the International Brachial Artery Reactivity Task Force (8), measurements of FMD were conducted 60 sec. after cuff deflation. Others have, however, reported that when using an upper arm cuff placement, as done in the present study, the vasodilator response may be somewhat delayed (3,4,22). Additionally, the cuff placement may induce brachial artery hypoxia which perhaps can compromise vasodilation as hypothesized by Berry et al. (3).

Furthermore, inter-volunteer variability may have affected our results. The volunteer median age was 51 years; it is therefore plausible that some volunteers had endothelial dysfunction during the test.

Others have also studied the effect of marine n-3 PUFA in healthy volunteers (Table 3). In a randomized, double blinded, 8-week dietary intervention study, 87 healthy males were randomized to one of three dietary invention groups to test whether intake of trans fatty acids (TFA), n-3 PUFA or control fat affected FMD (11). The groups were comparable and subjects were given a daily dose of 33 g fat, of which the TFA group was given 20 g industrially produced TFA, the n-3 PUFA group was given 4 g marine n-3 PUFA and the control group was only given control fat. The fat was isocalorically supplemented via baked products and cakes. Results did not reveal any alterations in FMD and differences between groups (11). In a cross section study, Leeson et al. (18) assessed FMD

and plasma n-3 PUFA in 326 healthy subjects. Looking at all subjects, no significant association between FMD and plasma EPA and DHA could be demonstrated. In contrast, when stratifying according to smoking status a positive and significant correlation between FMD and plasma DHA was seen in smokers (18). The present study only included 4 current smokers, and we are therefore unable to comment on this issue. Finally, Shah et al. (30), from a randomized, single-blinded, placebo-controlled trial including 26 non-smoking asymptomatic subjects, reported that a daily supplement of 1 g of n-3 PUFA (EPA: 0.4 g/day, DHA: 0.6 g/day) for 14 days significantly increased FMD within the fish oil group. The control group supplemented with 1 g of corn oil did not show any alterations.

FMD and its possible association with n-3 PUFA have also been investigated in subjects suffering from disease states linked with atherosclerosis. Most of these studies (12,16,26,35) have shown that marine n-3 PUFA have a beneficial effect on endothelial function (Table 3). Thus, in a placebo-controlled, double-blind trial, Goodfellow et al. (16) found that supplementing hypercholesterolemic subjects with fish oil for 120 days significantly increased FMD. There were 13 subjects in the n-3 PUFA group receiving a daily dose of 4 g n-3 PUFA and 15 subjects in the placebo group given 4 g corn oil/day. Criticism can be raised against the fact that information regarding FMD was reported as absolute values and not as percentage change.

Patients with systemic lupus erythematosus have a high prevalence of atherosclerosis (25). In a recent study of such patients, FMD was significantly improved from 3% to 8.9% when the patients were given a daily dose of 1.2 g DHA and 1.8 g EPA for 24 weeks (35). Furthermore, in 20 children with familial hypercholesterolemia or familial combined hyperlipidemia Engler et al. (12) found that 1.2 g DHA/day significantly improved their FMD. Finally, in a study of patients (n = 32) with intermittent claudication Schiano et al. (26) reported that supplementing the usual diet with n-3 PUFA (2 g/day) for 3 months increased FMD significantly by 3.3% in contrast to the control group continuing with their traditional diet.

Regarding mechanisms, an in vitro study by Goode et al. (15) reported that arteries from hypercholesterolemic patients given marine n-3 PUFA had a better endothelial function compared to arteries from matched controls. The content in membrane of DHA and EPA was correlated with this finding, and Goode et al. (15) hypothesized that marine n-3 PUFA affected the membrane fluidity, thereby promoting either increased synthesis and/or release of endothelium-derived nitric oxide (NO). This hypothesis is supported by another in vitro study, showing that EPA increased endothelial nitric oxide production (20). Furthermore, Lopez et al. (19) have reported that rats supplemented with fish oil had an increased content of aortic endothelial nitric oxide synthase (eNOS). Others have found that membrane EPA can induce dissociation of eNOS from caveolin-1, thereby increasing NO production (21). Also, NO production following increased shear stress correlated closely with phosphorylation of constitutive eNOS (9). Consequently, it seems plausible that EPA exerts a synergistic effect on the normal NO production in endothelial cells. Another mean by which EPA and DHA can interfere with vasomotion is that EPA and DHA can act as alternative substrates for both cyclooxygenase and lipoxygenase enzymes and, with the 3-series prostaglandins and thromboxanes and 5-series leukotrienes having a more favourable biological activity, result in cardiovascular benefits favouring vasodilation rather than vasoconstriction (2).

Incorporation of n-3 PUFA into cell membranes plays a role in the mechanism of action of n-3 PUFA (9,15,19,20,21). In the present study, sPL EPA and sPL DHA were used in our analysis. Studies have shown that dietary intake of EPA and DHA affects both plasma phospholipid (pPL) EPA and pPL DHA in parallel with changes in erythrocyte and platelets membrane EPA and DHA content (33,7). In a study with 20 healthy subjects Cao et al. (7) found that these changes correlated significantly with each other and that erythrocyte membrane EPA and DHA over time had a more stable concentration than pPL EPA and pPL DHA. Consequently, membrane n-3 PUFA might be a better index for monitoring long-term intake of n-3 PUFA.

In conclusion, the present study has shown that endothelial function neither correlates with dietary intake of fish nor sPL DHA and sPL EPA levels in healthy subjects.

Other articles in this theme issue include references (36-47).

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