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FATTY ACID COMPOSITION OF THE INTERNAL MAMMARY ARTERY IN RELATION TO DIETARY INTAKE OF MARINE N-3 POLYUNSATURATED FATTY ACIDS AND ASSOCIATION WITH FLOW-MEDIATED VASODILATION

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Abstract – Some evidence suggests that long-chain marine n-3 polyunsaturated fatty acids (n-3 PUFA) may increase production of vasodilatory nitric oxide from vascular endothelium. Fatty acids may therefore play a role for the left internal mammary artery (LIMA) graft function in coronary artery bypass grafting (CABG). However, little is known about the composition of fatty acids in the vessel wall of the LIMA. Using gas chromatography we investigated fatty acid composition in segments of the LIMA, in plasma nonesterified fatty acids (NEFA), in plasma phospholipid (PL) and in the pericardial adipose tissue (PAT) from 22 patients undergoing CABG. Furthermore, we investigated whether there was an association between the n-3 PUFA composition in LIMA and flow-mediated vasodilation (FMD). Self-reported fish consumption and supplementation of eicosapentaenoic acid and docosahexaenoic acids were reflected by the fatty acid composition in NEFA, PL and in PAT, but less so in the LIMA. There was no association between FMD and fatty acid composition of the LIMA.

Key words: Fatty acids, dietary fish intake, internal mammary artery, coronary artery bypass surgery, flow-mediated vasodilation.

INTRODUCTION

Dietary intake of n-3 polyunsaturated fatty acids (n-3 PUFA), especially eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), may play an important role in cardiovascular health and disease (3,10,21,22). Laboratory studies suggest that long-chain marine n-3 PUFA may increase production of vasodilatory nitric oxide from vascular endothelium (19) and may augment endothelium-dependent vasorelaxation by enhanced release of endothelium-derived relaxing factor and vasodilator prostaglandins (14). N-3 PUFA may therefore play a role for the excellent graft function of the left internal mammary artery (LIMA) in coronary artery bypass grafting (CABG) (24). However, virtually nothing is known about the composition of fatty acids in the vessel wall of the LIMA. The purpose of the present study was to test the hypothesis that the content of n-3 PUFA in the vessel wall of LIMA reflects dietary intake of fish and is associated to flow-mediated vasodilation (FMD). Furthermore, we compared the fatty acid composition in the vessel wall of LIMA in order to evaluate whether the content of n-3 PUFA correlates with the n-3 PUFA content in nonesterified fatty acid (NEFA) and phospholipid fatty acids (PL) in plasma, and with the n-3 PUFA content in pericardial adipose tissue (PAT).

Abbreviations: ACE, Angiotensin-converting enzyme; **BMI,** body mass index; **CABG,** coronary artery bypass grafting; **CI,** confidence interval; **DHA,** docosahexaenoic acid; **EPA,** eicosapentaenoic acid; **FMD,** flow-mediated vasodilation; **HDL,** high-density lipoprotein; **LDL,** low density lipoprotein; **LIMA,** left internal mammary artery; **n-3 PUFA,** n-3 polyunsaturated fatty acids; **NEFA,** nonesterified fatty acid; **PAT,** Pericardial adipose tissue; **PL,** Plasma phospholipids.

MATERIAL AND METHODS

The present study was approved by the Regional Committee on Biomedical Research Ethics and written informed consent was obtained from all patients.

Subjects

Twenty-five patients scheduled for primary, elective CABG in the Department of Cardiothoracic Surgery, Aalborg Hospital, Aarhus University Hospital, were included in the study, if the LIMA was planned to be used as graft material. Patients below 30 years of age and pregnant patients were not included.

All patients were seen after an overnight fast in the hospital on the last working day prior to scheduled surgery. At this time fasting blood samples were obtained and assessment of dietary intake of fish as well as measurements of FMD were carried out as described below. Surgery was cancelled in one patient due to several concomitant diseases and in two patients adequate tissue samples from the LIMA could not be obtained.

Blood samples and analyses

Blood samples were collected from a cubital vein after 10 minutes of supine rest. K-EDTA 1.6 mg/ml was used as anticoagulant. Serum and plasma were collected after centrifugation at 3000 x g for 20 min and stored at -80°C. Tubes for fatty acid analysis were filled with nitrogen to avoid oxidation until analysis.

Levels of plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triacylglycerols were measured in each patient according to standard methods in the hospital laboratory on a Modular, Roche Diagnostics, Basel, Switzerland. Total lipid was extracted from plasma according to Folch et al. (11), NEFA and PL were separated from other lipid classes according to Burdge et al. (4). Fatty acid composition was measured by gas chromatography in our lipid research laboratory and expressed as percentage of the total fatty acid content. Plasma (500µl) was mixed with 5 ml chloroform-methanol 2:1 ($CHCl_3$-MeOH) containing butylated hydroxytoluene (50 µg/ml) as antioxidant. After brief mixing, the tubes were shaken for 15 minutes, 750 µl sodium chloride 0.9% was added, briefly mixed and the tubes were centrifuged at 1600 x g for 10 minutes at 4°C for phase separation. The upper aqueous phase was discharged and the lower organic phase was collected. The protein disk was mixed with 5 ml $CHCl_3$-MeOH 2:1 and 1 ml sodium chloride 0.9%, centrifuged, and the organic phase combined with the initial $CHCl_3$ phase, the lipid phase was dried under nitrogen and the extract was dissolved in 1 ml $CHCl_3$, transferred to a Varian, Bond Elut, 200 mg, NH_2 column (Varian Middleburg, Nederland) which had been preconditioned with hexane. Fatty acid fractions separated according to Burdge et al. (4). The column was washed with 4 ml $CHCl_3$, PL were eluted with 2 ml $CHCl_3$-MeOH 3:2 v/v followed by 2 ml MeOH. NEFA were eluted with 4ml $CHCl_3$-MeOH-acetic acid 100:2:2. PL and NEFA fractions were dried under nitrogen. NEFA's were dissolved in toluene, methylated using 2% sulphuric acid in methanol, incubated at 50°C for 2 hours, dried with 1 ml $KHCO/K_2CO_3$ and extracted into hexane. This phase was collected, dried under nitrogen and dissolved in heptane. PL were dissolved in heptane and methylated at 50°C using 2M potassium hydroxide in methanol. After centrifugation the heptane phase was collected. The fatty acid compositions was analyzed by gas chromatography using a Varian 3900 GC with a CP-8400 auto sampler (Varian, Middleburg, Nederland) equipped with a flame ionization detector. Split injection mode, a CP-sil 88, 60 m x 0.25 mm ID capillary column (Varian, Middleburg, Nederland), temperature programming from 90°C to 210°C and constant flow was used. Helium was used as carrier gas. Commercially available standards (Nu-chek-Prep., Inc. Minnesota) were used to recognize the individual fatty acids. This approach permits quantification of fatty acids methyl esters with 12 to 24 carbon atoms. Interassay coefficients of variation for PL: EPA 0.5%, DHA 1.3% and for NEFA: EPA 3.5%, DHA 2.6%.

Dietary evaluation of fish intake

A dietary questionnaire was used to estimate fish intake and intake of fish oil among the patients. A score from 1-8 was given according to the reported intake of fatty fish at lunch and dinner, respectively, thus giving a minimum of 2 and a maximum of 16 points. As to lunch, the score corresponded to the number of slices of fatty fish, being, respectively, less than ½ a month, ½ a month, 1-1½ a month, ½ a week, 1-1½ a week, 2-3 a week, ½ a day, 1-1½ a day. Regarding dinner, the score indicated the frequency of warm meals with fatty fish, being, respectively, less than once a month, once a month, 2-3 times a month, once a week, 2-3 times a week, 4-6 times a week, once a day, twice a day. For further analysis, this estimate of the dietary fish consumption was combined with the potential supplementation of fish oils to give a crude estimate of daily intake of EPA and DHA, assuming average portion sizes. The intake is reported in mg.

Assessment of flow-mediated vasodilation

Ultrasonographic imaging of the right brachial artery was used to assess FMD and endothelium-independent vasodilation (9). The brachial ultrasound was performed by an experienced investigator using a Siemens Sonoline G50 vascular ultrasound apparatus (Siemens medical Solutions, Erlangen, Germany) with a 6.5-10 MHz multifrequency linear probe during simultaneous ECG-monitoring. After 15 minutes of rest in the supine position a baseline rest image was acquired. Endothelium-dependent FMD was induced by inflation of a blood pressure cuff on the forearm for exactly 5 minutes to 300 mmHg followed by a rapid release of the occlusive cuff. A subsequent scan was performed 45-90 seconds after deflation. In order to assess endothelium-independent vasodilation another scanning sequence was performed following 15 minutes of rest before and 3.5- 4.5 minutes after sublingual administration of a single dose of nitroglycerin spray (Nitrolingual® 0.4 mg/dose, G. Pohl Boskamp GmbH & Co, Germany). All ultrasonographic images were recorded digitally and transferred to a computer for off-line analysis of the brachial artery diameters using commercially available software (Brachial Analyzer, Medical Imaging Applications, Iowa, USA). The arterial diameter was measured at the onset of the R-wave from longitudinal images and measured as the distance between the two media layers. FMD was expressed as the actual change in post-stimulus diameter as a percentage of the baseline diameter.

Tissue sampling and analyses

Approximately 1 cm^3 of pericardial fat was obtained during surgery. The tissue sample was immediately transported to the laboratory in a dry tissue container. From the biopsies 5 mg were transferred into a 2 ml glass vial, over layered with Nitrogen and stored at -80ºC until analysis. The composition of fatty acids in the pericardial

adipose tissue was determined with gas chromatography using the same technique as described above and expressed as percentage of total fatty acids. Prior to gas chromatography the biopsies were thawed. Approximately 2 - 4 mg was removed to a glass-glass and prewarmed at 50°C for 10 minutes. Subsequently the fat was dissolved in heptane and methylated at 50°C using 2M potassium hydroxide in methanol. After centrifugation the heptane phase was collected and analysed. Interassey coefficients of variation for PAT: EPA 6.3% and DHA 5.2%.

Tissue rings from the distal part of the LIMA close to the bifurcation were obtained during surgery. If the surgeon decided to use the whole length of the harvested LIMA pedicle no tissue sample from the vessel was obtained as excess LIMA tissue was never harvested just for the purpose of this study. The vessel ring was cut open and the intima plus inner media part of the LIMA were peeled off with a pair of tweezers. The tissue sample was placed in saline at room temperature and immediately transported to the laboratory. The tissue, 2 - 10 mg, was mixed with 1.0 ml Methanol (MeOH) containing butylated hydroxytoluene (100 µg/ml) as antioxidant and stored at -80°C until analysis. At the time of analysis the samples were thawed, 0.5 ml $CHCl_3$ added and each sample was homogenised for 1 min by a Sonopuls Ultrasonic Homogenizer HD 2070 (Brandelin, Berlin, Germany) equipped with a MS 72 Titanium microtip. After addition of 0.4 ml H_2O the sample was mixed, another 0.5 ml $CHCl_3$ and 0.5 ml H_2O were added, mixed, centrifuged and the organic phase collected. The extraction was repeated by 0.5 ml $CHCl_3$, mix, centrifugation and collection of the organic phase, which was combined with the former organic phase and dried under Nitrogen. The lipid was dissolved in heptane and methylated at 50ºC using 2M potassium hydroxide in methanol. After centrifugation the heptane phase was collected and fatty acids analysed as mentioned above. Results are given as weight percentage of total fatty acids. Tissue samples were analysed in duplicate and intra-assay coefficients of variation was for EPA 13.0% and for DHA 20.4%.

Statistical analysis

Associations between estimated daily intake of marine n-3 PUFA and fatty acid proportions in LIMA, NEFA, PL and PAT were analyzed by univariate linear regression. Furthermore, we also analyzed the association between FMD and the n-3 PUFA content in the LIMA by univariate linear regression. Analyses were adjusted for mean arterial pressure (calculated as (systolic + 2 diastolic)/3), age, gender, LDL cholesterol and Body Mass Index (BMI). The associations between estimated daily intake of marine n-3 PUFA and fatty acid proportions in LIMA, PAT, NEFA and PL are reported as expected change of fatty acid proportion per increase of 100 mg n-3, with 95% confidence interval and P-value. Assumptions of normality and variance homogeneity over the range of covariates were checked by visual inspection of residuals. Correlations between proportions of EPA in LIMA, NEFA, PL and PAT were given by Pearson's coefficient of correlation, and likewise for DHA, and adjusted correlations by partial coefficients of correlation. Comparisons between Pearson and non-parametric Spearman correlations showed very similar results. As we also measure the association between variables by regression models assuming normality, we report the Pearson correlations. All the analyses were conducted using STATA statistical software, version 10.1.

RESULTS

Preoperative baseline patient characteristics are shown in Table 1. No fatty streaks were visible to the naked eye in any of the LIMAs. The compositions of the major fatty acids in LIMA, PAT, NEFA and PL are shown in Table 2. There was a highly statistically significant (P = 0.0001) correlation between the content of EPA in all the four compartments evaluated, while the content of DHA in LIMA did not correlate to the content of DHA in the other 3 compartments which correlated. Same conclusion is found after adjustment for mean arterial pressure, age, gender, LDL cholesterol and BMI. DHA was the most prominent n-3 PUFA in the LIMA. The association between dietary intake of EPA and DHA and the proportions of these n-3 PUFAs in the LIMA are shown in Table 3. Self-reported fish consumption and supplementation of EPA and DHA was reflected by the fatty acid composition in NEFA, PL and in PAT but less so in the LIMA. Figures 1-2 show scatterplots of n-3 PUFA in P-NEFA, P-Phospholipids, PAT and LIMA versus the estimated daily n-3 PUFA intake.

Table 1. Patient characteristics *(n = 22)*.

Age (years), mean ± SD	65 ± 9
Male gender (n, %)	20 (91%)
BMI (kg/m²), mean ± SD	28 ± 5
Blood pressure	
Systolic (mmHg), mean ± SD	151 ± 20
Diastolic (mmHg), mean ± SD	86 ± 13
Current smokers (n, %)	1 (5 %)
Previous smokers (n,%)	13 (59 %)
Diabetes mellitus (n, %)	2 (9 %)
Medications (n, %)	
Beta-blockers	14 (54 %)
Calcium antagonists	4 (18 %)
Diuretics	4 (18 %)
ACE inhibitors	11 (50%)
Angiotensin II receptor antagonists	3 (14%)
Statins	22 (100 %)
Long term nitrate therapy	10 (46%)
Laboratory parameters, mean ± SD	
Total-cholesterol (mmol/l)	4.3 ± 0.8
HDL-cholesterol (mmol/l)	1.4 ± 0.5
LDL-cholesterol (mmol/l)	2.2 ± 0.7
Triacylglycerols (mmol/l)	1.6 ± 1.2

BMI; Body mass index, ACE; Angiotensin-converting enzyme, HDL; High density lipoprotein, LDL; Low density lipoprotein

The mean FMD in the population was 2.5% (SD=2.5). Pearson's coefficient of correlation between FMD and the content of EPA+DHA in LIMA was 0.145 (95% CI: -0.294- 0.534), p=0.52. There was no association between FMD

and the composition of n-3 PUFA in the LIMA, whereas FMD was positively associated with DHA in NEFA (adjusted P = 0.019), in PL (adjusted P = 0.055) and in the PAT (adjusted P = 0.065). Pearson's coefficient of correlation between FMD and dietary intake of EPA+DHA was 0.049 (95% CI: -0.381 – 0.461), p= 0.83.

Table 2. Major fatty acid proportions of plasma non-esterified fatty acids, plasma phospholipids, pericardial adipose tissue and in the left internal mammary artery *(n = 22)*.

	p-NEFA	p-Phospholipids	PAT	LIMA
Palmitic acid, 16:0	24.51±1.40	30.14±1.49	24.54±1.54	20.70 ± 2.61
Stearic acid, 18:0	12.66±1.89	12.36±1.21	5.77±0.86	12.97 ± 4.10
Oleic acid, 18:1n-9	36.89±2.96	11.16±2.69	43.70±2.09	28.22 ± 9.12
Linoleic, 18:2n-6	9.04±1.42	19.00±3.11	8.93±1.50	6.91 ± 1.83
Arachidonic acid, 20:4n- 6	0.79±0.26	10.02±2.62	0.29±0.06	11.31 ± 6.94
α-linolenic, 18:3n-3	1.03±0.19	0.23±0.08	0.81±0.17	0.38 ± 0.24
Eicosapentaenoic acid, 20:5n-3	0.21±0.18	2.28±1.72	0.07±0.05	0.20 ± 0.14
Docosahexanenoic acid, 22:6n-3	1.06±0.60	4.56±1.55	0.24±0.14	2.42 ± 1.36

Values are presented in % (mean ± SD) of total. NEFA; nonesterified free fatty acid, PAT; Pericardial adipose tissue, LIMA; Left internal mammary artery.

Table 3. The association between n-3 fatty acids in the compartments P-NEFA, P-Phospholipids, PAT and LIMA and the estimated daily n-3 intake *(n = 22)*. The association is measured in expected difference in n-3 concentration, when comparing two individuals with a 100 mg difference in estimated daily n-3 intake.

	Crude Association (95% CI)	(P-value)	Adjusted* Association (95% CI)	(P-value)
p-NEFA				
EPA	0.0143 (0.0094-0.192)	<0.0005	0.0152 (0.0075-0.0228)	0.001
DHA	0.0444 (0.0263-0.0645)	<0.0005	0.0426 (0.0160-0.0692)	0.004
p-phospholipids				
EPA	0.1334 (0.0852-0.1816)	<0.0005	0.1360 (0.0602-0.2119)	0.002
DHA	0.1182 (0.0734-0.1630)	<0.0005	0.0788 (0.0270-0.1306)	0.006
PAT				
EPA	0.0033 (0.0018-0.0049)	0.001	0.0033 (0.0011-0.0054)	0.007
DHA	0.0092 (0.0044-0.0140)	0.001	0.0088 (0.0018-0.159)	0.018
LIMA				
EPA	0.0110 (0.0067-0.0151)	<0.0005	0.0086 (0.0029-0.0143)	0.006
DHA	0.0476 (-0.0108-0.1060)	0.105	0.0242 (-0.0627-0.1110)	0.560

*Adjustment for mean arterial blood pressure, age, gender, LDL cholesterol and body mass index.
CI; confidence interval, NEFA; nonesterified free fatty acid, PAT; Pericardial adipose tissue, LIMA; Left internal mammary artery, EPA; Eicosapentaenoic acid, DHA; Docosahexanenoic acid.

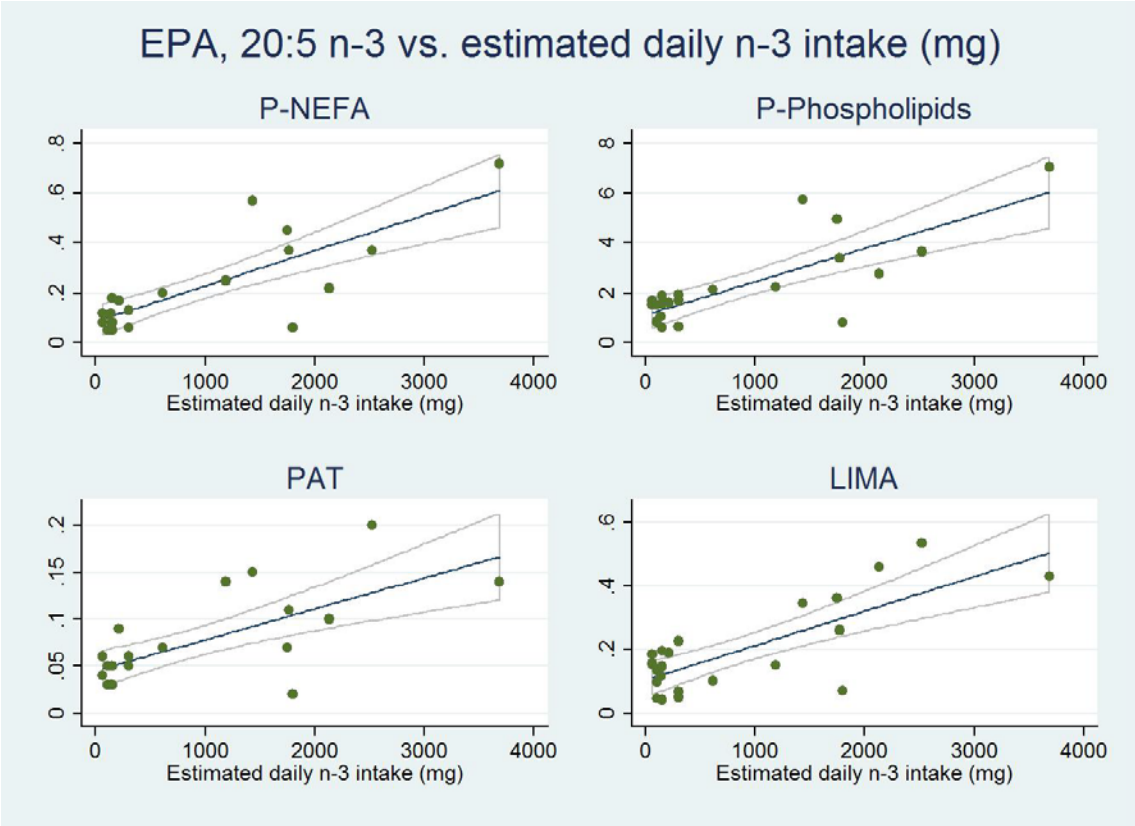

Figure 1. Scatterplot of EPA in P-NEFA, P-Phospholipids, PAT and LIMA versus the estimated daily n-3 PUFA intake.

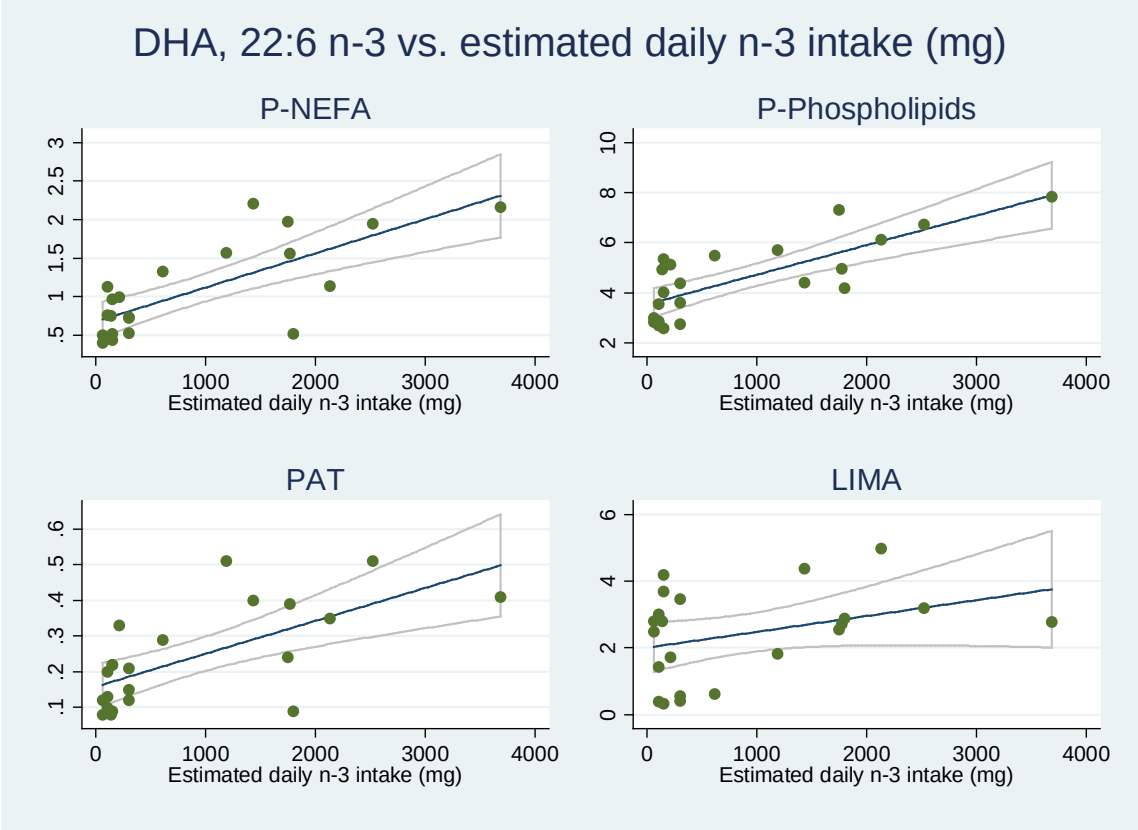

Figure 2. Scatterplot of DHA in P-NEFA, P-Phospholipids, PAT and LIMA versus the estimated daily n-3 PUFA intake.

DISCUSSION

The LIMA is frequently used during CABG due to the superior long-term patency rates compared with saphenous vein grafts and due to survival benefits (24). Differences in endothelium-derived vasoactive factors between LIMA and saphenous vein grafts may be important determinants of graft function, with LIMA having more pronounced relaxations than the saphenous vein (17). Fatty acids may play a role in relation to the LIMA graft function, and it has been reported that n-3 PUFA have a direct effect on vascular function through uptake and incorporation of n-3 PUFA into endothelial and smooth muscle cells (10). Although n-3 PUFA supplementation in humans has been reported to improve endothelium-dependent vasodilation (12,15), this study suggests that neither the content of EPA+DHA in the LIMA nor the dietary intake of EPA+DHA is associated with FMD in patients with severe coronary artery disease. The fatty acid composition in the LIMA only reflected self-reported fish consumption and supplementation of fish oils to a minor degree, although this was true with regard to the fatty acid composition in the other compartments evaluated. Therefore, we do not believe that dietary supplementation with fish oils and fish intake will have any direct clinical impact on the endothelial vasodilatory function of the LIMA among patients undergoing CABG. However, other favourable physiological effects of n-3 PUFA may be beneficial for patients undergoing CABG. These effects may include a favourable shift in the distribution of lipoprotein particles and a beneficial effect on cardiac arrhythmia including reduction of postoperative atrial fibrillation (2,5,10).

The fatty acid composition of the internal mammary artery has to our knowledge previously only once been described in one previous study (1). In general we found a very different fatty acid composition in the LIMA compared with that study. The proportion of EPA in the LIMA was e.g. ten times lower in our study population. Theses differences may be explained by the use of different laboratory methods and differences in the intake of marine n-3 PUFA as intake of marine n-3 PUFA differs considerably between populations (21). We were able to detect the major n-3 PUFA in the LIMA and believe that these fatty acids were predominantly derived from the cell membranes of the endothelium, although some smooth muscle fibers and mesenchymal cells from the intima and a part of the media layer of the LIMA may also have been present in the tissue samples.

In the present study we used well described and standardized methods for separation of plasma lipids for fatty acid analysis (4,11,) and as intra-assay variation was fairly acceptable for EPA (13.0%) and DHA (20.4%) in the LIMA, we consider the methods used in the present study reliable.

The content of fatty acids is expected to vary in different tissues and biological membranes and we confirmed this as shown in Table 2. We found a highly statistically significant correlation between the content of EPA in all the four compartments evaluated, but the content of DHA in LIMA did not correlate to the content of DHA in the other 3 compartments. These findings suggests different physiological roles of n-3 PUFA in various tissues and membranes, however, it is unknown by what mechanisms the content of fatty acids in various cells and tissues are regulated.

The proportion of marine n-3-PUFA in pericardial adipose tissue has to our knowledge never been reported before. The proportions of n-3 PUFA in the PAT seem to be comparable to the proportions of n-3 PUFA in subcutaneous gluteal adipose tissue (7). Thus, marine n-3 PUFA in PAT may be considered a marker of long term habitual fish consumption in the same way as the content of n-3 PUFA in subcutaneous adipose tissue (18).

In addition to a beneficial effect on endothelial vasodilatory function, n-3 PUFA may also be important in relation to atherogenesis (16,20,25). This is of interest since the incidence of atherosclerosis in the LIMA is very low (13,23). The LIMA content of EPA and DHA, which are higher than in PAT, may be protective against atherosclerosis in LIMA, however, we have only minor knowledge about the fatty acid composition in atherosclerotic plaques from different vascular sites in the same patient (1,6,8,20). More studies on the fatty acid composition of different arteries from the same patient are warranted in order to evaluate whether the fatty acid composition of LIMA differs from arteries that are more prone to development of arteriosclerosis.

In conclusion, self-reported fish consumption and supplementation of fish oils was reflected by the fatty acid composition in NEFA, PL and in the PAT, but less so in the

LIMA. There was no association between FMD and the fatty acid composition of the LIMA.

Other articles in this theme issue include references (26-37).

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