Association of n-3 polyunsaturated fatty acids with soluble thrombomodulin as a marker of endothelial damage: A cross-sectional pilot study

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Association of n-3 polyunsaturated fatty acids with soluble thrombomodulin as a marker of endothelial damage: A cross-sectional pilot study☆

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ABSTRACT

Background: Soluble thrombomodulin (sTM) is a useful marker of vascular endothelial damage. Although n-3 polyunsaturated fatty acids (n-3 PUFAs) (eicosapentaenoic acid: EPA; docosahexaenoic acid: DHA) have various cardiovascular protective effects, their effect in preventing vascular endothelial damage remains unclear. Furthermore, little is known about the association of EPA and DHA with sTM using the cross-sectional study method.
Methods and results: This pilot study was designed as a hospital-based cross-sectional study to investigate the relationships between serum n-3 PUFA levels and sTM level in patients with the presence of one or more risk factors for atherosclerosis. Of the 534 sequential patients who had routinely been registered to a study cohort of our institute, 324 patients without chronic kidney disease (because sTM is eliminated by renal excretion and the serum sTM level is increased by renal dysfunction) were enrolled in this study. In a multivariate analysis after adjustment for atherosclerotic risk factors, elevated EPA + DHA level was an independent variable of decreased sTM level ($\beta = -0.183$, $p = 0.0006$). The serum levels of EPA and DHA showed a strong correlation ($r = 0.736$, $p < 0.0001$); however, multivariate analysis including EPA and DHA revealed that serum DHA ($\beta = -0.243$, $p = 0.003$), but not serum EPA ($\beta = 0.049$, $p = 0.538$), was identified as an independent negative determinant of sTM level.
Conclusion: Although there are numerous unresolved issues in regard to the differences in the cardiovascular protective effects between EPA and DHA, DHA may be associated with a decrease in sTM. A large-scale trial would be warranted to demonstrate whether the beneficial effect of n3-PUFAs therapy on endothelial damage and improvement of endothelial function might also result in fewer clinical cardiovascular events.

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Introduction

Regular fish consumption has been shown to be correlated with lower mortality from coronary artery disease (CAD) [1], and the lower mortality has been ascribed to the beneficial effect of the two major n-3 polyunsaturated fatty acids (n-3PUFAs):

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that are found in fish [2,3].

Thrombomodulin (TM) is an integral membrane glycoprotein and high-affinity receptor for thrombin on the endothelial cell surface, and it has been implicated in the endothelial regulation of fibrinolysis and coagulation [4]. TM is widely distributed on the endothelium of human arteries, veins, capillaries, and lymphatics in all organs and tissues except the brain [5,6]. After proteolytic cleavage of TM from the endothelial surface, soluble TM (sTM) can be detected in the circulation [6,7]. The physiological role of sTM is unknown, but its concentration is thought to reflect the degree of endothelial damage [8,9].

Although many factors that affect the CAD risk may be impacted by n-3PUFAs intake, there have been few data regarding

the relationship between serum sTM levels as an indicator of endothelial damage and serum n-3PUFA levels. We hypothesized that serum sTM levels are lower in populations with higher serum levels of n-3PUFAs derived from fish consumption.

The purpose of this study was to elucidate the relationship between serum n-3PUFA levels and serum sTM levels by a cross-sectional study design, and furthermore, to evaluate the difference in the relationship between serum EPA, DHA levels, and serum sTM level.

Methods

Study design and study population

This pilot study was designed as a hospital-based cross-sectional study of the relationships between serum sTM levels as indicators of endothelial damage and serum n-3PUFA levels in patients who had one or more risk factors for atherosclerosis.

Because sTM is excreted by the kidneys and the serum sTM level rises when renal function is impaired [5], of the 534 sequential patients who had routinely been registered in a study cohort of our institution, the Cardiovascular Center, during the period from April to July 2013, we enrolled 383 patients (mean age: 63 years; men: 65%) who did not have stage 3 or more chronic kidney disease (CKD). The severity of CKD was determined on the basis of estimated glomerular filtration rate (eGFR) calculated using the abbreviated Modification of Diet in Renal Disease (MDRD) Study formula modified by a Japanese coefficient [10].

The diagnostic criteria for the diseases among the risk factors for atherosclerosis that we used in this study analysis were as follows: for hypertension, a systolic pressure of at least 140 mmHg or diastolic pressure of at least 90 mmHg, or taking antihypertensive medication; for diabetes, a fasting plasma glucose concentration $\geq$126 mg/dL and HbA1c $\geq$6.5% (according to the National Glycohaemoglobin Standardization Program), or current treatment with an antidiabetic agent; for dyslipidemia, a low-density lipoprotein cholesterol (LDL-C) level of at least 140 mg/dL, a triglyceride (TG) level of at least 150 mg/dL, or a high-density lipoprotein (HDL-C) level below 40 mg/dL, or current treatment with lipid-modifying medication; for history of CAD, meaning a documented history of myocardial infarction, coronary revascularization intervention (coronary artery bypass graft surgery or percutaneous coronary intervention), or a diagnosis of $\geq$50% stenosis in 1 or more coronary arteries diagnosed by cardiac catheterization.

Patients who met any of the following exclusion criteria were not enrolled: bleeding tendency, hepatic or renal dysfunction (CKD stage $\geq$3), serum alanine aminotransferase and aspartate aminotransferase levels $\geq$2 times the upper limit of their normal range of values, known malignant neoplasm, current treatment with an n-3PUFA, and acute coronary syndrome (ACS) within the preceding 3 months.

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of our institutions, and written informed consent was obtained from all participants.

Measurement of clinical laboratory parameters

Fasting blood samples were collected early in the morning after a 12-h fast. The serum sTM level was measured by an enzyme immunoassay (SRL Co., Ltd., Tokyo, Japan). Serum fatty acid levels were measured by capillary gas chromatography (SRL). Serum total cholesterol (TC), HDL-C, and TG levels were measured by the standard methods. LDL-C levels were estimated using the Friedewald formula [11]. The concentration of fibrin was measured using the Clauss assay (SRL). The serum amyloid A concentration was measured using a latex agglutination turbidimetric immunoassay (SRL). The high-sensitivity C-reactive protein (hs-CRP) level was measured by a nephelometric assay (Behring Diagnostic, Marburg, Germany).

Statistical analysis

We performed all the statistical analyses using the SPSS Window version 12.0 software program (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA). Data are expressed as the mean $\pm$ standard deviation (SD) for continuous variables and as percentages for discrete variables. Before performing the statistical tests, each variable was examined to ensure a normal distribution. In the subset analyses according to tertiles of serum sTM levels, we used an analysis of variance followed by the Bonferroni methods adjusting for covariate if differences were shown in patient characteristics and laboratory profile. If the values of a variable did not have a normal distribution, the Kruskal–Wallis and the Mann–Whitney U test were performed to test for statistically significant differences between the groups. Univariate and multivariate regression analyses were performed to identify independent variables that were associated with serum sTM level. All variables that correlated with the serum sTM at $p < 0.05$ in the univariate regression analysis were entered into the multivariate model. A p-value less than 0.05 was considered to indicate statistical significance.

Results

Patients

We excluded 2 subjects from the analysis because of missing laboratory data, and thus 381 subjects were ultimately included in the analysis. The serum sTM levels ranged from 1.4 FU/mL to 4.0 FU/mL (mean $\pm$ SD: 2.36 $\pm$ 0.49). The patients were divided into sTM level tertiles. The ranges of serum sTM levels according to the tertiles were tertile 1 ($n = 127$), 1.4–2.1 FU/mL; tertile 2 ($n = 127$), 2.2–2.5 FU/mL; and tertile 3 ($n = 127$), 2.6–4.0 FU/mL.

Comparisons between patient characteristics and laboratory profiles according to sTM tertiles

Age and number of risk factors were significantly increased with the tertiles of sTM levels, but there were no significant differences between the tertiles in gender, BMI, presence of hypertension, presence of dyslipidemia, presence of diabetes mellitus, presence of current smoking, or presence of hyperuricemia. The results of the analyses of relationships between current treatment with prescription drugs and sTM tertiles showed that current treatment with an antiplatelet drug was associated with elevated sTM levels. There were no significant differences between the tertiles of serum sTM levels according to whether the subjects were being treated with renin angiotensin system (RAS) inhibitors (angiotensin-converting inhibitor/angiotensin receptor blocker/direct renin inhibitor), β blockers, calcium-channel blockers, or statins.

The serum TC levels decreased significantly with the tertiles of sTM levels. There were no significant differences in the serum levels of LDL-C, HDL-C, TG, fibrinogen, amyloid A, or hs-CRP, or in leukocyte count between the tertiles of sTM levels. There were significant differences in the serum DHA levels and arachidonic acid (AA) between the tertiles of sTM levels; the highest sTM tertile (tertile 3) was associated with a significant decrease in sTM levels, but there were no significant differences in serum EPA levels,

the total serum EPA + DHA levels, EPA/AA ratio, DHA/AA ratio, or (EPA + DHA)/AA ratio between the tertiles of sTM levels (Table 1).

Univariate regression analysis to identify independent variables that were significantly associated with serum sTM levels

No significant associations were found between serum sTM levels and gender, BMI, presence of hypertension, current smoking, diabetes mellitus, dyslipidemia, hyperuricemia, or treatment with antiplatelet drugs, RAS inhibitors, β blockers, calcium-channel blockers, or statins. The serum sTM levels were significantly positively correlated with age, number of risk factors, and hs-CRP levels, and significantly negatively correlated with the serum LDL-C, DHA levels, total EPA + DHA levels, and AA levels, but there was no significant correlation between the sTM levels and any other n3-PUFA-related factors [EPA levels, EPA/AA, DHA/AA, or (EPA+DHA)/AA]. The serum sTM levels were also not correlated with the serum level s of HDL-C, TG, or LDL-C/HDL-C (Table 2).

Multivariate regression analysis to identify independent variables that were significantly associated with serum sTM levels

Next, in order to assess the relationship between the serum sTM levels and serum n-3PUFA and n-6PUFA levels from various viewpoints, we devised the following five multivariate analysis models in which we used the sTM concentration as the dependent variable and the serum n-3PUFA-related factors and AA.

- Model 1: Including total n-3PUFA concentration (EPA + DHA) as dependent variable.
- Model 2: Because there was a strong correlation between the serum EPA concentrations and serum DHA concentrations ($r = 0.736$, $p < 0.0001$; Fig. 1), they were mutually confounding factors. We devised a model that incorporated both EPA and DHA in order to minimize their influence.
- Model 3: A model in which EPA alone was incorporated as the serum n-3PUFA.

Table 1
Comparisons between patient characteristics and laboratory profiles according to sTM tertiles.

		sTM Tertile			
	All cases ($n = 381$)	T1 ($n = 127$)	T2 ($n = 127$)	T3 ($n = 127$)	*p* value
Male/female, *n* (%)	263 (69)/118 (31)	83 (66)/43 (34)	89 (70)/38 (30)	89 (70)/38 (30)	0.668
Age (years)	61 ± 12	58 ± 12	62 ± 11[2]	64 ± 12[3]	<0.0001[*]
BMI (kg/m^2)	24.4 ± 3.9	24.5 ± 3.7	24.3 ± 4.0	24.2 ± 3.9	0.931
CAD, *n* (%)	88 (23)	33 (26)	22 (17)	33 (26)	0.217
Hypertension, *n* (%)	259 (68)	83 (65)	83 (66)	90 (71)	0.514
Current smoking, *n* (%)	42 (11)	12 (10)	12 (10)	15 (12)	0.827
Diabetes mellitus, *n* (%)	80 (21)	22 (17)	30 (24)	29 (23)	0.293
FBS (mg/dL)[§]	105 (96/167)	104 (96/116)	106 (96/122)	105 (95/115)	0.523
HbA1c (%)[§]	5.8 (5.6/6.2)	5.8 (5.5/6.1)	5.8 (5.6/6.4)	5.8 (5.6/6.3)	0.256
HOMA-IR[§]	1.8 (1.0/3.3)	1.7 (1.1/2.9)	1.8 (1.0/4.0)	1.9 (0.9/3.2)	0.756
Dyslipidemia, *n* (%)	251 (66)	86 (68)	76 (60)	89 (70)	0.190
TC (mg/dL)	191 ± 32	195 ± 31	194 ± 33	185 ± 31[1]	0.026[*]
LDL-C(mg/dL)	108 ± 27	110 ± 26	110 ± 28	104 ± 26	0.122
HDL-C (mg/dL)	59 ± 16	60 ± 15	59 ± 17	58 ± 15	0.522
LDL-C/HDL-C ratio	1.93 ± 0.70	1.91 ± 0.68	1.99 ± 0.72	1.90 ± 0.72	0.578
TG (mg/dL)[§]	107 (77/153)	109 (77/159)	113 (81/166)	102 (72/144)	0.250
Hyperuricemia, *n* (%)	50 (13)	22 (17)	12 (10)	17 (13)	0.345
Number of risk factors	1.2 ± 0.8	1.1 ± 0.8	1.2 ± 0.8	1.4 ± 0.8[2]	0.026[*]
Inflammatory markers					
WBC count (mm^{-3})	6087 ± 1571	6115 ± 1520	6122 ± 1563	6009 ± 1652	0.810
Fibrinogen (mg/dL)[§]	279 (253/316)	277 (249/308)	279 (253/316)	288 (253/322)	0.212
Amyloid A (μg/mL)[§]	5.7 (4.2/9.3)	5.5 (3.6/8.6)	5.7 (4.4/9.4)	6.1 (4.1/9.5)	0.528
hs-CRP (mg/L)[§]	0.5 (0.3/1.1)	0.5 (0.2/0.8)	0.6 (0.3/1.2)	0.5 (0.3/1.3)	0.169
Concomitant drugs, n (%)					
Antiplatelete	95 (25)	34 (27)	20 (16)	41 (32)	0.014
RAS inhibitors	198 (52)	62 (49)	67 (53)	67 (53)	0.798
β blockers	91 (24)	33 (26)	24 (19)	32 (25)	0.381
Calcium channel blockers	209 (55)	209 (55)	72 (57)	67 (53)	0.808
Statins	206 (54)	67 (53)	62 (49)	75 (59)	0.315
n3-PUFAs-related factors					
EPA (μg/mL)[§]	69 (43/107)	75 (42/117)	64 (43/107)	67 (45/97)	0.428
DHA (μg/mL)[§]	138 (112/174)	143 (117/187)	144 (111/185)[4]	134 (112/153)[5]	0.024[#]
AA (μg/mL)	164 ± 46	174 ± 49	160 ± 44	158 ± 50[2]	0.018[*]
EPA + DHA (μg/mL)[§]	213 (162/277)	220 (165/306)	216 (164/293)	200 (160/254)	0.112
EPA/AA ratio[§]	0.44 (0.27/0.72)	0.44 (0.25/0.73)	0.46 (0.29/0.69)	0.44 (0.27/0.72)	0.923
DHA/AA ratio[§]	0.87 (0.68/1.17)	0.86 (0.66/1.17)	0.89 (0.69/1.19)	0.87 (0.68/1.12)	0.479
(EPA + DHA)/AA ratio[§]	1.37 (0.95/1.83)	1.37 (0.93/1.86)	1.48 (1.00/1.88)	1.30 (0.94/1.77)	0.709
Renal function					
eGFR (ml/min/1.73m^2)	76.2 ± 11.8	79.6 ± 12.8	74.7 ± 9.7[2]	74.5 ± 12.0[2]	0.0006[*]

In this analysis, risk factors were defined as: age ≥ 65 years, male, BMI ≥ 25 kg/m^2, current smoker, hypertension, diabetes mellitus, and dyslipidemia. We calculated the mean number of risk factors of the subjects of this study on the basis of the total numbers of risk factors that were present. [§]: Median; interquartile range in parentheses (all such values). [*]: ANOVA and post hoc tests with Bonferroni correction were performed to test between-group differences; [1]$p < 0.05$, [2]$p < 0.01$, [3]$p < 0.0001$ vs. T1; [#]: Kruskal–Wallis and post hoc tests with Mann–Whitney U test were performed to test between-group differences; [4]$p < 0.05$, vs. T1, [5]$p < 0.01$, vs. T2. BMI: Body mass index; CAD: coronary artery disease; FBS: fasting blood sugar; HB: hemoglobin; HOMA-IR: Homeostasis model assessment-insulin resistance and HOMA-IR was calculated as the fasting insulin level (μU/mL) × FBS level (mg/dL)/405. TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride; hs-CRP: high-sensitivity c-reactive protein; RAS (renin angiotensin system) inhibitors indicates angiotensin-converting inhibitor, angiotensin receptor blocker, and direct renin inhibitor; CCB: calcium channel blocker; WBC: white blood cell; n3-PUFA:n-3 polyunsaturated fatty acids: EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; AA: Arachidonic acid.

Table 2
Univariate and multivariate regression analyses to identify independent variables that were significantly associated with serum sTM levels.

Variable	Univariate		Multivariate									
			Model 1		Model 2		Model 3		Model 4		Model 5	
	r	p	β	p	β	p	β	p	β	p	β	p
Age	0.186	<0.001	0.209	0.002	0.206	0.0017	0.211	0.001	0.192	0.004	0.146	0.022
Male	0.081	0.114										
BMI	−0.018	0.722										
Hypertension	0.036	0.483										
Current smoking	0.049	0.346										
Diabetes mellitus	0.074	0.148										
FBS[a]	0.122	0.018	0.064	0.215	0.068	0.186	0.067	0.191	0.060	0.246	0.063	0.229
HbA1c[a]	0.079	0.124										
HOMA-IR[a]	0.027	0.616										
Dyslipidemia	0.011	0.830										
LDL-C	−0.107	0.037	−0.061	0.244	−0.038	0.474	−0.044	0.402	−0.077	0.144	−0.061	0.258
HDL-C	−0.067	0.195										
LDL-C/HDL-C	−0.034	0.512										
TG[a]	−0.037	0.139										
Hyperuricemia	−0.064	0.212										
Number of risk factors	0.119	0.022	−0.036	0.563	0.14	0.356	−0.036	0.557	−0.035	0.579	−0.042	0.511
Inflammatory marker												
WBC count	−0.033	0.521										
Fibrinogen[a]	0.071	0.165										
Amyloid A[a]	0.028	0.612										
CRP[a]	0.136	0.009	0.142	0.007	0.140	0.008	0.141	0.007	0.138	0.009	0.132	0.014
Concomitant drugs												
Anti-platelets	0.080	0.181										
RAS inhibitors	0.021	0.686										
β Blockers	−0.010	0.850										
CCBs	−0.015	0.764										
Statins	−0.001	0.986										
n3-PUFA-related												
EPA[a]	−0.057	0.273	–	–	0.049	0.538	−0.206	0.0001	–	–	–	–
DHA[a]	−0.139	0.007	–	–	−0.243	0.003	–	–	−0.133	0.013	–	–
AA	−0.124	0.016	–	–	–	–	–	–	–	–	−0.087	0.099
EPA[a] + DHA	−0.107	0.038	0.183	0.0006	–	–	–	–	–	–	–	–
EPA/AA[a]	0.005	0.925	–	–	–	–	–	–	–	–	–	–
DHA/AA[a]	−0.034	0.507	–	–	–	–	–	–	–	–	–	–
(EPA + DHA)/AA[a]	0.021	0.684	–	–	–	–	–	–	–	–	–	–
Renal function												
eGFR	0.195	0.0002	−0.189	0.0004	−0.192	0.0003	−0.191	0.0004	−0.191	0.0001	−0.192	0.0004

sTM, soluble thrombomodulin; BMI, body mass index; CAD, coronary artery disease; FBS, fasting blood sugar; Hb, hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance and HOMA-IRwas calculated as the fasting insulin level (μU/mL) × FBS level (mg/dL)/405; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; CRP, c-reactive protein; RAS (renin angiotensin system) inhibitors indicate angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, and direct renin inhibitor; CCB, calcium-channel blocker; WBC, white blood cell; n3-PUFAs, n-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; eGFR, estimated glomerular filtration rate.

[a] Tested after log-transformed. All variables that correlated with the serum sTM at $p < 0.05$ in the univariate regression analysis were entered into the multivariate model.

Fig. 1. Relationship between serum levels of EPA and DHA. Regression analysis was performed using linear regression and Spearman's rank correlation coefficient. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

- Model 4: A model in which DHA alone was incorporated as the serum n-3PUFA.
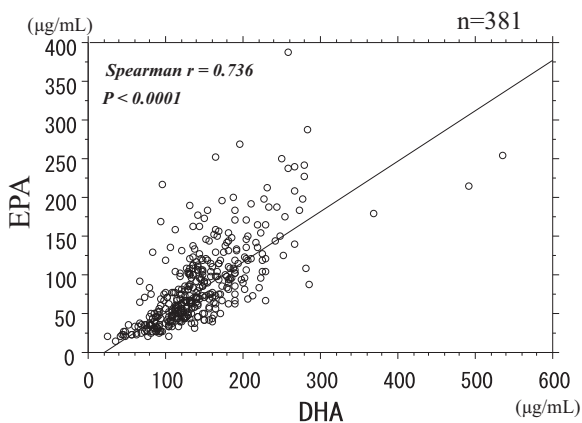- Model 5: A model in which AA alone was incorporated as the serum n-6PUFA.

In all of the multivariate regression models, higher age, the serum hs-CRP level, and eGFR were identified as independent variables of higher serum sTM levels. In model 1, the higher total EPA + DHA level was identified as an independent variable of lower serum sTM levels. In model 2, the higher level of DHA, but not a higher EPA level, was identified as a significant and independent variable of lower serum sTM levels. In models 3 and 4, the EPA level and the DHA level, respectively, were identified as independent variables of lower serum sTM levels. In model 5, although the AA level correlated negatively with serum sTM levels, the AA level was not identified as an independent variable of lower serum sTM levels in the multivariate regression analysis. The relationship between the serum n-3PUFA levels and the serum sTM levels is shown in Fig. 2.
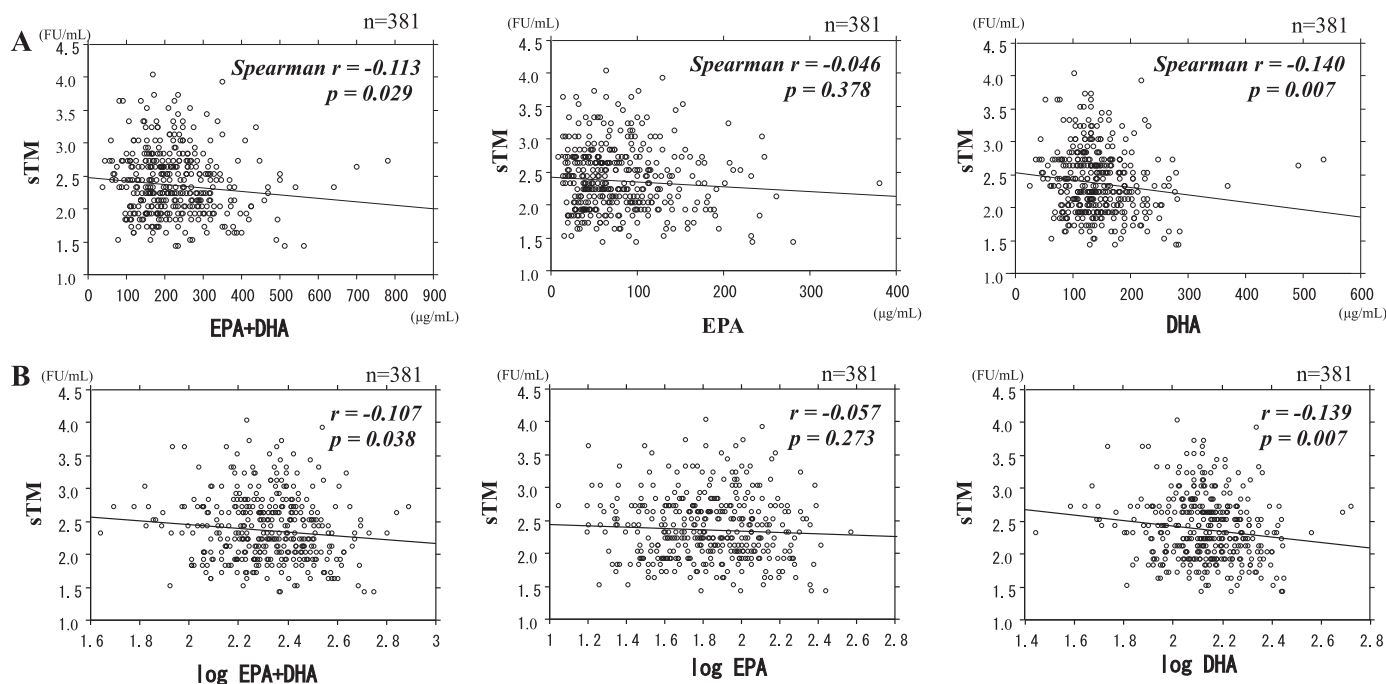
Fig. 2. (A and B) Relationship between n3-PUFAs and sTM. Regression analysis was performed using linear regression and Spearman's rank correlation coefficient. The serum sTM level was weakly correlated with serum total EPA + DHA level and serum DHA level, but not with serum EPA level. n-3PUFAs, n-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; sTM, soluble thrombomodaulin.

Discussion

We clarified the following points in this cross-sectional study. Increases in the serum concentration of n-3PUFAs obtained by consuming fish, in particular, in DHA, may be involved in decreases in the concentration of sTM, a marker of vascular endothelial damage. Many matters related to differences between the cardiovascular protective effects of EPA and DHA are unknown, but DHA may be more involved than EPA in the preventive effect of n-3PUFAs against vascular endothelial damage, with the serum sTM concentration used as a marker.

TM is present on the surface of vascular endothelial cells. It forms a complex with the thrombin that is present in the blood and activates the clotting inhibitor protein C [4]. The TM produced in endothelial cell damage is particularly degraded by intracellular proteases in damage and destruction of microvessel walls, and is released into the blood, where it becomes sTM, and is excreted in urine. Thus, since endothelial cell production function and the severity of the damage are inferred by measuring the concentration of sTM in the blood, sTM is useful as a marker of vascular endothelial cell damage [12].

The serum level of the endothelium-specific marker sTM increases with the severity of CAD [13], stroke [14], and peripheral occlusive arterial disease [15], and serum sTM levels are not elevated in healthy or asymptomatic subjects [16,17]. More specifically, since the degree of vascular endothelial damage was found to be small in a study of a population with few risk factors for atherosclerosis, the antithrombotic action of TM, which is its original function, was being exerted, and the TM was present bound to the vascular endothelium. However, it is easy to imagine that as vascular endothelial damage progresses in people who have risk factors for atherosclerosis, TM is released from the vascular endothelium and becomes a risk factor for atherosclerotic diseases [12]. Seeming to corroborate this, the results of the present study showed that the sTM levels tended to be high in the elderly subjects whose degree of atherosclerosis had progressed. An increase in serum sTM level was not found to be an independent factor in the multivariate analysis in this study, but there was a positive correlation between the sTM concentrations and the number of risk factors for atherosclerosis.

If sTM is a risk factor for atherosclerosis as stated above, we think that the fact that an increase in sTM and increase in hs-CRP [18,19], which detects the expansion of atherosclerotic plaques on the basis of evidence of inflammation, occurred at the same time as shown in the results of this study is a phenomenon that stands to reason. As evidence in support of this, high sTM levels and high hs-CRP levels were observed simultaneously in a population that shared having them as predictors of the onset of CAD in a prospective cohort study of 15,792 subjects in the Atherosclerosis Risk in Communities Study [20].

Ameliorating effects of n-3PUFA interventions have been reported on inflammatory markers, including serum amyloid A, interleukin-6, interleukin-8, and tumor necrosis factor-α as well as hs-CRP, and on vascular endothelial markers, including von Willebrand factor and soluble adhesion factors as well as sTM [21–23]. However, the n-3PUFA preparations that were used in those studies were mixtures of EPA and DHA, and they only assessed the effects of n-3PUFAs as a whole. There is a report of a study that demonstrated a decrease in hs-CRP when EPA alone was given [24], but there have been few reports of studies of the anti-inflammatory and anti-atherosclerotic effects of DHA alone. The molecular mechanism of the anti-inflammatory effect of n-3PUFAs investigated was reported in a basic study [25]. However, n-3PUFAs are said to exhibit physiological activity after being incorporated into cell membranes, but many aspects of the differences in physiological effects exhibited by EPA and DHA and their in vivo kinetics have yet to be clarified. Many matters concerning the relationship between serum n-3PUFA concentrations and their concentrations in cell membranes that exert physiological activity are also unknown.

Study limitations

First, we did not examine the relationship between increased serum sTM and actual endothelial dysfunction measured by

either flow-mediated vasodilatation or acetylcholine-induced flow increase. Second, AA acts as an inflammatory response mediator in vivo, and, in theory, AA values might show a positive correlation with serum sTM concentrations. However, the serum AA values and serum sTM values showed a negative correlation, but that may be related to the fact that there was a positive correlation between the serum AA values and the serum DHA values ($R = 0.344$, $p < 0.0001$), and they are mutually confounding factors. The serum AA values were not an independent predictor of the serum sTM values in the multivariate analysis (model 5). Third, this study was of cross-sectional design, which made it impossible to establish a cause–effect relationship. Further study will be necessary to investigate whether the results of this study can be applied to the entire population. Finally, this study did not investigate the relationship between the amount of fish the subjects consumed and their serum n-3PUFA levels, and since it was conducted on the basis of a one-point blood collection, it is impossible to rule out the possibility that a bias in routine fish consumption affected the results of the analysis.

Clinical implications

Since this cross-sectional study included only a small sample, further studies will need to be expanded, and at the same time longitudinal studies should also be conducted to examine the relationship between sTM levels, n-3PUFA levels, and prognosis of atherosclerotic disease. There are reports that serum sTM concentrations decrease as a result of n3 PUFA or statins consumption [21–23,26–28]. In the future their serum sTM concentration reducing effect and preventive effect on the occurrence of arteriosclerotic cardiovascular events should be verified by interventional studies with these drugs. The results of the present study may serve as the foundation for proper prospective studies. Even though a CKD < 60 mL/min/1.73 m^2 was an exclusion criterion for enrolled patients, an increase in eGFR was an independent predictor of an increase in serum sTM concentration. In the future it will be necessary to consider a method of predicting serum sTM concentrations that does not depend on renal function.

Conclusions

The higher serum concentration of n-3PUFAs, particularly DHA, achieved by consuming fish, may be involved in decreases in the lower serum concentration of sTM, a marker of endothelial damage. This was a cross-sectional study, and an interventional study that uses n-3PUFAs will be necessary to investigate the mechanism of the preventive effect of n-3PUFAs on vascular endothelial damage, with sTM as a marker, or differences between the effects of EPA and DHA.

Acknowledgment

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追記

1. 脂肪酸の分類（n-3 PUFA、n-6 PUFA、DHA、EPA、AA など）を図で説明。
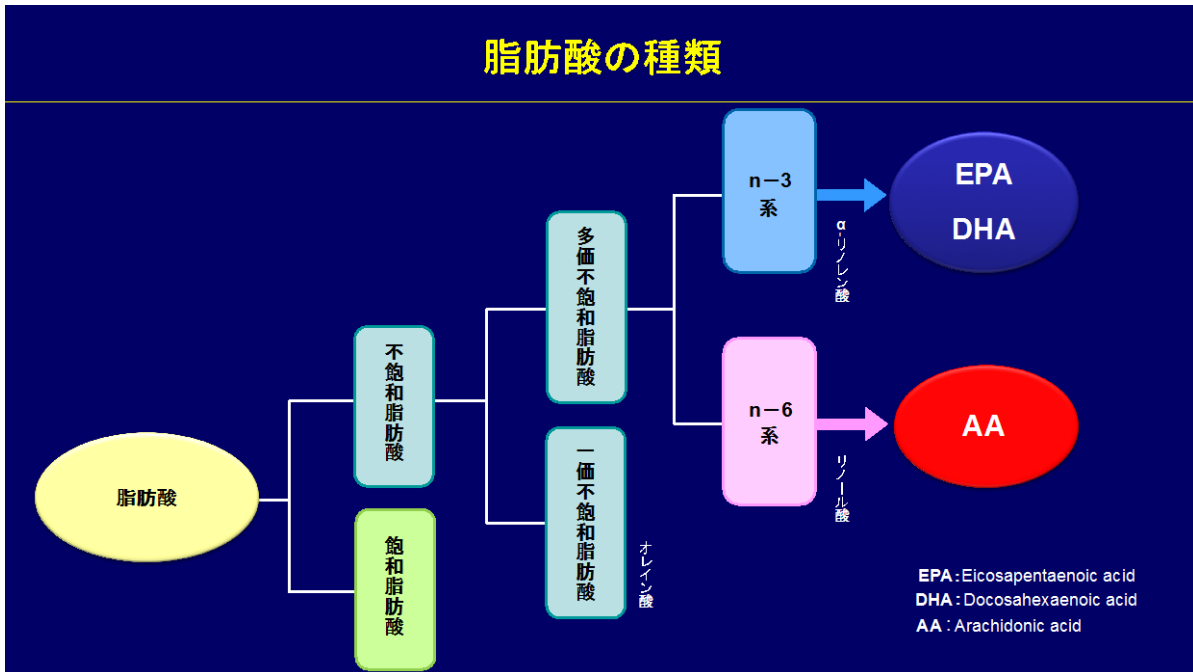
2. DHA, EPA, AA が動脈硬化に及ぼす影響または作用についての説明。

　　動脈硬化は慢性炎症性疾患であり、その進展、抑制には炎症性・抗炎症性脂質メディエーターが深く関与している。n-3 系の EPA 、DHA の AA 代謝系に対する作用は、ホスホリパーゼ A2 依存性にリン脂質からの AA の切り出しの抑制や、炎症性脂質メディエーター産生に関わるシクロオキシゲナーゼ、5-リポキシゲナーゼの AA 利用を競合的に阻害することで、プロスタグランジン群、トロンボキサン群、ロイコトリエン群などの起炎性エイコサノイドの産生と作用を抑制する。

　　近年さらに、 n-3 polyunsaturated fatty acid（PUFA）由来の新しいクラスの脂質メディエーターの存在が明らかとなり、抗炎症性脂質代謝物前駆体（Specialized proresolving mediators : SPM)と名付けられた。現在 EPA 由来のレゾルビン E1〜E3、DHA 由来のレゾルビン D1〜D4、プロテクチン D1 が同定されている。これらは炎症反応を収束させ、急性から慢性炎症への進展を阻止し組織の恒常性を維持する。炎症の際に n-3 PUFA 由来の脂質メディエーターおよび炎症促進性トロンボキサン、プロスタグランジンおよびロイコトリエンが並行して形成されるため、炎症が収束または慢性に進行するかは、これら 2 つの相反する効果のバランスが決定的である。

　　心血管疾患に関しては、最近、唾液中のレゾルビン対ロイコトリエン比が無症候性アテローム性動脈硬化症を予測するという報告がある。したがって、レゾルビン対ロイコトリエン比の決定は、n-3 および n-6 PUFA 誘導メディエーターの相対的な役割を決定するために重要であるだけでなく、アテローム性動脈硬化進行にとって重要である。

3. EPA/AA や DHA/AA が低いと予後が悪いとされる観察研究の補足。

1) Ratio of serum n-3 to n-6 polyunsaturated fatty acids and the incidence of major adverse cardiac events in patients undergoing percutaneous coronary intervention.

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待機的 percutaneous coronary intervention (PCI)をうける連続 284 人が登録され、n-6 PUFAs、n-3 PUFAs、血清 EPA/AA 比、血清 DHA/AA 比と主要心血管事故(心臓死、急性冠症候群、新規病変に対する PCI、バイパス手術)との関連を解析した。その結果、多変量解析にて高い EPA/AA 比のみが低い major adverse cardiac events (MACE)と相関していた。
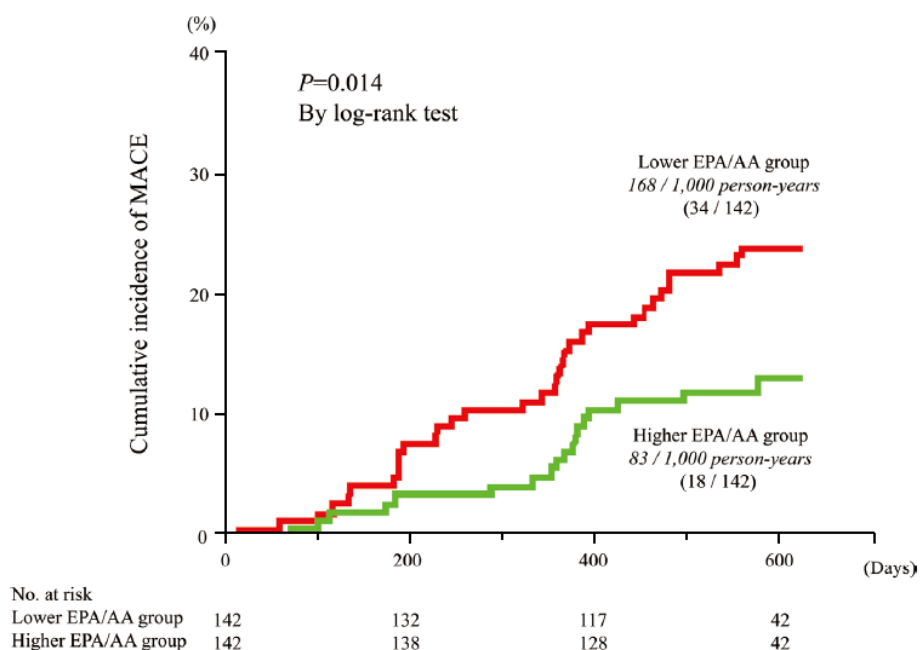

Figure. Kaplan-Meier survival curves: lower vs. higher eicosapentaenoic acid/arachidonic acid (EPA/AA) ratio. MACE, major adverse cardiac events.

Table 4. MACE Multivariate Analysis (Cox Proportional Hazards)

	Model A	Model B	Model C
Analysis 1			
Higher EPA (>62.0 µg/ml)			
HR (95%CI), P value	0.63 (0.33–1.16), 0.14	0.56 (0.29–1.07), 0.079	0.57 (0.30–1.07), 0.079
Higher DHA (>134.1 µg/ml)			
HR (95%CI), P value	0.71 (0.37–1.32), 0.28	0.78 (0.40–1.50), 0.46	0.74 (0.38–1.69), 0.81
Higher AA (>150.4 µg/ml)			
HR (95%CI), P value	1.16 (0.67–2.02), 0.60	1.08 (0.61–1.92), 0.78	0.93 (0.51–1.69), 0.81
Analysis 2			
Higher EPA/AA ratio (>0.4037)			
HR (95%CI), P value	0.52 (0.27–0.99), 0.048	0.51 (0.26–0.98), 0.043	0.49 (0.25–0.94), 0.033
Higher DHA/AA (>0.8716)			
HR (95%CI), P value	0.89 (0.47–1.66), 0.73	1.03 (0.54–1.94), 0.93	1.07 (0.55–2.03), 0.83

Model A, no adjusted factors; model B, adjusted for age, diabetes; model C, adjusted for age, gender, diabetes, hypertension, smoking, LDL-C.
Abbreviations see in Tables 1,2.

Table 5. MACE vs. EPA/AA Ratio

	Lower EPA/AA (≤0.4037)	Higher EPA/AA (>0.4037)	P value
MACE	34/142 (23.9)	18/142 (12.7)	0.0096
Cardiac death	2/142 (1.4)	0/142 (0)	0.16
ACS	2/142 (1.4)	1/142 (0.70)	0.56
PCI for de novo lesion	26/142 (18.3)	12/142 (8.5)	0.015
CABG	4/142 (2.8)	5/142 (3.5)	0.73

Data given as n (%).
ACS, acute coronary syndrome. Other abbreviations see in Table 1.
MACE include cardiac death, ACS, PCI for de novo lesion, and CABG.

2) The ratio of serum n–3 to n–6 polyunsaturated fatty acids is associated with diabetes mellitus in patients with prior myocardial infarction: a multicenter cross-sectional study.

心筋梗塞の既往のある糖尿病患者では EPA/AA 比、DHA/AA 比が低く、EPA/AA 比と DHA/AA 比の高感度 CRP（C-reactive protein)に対する相関は異なっていた。スタチンの使用は DHA/AA 比に影響を与える可能性があるが、EPA/AA 比には影響を与えなかった。そのため、EPA/AA 比は心血管イベントを評価するよりよい指標である。
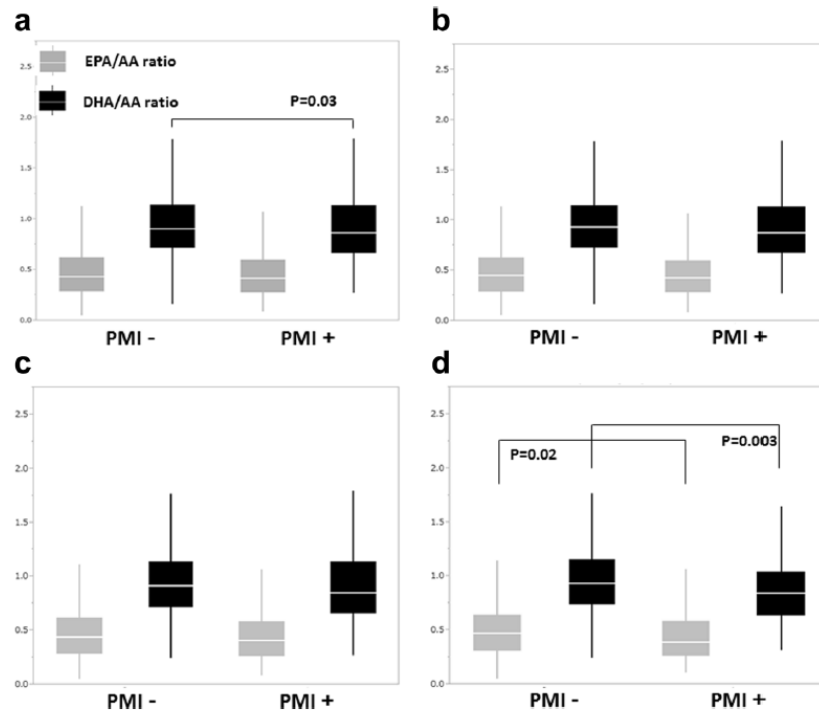
Fig. 1 Comparison of ratios of eicosapentaenoic acid (EPA) to arachidonic acid (AA) levels (EPA/AA) and docosahexaenoic acid (DHA) to arachidonic acid (AA) levels (DHA/AA) between the patients with and without prior myocardial infarction (PMI). Analysis for all patients (**a**) and patients with hypertension (HTN; **b**), dyslipidemia (DL; **c**), and diabetes mellitus (DM; **d**). A statistically significant difference in EPA/AA ratios was only present for the patients with DM, whereas that in DHA/AA ratios was present for all and DM patients

Table 4 Polyunsaturated fatty acids levels in all patients according to statin use

	Statin use		P value
	-	+	
N	813	920	
EPA, µg/mL	73.0 ± 43.9	74.6 ± 44.1	0.46
DHA, µg/mL	146.8 ± 57.4	140.5 ± 48.1	<0.05
AA, µg/mL	154.2 ± 57.6	160.3 ± 40.9	<0.01
DHLA, µg/mL	32.2 ± 12.8	34.1 ± 11.8	<0.05
EPA/AA	0.50 ± 0.30	0.49 ± 0.31	0.6
DHA/AA	0.99 ± 0.44	0.91 ± 0.32	<0.0001

Fatty acids (EPA, DHA, AA and DHLA) profile with or without statin use in all study patients
Abbreviations are listed in the footnote to Table 1

4. sTM と TM の違いについての補足説明。

　Thrombomodulin (TM)は血管内皮細胞表面に発現している抗血栓分子として発見されたが、近年、抗炎症作用も持ち合わせていることが明らかになった。TM の抗炎症作用は活性化プロテイン C（activated protein C : APC）による作用と、TM そのものによる作用とに大別される。TM の Epidermal growth factor（EGF) 様領域（D2）にトロンビンが結合すると、プロテイン C が結合し APC となる。APC は抗凝固作用の他に、白血球や血管内皮細胞表面のプロテアーゼ活性化受容体 (protease-activated receptor : PAR）-1 および血管内皮プロテイン C 受容体 (endothelial protein C receptor : EPCR) に作用することで、これらの細胞の遺伝子発現パターンを変化させ、抗炎症作用を発揮すると考えられている。次に、TM そのものによる抗炎症作用としては、レクチン様領域（D1）が担っている。D1 は APC 非依存的に抗炎症作用を発揮し、そのメカニズムとしては、HMGB1 (high mobility group box 1) を不活化すること、グラム陰性桿菌由来のエンドトキシンを中和すること、補体の活性化を抑制することなどが報告されている。

　Soluble thrombomodulin (sTM) は、内皮細胞の TM が細胞傷害部位に集積した好中球由来のエラスターゼにより分解され血中に遊離したものである。sTM は D4、D5 を欠くが、D1、D2 を保持しており、TM の 30~50%の生理活性を有している。sTM は播種性血管内凝固症候群、血栓性血小板減少性紫斑病、 糖尿病性血管障害、膠原病、および急性呼吸窮迫症候群など全身的な血管内皮細胞傷害を伴う病態において上昇すると報告されており、その血中濃度は血管内皮細胞傷害の程度を反映している。また、近年開発された、遺伝子組み換え型 TM（リコモジュリン®）は sTM と同じく D1～D3 ドメインを全て含んでおり、抗凝固、抗炎症などの多彩な生理活性を含むと考えられ DIC 治療に用いられている。

5. 多変量解析で冠動脈疾患の有無について検討を加えた表を追加。

本文中の Table 2 を改変

Variable	Univariate		Multivariate									
			Model 1		*Model 2*		*Model 3*		*Model 4*		*Model 5*	
	r	p	β	p	β	p	β	p	β	p	β	p
年齢	0.186	< 0.001	0.209	0.002	0.206	0.0017	0.211	0.001	0.192	0.004	0.146	0.022
性別	0.081	0.114										
BMI	-0.018	0.722										
高血圧	0.036	0.483										
冠動脈疾患の有無	0.043	0.402										
喫煙	0.049	0.346										
糖尿病	0.074	0.148										
空腹時血糖	0.122	0.018	0.064	0.215	0.068	0.186	0.067	0.191	0.060	0.246	0.063	0.229
*HbA1c	0.079	0.124										
*HOMA-IR	0.027	0.616										
脂質異常症	0.011	0.830										
LDL-C	-0.107	0.037	-0.061	0.244	-0.038	0.474	-0.044	0.402	-0.077	0.144	-0.061	0.258
HDL-C	-0.067	0.195										
LDL-C/HDL-C	-0.034	0.512										
*TG	-0.037	0.139										
高尿酸血症	-0.064	0.212										
冠危険因子数	0.119	0.022	-0.036	0.563	0.14	0.356	-0.036	0.557	-0.035	0.579	-0.042	0.511
炎症マーカー												
白血球数	-0.033	0.521										
フィブリノゲン	0.071	0.165										
アミロイドA	0.028	0.612										
*CRP	0.136	0.009	0.142	0.007	0.140	0.008	0.141	0.007	0.138	0.009	0.132	0.014
併用薬												
抗血小板剤	0.080	0.181										
RAS阻害剤	0.021	0.686										
βブロッカー	-0.010	0.850										
Ca拮抗薬	-0.015	0.764										
スタチン	-0.001	0.986										
n3-PUFAs 関連因子												
*EPA	-0.057	0.273	—	—	0.049	0.538	-0.206	0.0001	—	—	—	—
*DHA	-0.139	0.007	—	—	-0.243	0.003	—	—	-0.133	0.013		
AA	-0.124	0.016	—	—	—	—	—	—	—	—	-0.087	0.099
*EPA + DHA	-0.107	0.038	-0.183	0.0006	—	—	—	—	—	—	—	—
*EPA/AA	0.005	0.925	—	—	—	—	—	—	—	—	—	—
*DHA/AA	-0.034	0.507	—	—	—	—	—	—	—	—	—	—
*(EPA + DHA) /AA	0.021	0.684	—	—	—	—	—	—	—	—	—	—
腎機能												
eGFR	0.195	0.0002	-0.189	0.0004	-0.192	0.0003	-0.191	0.0004	-0.191	0.0001	-0.192	0.0004

6．FMD などの内皮機能検査の結果と n-3 PUFA との関連を示すこれまでの研究。

　　Flow Mediated Dilation（FMD）が低下し（FMD ≦ 6%）、かつ主要冠動脈近位部に 75%以上の器質的狭窄を有す冠動脈疾患症例 127 例を、従来の治療を継続した群（コントロール群：n ＝ 71）、と従来の治療に高純度 EPA 製剤 1,800 mg/日を追加投与した群（EPA 群：n ＝ 56）に分けて 12 カ月間観察し、前後の諸指標と FMD の変化を検討した。

12 カ月間で総コレステロールと LDL　（low density lipoprotein）コレステロールはコントロール群，EPA 群共に有意に減少したが、トリグリセリドは EPA 群でのみ有意に減少した。血清尿酸値、高感度 CRP（C-reactive protein）値は両群で有意な変化を示さなかった。心拍数と収縮期血圧は両群で有意な変化を示さなかったが、拡張期血圧は EPA 群でのみ有意に低下した。上腕動脈内径や最大拡張到達時間は両群で有意な変化を示さなかった。FMD はコントロール群で 3.29 ± 1.45%から 3.27 ± 1.49%へと有意な変化を示さなかったが、EPA 群では 3.04 ± 1.50%から 5.54 ±2.55%へと有意（p ＜ 0.0001）に増加した。

FMD の低下した冠動脈疾患患者において高純度 EPA 製剤の 12 カ月間の服用で FMD の有意な増加が見られ、EPA の血管内皮機能改善作用が推測された。

コントロール群　　　　　　　　　　　　　　　EPA 群