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Serum long-chain n-3 polyunsaturated fatty acids and aortic calcification in middle-aged men: The population-based cross-sectional ERA-JUMP study

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Abstract *Background and aim:* Few studies have examined the association of long-chain n-3 polyunsaturated fatty acids (LCn-3PUFAs) with the measures of atherosclerosis in the general population. This study aimed to examine the relationship of total LCn-3PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) with aortic calcification.
Methods and results: In a multiethnic population-based cross-sectional study of 998 asymptomatic men aged 40–49 years (300 US-White, 101 US-Black, 287 Japanese American, and 310 Japanese in Japan), we examined the relationship of serum LCn-3PUFAs to aortic calcification (measured by electron-beam computed tomography and quantified using the Agatston method) using Tobit regression and ordinal logistic regression after adjusting for potential confounders. Overall 56.5% participants had an aortic calcification score (AoCaS) > 0. The means (SD) of total LCn-3PUFAs, EPA, and DHA were 5.8% (3.3%), 1.4% (1.3%), and 3.7% (2.1%), respectively. In multivariable-adjusted Tobit regression, a 1-SD increase in total LCn-3PUFAs, EPA, and DHA was associated with 29% (95% CI = 0.51, 1.00), 9% (95% CI = 0.68, 1.23), and 35% (95% CI = 0.46, 0.91) lower

List of abbreviations: ALA, α-linolenic acid; AoCaS, Agatston aortic calcification score; ARA, arachidonic acid; BMI, body mass index; CAC, coronary artery calcification; CI, confidence interval; CIMT, carotid intima-media thickness; CHD, coronary heart disease; CRP, C-reactive protein; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EBCT, Electron beam computed tomography; EPA, eicosapentaenoic acid; HDL-C, high-density lipoprotein cholesterol; LA, linoleic acid; LCn-3PUFAs, long-chain n-3 polyunsaturated fatty acids; LDL-C, low-density lipoprotein cholesterol; RCT, randomized controlled trial; SD, standard deviation; SEM, standard error of mean; TR, tobit ratio.

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AoCaS, respectively. Results were similar in ordinal logistic regression analysis. There was no significant interaction between race/ethnicity and total LCn−3PUFAs, EPA or DHA on aortic calcification.
Conclusions: This study showed the significant inverse association of LCn-3PUFAs with aortic calcification independent of conventional cardiovascular risk factors among men in the general population. This association appeared to be driven by DHA but not EPA.

Introduction

Meta-analyses of prospective observational studies and few randomized controlled trials (RCTs) documented a protective effect of long-chain n-3 polyunsaturated fatty acids (LCn-3PUFAs) [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] on cardiovascular health, particularly lowering the risk of cardiac mortality [1−5]. The beneficial effect of LCn-3PUFAs on cardiac mortality was mainly attributed to their antiarrhythmic activity [2,6]. Other cardiovascular benefits of LCn-3PUFAs include the lowering of triglycerides, blood pressure, resting heart rate, cytokine formation, platelet aggregation, and inflammatory markers; and improving endothelial dysfunction, arterial compliance, and vascular reactivity [2]. It is also speculated that LCn-3PUFAs inhibit the atherosclerosis process (the major underlying pathophysiological process of coronary heart disease (CHD)) [7−10] by lowering inflammation, improving endothelial function, and increasing atherosclerotic plaque stability [3,11−13]. Animal studies [14−16] and basic research [17] strongly support the anti-atherosclerotic properties of LCn-3PUFAs. However, the limited number of studies conducted in a healthy human population reported mixed findings with some documenting no significant association [18−20] and another reporting a significant inverse association [21].

Aortic calcification is a reliable biomarker of generalized atherosclerosis [22] and has a graded and consistent relationship with CHD beyond traditional cardiovascular risk factors [23,24]. Aortic calcification may be a better measure of atherosclerosis than coronary artery calcification (CAC) - a well-established biomarker of atherosclerosis; because it adds valuable prognostic information of cardiovascular risk beyond CAC [25,26]; develops earlier and more extensively than in any other vascular bed [27]; and may have a stronger association with cardiovascular risk factors than CAC [25,26,28]. To our knowledge, however, no previous study has examined the association of serum biomarkers of LCn-3PUFAs and aortic calcification in the general population.

We aimed to explore the relationship of LCn-3 PUFAs to aortic calcification in asymptomatic middle-aged men in the ERA-JUMP Study [the Electron Beam Computed Tomography (**E**BCT), **R**isk-Factor **A**ssessment among **J**apanese and the **U**nited States (US) **M**en in the **P**ost-World-War-II birth cohort]. Based on our previous finding of a significant inverse association of LCn-3PUFAs with CAC and carotid intima-media thickness (CIMT) [21]; and a differential significant association of DHA compared to EPA with endothelial dysfunction (a precursor of atherosclerosis) in mechanistic studies [3], we hypothesized that serum total LCn-3PUFAs especially DHA but not EPA would have a significant inverse association with aortic calcification. It is important to examine the relationship between LCn-3PUFAs and atherosclerosis in the general population to gain further insight into the relationship between LCn-3PUFAs and cardiovascular disease (CVD) outcomes.

Methods

Participants

The ERA-JUMP Study is a population-based study of 1033 men aged 40−49 years comprising US-White, US-Black, Japanese American, and Japanese in Japan. The details of the study protocol have been described previously [29]. Briefly, during 2002−2006, a population-based sample of 1033 men aged 40−49 years, without clinical CVD or other severe illnesses, was obtained from 3 centers: 310 White and 107 Black from Pittsburgh, Pennsylvania, US; 303 Japanese American from Honolulu, Hawaii, US; 313 Japanese from Kusatsu City, Shiga, Japan. The study protocol complied with the Helsinki Declaration as revised in 1983. We obtained study approvals from the Institutional Review Boards of University of Pittsburgh, Pittsburgh, US; Kuakini Medical Center, Honolulu, US; Shiga University of Medical Science, Otsu, Japan. All participants gave written informed consent. We excluded participants with missing data for aortic calcification (n = 27) and LCn-3PUFAs (n = 8). Our final sample size was 998 with 300 US-White, 101 US-Black, 287 Japanese American, and 310 Japanese in Japan.

Risk factor assessment

As published elsewhere, participants underwent a physical examination, completed a set of lifestyle questionnaire,

and a laboratory assessment [30,31]. Body weight and height were measured while the participant was wearing minimal light clothing without shoes. Participants with systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or use of antihypertensive medications were considered hypertensive. Medication use (antihypertensive, antidiabetic, or lipid-lowering) was reported as 'yes/no'. Participants were classified as smokers if they reported current use of cigarettes or having stopped smoking within the past 30 days. The formula used to calculate pack-years of smoking was 'years of smoking multiplied by the number of cigarettes per day divided by 20'. Meat intake was defined as individuals who ate beef, pork, or sausage ≥2 times per week. Self-reported physical activity related to the current job was categorized into sedentary, light, medium, and heavy physical activity.

Venipuncture was performed early in the clinic visit after a 12-h fast. Blood samples were stored at −70 °C and shipped on dry ice from all the centers to the University of Pittsburgh. Serum/plasma samples were assayed for glucose, lipids [including total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)], fibrinogen, and C-reactive protein (CRP) as described previously [32]. Participants with fasting glucose ≥7.0 mmol/L or using medications for diabetes were recorded as diabetics.

n-3 and n-6 polyunsaturated fatty acids assessment

Serum levels of n-3 PUFAs [EPA (20:5n-3), docosapentaenoic acid (DPA (22:5n-3)), DHA (22:6n-3), and α-linolenic acid (ALA (20:3n-3), and n-6 PUFAs [linoleic (LA (18:2n-6)) and arachidonic (ARA (20:4n-6)) acids] were measured using Capillary Gas-Liquid chromatography [29]. Serum fatty acids were reported as weight percentages of total fatty acids. The coefficients of variation between tests in the ERA-JUMP study for EPA, DPA, DHA, ALA, LA, ARA, and total fatty acids were 4.5%, 4.5%, 7.2%, 1.6%, 7.9%, 2.8%, and 5.7%, respectively. Total LCn-3PUFAs was defined as the sum of EPA, DPA, and DHA.

Aortic calcification assessment

To evaluate aortic calcification, 6 mm EBCT images using a GE-Imatron C150 scanner (GE Medical Systems, South San Francisco, US) [32,33], were acquired from the aortic arch to the iliac bifurcation. Readings of the scans from all centers were performed centrally at the Cardiovascular Institute, University of Pittsburgh, using a DICOM (Digital Imaging and Communications in Medicine) workstation and software by AccuImage (AccuImage Diagnostic Cooperation, San Francisco, US). The software program implements the widely accepted Agatston scoring method [34]. Trained radiology technicians-blinded to each participant's characteristics and to the study centers, evaluated the readings. The reproducibility of non-zero Agatston aortic calcification score (AoCaS) had an intra-class correlation of 0.98.

Statistical analysis

Continuous variables with approximately normal distribution: total LCn-3PUFAs, EPA, DHA, ALA, LA, ARA, age, BMI, LDL-C, and HDL-C were standardized. Distribution of AoCaS was highly skewed, therefore we created four categories of AoCaS: 0, 1−99, 100−299, and ≥300. We also created race/ethnicity-specific quartiles of total LCn-3PUFAs, EPA, and DHA. Across different quartiles of total LCn-3PUFAs as well as categories of AoCaS (i) age and race/ethnicity adjusted BMI, LDL-C, and HDL-C were expressed as means ± standard error of mean (SEM); (ii) age and race/ethnicity adjusted triglycerides, years of education, and the pack-years of smoking were expressed as medians and interquartile range; (iii) age and race/ethnicity adjusted categorical variables were expressed in percentages. *P*-values for trend across different quartiles of total LCn-3PUFAs and AoCaS were determined using: linear regression when a response variable was continuous with a normal distribution; quartile regression when a response variable was continuous with skewed distribution; and logistic regression when a response variable was categorical.

We used Tobit conditional regression to determine the independent association of total LCn-3PUFAs, EPA, or DHA with aortic calcification adjusting for potential confounders. For Tobit regression, outcome variable AoCaS was log transformed after the addition of one unit [natural log of (AoCaS + 1)]. We considered Tobit regression because it is suited to the uncommon distribution of AoCaS (right-sided skewness and many participants with zero AoCaS) [35,36]. Secondarily, we performed ordinal logistic regression to assess the likelihood of study participants being in a higher category of AoCaS. We used the Brant test to evaluate the proportional odds assumption of ordinal regression. For both Tobit regression and ordinal regression: Model I was adjusted for socio-demographic variables (age, race/ethnicity, and years of education); Model II was further adjusted for potential confounders (pack-years of smoking, alcohol consumption, BMI, diabetes, lipid-lowering medications, LDL-C, physical activity related to current job, and meat intake); In model II, we tested for an interaction between race/ethnicity and total LCn-3PUFAs (or EPA or DHA) on aortic calcification. In model III, we additionally adjusted for intermediary variables (hypertension, HDL-C, triglycerides, CRP, and fibrinogen) in the relationship between LCn-3PUFAs and atherosclerosis/CHD. In regression models, we treated total LCn-3PUFAs, EPA, and DHA as categorical variables (race/ethnicity-specific quartiles) or continuous variables, separately. In a supplementary analysis, we assessed the independent association of ALA, LA, and ARA using similar regression techniques and models as mentioned above. The inclusion of variables in the regression models was mainly based on previously published literature on the relationship of LCn-3PUFAs to atherosclerosis/CHD. In Tobit regression and in ordinal regression, a *p*-value for

linear trend across the quartiles of LCn-3PUFAs was calculated using contrast.

Sensitivity analyses include: (i) Excluding Japanese study participants, as serum median levels of LCn-3PUFAs among Japanese in Japan were ≥2 times higher than in the other study participants [29]; (ii) Stratifying the analysis by race/ethnicity. In the race/ethnicity stratified analysis, we treated total LCn-3PUFAs, EPA, and DHA as continuous variables only. All *p*-values were two-tailed and a *p*-value <0.05 was considered as significant. SAS version 9.4 (SAS Institute, Cary, NC, US) and STATA version 14.0 (StataCorp LP, College Station, TX, US) were used for all statistical analyses.

Results

Overall mean (SD) age of study participants was 45.3 (2.8) years. The mean (SD) of LCn-3PUFAs (%) among individual race/ethnicity was: US-White 3.8 (1.7), US-Black 3.8 (1.5), Japanese American 4.8 (2.2), and Japanese in Japan 9.3 (3.0). Except for serum levels of LDL-C and HDL-C, there was a significant decreasing trend in age and race/ethnicity-adjusted: BMI, triglycerides, the proportion with diabetes, CRP, and AoCaS with increasing in quartile of total LCn-3PUFAs (Table 1).

Total number (%) of participants with AoCaS >0 was 564 (56.5): US-White 206 (68.7), US-Black 70 (69.3), Japanese American 177 (61.7), and Japanese in Japan 111 (35.8). Except for HDL-C, total LCn-3PUFAs, EPA, and DHA, participants with AoCaS >0 had a higher age and race/ethnicity-adjusted: BMI, pack-years of smoking, LDL-C, triglycerides, CRP, and fibrinogen; had a greater proportion with diabetes and hypertension; and were more likely to be on lipid-lowering medications compared to zero AoCaS category (Table 2).

In Tobit regression, participants in the fourth quartile compared to the first quartile of total LCn-3PUFAs had a 49% lower AoCaS [Model II: Tobit ratio (TR) (95% CI) = 0.51 (0.26, 0.97)] (Table 3). After further adjustment for intermediary variables, this significant inverse association was attenuated and became nonsignificant [Model III: TR (95% CI) = 0.55 (0.28, 1.08)]. In model II, a 1-SD (3.3%) increase in total LCn-3PUFAs was associated with a 29% lower AoCaS [TR (95% CI) = 0.71 (0.51, 1.00)]. EPA (either categorical or continuous variable) was not significantly associated with aortic calcification. In model II, a 1-SD increase in EPA (1.3%) was associated with a 9% lower AoCaS [TR (95% CI) = 0.91 (0.68, 1.23)]. Participants in the fourth quartile of DHA compared to the first quartile had significantly lower AoCaS in all models− from unadjusted to fully adjusted model III. There was a significant dose−response relationship between DHA and aortic calcification (*p* for linear trend <0.05). In model II, a 1-SD (2.1%) increase in DHA was associated with a 35% lower AoCaS [TR (95% CI) = 0.65 (0.46, 0.91)]. This significant inverse association remained after further adjustment for intermediary variables [Model III: TR (95% CI) = 0.69 (0.49, 0.98)] (Table 3) and ALA, LA, and ARA [TR (95% CI) = 0.68 (0.47, 0.98)] (Supplementary

Table 1 Age and race/ethnicity adjusted demographic and clinical characteristics by race/ethnicity specific quartiles of total LC n-3 PUFAs for the ERA JUMP Study, 2002−2006 (n = 998).

Total LCn-3PUFAs quartiles	Q1	Q2	Q3	Q4	*p*-trend[d]
Total number (%)	251 (25.3)	249 (24.9)	249 (24.9)	249 (24.9)	–
LCn-3PUFAs (%)[b]	2.7 (2.3, 5.0)	3.8 (3.1, 7.7)	4.9 (4.0, 9.0)	7.3 (5.9, 11.8)	–
Age[a] (years)	45.1 (0.2)	45.5 (0.2)	45.0 (0.2)	45.8 (0.2)	0.01
BMI[a] (kg/m^2)	28.3 (0.3)	28.3 (0.3)	27.7 (0.3)	27.1 (0.3)	0.01
Pack-years of smoking[b]	0.0 (0.0, 6.2)	0.0 (0.0, 2.7)	0.0 (0.0, 2.7)	0.0 (0.0, 2.6)	0.10
Alcohol[b] (gm/day)	2.8 (1.0, 10.8)	5.1 (1.0, 16.8)	6.1 (1.0, 16.1)	5.6 (1.0, 16.3)	0.20
LDL-C[a] (mg/dL)	131.3 (2.8)	132.5 (2.8)	135.9 (2.8)	140.6 (2.8)	0.01
HDL-C[a] (mg/dL)	44.7 (1.1)	48.8 (1.1)	48.5 (1.1)	49.5 (1.1)	0.01
Triglycerides[b] (mg/dL)	154.1 (104.2, 214.3)	126.1 (93.2, 181.4)	122.1 (90.2, 158.1)	120.8 (82.2, 163.0)	0.01
Hypertension[c]	40 (15.6)	37 (14.8)	32 (12.9)	35 (13.9)	0.39
Diabetes[c]	12 (4.8)	9 (3.4)	6 (2.3)	4 (1.7)	0.01
Anti-lipid med[c]	30 (11.8)	32 (12.6)	35 (13.8)	26 (10.2)	0.68
Meat intake[c]	210 (83.4)	201 (80.5)	194 (77.9)	166 (66.7)	0.01
Years of education[b]	17.0 (16.0, 18.0)	16.0 (16.0, 18.0)	17.0 (16.0, 18.0)	17.0 (16.0, 18.0)	0.32
CRP[b], (mg/dL)	1.1 (0.6, 1.8)	1.0 (0.5, 1.9)	1.0 (0.5, 1.6)	0.9 (0.5, 1.7)	0.05
Fibrinogen[a], (mg/dL)	290.5 (5.6)	292.7 (5.6)	288.2 (5.7)	294.8 (5.6)	0.66
AoCaS[b]	9.2 (0.0, 91.7)	8.7 (0.0, 67.0)	8.2 (0.0, 67.0)	6.7 (0.0, 57.0)	0.35

Q1, Q2, Q3, Q4: race/ethnicity specific quartiles of total LCn-3PUFAs; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; AoCaS, aortic calcification score; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; Total LCn-3PUFAs (%), total long-chain n-3 polyunsaturated fatty acids defined as sum of EPA, DPA, and DHA; n(%): number(%).

Values for all variables except age and LCn-3PUFAs were adjusted for 'age' and 'race/ethnicity': value of age was fixed at 45.3 years and race/ethnicity was fixed as 'US-White'.

SI conversion factors: To convert LDL-C and HDL-C to mmol/L, multiply values by 0.0259. To convert triglycerides to mmol/L, multiply values by 0.01129. To convert fibrinogen to μmol/L, multiply values by 0.0294. To convert CRP to nmol/L, multiply values by 9.524.

[a] Continuous normally distributed variables were expressed as mean (standard error).

[b] Continuous variables with skewed distribution were expressed as median (inter-quartile range).

[c] Categorical variables were expressed as numbers (%).

[d] *p*-trend shows a *p*-value for linear trend across the quartiles of total LCn-3PUFAs.

Table 2 Age and race/ethnicity adjusted demographic and clinical characteristics by AoCaS categories for the ERA-JUMP Study, 2002–2006 (n = 998).

AoCaS categories	AoCaS = 0	AoCaS 0 - 99	AoCaS 100-299	AoCaS ≥300	*p*-trend[d]
Total number (%)	434 (43.5)	367 (36.8)	89 (8.9)	108 (10.8)	–
Age[a] (years)	44.8 (0.1)	45.4 (0.2)	46.2 (0.3)	46.3 (0.3)	0.01
BMI[a] (kg/m^2)	26.2 (0.3)	28.7 (0.3)	29.0 (0.5)	27.3 (0.5)	0.01
Pack-years of smoking[b]	0.0 (0.0, 0.9)	0.0 (0.0, 1.0)	0.0 (0.0, 12.8)	11.0 (0.8, 19.7)	0.01
Alcohol[b] (gm/day)	5.0 (1.0, 15.9)	3.6 (1.0, 13.9)	3.5 (1.0, 11.5)	24.5 (2.1, 35.0)	0.01
LDL-C[a] (mg/dL)	130.6 (2.6)	137.9 (2.3)	131.8 (4.1)	138.2 (3.8)	0.16
HDL-C[a] (mg/dL)	50.4 (1.0)	46.3 (0.9)	46.2 (1.5)	49.8 (1.5)	0.71
Triglycerides[b] (mg/dL)	114.7 (80.7, 154.4)	130.7 (95.7, 184.9)	148.7 (105.7, 207.9)	138.7 (95.7, 240.5)	0.01
Hypertension[c]	42 (9.5)	59 (16.1)	18 (19.9)	17 (15.2)	0.02
Diabetes[c]	9 (2.1)	10 (2.7)	6 (6.5)	5 (4.6)	0.01
Anti-lipid med[c]	39 (9.0)	41 (11.0)	23 (25.8)	16 (14.5)	0.01
Meat intake[c]	324 (74.7)	287 (78.0)	72 (80.0)	83 (76.4)	0.61
Years of education[b]	17.0 (16.0, 18.0)	17.0 (16.0, 18.0)	16.0 (16.0, 18.0)	16.0 (16.0, 18.0)	0.01
CRP[b] (mg/dL)	0.8 (0.4, 1.6)	1.0 (0.6, 1.9)	1.0 (0.5, 2.0)	1.0 (0.5, 1.9)	0.05
Fibrinogen[a] (mg/dL)	284.6 (5.3)	293.0 (4.8)	300.0 (8.2)	298.6 (7.7)	0.05
Total LCn-3PUFAs[a] (%)	4.2 (0.2)	3.7 (0.2)	3.6 (0.3)	3.7 (0.3)	0.08
EPA[a] (%)	0.9 (0.1)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.74
DHA[a] (%)	2.6 (0.1)	2.3 (0.1)	2.2 (0.2)	2.3 (0.2)	0.01
ALA[a] (%)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.63
LA[a] (%)	30.3 (0.3)	30.0 (0.3)	29.2 (0.5)	28.9 (0.5)	0.01
ARA[a] (%)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.3 (0.0)	0.17

AoCaS, aortic calcification score; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acids; Total LCn-3PUFAs (%), total long chain omega 3 polyunsaturated fatty acids defined as sum of EPA, DPA, and DHA; n(%), number(%).

Values for all variables except age were adjusted for 'age' and 'race/ethnicity': value of age was fixed at 45.3 years and race/ethnicity was fixed as 'US-White'.

SI conversion factors: To convert LDL-C and HDL-C to mmol/L, multiply values by 0.0259. To convert triglycerides to mmol/L, multiply values by 0.01129. To convert fibrinogen to μmol/L, multiply values by 0.0294. To convert CRP to nmol/L, multiply values by 9.524.

[a] Continuous normally distributed variables were expressed as mean (standard error).

[b] Continuous variables with skewed distribution were expressed as median (inter-quartile range).

[c] Categorical variables were expressed as numbers (%).

[d] *p*-trend shows a *p*-value for linear trend across the AoCaS categories.

Table 2-B). When the analysis was repeated using ordinal regression, the findings remained materially the same. There was no significant interaction between race/ethnicity and total LCn−3PUFAs, EPA, DHA, ARA, ALA, or LA on aortic calcification.

In model II, ARA but not ALA or LA had a significant inverse association with aortic calcification [ARA: TR (95% CI) = 0.64 (0.48, 0.86); OR (95% CI) = 0.81 (0.70, 0.95)] (Supplementary Table 2-A). In a sensitivity analysis excluding Japanese in Japan, results were similar to the analysis for all study participants; A 1-SD increase in total LCn-3PUFAs (1.9%) and DHA (1.3%) but not EPA (0.7%) was significantly and inversely associated with aortic calcification (Supplementary table 3).

In a race/ethnicity-stratified analysis, in US-White, in multivariable-adjusted Tobit regression, there was a significant inverse association of total LCn-3PUFAs [Model II: TR (95% CI) = 0.48 (0.25, 0.92)] and DHA [Model II: TR (95% CI) = 0.54 (0.31, 0.94)] but not of EPA [Model II: TR (95% CI) = 0.57 (0.26, 1.26)] with aortic calcification. In US-Black, Japanese in Japan, and Japanese American, total LCn-3PUFAs and DHA were inversely and nonsignificantly associated with aortic calcification. When the analysis was repeated using ordinal regression, the findings remained materially the same (Table 4).

Discussion

In this community-based sample of asymptomatic middle-aged men, blood levels of total LCn-3PUFAs were significantly and inversely associated with aortic calcification independent of cardiovascular risk factors; This significant inverse association appeared to be driven by DHA. DHA was significantly and inversely associated with aortic calcification after adjustment for conventional cardiovascular risk factors and other fatty acids including ALA, LA, and ARA. There was no significant interaction between race/ethnicity and total LCn−3PUFAs, EPA or DHA on aortic calcification. To our knowledge, this is the first community-based study examining the relationship between blood biomarkers of LCn-3PUFAs and aortic calcification in asymptomatic middle-aged men across different races/ethnicities from two countries in a standardized manner.

In contrast to our findings, He et al. in the Multi-Ethnic Study of Atherosclerosis [18] of 5488 healthy US adults from four different races/ethnicities, aged 45–84 years without clinical CVD and Heine-Broring et al. in the Rotterdam Study [19] of 1570 asymptomatic participants aged >55 years reported no significant association of dietary intake of LCn-3PUFAs with CAC as measured by EBCT.

Table 3 Association between LCn-3PUFAs and aortic calcification: Tobit ratio/odds ratio (95% confidence interval) for AoCaS by the quartiles and per 1-SD of total LCn-3PUFAs/EPA/DHA (n = 998).

LCn-3PUFAs quartiles	Q1	Q2	Q3	Q4		LCn-3PUFAs (per SD change)
			Tobit Regression			
	TR	TR (95% CI)	TR (95% CI)	TR (95% CI)	*p*-trend[a]	TR (95% CI)
Total LCn-3PUFAs						
Model I	1	0.74 (0.38, 1.46)	0.73 (0.37, 1.44)	0.38 (0.19, 0.75)	0.01	0.62 (0.43, 0.88)
Model II	1	0.85 (0.45, 1.60)	0.91 (0.48, 1.72)	0.51 (0.26, 0.97)	0.06	0.71 (0.51, 1.00)[b]
Model III	1	0.87 (0.46, 1.65)	1.03 (0.54, 1.98)	0.55 (0.28, 1.08)	0.14	0.76 (0.54, 1.08)
EPA						
Model I	1	0.59 (0.30, 1.17)	0.64 (0.32, 1.28)	0.57 (0.29, 1.12)	0.14	0.92 (0.67, 1.26)
Model II	1	0.60 (0.32, 1.13)	0.59 (0.31, 1.11)	0.58 (0.30, 1.11)	0.10	0.91 (0.68, 1.23)
Model III	1	0.61 (0.32, 1.15)	0.65 (0.34, 1.24)	0.64 (0.33, 1.24)	0.22	0.96 (0.71, 1.29)
DHA						
Model I	1	0.80 (0.41, 1.58)	0.59 (0.30, 1.17)	0.30 (0.15, 0.60)	0.01	0.52 (0.36,0.74)
Model II	1	0.85 (0.46, 1.60)	0.74 (0.39, 1.40)	0.43 (0.22, 0.83)	0.01	0.65 (0.46, 0.91)
Model III	1	0.86 (0.45, 1.62)	0.83 (0.43, 1.57)	0.47 (0.24, 0.93)	0.03	0.69 (0.49, 0.98)
			Ordinal Logistic Regression[c]			
	OR	OR (95% CI)	OR (95% CI)	OR (95% CI)	*p*-trend[a]	OR (95% CI)
Total LCn-3PUFAs						
Model I	1	0.86 (0.62, 1.20)	0.85 (0.61, 1.19)	0.61 (0.43, 0.85)	0.83	0.77 (0.65, 0.92)
Model II	1	0.91 (0.65, 1.29)	0.95 (0.67, 1.35)	0.69 (0.48, 0.99)	0.93	0.84 (0.70, 1.01)[d]
Model III	1	0.94 (0.66, 1.33)	1.04 (0.73, 1.48)	0.74 (0.52, 1.07)	0.81	0.88 (0.73, 1.06)
EPA						
Model I	1	0.78 (0.56, 1.09)	0.82 (0.59, 1.14)	0.72 (0.52, 1.01)	0.25	0.94 (0.81, 1.09)
Model II	1	0.78 (0.55, 1.10)	0.76 (0.54, 1.07)	0.73 (0.51, 1.04)	0.19	0.95 (0.81, 1.12)
Model III	1	0.78 (0.55, 1.11)	0.80 (0.56, 1.14)	0.78 (0.55, 1.12)	0.21	0.98 (0.84, 1.16)
DHA						
Model I	1	0.91 (0.65, 1.26)	0.78 (0.56, 1.08)	0.55 (0.40, 0.78)	0.93	0.72 (0.60, 0.86)
Model II	1	0.94 (0.67, 1.33)	0.87 (0.62, 1.23)	0.65 (0.45, 0.92)	0.83	0.80 (0.67, 0.97)
Model III	1	0.95 (0.67, 1.35)	0.93 (0.65, 1.32)	0.70 (0.49, 1.01)	0.82	0.84 (0.70, 1.02)

AoCaS, aortic calcification score; Q1, Q2, Q3, Q4: race/ethnicity specific quartiles of LCn-3PUFAs/EPA/DHA; IQR, interquartile range; TR, Tobit ratio; OR, odds ratio; CI: confidence interval; SD: standard deviation; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; Total LCn-3PUFAs, total long chain omega 3 polyunsaturated fatty acids defined as the sum of EPA, DPA, and DHA.

For *total LCn-3PUFAS (%)*, median (IQR) for Q1, Q2, Q3, and Q4 equals to 2.7 (2.3, 5.0), 3.8 (3.1, 7.7), 4.9 (4.0, 9.0), and 7.3 (5.9, 11.8) respectively; For *EPA (%)*, median (IQR) for Q1, Q2, Q3, and Q4 equals to 0.4 (0.4, 0.9), 0.6 (0.6, 1.8), 0.9 (0.7, 2.4), and 1.9 (1.2, 3.6) respectively; For *DHA (%)*, median (IQR) for Q1, Q2, Q3, and Q4 equals to 1.6 (1.3, 3.4), 2.5 (1.8, 4.9), 3.2 (2.6, 5.9), and 4.9 (4.0, 7.3) respectively; One SD of total LCn-3PUFAs, EPA, and DHA equals to 3.3%, 1.3%, and 2.1% respectively.

Model I: Fatty acids (Total LCn-3PUFAs or EPA or DHA), age, race/ethnicity, years of education.

Model II: Model I + pack-years of smoking, alcohol consumption, BMI, diabetes, lipid-lowering medications, LDL-C, physical activity at the job, and meat intake.

Model III: Model II + hypertension, HDL-C, triglycerides, CRP, and fibrinogen.

[a] *p*-trend shows a *p*-value for linear trend across the quartiles of total LCn-3PUFAs/EPA/DHA calculated using contrast.

[b] Tobit ratio of 0.71 means that with every SD (3.3%) increase in total LCn-3PUFAs, there is a 29% decrease in the AoCaS.

[c] For ordinal logistic regression, AoCaS categories used were 0, 1–99, 100–299, and ≥300.

[d] OR of 0.84 means that a 1-SD (3.3%) increase in total LCn-3PUFAs made a participant in this study 16% less likely to be in a higher AoCaS category.

Similarly, Shang et al. in the Melbourne Collaborative Cohort Study of 312 asymptomatic participants aged 45–64 years reported no significant association between dietary intake of LCn-3PUFAs and aortic calcification as measured by lateral thoracolumbar radiography and dual-energy X-ray absorptiometry [20]. Several plausible explanations for the contrasting findings between these studies [18–20] and ours may include differences in the age distribution, subclinical atherosclerosis assessment techniques, vascular bed examined, and the use of blood biomarkers of LCn-3PUFAs in our study as opposed to self-reported dietary assessment of fatty acids intake-which may lead to LCn-3PUFAs misclassification [2].

In our study, the association of total LCn-3PUFAs, EPA, and DHA with aortic calcification was partly attenuated after adjusting for intermediary variables suggesting that the relationship was partly mediated through the effects of LCn-3PUFAs on blood pressure, lipids, and inflammation. Atherosclerosis is a systemic chronic inflammatory disease of the vessel walls. Inflammation resulting from the interaction of modified atherogenic lipoproteins, inflammatory cells, and smooth muscle cells of vessel wall plays a major role in the initiation and progression of atherosclerotic plaque. Available evidence from epidemiological and experimental studies suggest that LCn-3PUFAs exerts their antiatherosclerotic effect through several anti-inflammatory pathways including lowering expression of nuclear factor κ-B, regulators of inflammation, and oxidative stress, improving endothelial function, and increasing atherosclerotic plaque stability [3,10–13].

Table 4 Association between LCn-3PUFAs and aortic calcification by races/ethnicities: Tobit ratio/odds ratio (95% confidence interval) for AoCaS per 1-SD of total LCn-3PUFAs/EPA/DHA.

Races/Ethnicities	Regression Technique	Regression Models	Polyunsaturated Fatty Acids		
			Total LCn-3PUFAs	EPA	DHA
US-White (n = 300)	Tobit Regression	Model I	0.34 (0.18, 0.65)	0.42 (0.19, 0.93)	0.40 (0.23, 0.71)
		Model II	0.48 (0.25, 0.92)[b]	0.57 (0.26, 1.26)	0.54 (0.31, 0.94)
		Model III	0.43 (0.22, 0.86)	0.60 (0.27, 1.34)	0.47 (0.26, 0.85)
	Ordinal Logistic Regression[a]	Model I	0.48 (0.31, 0.74)	0.56 (0.33, 0.94)	0.54 (0.37, 0.78)
		Model II	0.58 (0.36, 0.92)[c]	0.66 (0.37, 1.16)	0.63 (0.43, 0.93)
		Model III	0.60 (0.35, 0.94)	0.70 (0.40, 1.28)	0.61 (0.40, 0.94)
US-Black (n = 101)	Tobit Regression	Model I	0.27 (0.07, 1.06)	0.34 (0.06, 1.85)	0.31 (0.10, 0.95)
		Model II	0.42 (0.11, 1.56)	0.56 (0.10, 3.09)	0.45 (0.16, 1.28)
		Model III	0.35 (0.09, 1.29)	0.44 (0.08, 2.50)	0.41 (0.14, 1.20)
	Ordinal Logistic Regression	Model I	0.52 (0.22, 1.19)	0.56 (0.21, 1.52)	0.54 (0.26, 1.11)
		Model II	0.62 (0.24, 1.60)	0.75 (0.22, 2.52)	0.62 (0.29, 1.33)
		Model III	0.54 (0.20, 1.45)	0.63 (0.17, 2.29)	0.59 (0.26, 1.32)
Japanese American (n = 287)	Tobit Regression	Model I	0.54 (0.27, 1.03)	1.25 (0.67, 2.32)	0.47 (0.25, 0.90)
		Model II	0.72 (0.38, 1.35)	0.92 (0.51, 1.64)	0.66 (0.35, 1.24)
		Model III	0.71 (0.38, 1.33)	0.85 (0.48, 1.51)	0.68 (0.36, 1.27)
	Ordinal Logistic Regression	Model I	0.73 (0.51, 1.03)	0.90 (0.65, 1.23)	0.69 (0.49, 0.96)
		Model II	0.83 (0.58, 1.19)	0.94 (0.67, 1.30)	0.81 (0.57, 1.15)
		Model III	0.83 (0.58, 1.19)	0.90 (0.65, 1.26)	0.82 (0.57, 1.18)
Japanese in Japan (n = 310)	Tobit Regression	Model I	0.88 (0.41, 1.90)	1.10 (0.60, 2.01)	0.73 (0.31, 1.71)
		Model II	0.82 (0.40, 1.68)	0.94 (0.53, 1.65)	0.75 (0.34, 1.64)
		Model III	0.95 (0.46, 1.96)	1.08 (0.61, 1.92)	0.86 (0.38, 1.91)
	Ordinal Logistic Regression	Model I	0.96 (0.75, 1.23)	1.02 (0.83, 1.24)	0.93 (0.70, 1.22)
		Model II	0.96 (0.72, 1.27)	0.98 (0.79, 1.23)	0.94 (0.69, 1.28)
		Model III	1.02 (0.75, 1.34)	1.04 (0.83, 1.31)	0.98 (0.71, 1.34)

AoCaS, aortic calcification score; IQR, interquartile range; TR, Tobit ratio; OR, Odds ratio; CI: confidence interval; SD: standard deviation; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; Total LCn-3PUFAs, total long chain omega 3 polyunsaturated fatty acids defined as the sum of EPA, DPA, and DHA.
For US-White, a 1-SD of total LCn-3PUFAs, EPA, and DHA equals to 1.7%, 0.5%, and 1.2%, respectively.
For US-Black, a 1-SD of total LCn-3PUFAs, EPA, and DHA equals to 1.5%, 0.5%, 1.1%, respectively.
For Japanese in Japan, a 1-SD of total LCn-3PUFAs, EPA, and DHA equals to 3%, 1.4%, and 1.7%, respectively.
For Japanese American, a 1-SD of total LCn-3PUFAs, EPA, and DHA equals to 2.2%, 0.9%, and 1.4%, respectively.
Model I: Fatty acids (Total LCn-3PUFAs or EPA or DHA), age, and years of education.
Model II: Model I + pack-years of smoking, alcohol consumption, BMI, diabetes, lipid-lowering medications, LDL-C, physical activity at the job, and meat intake.
Model III: Model II + hypertension, HDL-C, triglycerides, CRP, and fibrinogen.
[a] For ordinal logistic regression, four AoCaS categories used were 0, 1–99, 100–299, and ≥300.
[b] Tobit ratio of 0.48 means that in US-White for every SD (1.7%) increase in total LCn-3PUFAs, there is a 52% decrease in the AoCaS.
[c] OR of 0.58 means that a 1-SD (1.7%) increase in total LCn-3PUFAs made a US-White participant in this study 42% less likely to be in a higher AoCaS category.

Our study shows a significant inverse association of DHA but not of EPA with aortic calcification. This finding concords with our previous observation of a significant inverse association of DHA but not of EPA with CIMT [21] as well as the results of a prospective cohort study among postmenopausal women with CHD where DHA but not EPA was significantly associated with less progression of coronary atherosclerosis [37]. Additionally, indirect evidence from short-term clinical trials in humans reported that DHA compared to EPA was more potent in lowering blood pressure [38,39], resting heart rate [40], the expression of pro-inflammatory cytokines, cell adhesion molecules, and monocyte adhesion to endothelial cells [41].

To date, no RCT has assessed the effect of pure DHA or compared the effect of pure DHA with EPA on cardiovascular outcomes or atherosclerosis. RCTs of pure EPA conducted among diabetics [8], patients with stable angina [9], and CHD [7] reported an inverse association of EPA with atherosclerosis. The REDUCE-IT trial of pure EPA (at a dose of 4.0 g per day) showed a significant 25% relative risk reduction in CVD outcome among 8000 statin-treated patients with CHD or diabetes [5]. The VITAL trial of a fish-oil capsule containing 840 mg of LCn-3PUFAs (460 mg of EPA + 380 mg of DHA) among men ≥50 years and women ≥55 years in the US reported a 28% hazard ratio reduction in MI [42]. The RCTs in diabetics [8] and CHD patients [7] reported an increase in serum EPA but not in DHA (although EPA theoretically can be metabolized to DHA) among intervention groups supporting an anti-atherogenic effect for EPA. The significant inverse association of DHA but not of EPA with aortic calcification in our study may imply that DHA may be more antiatherogenic than EPA. RCTs are warranted to disentangle the differential association of EPA and DHA with cardiovascular outcomes and atherosclerosis.

In our study, in US-White, total LCn-3PUFAs and DHA were significantly and inversely associated with aortic

calcification but not in US-Black, Japanese American or Japanese in Japan. This finding suggests that US-White may respond differently to LCn-3PUFAs compared to other races/ethnicities (although there was no significant interaction between race/ethnicity and LCn−3PUFAs on aortic calcification). The differential association of LCn-3PUFAs by races/ethnicities with aortic calcification in this study may be a random finding due to cross-sectional study design and small sample size. The lack of association in Japanese in Japan could be due to saturation in a dose−response curve due to a large fish intake resulting in high levels of blood LCn-3PUFAs [43].

Our study also shows a significant inverse association of ARA with aortic calcification. This finding concords with findings reported in a meta-analysis of prospective observational studies and RCTs [44]. Although ARA-derived eicosanoids such as leukotriene-B4 and thromboxane-A2, are thought to be pro-inflammatory, other ARA-derived eicosanoids such as epoxyeicosatrienoic acid and lipoxins are considered to lower inflammation [45]. The anti-inflammatory mechanism of ARA is also supported by animal studies and observational studies in humans showing an inverse association of ARA metabolites with cardiovascular risk [2].

Our study has several limitations. First, the serum fatty acids composition has a lot of day-to-day variations and reflect recent intake of fat (and may not reflect long-term dietary intake) - and thus more likely to lead to misclassification bias than more stable markers of intake such as red blood cell fatty acids or adipose tissue fatty acids; As the serum levels of LCn-3PUFAs vary randomly, the actual association between blood levels of LCn-3PUFAs and aortic calcification may be stronger than reported in the current study. Second, we examined healthy men aged 40−49 years in Japan and the US; therefore, the results of the study cannot be generalized to females, other populations, or age groups. Third, although we have controlled for a variety of sociodemographic and clinical characteristics, the possibility of residual confounding cannot be excluded; for example, we adjusted for physical activity related to the current job and not the physical activity during leisure time. Fourth, we cannot establish a causal association (temporality) between blood levels of LCn-3PUFAs and aortic calcification based on the cross-sectional analysis. However, it is unlikely that symptoms of atherosclerosis have led to exposure to LCn-3PUFAs.

Strengths of the current study include: (i) the community-based nature of the study design with randomly selected study participants-increasing the external validity of the study findings; (ii) Standardized measurement techniques across all study-centers; (iii) The use of EBCT to detect aortic calcification, allowing the accurate visualization of small calcific deposits without image blurring; (iv) The objective measurements of exposure to LCn−3PUFAs and the radiology technicians were blinded to participant's exposure to LCn−3PUFAs limits the differential misclassification of the outcome. Also, serum levels of LCn−3PUFAs are a short-term biomarker of intake of LCn−3PUFAs and metabolism. Thus, levels of LCn−3PUFAs reflect endogenous exposure to LCn−3PUFAs and therefore presumably a more biologically relevant measure of exposure to LCn−3PUFAs than the intake of LCn−3PUFAs.

Our study findings have public health significance. All over the world, the commercialization of the use of fish oil or LCn-3PUFAs is rapidly expanding. This growing enthusiasm needs to be supported by the robust scientific data on intake of LCn-3PUFAs. Evidence concerning LCn-3PUFAs and atherosclerosis is limited in the general population. Evidence generated from this study adds to the evidence on the anti-atherosclerotic property of LCn-3PUFAs especially DHA in healthy middle-aged men. The findings of this study, if replicated in larger and longer follow-up studies, would help support intake of LCn-3PUFAs policy in the general population.

Conclusions

Our study demonstrated for the first time that in the general male population, LCn-3PUFAs were significantly inversely associated with subclinical atherosclerosis, defined by aortic calcification, independent of conventional cardiovascular risk factors. This significant inverse association was mainly attributed to DHA. Follow-up population-based studies are needed to further clarify the effect of LCn-3PUFAs on the incidence and progression of atherosclerosis as well as to disentangle the differential effect of EPA and DHA, and on the underlying biological mechanisms.

Authors' contribution

HM designed the research question, analyzed, and interpreted the data, drafted the manuscript. K Masaki, BW, K Miura, AF, AK, HU, RE, and EB-M collected the data and critically reviewed the manuscript for important intellectual content. JC, CS, JG, and SS critically reviewed the manuscript for important intellectual content. LK designed the research question, collected the data, and critically reviewed the manuscript for important intellectual content. AS designed the research question, analyzed, and interpreted the data, drafted the manuscript, critically reviewed the manuscript for important intellectual content, and provided administrative and material support. HM and AS have full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.04.011.

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