n-3 AND n-6 POLYUNSATURATED FATTY ACIDS AND VITAMIN D RELATED TO SUBCLINICAL ATHEROSCLEROSIS IN THE ERA-JUMP STUDY

by

Sunghee Lee

BS, Kyungwon University, S. Korea 1996

MPH, Korea University, S. Korea 2001

MS, University of Michigan 2006

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This dissertation was presented

by

Sunghee Lee

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and approved by

Dissertation Advisor
Akira Sekikawa, MD, PhD, PhD
Assistant Professor, Department of Epidemiology
Graduate School of Public Health, University of Pittsburgh

Evelyn Talbott, PhD
Professor, Department of Epidemiology
Graduate School of Public Health, University of Pittsburgh

Daniel Edmundowicz, MS, MD
Associate Professor, School of Medicine, University of Pittsburgh
Director of Preventive Cardiology, Cardiovascular Institute,
University of Pittsburgh Medical Center

Rhobert W. Evans, PhD
Associate Professor, Department of Epidemiology
Graduate School of Public Health, University of Pittsburgh

Emma Barinas-Mitch, PhD
Assistant Professor, Department of Epidemiology
Graduate School of Public Health, University of Pittsburgh

Akira Sekikawa, MD, PhD, PhD

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Sunghee Lee, PhD

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Cardiovascular disease is of public health significance due to its highest rates of mortality. Atherosclerotic cardiovascular events can often result in fatal or disabling non-fatal events. More than half of CHD fatal events do not show earlier symptoms. Early identification of subclinical atherosclerosis and establishment of preventive control are important to reduce CHD mortality and morbidity.

The present study aimed to examine: 1) whether n-6 fatty acids are inversely associated with plasminogen activator inhibitor-1 (PAI-1) and fibrinogen; 2) whether vitamin D deficiency is associated with subclinical atherosclerosis; and 3) whether Japanese men have lower incidence or progression of CAC than Caucasian men, and further if marine n-3 fatty acids are inversely associated with incidence or progression of CAC. To test these aims, the **E**lectron-Beam Tomography, **R**isk Factor **A**ssessment among **J**apanese and **U**.S. **M**en in **P**ost-World War II Birth Cohort (ERA-JUMP) study was utilized.

The findings were: 1) in a population-based cross-sectional-sample of 915 men aged 40-49, serum n-6 fatty acids were inversely and significantly associated with PAI-1 but not with fibrinogen; 2) in 295 middle-aged men of a population-based cross-sectional sample, Japanese

men showed lower levels of serum vitamin D, despite their habitual fish intake as a major dietary intake, than Caucasian or Japanese-American men. Further, vitamin D deficiency was not associated with subclinical atherosclerosis as measured by intima-media thickness (IMT) and CAC, except for significant associations on IMT in an univariate model among Caucasian men, and on CAC in both univariate and multivariate models among Japanese-American men; and 3) in the follow-up study of 472 men, Japanese men had a significantly lower incidence and progression of CAC than Caucasian men. Japanese men showed significant risk reduction on incident CAC associated with marine n-3 PUFA. However, Japanese and Caucasian men showed no significant associations of marine n-3 PUFA on the progression of CAC. Future studies to examine the causal associations as well as underlying mechanisms are warranted. From the public health importance, these findings extend our understanding of n-3 and n-6 polyunsaturated fatty acids and vitamin D related to subclinical atherosclerosis as well as help to establish public health guidelines.

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1.0 INTRODUCTION

1.1 SPECIFIC AIMS

Cardiovascular disease, the highest mortality worldwide[1], occurs in different geographic patterns of distribution. Coronary heart disease (CHD) is more prevalent in Western countries, including the United States, whereas stroke is more prevalent in Asian countries.[2, 3] Japan has a lower CHD mortality than Western countries.[4] Although Japan have a high smoking prevalence, high levels of blood pressure, and similar serum total cholesterol levels, among the post-World War II birth cohort due to Westernized lifestyle, the CHD mortality rate in Japanese men is still very low; scientists refer to this low CHD mortality in the Japanese as 'Japanese Paradox'.[2] Identification on the determinants of different geographical patterns for CHD risks across Western and Asian countries may help establish preventive strategies for reducing CHD morbidity and mortality. The present study will evaluate the associations of serum n-3 and n-6 fatty acids with subclinical atherosclerosis, as measured by carotid intima-media thickness (IMT) and coronary artery calcification (CAC), among three populations (Japanese men, Caucasian men, and Japanese-American men) in the **E**lectron-Beam Tomography, **R**isk Factor **A**ssessment among **J**apanese and **U**.S. **M**en in **P**ost-World War II Birth Cohort (ERA-JUMP) study.

This ERA-JUMP study was initiated to examine the different prevalence rates of subclinical cardiovascular disease in multi-study sites. The baseline survey (from 2002 to 2006)

was extended by the follow-up survey (from 2007 to 2012). From 2002 to 2006, men ages 40-49 were randomly selected from four study sites (Otsu, Shiga, Japan; Pittsburgh, Pennsylvania; Honolulu, Hawaii; and Ansan, South Korea) within a population-based cross-sectional study. Some major findings of the ERA-JUMP study have shown that Japanese men have a lower prevalence of subclinical atherosclerosis measured by IMT and CAC than Caucasian men.[5] Also, serum n-3 fatty acids have strong inverse associations with the prevalence of IMT and CAC.[6] The extremely low prevalence of subclinical atherosclerosis in the Japanese men has been shown, despite the similar or even higher risk factors for cardiovascular disease from their higher prevalence of smoking and higher alcohol consumption, as compared to Caucasian men.[6] Independent of traditional cardiovascular risk factors, the very low prevalence of subclinical atherosclerosis can be explained by their habitual intake of marine n-3 fatty acids, which may suggest anti-atherogenic effect.[6] By comparing three study populations of Japanese, Caucasian, and Japanese American men from multi-study centers, the current study, which controls for genetic differences, assesses the environmental risk factors.

The present study has the following three specific aims and associated hypotheses:

Specific aim 1: To examine the association of serum n-6 polyunsaturated fatty acids (PUFA), including linoleic (18:3n6, LA) and arachidonic (20:4n6, AA) acids with plasma PAI-1.

Hypothesis 1. We hypothesized that higher serum n-6 fatty acids, including LA and AA, are associated with lower levels of PAI-1 in the ERA-JUMP cohort.

Specific aim 2: To examine if serum vitamin D level in the Japanese men in Japan is higher, due to habitual fish intake as a major dietary source of vitamin D, than in the other two study populations of the Caucasian men and the Japanese American men; and to explore whether vitamin D deficiency (defined by 25(OH)D<20 ng/mL) is associated with subclinical

atherosclerosis measured by IMT and CAC in a population-based cross-sectional sample of 295 participants.

Hypothesis 2. We hypothesized that the Japanese men in Japan showed higher serum 25(OH)D level than the men in the other two study populations, Caucasian and Japanese Americans. We also hypothesized that IMT and CAC are associated with vitamin D deficiency, defined as serum 25(OH)D<20ng/mL in 295 men aged 40 to 49.

Specific aim 3: To examine whether the incidence or progression of CAC is lower in the Japanese man in Japan than in the Caucasian men in the U.S.; and to examine whether marine n-3 fatty acids are inversely associated with the incidence or progression of CAC.

Hypothesis 3. We hypothesized that Japanese men in Japan have a lower incidence and progression of CAC, as compared to the Caucasian men in the U.S. We additionally hypothesized that higher marine n-3 fatty acids at baseline are associated with at lower risk for developing or progressing CAC in the ERA-JUMP follow-up study of 495 men.

1.2 BACKGROUND

1.2.1 Epidemiology of cardiovascular disease (CVD)

According to the World Health Organization (WHO) in 2004, cardiovascular disease (CVD) is the leading cause of mortality throughout the world.[1, 7-9] CVD occurs in different geographical patterns between Western and Asian countries. Western countries experience higher coronary heart disease (CHD) mortality, whereas Asian countries have more deaths from stroke than CHD.[2, 3] For example, according to a recent update reported in the *Circulation* (2009)[10], CHD

deaths between the U.S. (2005) and Japan (2004) for men ages 35 to 74 were 169.4 and 50.4 (per 100,000 population) respectively.

Atherosclerotic cardiovascular events can often be fatal or disabling nonfatal.[10] The early identification of the presence or progression of subclinical atherosclerosis can result in a more effective prevention and target treatment. In the Cardiovascular Health Study (CHS), 38.7% of men and 36% of women among 5,201 participants ages ≥65 showed subclinical atherosclerosis.[11]

1.2.2 Subclinical atherosclerosis

Atherosclerosis is a progressive condition in which a fatty build-up accumulates and hardens, resulting in the narrowing or blocking of blood vessels. This fatty build-up grows and progresses into a plaque embedded in the artery walls. As the plaque enlarges, the artery walls expand to have the same amount of blood flow. When the elastic layers cannot stretch any more, the subsequent plaque rupture may occur depending on the lipid size as well as the thin fibrous cap. A larger lipid core or more vulnerable fibrous cap makes a plaque rupture more likely; a heart attack or stroke often results. This process may involve inflammatory and hemostatic responses.

Researchers agree that an injury to the inner artery walls in the atherosclerotic progression initiates an inflammatory response.[12] The injury attracts macrophage white blood cells and may also lead to blood clots.[12] In response to the damaged site, monocytes traveling in the blood stream convert into macrophages by such oxidized cholesterol as LDL.[12] Once macrophages eat and digest cholesterol molecules, they transform into a foam cell or plaque.[12] The artery wall injury usually leads to thrombotic and fibrinolytic responses on the endothelium.[12] As macrophage-derived foam cells progress, they secrete pro-inflammatory cytokines.[13] Simultaneously, smooth muscle cells within the arterial wall start proliferating.

In the next section, two non-invasive methods (i.e., IMT and CAC) are reviewed as measurements of subclinical atherosclerosis.

1.2.2.1 Carotid intima-media thickness (IMT)

Carotid intima-media thickness (IMT), as measured by ultrasound technique, is a highly reliable, sensitive, and non-invasive measurement to identify the presence or severity of subclinical atherosclerosis, by measuring the first two layers of the carotid artery, even in an early stage.[14-16] Many large epidemiological studies, including a systemic review and meta-analysis review that combined eight other studies by Lorenz and colleagues,[14] utilized IMT to quantify atherosclerotic burden and demonstrated strong associations with cardiovascular disease. As compared to the Framingham Risk Score, IMT has demonstrated a significant improvement to the prediction of CHD risk.[17]

Table 1. Associations between intima-media thickness (IMT) and cardiovascular disease

Author, year, study name	Study population	Follow -up (yr)	Exposure variable	Outcome and results
Hodis et al. (1998)[18] CLAS	146 men with previous coronary artery (40-59 years)	2	0.03mm IMT increase per year	Nonfatal MI or coronary death, RR=2.2 (95% CI, 1.4-3.6) Any coronary event, 3.1 (2.1-4.5)
O'Leary (1999)[19] CHS	Longitudinal, 5,858 participants aged ≥65 without a history of CVD	6.2	IMT	Incident MI or stroke, RR= 3.87 (2.72-5.51) (highest quintile vs. lowest, with age and sex were adjusted)
Chambless LE, et al. (1997)[20] ARIC	Longitudinal, 12,841 aged 45-64 without CHD at baseline	5.2	IMT (≥1mm vs. <1mm)	Predictor of CHD incidence Hazard rate ratio (HRR) =5.07 (3.08-8.36) for women HRR=1.85 (1.28-2.69) for men
Chambless LE, et al. (2000)[21] ARIC	14,214 aged 45-64	7.2	IMT (≥1mm vs. <0.6mm)	Predictor of ischemic stroke incidence HRR=8.5 (3.5-20.7) for women HRR=3.6 (1.5-9.2) for men

Table 1 (Continued).

Author, year, study name	Study population	Follow -up (yr)	Exposure variable	Outcome and results
Mikkila *et al.* (2009)[22] Young Finns	A prospective cohort, 785 subjects	21	Long-term scores of a traditional dietary pattern	IMT- in multivariate analyses for men ($p<0.01$), for women ($p=0.66$)
Lorenz MW, et al. (2006) CAPS	Longitudinal, Among 5,056 aged 19-90 years)	4.2	IMT at all carotid segments	Per 1 SD IMT increase HRR=1.43 (1.35-1.51) for MI HRR=1.47 (1.35-1.60) for stroke HRR=1.45 (1.38-1.52) for all MI, stroke or death
Salonen JT, et al. (1993)[23] KIHD	Longitudinal, 1,257 men	3	IMT	For each 0.1mm of IMT, AMI risk increases by 11% (95% 6%-16%) Hazard ratio=2.1(0.8-5.2) for AMI
Bots ML, et al. (1997)[24] Rotterdam Study	Nested case control, ≥55 years, Case-193 (98 MI, 95 stroke) Control-1,373	2.7	IMT	(Adjusted for age and sex) For stroke per 1SD increase (0.163mm) OR=1.41(1.25-1.82) For MI, OR=1.43(1.16-1.78)
van der Meer et al. (2004)[25] Rotterdam Study	6,389 participants, >55 years	7-10	IMT	For MI, Hazard ratio=2.91 (1.80-4.70) (severe vs. no atherosclerosis based on the quartile of composite scores)
Johnson HM, et al. (2007)[26] Bogalusa Heart Study	336 young adults (25-37years) in rural community	5.8	Progression of IMT	CIMT progression rates in men (0.020±0.027mm/year) Smoking as an independent predictor ($p=0.03$)
Kitamura A, et al.(2004)[27] Yao city	1,289 Japanese men ages60-74 years	4.5	IMT (quartile)	For stroke, RR=3.0 (1.1-8.3) (IMT≥1.07 vs. ≤0.77mm)
Murakami S, et al. (2005)[28] LILAC study	298 Japanese men and women ages≥75 years	4.3	IMT	For all cause mortality, per 0.3mm increase in left IMT, RR=1.65 (1.08-2.52), in right IMT, RR=3.33 (1.43-7.75) For cardiovascular mortality, per 0.3mm increase in left IMT, RR=2.35(1.03-5.37), in right IMT, RR=2.89 (1.06-7.89)

ARIC, Atherosclerosis Risk in Communities Study; MESA, Multi-Ethnic Study of Atherosclerosis; CAPS, Carotid Atherosclerosis Progression Study; CHS, Cardiovascular Heart Study; CLAS, Cholesterol Lowering Atherosclerosis Study; KIHD, Kuopio Ischemic Heart Disease Risk Factor Study; LILAC study, Longitudinal Investigation for the Longevity and Aging in Hokkaido County study

1.2.2.2 Coronary artery calcification (CAC)

Both Electron Beam Computed Tomography (EBCT) and Multi-Detector Computed Tomography (MDCT) are non-invasive and reliable measurements to identify the presence or progression of subclinical atherosclerosis.[29] [30, 31] Because the first manifestations of CHD events in asymptomatic individuals are often fatal without any previous symptoms, early identification of atherosclerosis is imperative.[4]

Coronary artery calcification (CAC), as measured by either EBCT or MDCT, has demonstrated strong associations with cardiovascular events.[32] A study in a general population ages 45-84 years without clinical cardiovascular disease showed a dose-response increase in risks of coronary events, according to the CAC scores of 1-100, 101-300, and >300 (Hazard ratios (HR) 3.9[1.7-8.8], 7.1[3.01-16.5], and 6.8[2.9-16.0], as compared to a zero CAC).[32]

Previous studies showed that CAC improves CHD risk assessment, specifically for those in the intermediate category, based on a Framingham risk assessment using seven traditional cardiovascular risk factors (age, gender, smoking, total cholesterol, HDL, blood glucose, and blood pressure).[32, 33] No racial/ethnic differences were observed in this improvement of the CHD risk assessment (Caucasian, Chinese, African-American, and Hispanic individuals).[32]

However, several limitations on CAC make difficult in adopting into a routine screening practice: radiation exposure, lack of insurance coverage, and lack of risk stratification on clinical implication.

With regards to controlling LDL cholesterol as a major fundamental risk factor, lipid-lowering treatments (i.e., statins) have been examined to determine whether statins can hold off atherosclerotic progression. Several earlier trials found the effectiveness of statins on atherosclerotic progression,[34, 35] but more recent trials did not show the effectiveness.[36-40]

Table 2. Coronary artery calcification (CAC) on Cardiovascular disease

Study name	Author, year	Study population	Follow-up(yr)	Exposure variable	Outcome and results
MESA	Wang L., et al. (2006)[41]	A cross-sectional study, 222 men and women ages 45-84 without diagnosis of CHD, in the Minnesota field of the MESA cohort		Coronary artery calcification (CAC)	Myocardial perfusion, Odds Ratio (95% CI) of CAC 0 : 1 (reference) 0.1-99.9 : 2.16 (0.96-4.84) 100-399 : 2.81 (1.04-7.58) ≥400 : 4.99 (1.73-14.4)
	Berry JD, et al. (2009)[42]	2,988 participants ages ≤50 years in the follow-up Year 15th CARDIA study, 1076 participants ages ≤50 years from MESA		As compared to low 10-year and low lifetime risk, those with a high lifetime risk and low 10-year CVD risk	In CARDIA, CAC progression OR(95%CI) 1.60 (1.15-2.24) for men 1.68 (1.09-2.59) for women In MESA, CAC progression OR(95%CI) 1.78 (0.99-3.22) for men 1.52 (0.78-2.97) for women
	Kestenbaum BR., et al. (2009)[43]	562 chronic kidney disease patients (mostly stage 3) without cardiovascular disease	1.6-3.2	CAC	Incidence of CAC – 6.1% per year in women and 14.8% per year in men Progression – 17% per year in all participants, 65% greater adjusted risk of progression in diabetic participants
Detrano, et al. (2008)[32]		6,722 men and women in a population-based sample without a cardiovascular disease at entry (four racial/ethnic groups (Caucasian, black, hispanic, chinese)	3.8	CAC Doubling CAC	All four racial/ethnic groups; -15-35% increased risk for a major coronary event -18-39% for any coronary event

ARIC, Atherosclerosis Risk in Communities Study; MESA, Multi-Ethnic Study of Atherosclerosis; CAPS, Carotid Atherosclerosis Progression Study; CHS, Cardiovascular Heart Study; CLAS, Cholesterol Lowering Atherosclerosis Study

Table 3. Clinical trials with EBCT

Author, year	Study population	Follow-up	Exposure variable	Outcome and results
Achenbach S, et al. (2002) [34]	66 patients with coronary calcifications in EBT, LDL>130mg/dL	14	Cervastatin (0.3mg/d)	Significantly difference (p=0.01) in the median annualized absolute and relative coronary calcium scores
Nissen SE, et al (2004) [35]	A double-blind, randomized active control multicenter trial, in 654 patients	18	Intensive (atorvastatin 80mg) vs. Moderate (pravastatin 40mg)	Intensive treatment with atorvastatin (no progression, -0.4%[-2.4%~1.5%, p=0.98]) reduced CAC progression as compared with pravastatin (2.7% progression [0.2%~4.7%], p=0.001)
Arad Y, et al. (2005) [36]	A double-blind, placebo-controlled, in 1,005 healthy adults ages50-70 years with CAC≥80th for age & gender	51	Atorvastatin20mg/d+VitC1g/d+VitE1000IU/d+Aspirin81mg/d vs. placebo+aspirin 81mg/d	No difference on CAC progression (P=0.80), no significant reduction of all atherosclerotic cardiovascular disease (6.9% vs. 9.9%, p=0.08)
Hecht HS, et al. (2003) [37]	A prospective study, 182 asymptomatic patients without cardiovascular disease	14	More (≤80) vs. less (>80 mg/dl) aggressive LDL cholesterol lowering txts	No difference in calcified plaque progression (9.3%/year vs. 9.1%/year)
Schmermund A, et al. (2006) [40]	A multicenter, randomized, double-blind trial, 471 patients with no history of cardiovascular disease	12	Intensive (atorvastain 80mg/d) vs. standard (atorvastatin 10mg/d)	No difference on the CAC progression (27% vs. 25%, p=NS)
Raggi P, et al. (2005)[39]	A double-blind, multicenter trial in 615 hyperlipidemic, postmenopausal women	12	Intensive (atorvastatin 80mg/d) vs. moderate (pravastatin 40mg/d)	No difference - Calcium volume scores (CVS) (median 15.1% vs. 14.3%, p=NS)
Houslay ES, et al. (2006) [38]	Double-blind randomized controlled trial, 102 patients with calcific aortic stenosis and CAC	24	Atorvastatin 80mg/d vs. placebo	No difference in the rate of change in CAC (26%/yr vs. 18%/yr) (95%CI -3% to 18%, p=0.18)

1.2.3 n-3 and n-6 Polyunsaturated fatty acids

Two essential fatty acids, which are alpha-linolenic acid (18:3n-3, n-3 fatty acid) and linoleic acid (18:2n-6, n-6 fatty acid), transform via desaturation (adding double bonds) and elongation (adding carbon atoms) into the longer-chain of polyunsaturated fatty acids. As noted from the name, the human body cannot produce and needs to obtain from foods. These essential fatty acids have imperative biological functions in developing and growing cell membranes, skin,

brain, and heart, as well as in producing local hormone, including prostaglandins and leukotrienes.[44]

The main food sources for alpha-linolenic acid (n-3 fatty acid) are usually plant oils, for example, flaxseed, walnut, or dark green leafy vegetables. EPA and DHA, the longer carbon chain of polyunsaturated fatty acids, come from having salmon, tuna, halibut, and trout. The main sources of linoleic acid (n-6 fatty acid) are plant seed oils (e.g. oils of safflower, corn, and sunflower).

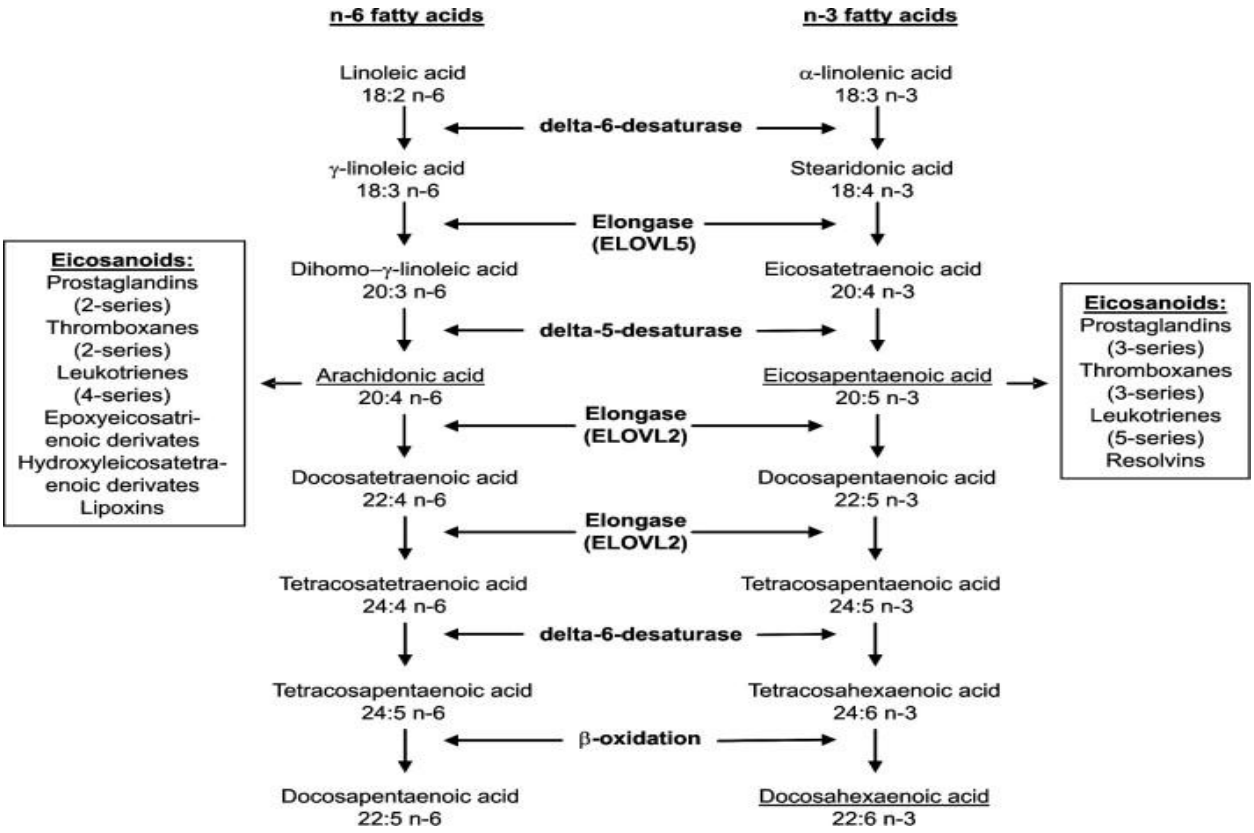

Figure 1. n-3 and n-6 fatty acids
[From *Prog Lipid Res* 2008;47:147-55 with permission from Dr. Gerd Schmitz[45]]

1.2.4 CHD associated with n-3 and n-6 fatty acids

1.2.4.1 Marine n-3 fatty acids and CHD

Many previous studies have shown that marine n-3 fatty acids (i.e. EPA or DHA) have beneficial and therapeutic effects against CHD, via modulating dyslipidemia such as reducing triglyceride[46], lowering blood pressure[47], reducing thrombosis[48], and inhibiting vascular smooth muscle cell proliferation[49].

Several clinical trials of n-3 fatty acids, the Diet and Reinfarction Trial (DART), the Diet and Angina Randomized Trial (DART2), the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico-Prevenzione Trial (GISSI-Prevenzione Study), the Japan EPA Lipid Intervention Study (JELIS), and one observational study, the Japan Public Health Center-based study cohort I (JPHC) are summarized as following.

The DART, a secondary prevention trial among 2,033 men in Wales who were diagnosed with MI, studied whether physicians' advice to eat oily fish such as mackerel, herring, kipper, pilchard, sardines, salmon, or trout, by more than two servings per week (200-400 g/week) or to take fish oil supplementation (called 'Maxepa' capsule of 170mg EPA+115mg DHA) by three capsules per day could reduce the risk of cardiovascular events. For its two years of follow-up, the treatment group showed risk reduction on all-cause mortality by 29% (95%CI 0.54-0.92) and on ischemic heart disease by 16% (0.67-0.93), as compared to no dietary advice.[50] On the other hand, the DART2, a subsequent secondary prevention trial with a 3-9 year follow-up, did not show benefits from dietary advice,[51] with lower compliance over a longer follow-up period.

The GISSI-Prevenzione Study, a large multi-center secondary prevention trial, found benefits of n-3 fatty acid in 11,323 men in Italy within three months of a heart attack event. The main finding showed significant risk reduction in all-cause mortality or non-fatal MI/stroke

[relative risk (RR)=0·85(0·74–0·98)].[52] A time-course analysis, to examine how quickly this benefit could be effective to reduce the risks of total mortality and sudden death, found that even only after three months with n-3 PUFA exerted to reduce 41% of the risk for total mortality, and after four months 53% of the risk for sudden death.[53] These two trials of the DART and GISSI studies suggested benefits of reducing the risk for arrhythmia and sudden cardiac death, which are fatal CHD events.

Two recent large clinical trials in Japan indicated that habitual marine-derived n-3 fatty acids could reduce the risk for non-fatal events, which suggested anti-atherosclerotic effects. The Japan EPA Lipid Intervention Study (JELIS) was conducted among 18,645 hypercholesterolemic patients (with and without coronary artery disease (CAD)) for 4.6 years to examine whether either EPA (1800 mg/day) plus statin vs. statin only could prevent major coronary events. The treatment group showed a 19% risk reduction on major coronary events as compared to those with only a statin. Since both groups took stains, the finding was independent of cholesterol changes. Those who had a CAD history appeared to reduce the risk by 19%, and those without CAD at their enrollment showed risk reduction by 18%.[54]

The other Japan study, the Japan Public Health Center-based study cohort I (JPHC), was a large prospective study for 5 years and examined whether high habitual fish intake could reduce CHD risk, in 41,578 Japanese adults ages 40-59 with no cardiovascular disease and cancer at enrollment.[55] The highest quintile of fish intake (180 g/d vs. lowest 23g/d), as compared to the lowest quintile, showed a significant dose-response relationship on the risk reduction for nonfatal coronary events [0.43, (0.23-0.81)] but not for fatal events [1.08, (0.42-2.76)]. Together, many clinical trials and epidemiological observational studies consistently demonstrated cardioprotective effects of n-3 fatty acids, including fatal as well as non-fatal events.

Table 4. n-3 PUFA and cardiovascular disease in clinical trials

Author, year, Study name	Study population/ Study design	Follow-up(year)	Exposure/ Supplementation	Outcome
Burr ML, et al. (1989)[50] DART	2,033 men who recovered from MI under 70 years	2	1) Fish advice- to intake at least two weekly servings of oily fish (200-400g/wk) or three fish oil capsules/day 2) fat advice – to reduce fat intake up to 30% of total energy and to increase PUFA/saturated ratio to 1.0 3) fiber advice – to increase cereal fiber to 18g/d 4) as compared to no advice	All-cause mortality 29% reduction (95%CI 0.54-0.92) as compared to no dietary advice group Ischemic heart disease 16% reduction (0.67-0.93)
Burr ML, et al. (2003)[51] DART2	3,114 men with angina under the age of 70 (Recruiting phase I: 1,111, phase II: 2,003)	3-9		* Higher risk in the fish advice group than that in no advice Cardiac death HR=1.26 (1.00-1.58) Sudden cardiac death 1.54(1.06-2.23)
GISSI-Prevenzione Investigators (1999) [52] GISSI-Prevenzione Trial	11,324 survivors from recent MI (≤3months) / a open-label design	3.5	Four groups + pharmacological treatment and lifestyle advice 1) n-3 PUFA 1g/d (850-882 mg/d EPA+DHA) (n=2,836) 2) vitamin E 300mg/d (n=2830) 3) both (n=2,830) 4) none (n=2,828)	Death, non-fatal MI, and non-fatal stroke 15% risk reduction (95%CI 0.74-0.98) CVD death, non-fatal MI, and non-fatal stroke 20% risk reduction (0.68-0.95)
Marchioli R. et al.(2002)[53] GISSI-Prevenzione Trial	11,324 post-MI (≤3months) patients / a open-label design	3.5		After 3 months of treatment Total mortality RR=0.59 (95%CI, 0.36-0.97) After 4 months Sudden death RR=0.47 (0.22-0.99)
GISSI-HF investigators (2008)[56] GISSI-HF	6,975 patients with chronic heart failure in Italy / placebo-controlled for all participants blinding	3.9	3,494 patients with HF with 1g/d EPA+DHA (850mg), vs. 3,481 placebo patients	All-cause mortality HR=0.91 (0.833-0.998) Death or admission to hospital for cardiovascular reasons HR=0.92 (0.849-0.99)
Yokoyama M, et al. (2007) [54] JELIS	18,645 hypercholesterolemic (total-C ≥6.5 mmol/L or 251mg/dl) Japanese men/ a prospective, open-label, blinded end point evaluation	4.6 (1996-2004)	EPA 1.8g/d plus stain vs. statin-alone therapy 3,664 CAD patients, of these, 1,050 with a MI event	In all participants, for major coronary events 19% risk reduction [Secondary prevention] Coronary events - 19% risk reduction HR=0.81 (0.69-0.95) [Primary prevention] HR= 0.82 (0.63-1.06)
Iso H. et al. (2006)[55] JPHC	41,578 Japanese men and women ages40-59 years without cardiovascular disease and cancer	477,325 person-year (From 1990-1992 to 2001)	High fish intake [(highest quintile, 180 g/d or 8times/week) vs. (lowest, 23g/d or once a week)]	*Quintile category 1) Total CHD HR=0.63 (0.38-1.04) 2) Definite MI 0.44 (0.24-0.81) 3) Sudden death 1.14 (0.36-3.63) [Nonfatal coronary events] HR=0.43 (0.23-0.81) [Fatal coronary events] HR=1.08 (0.42-2.76) *Continuous-Inverse associations 1) Definite MI HR=0.35 (0.18-0.66) 2) Non-fatal coronary events HR=0.33 (0.17-0.63)

HR=Hazard ratio; OR=Odds Ratio; MI=myocardial infarction; EPA, eicosapentaenoic acid (20:5n3); DHA, docosahexaenoic acid (22:6n3); DART=Diet And Reinfarction Trial; DART2=Diet and Angina Randomized Trial; GISSI=Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico-Prevenzione trial; HF=Heart Failure; JELIS=the Japan EPA Lipid Intervention Study; JPHC=Japan Public Health Center-Based study cohort

Table 5. n-3 PUFA and cardiovascular disease in observational studies

Author, year, Study name	Study design/ Study population	Exposure	Outcome
Hu F. et al. (2002)[57] Nurses' health study	Prospective cohort study (16 yrs), 84,688 women ages34-59 years without known cardiovascular risk factors	Fish intake frequency (high intake vs. rare fish <1 per month [reference group])	Significantly reduced relative risks for CHD death relative risk (RR) • 1-3 times/month 0.79 (0.64-0.97) • Once/week 0.71 (0.58-0.87) • 2-4 times/week 0.69 (0.55-0.88) • ≥5 times/week 0.66 (0.50-0.89) Trend in the quintiles of fish intake (p<0.001)
Guallar E, et al. (1995)[58] Physicians' Health Study	Nested case-control study/ 14,916 participants Case: one with MI within first 5 years of follow-up Control: ones without CHD who were matched by smoking and age	Plasma levels of fish oils	No significant difference on relative risks of EPA+DHA • in cholesterol esters 1.05(0.92-1.19) • in phospholipids 1.06 (0.80-1.40)
Albert CM, et al (1998)[59] Physicians' Health Study	Prospective cohort study (11 years of follow-up), 20,551 male physicians ages40-84 years without MI, cerebrovascular disease, and cancer at baseline	Regular fish consumption (≥1 meals of fish/week) vs. less than once a month	Sudden deaths 52% reduction (RR=0.48, 95%CI 0.24-0.96): Fish intake (at least once/week) was significantly associated with reduced risk of total mortality, but not for total MI, non-sudden death, or total cardiovascular mortality
Ascherio A, et al. (1995)[60] Health Professional follow-up Study	Prospective cohort study (6 years of follow-up) 44,895 male health professionals ages40-75 without known cardiovascular disease	n-3 fatty acid intake from dietary questionnaires	No significant association • (highest quintile vs the lowest of n-3 fatty acid intake) CHD relative risk=1.12 (0.96-1.31) • (≥6servings of fish/week vs. ≤1serving/month) CHD 1.14 (0.86-1.51) • Death from coronary disease (eat fish vs. eat no fish) 0.74 (0.44-1.23)
Simon JA, et al. (1995)[61] MRFIT	Nested case-control study, in the usual Care group Case:94 men with incident CHD during average 6.9 yrs of follow-up Control: 94 men without incident CHD matched on age, date of randomization, and clinic center	Phospholipid n-3 fatty acid docosapentaenoic acid	[n-3 fatty acid doxosapentaenoic acid] CHD risk OR=0.58 (95%CI 0.38-0.89), with adjusting for total plasma cholesterol [Docosahexaenoic acid] CHD risk OR=0.57 (95%CI 0.36-0.90), with adjusting for HDL/LDL
Daviglus ML, et al. (1997)[62] Western Electric Study	Prospective cohort study (30years of follow-up), 1822 men ages40-55 years without cardiovascular disease at baseline	Fish consumption from a detailed dietary history	[Fish intake ≥35g/day vs. 0g/day] CHD death HR=0.62(0.40-0.94) MI death 0.56 (0.33-0.93) [Fatal/non-fatal] Non-sudden death from MI - significant 0.33 (0.12-0.91) / Sudden death from MI 0.68 (0.37-1.25)

OR=odds ratio; RR=relative risk; CI=confidence interval; MRFIT=Multiple Risk Factor Intervention Trial;

1.2.4.2 n-6 Polyunsaturated fatty acids and CHD

In a Western diet, the most part of PUFA (about 90%) is comprised of n-6 fatty acids.[63] High n-6 fatty acids levels have been controversial, because several eicosanoids produced from arachidonic acids have proinflammatory and prothrombotic effects. However, recent studies found that arachidonic acid also produces eicosanoids with beneficial effects, including vasodilation, inhibition of platelet aggregation, and anti-inflammatory actions.[64] In addition, recent researchers suggested that AA does not have a direct link to increase CHD risk, because it may be tightly regulated in the human body.[65-67]

Several epidemiological studies showed the beneficial effects of n-6 fatty acids on CHD events. Prospective studies of the Kupio Heart Study[68] and the Nurses' Health Study[69] found risk reductions with higher linoleic acid on cardiovascular events. Based upon a 20-year follow-up study, the Nurses' Health Study reported the cardioprotective effect of linoleic acids.[69]

Table 6. Association between n-6 and CHD

Author/Year	Study design/ Study population	Follow-up(year)	Exposure	Results
Oh K, et al. (2005) [69] Nurses' Health Study	Prospective cohort study, 78,778 US women without cardiovascular disease and diabetes at baseline	20	With food frequency, Linoleic acid (the highest vs. the lowest quintile)	CHD risk 25% reduction (95% CI 0.62-0.95) (p-value for trend =0.01) [Stronger inverse associations] • Younger than 65 years • ≥25kg/m^2 BMI
Mozaffarian D, et al. (2005)[70] Health Professionals Follow-up Study	Prospective cohort study, 45,722 men without cardiovascular disease at baseline	14	With food frequency questionnaires	n-3 fatty acids to decrease 40-50% risk of sudden death, regardless of n-6 fatty acids. n-6 PUFA intake does not have adverse or opposite effect of n-3 PUFA on the risk of CHD events
Laaksonen DE, et al. (2005)[68] Kupio Ischemic Heart Disease Risk Factor (KIHD) study	Prospective cohort study, 1,551 middle aged men without a history of cardiovascular disease, diabetes, or cancer at baseline	1	4-day food record & serum fatty acid	Linoleic acid RR=0.39 (0.21-0.71) *Both serum and dietary fatty acids ascertainments
Iso H, et al. (2002)[71]	Prospective nested case-control study, 7,450 Japanese women and men ages40-85, 3 controls per case by matching for sex, age, community, year of serum storage, and fasting status	By 1998, (from 1984-1989, from 1989-1992)	Use of frozen serum samples	Multivariate odds ratios 1-SD increase in linoleic acid Total stroke OR=0.72 (0.59-0.89) Ischemic stroke 0.66 (0.49-0.88) Lacunar infarction 0.63 (0.46-0.88) Hemorrhagic stroke 0.81 (0.59-1.12)
Dolecek TA. (1992) [72] MRFIT	Nested a clinical trial, 12,866 middle-aged men in high risk for developing CHD, based upon smoking, diastolic blood pressure, and serum cholesterol From the usual care arm in the MRFIT study	10.5	Four dietary recall interviews at baseline and follow-up years 1,2,3. Linoleic acid (the highest vs. the lowest quintile)	No significant association with mortality for linoleic acid HR=0.58 (P<0.10)
Pietinen P, et al. (1997)[73] Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study	Nested in a randomized double-blind placebo-controlled trial, 21,930 smoking men ages50-60 without cardiovascular disease at baseline	6.1	Dietary questionnaire	No significant association between linoleic acid and CHD – Multivariate relative risk (RR) (the highest vs. the lowest quintile) RR=0.92 (95%CI 0.56-1.50) p-value for trend = 0.674

TG=triglycerides; Tx=treatment; RCT=randomized clinical trial; PUFA=polyunsaturated fatty acids; DHA=docoxahexanoic acids; EPA=eicosapentaenoic acids; OR=odds ratio; RR=relative risk; CI=confidence interval; MRFIT=Multiple Risk Factor Intervention Trial; KIHD=the Kuopio Ischemic Heart Disease Risk Factor study

1.2.5 PAI-1 associated with n-6 fatty acids

Plasminogen activator inhibitor-1 (PAI-1), which is a primary inhibitor of plasminogen activators, has an anti-fibrinolytic function.[74] Thogersen *et al.* demonstrated that high plasma PAI-1 levels are associated with a high risk for a first acute myocardial infarction (AMI) in middle-aged men and women,[75] suggesting that PAI-1 is an independent risk factor for CHD. PAI-1 levels are involved in local platelet activation or aggregation as an acute reactant. Additionally, PAI-1 is associated with obesity[76] and type 2 diabetes[77].

Table 7. Association between n-6 fatty acids and PAI-1 in clinical trials

Author/Year	Study population/ Study design	Regimen	Results
Fleischman AI, et al. (1975)[78]	20 men and 46 women from a geriatric center ages 60-109 years	- 32g/day (actual fat daily 25/6g) - Unsaturated diet in highly unsaturated margarine for 2 weeks vs. a saturated diet in butter for 2 weeks	Linoleic acid intake from 2.89±0.11 (% of energy) for 2 wks to 5.00±0.26 (% of energy) for 2 wks was associated with different aggregation time (doubling, p-value<0.05) and disaggregation time (halving, p<0.01) ➔Significant platelet aggregation activity differences associated with linoleic acid
O'Brien JR, et al. (1976)[79]	39 men ages 23-53 (case-19, control-20)	Partial replacement by linoleic acid (with sun-flower based product upto 65% linoleic acid) for 8 weeks	Decreased platelet activity • Platelet count between PUFA group (case) and control (p-value: 0.01) • Cholesterol (mg/dl) between PUFA group (case) and control (p-value: 0.01)
Hornstra et al. (1973) [80]	Case- 69 men (with high polyunsaturated and low saturated fatty acids) Control -79 men (normal)	One group with a polyunsaturated diet (linoleic acid approx. 12 % of total calorie) vs. The other group with a saturated diet (normal finnish diet, i.e., linoleic acid 4%)	Significantly decreased aggregatability of platelets (including Aggregation-times) observed in those who had a regimen of high polyunsaturated and low saturated fat diets (p-value <0.001)

Table 8. Association between arachidonic acid (AA) and PAI-1

In vitro studies			
Author /Year	Experimental material	Regimen	Results
Bates EJ, et al. (1995)[81]	Neutrophils from the heparinised blood of healthy volunteers	18 carbon fatty acids containing 1-4 double bonds	AA may have a pro-inflammatory effect →The bigger number of double bonds and the longer chain fatty acids, the more promoted in neutrophil adherence to endothelial cells →The n-3 fatty acids stimulate less neutrophil adherence than the n-6 isomers
Badwey JA, et al (1981)[82]	Human neutrophils were purified from blood	Adding the concentration of Arachidonate (110μM) to human neutrophils result in the maximum rate of superoxide (O_2^-) production	AA stimulated human neutrophils to generate increased superoxide production
Human clinical trials			
Author /Year	**Study population/ Study design**	**Regimen**	**Results**
Nelson GJ, et al. (1997)[83]	A single blind crossover study, 10 Healthy male subjects	1.5 g/days dietary AA supplements for 50 days and 210mg/day normal diet for 65 days	No changes in blood coagulation and thrombotic tendencies, as compared to those without supplement diet (non-significances)
Katsumoto et al. (2007)[84]	In a double-blind, placebo-controlled study, 24 healthy Japanese men with high habitual intake of fish oils	860 mg/d AA for 4 wks	After 2weeks, serum AA concentration 9.6 to 13.7 g/100g total fatty acids ($p<0.001$) Until 4 weeks, this increase was maintained but after wash-out period came back to baseline level
Lahoz C, et al. (1997)[85]	42 healthy subjects (18 women and 24 men) 17-71 years, 5 weeks	According to different fat saturation, 1) Saturated fatty acid 2) Monounsaturated fatty acid 3) Polyunsaturated fatty acid (PUFA, n-6) 4) PUFA, n-3	Effects of dietary fat saturation on • eicosanoid urinary excretion –no difference on prostaglandin, but significant difference on the excretion of thromboxane B_2 ($p=0.0001$) • platelet aggregation (PA) –a significant difference ($p<0.05$) • Systolic and diastolic BP were both significantly higher on saturated fatty acid than other three diets ($p<0.0001$)
Animal studies			
Author /Year	**Study population/ Study design**	**Regimen**	**Results**
Koskelo et al. (1997)	Sprague-Dawley rats (20/sex/group) for 90 days, 1.0 & 2.5 g/kg/d	In the ester form	No signs of toxicity in rats supplemented with supplements with high as 2.5 g/kg/d
Whelan J, et al. (1993)[86]	24 male Golden Syrian hamsters (85-110 g) into 4 groups (6 hamsters per group)	10g/day for 3 weeks (~102g fat/kg diet) 1) oleic acid (served as control) 2) linoleic acid 3) arachidonic acid 4) eicosapentaenoic acid	No effect on platelet aggregation according to fatty acid composition of the diet

1.2.6 Vitamin D and cardiovascular diseases

Vitamin D is stored in the body fat as fat-soluble and metabolized in the liver.[87] Vitamin D undergoes two hydroxylation processes to activate in the body: 1) in the liver, vitamin D from dietary sources and sun exposure undergoes hydroxylation into 25(OH)D circulation in the blood; and 2) in the kidney, this 25(OH)D form is again hydroxylated into $1,25(OH)_2D$.[88] These tightly regulated steps ensure that the excessive intake does not reflect a high degree of cumulative production.[89]

Habitual fish intake is the major source of vitamin D among the Japanese men in Japan.[90] Many cross-sectional studies have shown strong associations between vitamin D deficiency and CHD risk. Vitamin D could have an enormous impact on decreasing CHD risk since it is inexpensive, accessible, and safe to use.

Table 9. Associations between vitamin D and both subclinical atherosclerosis as well as risk factors for cardiovascular disease in cross-sectional studies

Author, year, study location	Study population	Exposure	Outcome	Results
Reis JP, et al.[91] (2009), within the Rancho Bernardo Study	654 adults ages55-96 years without a history of coronary heart disease, revascularization, or stroke	Serum 25(OH)D (quintile)	IMT	Internal carotid IMT (*P*trend=0.022) but not common carotid IMT (*P*trend=0.834) decreased in a dose-response relationship as 25(OH)D was increased → No associations of $1,25(OH)_2D$ with internal and common carotid IMTs
Michos ED, et al. (2009)[92] in the Old Order Amish (OOA)	650 Amish participants	Serum 25(OH)D (quartile)	IMT CAC C-RP	No association of serum 25(OH)D with IMT, CAC or C-RP → 25(OH)D deficiency (defined as <20ng/ml) prevalence - 38.2% (observed in winter)
Pilz et al.[93] (2009) In the Hoorn Study	Population-based 614 persons from a follow-up visit of the Hoorn study 2000-2001	Serum 25(OH)D (quartile)	IMT	No significant associations between 25(OH)D and IMT in both partial and full adjusted models
Scragg R, et al. (2007)[94] in the third NHANES III 1988-1994	12,644 people aged ≥20 years, in community setting, 1988-1994, excluding those on hypertensive medication	Serum 25(OH)D	BP	Significant inverse association for systolic BP, after adjusting for BMI ($p<0.05$) → This inverse association became greater in participants aged ≥50 years than younger ($p=0.021$)
Martins et al.[95] (2007) in the third NHANES 1988-1994	7186 men and 7902 women adults ages ≥20 years	Serum 25(OH)D (fourth quartile vs. first quartile)	Hypertension, Diabetes, Obesity, triglycerides	Hypertension OR = 1.30 Diabetes OR = 1.98 Obesity OR = 2.29 High triglyceride OR = 1.47 → Increased cardiovascular risk factors in lower vitamin D
Ginde et al. 2009 Comparison of two NHANES	18,883 adults in NHANES III and 13,369 adults in NHANES 2001-2004	25(OH)D		Decreased by 6 ng/mL from 1988-1994 to 2001-2004

IMT, intima media thickness; CAC, coronary artery calcification; C-RP, c-reactive protein; NHANES, national health and nutri1tion examination survey; OR, odds ratio;

Table 10. Associations between vitamin D and cardiovascular disease in case-control and prospective studies

Author, year, study location	Study population	Follow-up(yr)	Exposure	Outcome	Results
Melamed et al.[96] (2008) in the third NHANES 1988-1994	13,311 health participants in community	8.7	25(OH)D (highest quartile vs lowest)	All-cause mortality (1806 deaths including 777 from CVD)	26% increased rate of all-cause mortality (mortality rate ratio 1.26(95% CI 1.08-1.46))
de Boer IH, et al.[97] (2009) In the Multi-Ethnic Study of Atherosclerosis	1370 (394 with and 976 without chronic kidney disease)	3	25(OH)D	Coronary artery calcification (CAC) -At baseline CAC prevalence, 53% (723 out of 1370 participants)	-Among those without CAC at baseline, 21% (135 out of 647) developed incident CAC -Lower 25(OH)D associate with increased risk for incident CAC Adjusted RR=1.23 (1.00-1.52),
Giovanucci et al.[98] (2008) In Health Professionals Follow-up Study	A nested case-control study (1:2 matching), 18225 men 40-75 yr, without previous CVD	10	Plasma 25(OH)D	Myocardial infarction (case: 454 non-fatal MI or fatal CHD Control: 900 matched for age, date of blood collection, and smoking)	Adjusted hazards ratio HR= 2.09 [95% CI 1.24-3.54, P_{trend}=0.02] (as compared with ≥30 vs ≤15 ng/ml), after adjusting for family history of MI, BMI, alcohol, physical activity, history of diabetes and hypertension, ethnicity, region, marine n-3 intake, LDL & HDL cholesterol, triglyceride
Wang et al,[99] (2008), in Framingham Offspring Study	1739, with no history of CVD	5.4	Serum 25(OH)D	A first cardiovascular event (120 incident event)	HR 1.62 (95% CI 1.11-2.36), (≥15 vs. <15) low serum 25(OH)D levels were associated with incident cardiovascular disease
Forman JP, et al.[100] (2008), in the Nurses' Health Study	A nested case-control study, 1484 women ages32 to 52 years,		Plasma 25(OH)D (quartile)	Incident hypertension (742 cases and 742 controls) -matched on age, race, and month of blood collection	-(highest quartile vs. lowest) Odds ratio 1.66 (95%CI 1.11-2.48, P_{trend}=0.01) -With adjusting for BMI, physical activity, family history of hypertension, oral contraceptive, parathyroid hormone, calcium, phosphorous, creatinine, and uric acid, vitamin D deficiency (<30 ng/mL) OR=1.47 (95%CI 1.10-1.97)
Forman JP, et al.[101] (2007), From 2 prospective studies of the Health Professionals' follow-up study and the Nurses' Health Study	613 men from the Health Professionals' follow-up study and 1198 women from the Nurses' Health Study	4-8	Plasma 25(OH)D (<15 ng/ml vs. ≥30 ng/ml)	Incident hypertension	-Measured plasma 25(OH)D : 6.13 (95%CI 1.00-37.8) in men, 2.67 (1.05-6.79) in women, 3.18(95%CI 1.39-7.29) in the pooled relative risk combined men and women using random-effects model

CVD, cardiovascular disease; RR, relative risk;

Table 11. Associations between Vitamin D and Cardiovascular disease in clinical trials

Author, year, study location	Study population	Follow-up	Exposure	Outcome	Results
Krause et al.[102] (1998)	Randomized 18 subjects with stage I hypertension (8 women, ages 26-66 years)	6 weeks	Three times a week full body UVB or UVA	25(OH)D	Significant reduction in 24-h ambulatory systolic and diastolic blood pressure in the UVB but not in the UVA group. In the UVB group, 162% increase in plasma 25(OH)D, but in the UVA group, relatively unchanged
Margolis KL, et al.[103] (2008) In the Women's Health Initiative Study	Randomized trial, 36,282 postmenopausal women	Median 7 years	1g calcium plus 400 IU of vitamin D3 daily or placebo	-systolic and diastolic blood pressure change - incidence of hypertension	No effect of the intervention on BP or risk of hypertension
Hsia J, et al.[104] (2007) Women's Health Initiative Study	36,282 postmenopausal women ages50-79 years	7 years	500mg calcium carbonate with 200 IU vitamin D twice daily	Coronary or cerebrovascular risk	MI or CHD death - Hazard ratio 1.04 (95%CI, 0.92-1.18) Stroke – HR 0.95 (0.82-1.10) ➔ No significant difference

1.2.7 Associations between marine n-3 fatty acids and subclinical atherosclerosis

Table 12. Associations between marine n-3 fatty acids and subclinical atherosclerosis

Study	Study design (yr)	Study population	Outcome	Results
ERA-JUMP	Population-based cross-sectional study (2008)	40-49 years old men the Japanese, Caucasian and Japanese American men without any clinical CVD and cancer	CCS≥10	Only IMT in the Japanese in Japan appeared significant associations with marine n-3 fatty acids but not in the other two populations or for CAC in all three populations
Rotterdam[105]	Cross-sectional study (2010)	1,570 asymptomatic participants aged ≥ 55 years (average age was about 64 years old) from a population-based prospective cohort study in the Netherlands	CCS>10	Weak inverse associations between and CAC prevalence as compared to those with no fish intake (prevalence rate 0.87, 0.78-0.98 of mild and intermediate calcification associated with >19g/day of a fish intake). However, EPA and DHA intake did not show significant associations (PR 0.93 and 0.97, p>0.05)
MESA (Multi-Ethnic Study Atherosclerosis) [106]	Cross-sectional (2008)	a population-based study of 6814 men and women ages45-84 years who are without clinical CVD from 6 U.S. communities	CCS>0	Inverse association of dietary marine n-3 PUFA intake (220 vs 40 mg/d) with common carotid IMT (highest quartile vs. lowest quartile OR=0.69 [0.55-0.86]) but not with CAC score (1.14 [0.94-1.38])
CARDIA (Coronary Artery Risk Development in Young Adults)[107]	Prospective population-based multicenter cohort study (2007)	5,115 African American and Caucasian participants ages33 to 45 years at baseline recruited from 4 U.S. cities		Younger adults demonstrated risk factors for CAC as much as those for the elders.These young but being with higher risk factors had 2-3 times more likely to have CAC. Particularly, smoking, LDL cholesterol, systolic blood pressure, and glucose were found significant risk factors for having CAC.

CCS, coronary calcium score; CAC, coronary artery calcification;

1.2.8 ERA-JUMP study

The ERA-JUMP study has two surveys: the baseline (2002-2006) and the follow-up (2007-2012). This population-based study was initiated to measure prevalence and risk factors for subclinical atherosclerosis in three different populations (i.e., Japanese, Caucasian, and Japanese-American men) in order to reduce CHD. From 2002 to 2006, 926 participants aged 40-49 years were randomly selected from three study sites: 313 Japanese men from Kusatsu, Shiga, Japan; 310 Caucasian men from Allegheny County, Pennsylvania, U.S.; and 303 Japanese-Americans from Honolulu, Hawaii, U.S. Excluded were those who had clinical cardiovascular disease or other severe illnesses. The Japanese men ages 40-49 years in Kusatsu city were randomly enrolled from the residents' registry for all Japanese nationals.[5] The Caucasian men ages 40-49 years were randomly enrolled from the voter registration list of Allegheny County [5] The Japanese-American men in the Hawaiian site, representing the third/fourth generations of Japanese immigrants with no ethnic admixture, were randomly selected among the offspring whose father participated in the Honolulu Heart Program[108], a longitudinal follow-up study among Japanese-American men.[109]

2.0 MANUSCRIPT I: SIGNIFICANT INVERSE ASSOCIATIONS OF SERUM N-6 FATTY ACIDS WITH PLASMA PLASMINOGEN ACTIVATOR INHIBITOR-1

A manuscript in preparation for publication

Sunghee Lee[1]; J. David Curb[245]; Takashi Kadowaki[2]; Rhobert Evans[1]; Katsuyuki Miura[2]; Tomoko Takamiya[1]; Chol Shin[6]; Aiman El-Saed[1]; Jina Choo[7]; Akira Fujiyoshi[2]; Teruo Otake[1]; Sayaka Kadowaki[2]; Todd Seto[4]; Kamal Masaki[4]; Daniel Edmundowicz[3]; Hirotsugu Ueshima[2]; Lewis Kuller[1]; Akira Sekikawa[12]

Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA[1]
Department of Health Science, Shiga University of Medical Science, Otsu, Shiga, Japan[2]
Cardiovascular Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, USA[3]
John A Burns School of Medicine, University of Hawaii, Honolulu, Hawaii[4]
Pacific Health Research Institute, Honolulu, Hawaii [5]
Department of Internal Medicine, Ansan Hospital, Korea University, Ansan, South Korea[6]
College of Nursing, Korea University, Seoul, South Korea[7]

2.1 ABSTRACT

Objective: Epidemiological studies suggested that n-6 fatty acids, especially linoleic acid (LA), have beneficial effects on coronary heart disease (CHD), whereas some *in vitro* studies suggested that n-6 fatty acids, specifically arachidonic acid (AA), may have harmful effects. We examined the association of serum n-6 fatty acids with plasminogen activator inhibitor-1 (PAI-1).

Methods and Results: A population-based cross-sectional study recruited 926 randomly selected men aged 40-49 without cardiovascular disease during 2002 to 2006 (310 Caucasian, 313 Japanese, and 303 Japanese-American men). Plasma PAI-1 was analyzed in free form, both active and latent. Serum fatty acids were measured with gas-capillary-liquid-chromatography. To examine the association between total n-6 fatty acids (including LA and AA, respectively) and PAI-1, multivariate regression models were used. After adjusting for confounders, total n-6 fatty acids, LA, and AA were inversely and significantly associated with PAI-1 levels. These associations were consistent across three populations.

Conclusions: Among 915 middle-aged men, serum n-6 fatty acids had significant inverse associations with PAI-1.

Key words: plasminogen activator inhibitor-1; linoleic acid; fatty acids

2.2 INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1), a primary inhibitor of plasminogen activators, has an anti-fibrinolytic function.[74] High levels of PAI-1 are associated with an increased risk for developing coronary heart disease (CHD) or stroke.[75, 110, 111] This increased risk of CHD may be due to promoting platelet adhesion and acute thrombus formation.[75]

Epidemiological studies suggest that linoleic acid (LA), a major component of n-6 fatty acid, has beneficial effects on both CHD and its risk factors, whereas some *in vitro* studies suggest that another n-6 fatty acid, arachidonic acid (AA), may have adverse effects. The Nurses' Health Study showed that a high dietary intake of LA has a strong inverse association with CHD.[69] Additionally, a recent meta-analysis found that dietary intake of LA had a strong inverse association with non-fatal cardiovascular events.[112] The cardioprotective benefits[68] of n-6 fatty acids may be due to decreasing blood pressure,[113] reducing thrombosis,[114] and improving insulin sensitivity.[115] In contrast, several eicosanoids derived from AA are pro-inflammatory and pro-thrombotic, promoting vasoconstriction and enhance platelet aggregation. Thus, AA has been postulated to adversely affect CHD. However, recent studies identified AA-derived eicosanoids to have several beneficial attributes including vasodilation, platelet aggregation inhibition, and anti-inflammatory effects.[64] Interestingly, a recent meta-analysis showed that AA was not associated with fatal or non-fatal cardiovascular events.[112] These contradictory findings suggest that dietary or serum levels of AA have little association with CHD risk, possibly because AA levels are tightly regulated in the human body.[65-67] Although PAI-1 is known to be involved in developing atherothrombosis,[116, 117] very few studies reported their associations with n-6 fatty acids.

The purpose of this study was to test whether higher levels of serum n-6 fatty acids are associated with lower levels of PAI-1 in men aged 40-49. Additionally, we investigated whether higher levels of specific n-6 fatty acids, i.e., LA and AA, are associated with lower levels of PAI-1. To test these hypotheses, we examined data from a population-based cross-sectional study of 926 Caucasian, Japanese, and Japanese-American men aged 40-49 in the Electron-Beam Tomography, Risk Factor Assessment among Japanese and U.S. Men in the Post-World War II Birth Cohort (ERA-JUMP) study.[5]

2.3 METHODS

2.3.1 Study population

Participants were a randomly selected population-based sample of 926 men aged 40-49 between 2002 and 2006 from three centers: 310 Caucasian men from Allegheny County, Pennsylvania, 313 Japanese men from Kusatsu, Shiga, Japan, and 303 Japanese-American men from Honolulu, Hawaii. Those with clinical cardiovascular or other severe diseases were excluded. Detailed descriptions of the study population were previously published.[5, 108, 109] Our final sample was 915 men (304 Caucasian, 313 Japanese, and 298 Japanese-American men) due to 11 missing data. Informed consent from each participant was obtained. The protocol for the study was approved by the Institutional Review Boards of the University of Pittsburgh (Pennsylvania), Shiga University of Medical Science (Otsu, Japan), and the Kuakini Medical Center (Honolulu, Hawaii).

Venipuncture was performed in all participants in the early morning after a 12-hour fast, as previously described.[5] Fasting serum and plasma samples were stored at -80°C, and shipped on dry ice to the Heinz Laboratory at the University of Pittsburgh to examine levels of low-density lipoprotein cholesterol (LDLc), high-density lipoprotein cholesterol (HDLc), total cholesterol, triglycerides, insulin, and glucose, as published elsewhere.[5] A calorimetric-competitive-enzyme-linked immunosorbent assay was utilized to assess CRP (C-reactive protein). Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or anti-hypertensive medication usage. Diabetes mellitus was defined as

fasting glucose level ≥7mmol/L (126mg/dL) or anti-diabetic medication usage. 'Alcohol drinker' was defined as a man who drinks alcohol two days or more per week. 'Current smoking' was defined as a man who smoked during the prior month.

2.3.2 Measurement of plasminogen activator inhibitor-1

As previously reported,[118, 119] plasma PAI-1 was measured at the clinical biochemistry research laboratory of the University of Vermont. Briefly, plasma PAI-1 levels were analyzed in citrated plasma[118] by a two-site ELISA, which was sensitive to free PAI-1 form but not to complexes between t-PA and PAI-1[119], which was developed by Dr. Collen and colleagues.[120] Inter-assay coefficient of variations (CVs) for PAI-1 was 7.7%.

2.3.3 Measurements of serum fatty acids

To measure serum fatty acids in a percentage of total fatty acid amounts, gas-capillary-liquid chromatography (PerkinElmer Clarus 500; PerkinElmer, Waltham, MA) was performed.[6] The intra-assay CVs of LA (18:2n-6) and AA (20:4n-6) in serum n-6 fatty acids were 1.6% and 2.8%, respectively.[6] The CVs for other fatty acids ranged from 2.5% to 9.8%.[6]

2.3.4 Statistical analyses

Log-transformed PAI-1 was used for the analyses, because the distribution of PAI-1 was skewed. To compare descriptive distributions across centers, an analysis of variance (ANOVA) for a continuous variable and a Mantel-Haenszel test for a categorical variable were performed. When

a significant difference among the three groups exists, we examined multiple comparison tests using a Bonferroni test. To pool the data, we tested interactions according to centers on associations of PAI-1 with LA as well as AA. We also assessed the center-specific associations of n-6 fatty acids on PAI-1 according to the three study centers (Table 15). After confirming no interaction and the same direction of the associations by centers, we pooled the data. To estimate the association between serum n-6 fatty acids and PAI-1 levels, we performed multivariate linear regression analyses, adjusting for covariates as followings: in the model I, we adjusted for age and center; in the model II, we further adjusted for body mass index (BMI), current smoking, alcohol drinker, hypertension, and diabetes; in the model III, we further adjusted for LDLc, HDLc, triglycerides, and CRP; in the model IV, we further adjusted for marine n-3 and *trans* fatty acids. Because some eicosanoids of n-3 fatty acids were reported to inhibit the production of AA derived eicosanoids,[64] we tested marine n-3 as well as *trans* fatty acids as covariates. The level of significance was considered to be $p<0.05$. All reported *p*-values were based on two-sided tests. All statistical analyses were performed using SAS 9.2 for Windows (SAS Institute, Inc., Cary, North Carolina, U.S.).

2.4 RESULTS

General characteristics of the 915 study participants are shown in Table 13. The average age was 45 years old. In the total study population, participants with hypertension and diabetes were 24.9% and 7.5%, respectively. Median level (interquartile range) of PAI-1 was 37.4 ng/mL (23.3 to 58.4).

Serum proportions of fatty acids are listed in Table 14. Total n-6, total n-3, saturated, and monounsaturated fatty acids made up 39.0%, 6.5%, 31.2%, and 20.4%, respectively. LA and AA were 28.8% and 8.1%, respectively. A correlation coefficient between LA and AA was 0.06 (p=0.0596).

Table 13. General characteristics of the study participants

	Total (n=915)	Caucasian men (n=304)	Japanese men (n=313)	Japanese-American men (n=298)
Age (year)	45.4 ± 2.8	45.0 ± 2.8	45.1 ± 2.8 †	46.1 ± 2.8 ‡
BMI (kg/m^2)	26.5 ± 4.5	27.9 ± 4.3 *	23.7 ± 3.1 †	28.0 ± 4.6
Waist circumference (cm)	92.6 ± 12.0	98.7 ± 11.8 *	85.2 ± 8.1 †	94.0 ± 11.7 ‡
Systolic blood pressure (mmHg)	125.1± 13.6	122.6 ± 11.2	125.0 ± 16.1	127.6 ± 12.6 ‡
Diastolic blood pressure (mmHg)	75.8 ± 10.2	73.1 ± 8.6 *	76.5 ± 11.9	77.7 ± 9.3 ‡
Hypertension (%)	24.9	15.1 *	26.5	33.2 ‡
LDL cholesterol (mg/dL)	129.5 ± 34.5	134.7 ± 33.6	132.2 ± 35.9 †	121.4 ± 32.5 ‡
Triglyceride (mg/dL)§	140.5 (96.0-224.0)	128.0 (92.0-185.5)	137 (103-182)	140.5 (96.0-224.0)
HDL cholesterol (mg/dL)	50.9 ± 13.1	47.8 ± 12.8 *	54.1 ± 13.6 †	50.7 ± 12.2
Total cholesterol (mg/dL)	212.0 ± 36.9	212.1 ± 37.7	217.2 ± 36.0 †	206.5 ± 36.4
Glucose (mg/dL)	106.7 ± 18.4	101.3 ± 13.7 *	106.8 ± 18.7 †	112.0 ± 20.7 ‡
Insulin (μIU/mL)	13.5 ± 7.9	15.2 ± 8.3 *	10.3 ± 4.4 †	15.1 ± 9.2
Diabetes (%)	7.5	3.3	6.1 †	13.4 ‡
Current smoker (%)	23.5	7.2 *	49.2 †	13.1
Alcohol drinker (%)	49.6	44.1 *	67.1 †	36.9
C-reactive protein (mg/l)§	0.6 (0.3-1.3)	1.0 (0.5-1.8)	0.3 (0.2-0.7)	0.7 (0.3-1.3)
PAI-1 (ng/mL)§	37.4 (23.3-58.4)	27.4 (15.7-41.3) *	41.2 (24.3-67.3)	45.9 (31.6-61.7) ‡

Mean ± SD. S.D.=standard deviation;
Significance test was based on ANOVA, followed by Bonferroni test if the overall ANOVA was significant.
* Under Bonferroni test, significant difference between Caucasian and Japanese men, $p<0.01$
† Under Bonferroni test, significant difference between Japanese and Japanese-American men, $p<0.01$
‡ Under Bonferroni test, significant difference between Caucasian and Japanese-American men, $p<0.01$
BMI=body mass index; LDL=low density lipoprotein; HDL=high density lipoprotein;
§ Median (interquartile range)

Table 14. Distribution of serum fatty acids (%)

	Total (n=915)	Caucasian men (n=304)	Japanese men (n=313)	Japanese-American men (n=298)
Polyunsaturated fatty acids	45.4 ± 4.4	45.6 ± 4.5 *	44.3 ± 3.8 †	46.5 ± 4.7
Total n-6 fatty acids	39.0 ± 5.2	41.3 ± 4.1 *	34.7 ± 4.2 †	41.1 ± 4.3
Linoleic acid	28.8 ±4.5	29.9 ± 4.1 *	26.5 ± 4.1 †	30.0 ± 4.3
Arachidonic acid	8.1 ± 2.2	9.0 ± 1.9 *	6.6 ± 1.3 †	8.9 ± 2.3
Total n-3 fatty acids	6.5 ± 3.4	4.2 ± 1.8 *	9.6 ± 3.0 †	5.4 ± 2.3 ‡
Marine n-3 fatty acids	6.0 ± 3.4	3.8 ± 1.8 *	9.3 ± 3.0 †	4.9 ± 2.2 ‡
α-linolenic fatty acids	0.3 ± 0.3	0.3 ± 0.3 *	0.2 ± 0.2 †	0.4 ± 0.4 ‡
Monounsaturated fatty acids	20.4 ± 3.4	20.3 ± 3.2 *	21.2 ± 3.1 †	19.6 ± 3.6
Saturated fatty acids	31.2 ± 2.3	30.9 ± 2.4 *	31.7 ± 2.2 †	30.9 ± 2.2
Trans fatty acids	0.8 ± 0.4	1.0 ± 0.5 *	0.6 ± 0.2 †	0.9 ± 0.4 ‡

Mean ± S.D. (standard deviation)
Total n-6 fatty acids indicate the sum of linoleic acid (18:2n-6), gamma-linoleic acid (18:3n-6), dihomo-gamma-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6).
Marine-derived n-3 fatty acids were defined as eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3).
Total n-3 fatty acids indicate marine-derived n-3 fatty acids, eicosatetraenoic acid (20:4n-3) and α-linolenic acid (22:18n-3).
Saturated fatty acids indicate the sum of myristic aicd (14:0), palmitic acid (16:0) and stearic acid (18:0).
Monounsaturated fatty acids indicate the sum of palmitoleic acid (16:1n-7), oleic acid (18:1n-9), and cis-vaccenic acid (18:1n-7).
Trans fatty acids indicate the sum of palmitelaidic acid (16n-7:1t), trans 9-octadecanoic acid (18n-9:1t) and linolelaidic acid (18n-6:2tt).
Significance test was based on ANOVA followed by Bonferroni test if the overall ANOVA was significant.
* Under Bonferroni test, significant difference between Caucasian and Japanese men, $p<0.01$
† Under Bonferroni test, significant difference between Japanese and Japanese-American men, $p<0.01$
‡ Under Bonferroni test, significant difference between Caucasian and Japanese-American men, $p<0.01$

Pooled and center-specific analyses reveal that serum n-6 fatty acids were inversely associated with PAI-1 in the total population as well as in each of three different populations (Table 15). Pooled analyses showed that serum total n-6 fatty acids, LA, and AA had significant inverse associations with PAI-1 levels, even after adjusting for covariates. In center-specific analyses, PAI-1 had significant inverse associations with total n-6 fatty acids and LA over three study populations. These significant associations remained after multivariate adjustments. PAI-1 was inversely associated with AA. No significant interaction existed in the associations of serum n-6 fatty acids, LA, and AA with PAI-1 according to the study centers (p=0.0857, 0.0832, and 0.2373, respectively). Standard parameter estimates indicate a standard deviation (SD) unit change in log-transformed PAI-1 per a 1 SD unit increase in serum n-6 fatty acids.

Table 15. Center-specific and pooled associations between n-6 fatty acids and log(PAI-1)

	Standardized parameter estimates			
	Total (n=915)	Caucasian men (n=304)	Japanese men (n=313)	Japanese-American men (n=298)
Total n-6 Fatty acids				
Univariate	-0.2674*	-0.3185*	-0.2452*	-0.2711*
Model I	-0.3224*	-0.3184*	-0.2412*	-0.2711*
Model II	-0.2390*	-0.1807*	-0.2006*	-0.2069*
Model III	-0.1315*	-0.0642*	-0.1150*	-0.0996*
Model IV	-0.1354*	-0.0899*	-0.1169*	-0.0903*
Linoleic acid				
Univariate	-0.2346*	-0.2862*	-0.1952**	-0.2306*
Model I	-0.2392*	-0.2870*	-0.1912**	-0.2306*
Model II	-0.1708*	-0.1542*	-0.1494*	-0.1779*
Model III	-0.1015*	-0.0659*	-0.0704*	-0.1180*
Model IV	-0.1014*	-0.0819*	-0.0441*	-0.1140*
Arachidonic acid				
Univariate	-0.1568*	-0.1299***	-0.1852**	-0.1081
Model I	-0.1424*	-0.1314	-0.1849**	-0.1082
Model II	-0.1010*	-0.0826*	-0.1680*	-0.0669*
Model III	-0.0087*	-0.0031*	-0.0974*	0.0401*
Model IV	-0.0087*	-0.0029*	-0.0966*	0.0372*

*p<0.001; **p<0.01; ***p<0.05

Total n-6 fatty acids indicate the sum of linoleic acid (18:2n-6), gamma-linoleic acid (18:3n-6), dihomo-gamma-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6).

Marine-derived n-3 fatty acids were defined as eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3).

Total n-3 fatty acids indicate marine-derived n-3 fatty acids, eicosatetraenoic acid (20:4n-3) and α-linolenic acid (22:18n-3).

Trans fatty acids indicate the sum of palmitelaidic acid (16n-7:1t), trans 9-octadecanoic acid (18n-9:1t) and linolelaidic acid (18n-6:2tt).

Model I: adjusted for age;

Model II: additionally adjusted for BMI, current smoking, alcohol drinker, hypertension, and diabetes;

Model III: further adjusted for LDL cholesterol, HDL cholesterol, triglycerides, and CRP (C-reactive protein);

Model IV: continuously adjusted for marine n-3 fatty acids, and trans fatty acids.

2.5 DISCUSSION

This population-based cross-sectional study found that total serum n-6 fatty acids were inversely and significantly associated with PAI-1 among 915 men, aged 40-49. Additionally, both LA and AA showed significant inverse associations with PAI-1 levels (both, $p<0.0001$).

Our present study may provide a novel mechanism on the cardioprotective benefits of n-6 fatty acids by improving the fibrinolytic response, such as reducing PAI-1. Our finding of an inverse association between serum n-6 fatty acids and PAI-1 is consistent with the results of several previous studies,[78, 79] but not all.[121] These findings of n-6 fatty acids suggest a favorable fibrinolytic response on vascular thrombosis, including a decrease in platelet aggregation. Fleischman *et al*. found an increased platelet aggregation time ($p<0.05$) and a decreased disaggregation time ($p<0.01$) on a dietary LA in each for two weeks from about 2.9% to about 5.0% of energy among 66 subjects.[78] A crossover study by Thijssen *et al.* also demonstrated an increased platelet aggregation time while on a LA diet in comparison to on a saturated fatty acid diet in 18 men ($p=0.04$).[122] O'Brien *et al*. conducted a clinical trial in 39 healthy men for six weeks with either a PUFA diet (sunflower oil based foods, 65% LA) replaced for saturated fat or a normal diet.[79] They found the decreased platelet count ($p=0.01$), and the increased bleed time ($p=0.05$).[79] Further, previous studies, including the Nurses' Health Study,[69] have suggested that n-6 fatty acids lower the CHD risk, through a decrease in blood pressure,[113] a reduction of thrombosis,[114] and an improvement in insulin sensitivity.[115]

Our results of the inverse association between serum n-6 fatty acids and PAI-1 are partially inconsistent with the results of a previous study. Byberg *et al.* showed that PAI-1 activity has a significant inverse association with serum LA but a significant positive association with serum AA in their sub-analysis with 381 men from a population-based cross-sectional sample of 871 men aged 70 years.[121] The discrepancy in the association of PAI-1 with AA may be attributed to different measurements of PAI-1 and fatty acids or to different ages of participants. In measurements of PAI-1 and fatty acids, Byberg *et al.* measured PAI-1 activity (i.e., a free active form) and serum cholesterol ester for fatty acid measurements in older participants (mean average 70 years), whereas we measured total plasma PAI-1 levels (i.e., free active, free latent, and complex with t-PA forms) and fatty acids in serum cholesteryl ester, phospholipids, and triglycerides, in middle-aged men (ages 40-49). Although the previous study demonstarted a linear association between PAI-1 activity and PAI-1 antigen (r=0.80 in platelet-poor plasma; r=0.88 in platelet-rich plasma), about 66.7% of PAI-1 antigen in plasma was active.[120] Considering very short half-life of PAI-1 levels, and various processes (e.g., temperature, time, or pH) for handling the blood samples, as well as significant diurnal change of PAI-1, the PAI-1 antigen measurement as in our study may have the advantage of detecting comprehensive forms of relatively unstable total plasma PAI-1, rather than measuring only an active form.

Future studies are required in order to elucidate possible reasons of the discrepancy between *in vitro* and the population studies. Several *in vitro* studies have shown that LA increases the secretion of PAI-1 in HepG2 cells.[123, 124] *In vitro* studies reported that AA-produced eicosanoids promoted neutrophil adhesion[81] and IL-1β production by human monocytes[125]. However, more recent studies demonstrated no effect or a beneficial effect. A double-blind

placebo-controlled study with an AA supplementation of 840 mg/day for four weeks demonstrated no effect on platelet aggregation in 24 healthy Japanese men who had relatively high levels of fish oil consumption.[84] In another clinical trial of 10 healthy men taking a 200mg/day vs. 1,500mg/day AA regimen, Nelson *et al*. found a borderline significance between higher AA intake and prolonged bleeding time (p=0.06).[83] Although several AA-derived eicosanoids may indeed have a pro-inflammatory role, recent studies suggest that several AA-derived eicosanoids may play an anti-inflammatory role.[45]

Mechanisms responsible for the association of n-6 fatty acids with PAI-1 require future studies. However, two possibilities exist. First, n-6 fatty acids may delay platelet aggregation so that PAI-1, acting as an acute-phase reactant, is decreased within hemodynamic balance and thrombotic response, during vascular injury, in atherosclerosis and CHD. Several previous studies have shown that LA reduces platelet aggregation.[78, 79] Second, LA may reduce PAI-1 levels through its cholesterol lowering effect. A previous study from ERA-JUMP found that higher levels of serum LA and AA were associated with lower levels of LDL and VLDL.[126] An *in vitro* study showed that VLDL led to increased PAI-1.[127] These reduced cholesterol levels may improve or modulate the fibrinolytic response.

The strengths of the present study include the following: a) the association was examined in a randomly selected population-based sample; and b) the sample size was relatively large. However, this study also has several limitations: a) the cross-sectional study design could not assess a causality; b) the study population included only men aged 40-49 years, which may limit the generalizability to other populations; and c) as an observational study, this present study may include residual confounding or potentially unmeasured factors, such as total energy intake.[128]

In conclusion, serum n-6 fatty acids were inversely and significantly associated with PAI-1 levels in a population-based cross-sectional study. Total n-6 fatty acids, especially LA and AA, were inversely and significantly associated with PAI-1 levels in both univariate and multivariate models. These findings suggest that n-6 fatty acids may have favorable effects on fibrinolysis. A future study to examine the causality between n-6 fatty acid and PAI-1 is warranted.

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3.0 MANUSCRIPT II: SERUM 25(OH)D LEVELS ARE NOT ASSOCIATED WITH SUBCLINICAL ATHEROSCLEROSIS IN MIDDLE-AGED MEN

A manuscript in preparation for publication

Sunghee Lee[1]; Takashi Kadowaki[2]; J. David Curb[345]; Rhobert W. Evans[1];Katsuyuki Miura[3]; Robert D. Abbott[5]; Emma J.M. Barinas-Mitchell[1]; Hirotsugu Ueshima[3]; Chol Shin[7]; Jina Choo[6]; Daniel Edmundowicz[2]; Lewis H. Kuller[1]; Akira Sekikawa[13]

Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, USA[1]
Cardiovascular Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, USA[2]
Department of Health Science, Shiga University of Medical Science, Otsu, Shiga, Japan[3]
John A Burns School of Medicine, University of Hawaii, Honolulu, Hawaii[4]
Pacific Health Research Institute, Honolulu, Hawaii [5]
Korea University College of Nursing, Seoul, South Korea[6]
Department of Internal Medicine, Korea University Ansan Hospital, Ansan, South Korea[7]

3.1 ABSTRACT

Background: Previous studies have shown that vitamin D deficiency increases the risk for coronary heart disease (CHD). However, results on the association on subclinical atherosclerosis associated with vitamin D deficiency are inconsistent. We hypothesized that the vitamin D levels among Japanese men would be higher than the levels among Caucasian and Japanese-American men, due to the Japanese habitual intake of fish, which is a major food source of vitamin D. We further hypothesized that vitamin D deficiency would be associated with subclinical atherosclerosis.

Methods and Results: In a population-based cross-sectional study, 295 men aged 40-49 years without cardiovascular and other severe diseases, were randomly selected from three study centers (99 Japanese men from Shiga, Japan, 98 Caucasian men from Allegheny County, PA, and 98 Japanese-American men from Honolulu, HI) from 2002 to 2006. We explored the vitamin D levels of the three populations and the association between vitamin D deficiency and subclinical atherosclerosis. A liquid chromatography-tandem mass spectrometry was utilized to measure the serum vitamin D, including both 25(OH) vitD_2 and 25(OH) vitD_3. Two measurements of subclinical atherosclerosis, intima-media thickness (IMT) by an ultrasound scan and coronary artery calcification (CAC) by an Electro-Beam-Computed-Tomography (EBCT), were examined in standardized protocols across all three centers. We utilized multivariate regression models to estimate the association of vitamin D deficiency (defined as 25(OH)D<20 ng/mL) with IMT and logistic regression to estimate odds ratios on CAC (defined as coronary calcium score ≥10). The prevalence rates of vitamin D deficiency were 43.4% (43 of 98) in Japanese men, 11.2% (11 of 98) in Caucasian men, and 9.2% (9 of 98) in Japanese-

American men. In a univariate model, vitamin D deficiency showed a significant positive association with IMT only in Caucasian men (p=0.022). However, in multivariate models, vitamin D deficiency no longer showed a significant association with IMT. For the Japanese-American men, vitamin D deficiency was significantly associated with CAC, even after adjusting for covariates (adjusted OR=8.02, 95%CI 1.38-46.62).

Conclusion: In this population-based cross-sectional study, we found that the Japanese men showed lower serum vitamin D level than the Caucasian or the Japanese-American men. We further observed that vitamin D deficiency was not associated with either IMT or CAC, except for a few significant associations: Caucasian men had a significant association between vitamin D deficiency with IMT in a univariate model but not in multivariate models, and Japanese-American men had significant associations between vitamin D deficiency and CAC in multivariate models.

3.2 INTRODUCTION

Intima media thickness (IMT) of the carotid artery and coronary artery calcification (CAC), both highly reliable and non-invasive methods to measure subclinical atherosclerosis, have demonstrated strong associations with cardiovascular events.[15, 16, 129] As a sensitive measurement, IMT can detect the relatively early stage of atherosclerosis.[19, 20] CAC, based on a coronary artery calcium score as an independent predictor for CHD events,[130] demonstrated strong associations with CHD risk factors.[129]

Recent epidemiological evidence shows that vitamin D deficiency may increase the CHD risk. Cross-sectional studies with the National Health and Nutrition Examination Survey (NHANES) data[94-96, 131, 132] and many prospective studies have reported that low 25(OH)D levels are associated with high CHD risk factors, including hypertension[94, 100, 101, 133], glucose intolerance[134], diabetes mellitus[135], and obesity[136, 137]. Despite this impressive evidence linking vitamin D deficiency with CHD, the association between vitamin D deficiency and subclinical atherosclerosis, as measured by IMT and CAC, remains inconclusive.[92, 93] IMT of the internal carotid but not of the common carotid had a significant association in 654 adults, aged 55-96.[91] CAC showed an inverse association with serum vitamin D levels in 173 adults[138] but no association among 50 coronary angiography reported patients.[139]

Despite consistent evidence on the associations between vitamin D deficiency and both CHD and its risk factors, for example high blood pressure, few studies on the association between subclinical atherosclerosis and vitamin D deficiency were examined. Further evaluations are needed among different populations. For example, Japanese men have very low

prevalence of atherosclerosis as compared to Caucasian men.[5] To examine and explore the association between subclinical atherosclerosis and vitamin D deficiency among three different populations will extend our understanding of the relationship between vitamin D deficiency and CHD.

The present study aimed to examine whether the vitamin D levels in Japanese men are higher due to habitual fish intake (major food source of vitamin D) than the levels in the other two study populations, Caucasian and Japanese-American men. Additionally, we examined whether vitamin D deficiency (defined as 25(OH)D<20ng/mL) is associated with subclinical atherosclerosis as measured by IMT and CAC. To test these hypotheses, a population-based cross-sectional study of 295 participants (99 Japanese, 98 Caucasian, and 98 Japanese-American men) from three study centers in the 'Electron-Beam Tomography, Risk Factor Assessment among Japanese and U.S. Men in the Post-World War II Birth Cohort' (ERA-JUMP) study was examined.

3.3 METHODS

3.3.1 Study population

Detailed descriptions of the ERA-JUMP study population and measurements have been previously published.[5, 140] Briefly, between 2002 and 2006, 926 men aged 40-49 years, were randomly selected from three study centers (313 Japanese men from Shiga, Japan, 310 Caucasian men from Pennsylvania, U.S., and 303 Japanese-Americans men from Honolulu, Hawaii). All participants were without cardiovascular or other severe diseases. For this vitamin D study, 300 men, comprised of 100 men from each of the three study centers, were randomly chosen. After excluding five participants due to missing data, the final sample totaled 295 men (99 Japanese, 98 Caucasians, and 98 Japanese-Americans). All participants signed informed consent forms. This study was approved by the Institutional Review Boards of the three institutions (Shiga University of Medical Science, Japan; the University of Pittsburgh, U.S.; and the Kuakini Medical Center, Honolulu).

All participants, wearing light clothing and no shoes, took a physical examination to measure body weight and height. After empting their bladder and sitting quietly for five minutes, they had their blood pressure measurements taken twice and then averaged to use for analyses.

Venipuncture was performed after the participants fasted for 12 hours. Blood samples were stored at -80°C and then shipped on dry ice to the Heinz Laboratory, University of Pittsburgh, where using the standardized methods, fatty acids, insulin, and glucose were measured. The protocols standardized by the Centers for Disease Control and Prevention (CDC)

were utilized to measure serum lipids.[141] Particularly, gas-capillary-liquid chromatography (PerkinElmer Clarus 500; PerkinElmer, Waltham, MA) was used to measure serum fatty acids in the percentage unit of total fatty acid amount.[6] Marine n-3 fatty acids were defined as the sum of eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3). The intra-assay coefficients of variation (CV) were 2.5% for EPA, 2.5% DPA, and 7.0% DHA.[6] C-reactive protein (C-RP) was measured by a colorimetric competitive enzyme-linked immunosorbent assay.[142]

To gather information on the participants' demographic factors, alcohol and smoking habits, and medication usage, each participant completed a self-administered questionnaire. 'Alcohol drinker' was defined as an individual who drank alcohol more than two days per week. 'Current smoking' was referred to an individual who smoked cigarettes during the prior month. Hypertension was defined if a participant who had either a systolic blood pressure of ≥140 mmHg or a diastolic blood pressure of ≥90 mmHg, or used anti-hypertensive medications. Diabetes mellitus was defined as a fasting serum glucose level ≥7 mmol/L (126 mg/dL) or medication usage for diabetes.

3.3.2 Vitamin D measurement

Serum 25(OH)D levels were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS), including both 25(OH) vitD2 and 25(OH) vitD3, at the Mayo medical laboratory.[143] Circulating serum 25(OH)D, which can reflect total levels of vitamin D from both sun exposure as well as dietary intake, has been used to measure vitamin D levels in body.[88]

3.3.3 Intima-media thickness (IMT)

The descriptions of detailed procedures to measure subclinical atherosclerosis were previously reported.[5, 144] Trained sonographers used standardized procedures to measure IMT. They examined both far and near walls of thicknesses between lumen intima and media-adventitia, as well as the common carotid artery, which was 1cm proximal to the bifurcation. The scans were sent to read at the Ultrasound Research Laboratory, University of Pittsburgh. Measurements were averaged with each of the locations of right and left scorings. When trained readers performed scoring, they were blinded about the detailed information on the study subjects. The IMT scores have high reproducibility with high correlations (r=0.96) between sonographers and (r=0.99) between readers.[144]

3.3.4 Electron beam computed tomography (EBCT)

Scanning methods were standardized at each of three study centers by a GE-Imatron C150 Electron Beam Tomography scanner (GE Medical Systems, South San Francisco, US). Scanned images were sent to the Cardiovascular Institute, University of Pittsburgh. One trained scan reader used a Digital-Imaging-and-Communications-in-Medicine (DICOM) workstation and software by Accu-Image (AccuImage Diagnostic Corporation, San Francisco, U.S.). The Agatston scoring method was used to calculate the coronary calcium score. The reproducibility of the Agatston calcium scores was 0.98 of the intraclass correlation.[145]

3.3.5 Statistical analyses

To compare characteristics of risk factors among three population groups, an analysis of variance (ANOVA) test was used. Further, Bonferroni pair-wise comparisons were conducted for testing differences, which were considered significant at $p<0.01$. According to the literature review and the distribution of 25(OH)D within each study population, vitamin D deficiency was defined as less than 20 ng/mL of 25(OH)D.[146 147 92 148] To compare the vitamin D deficient and sufficient groups, a t-test and a chi-square test were utilized. For several variables with skewed distribution, a Kruskal-Wallis test for the three populations and a Mann-Whitney U test for the two group comparisons were utilized. To evaluate linear correlations between vitamin D deficiency and subclinical atherosclerosis measurements by IMT and CAC, correlation coefficients were examined. In order to assess the association between IMT and vitamin D deficiency, generalized linear models were utilized with log-transformed 25(OH)D levels. For the multivariate model, the following covariates were included: age (continuous), Body Mass Index (BMI) (continuous), hypertension (yes/no), diabetes (yes/no), alcohol (yes/no), current smoking (yes/no), LDLc (continuous), triglyceride (continuous), C-RP (continuous), and marine n-3 fatty acids (continuous). We had similar results with the categorical form of 25(OH)D on IMT in multivariate models. To examine the association between CAC and vitamin D deficiency, logistic regression models were tested. The CAC was defined as equal to or greater than 10 of a coronary calcium score. Additionally, no interactions were observed with several covariates, such as hypertension (yes/no), diabetes (yes/no), and obesity (obese/overweight/normal). All reported p-values were based on two-sided tests with the significance of less than 0.05. All statistical analyses were performed using SAS 9.2 for Windows (SAS Institute, Inc., Cary, North Carolina, U.S.).

3.4 RESULTS

3.4.1 Comparisons of risk factors among three study populations

Table 16 shows the general characteristics of the study participants. The Japanese men were less obese, had a higher HDLc level, had a higher smoking, had a high alcohol consumption, and had more than twice the amount of marine n-3 fatty acids than the Caucasian men. The Japanese men had lower systolic blood pressure, triglycerides, hypertensive and diabetic rates, than the Japanese-American men, but higher LDLc levels.

The serum vitamin D levels among the Japanese men were lower than the levels in the other two populations. Prevalence rates of vitamin D deficiency (defined as <20ng/ml) were 43.4% (43 of 98) in Japanese men, 11.2% (11 of 98) in Caucasian men, and 9.2% (9 of 98) in Japanese-American men. Caucasian men appeared to have as much 25(OH)D as the Japanese-American in Hawaii. The Japanese men also had lower IMT and CAC prevalence than the Caucasian and the Japanese-American men. The Japanese-American men had higher vitamin D levels than the other two populations. However, they also showed higher IMT levels and CAC percentages.

Table 16. Characteristics of the study participants during 2002-2006 (n=295)

	Caucasian (n=98)	Japanese (n=99)	Japanese-American (n=98)
Age (year)	45.5 ± 2.8	45.0 ±2.9 [b]	46.4 ± 2.6
BMI (kg/m^2)	27.5 ± 4.5[a]	23.6 ±3.0 [b]	28.2 ± 4.2
Systolic BP (mmHg)	122.7 ± 10.6	124.7±17.1	129.4 ±14.1[c]
Diastolic BP (mmHg)	73.3 ± 8.1	75.2 ±11.7	78.9 ± 10.3 [c]
Hypertension (n[%])	13 (13.3)	22 (22.2) [b]	41 (41.8) [c]
LDLc (mg/dL)	133.4 ± 34.4	134.1 ±34.4[b]	116.7 ±28.1[c]
Triglycerides (mg/dL) §‡	119 (92-163)	132 (96-176)	163 (96-286)
HDLc (mg/dL)	47.7 ± 13.4[a]	53.4 ± 10.8	51.4 ± 13.9
Total cholesterol (mg/dL)	207.8 ± 37.6	216.3 ± 39.5	206.9 ± 31.0
Diabetes (n[%])	3 (3.1)	6 (6.1)	17 (17.4) [c]
Current smoker (n[%])	5 (5.1)[a]	55 (55.6) [b]	16 (16.3)
Alcohol drinker (n[%])	42 (42.9)[a]	67 (67.7) [b]	36 (36.7)
n-3 fatty acids	4.5 ± 1.9 [a]	9.3 ± 3.1 [b]	4.7 ± 1.7
Plant fatty acid (α-linolenic acid)	0.2 ± 0.2	0.2 ± 0.1 [b]	0.4 ± 0.4 [c]
Marine n-3 fatty acids	4.3 ± 1.9 [a]	9.1 ± 3.1 [b]	4.3 ± 1.7
Serum vitamin D (25(OH)D) (ng/mL)			
(Mean, SD)	28.9 ± 8.5 [a]	20.8 ± 6.8 [b]	29.2 ± 8.0
(Median, interquartile range) §‡	28 (24-35)	21 (16-24)	28 (24-33)
Carotid intima-media thickness (IMT)			
Common carotid IMT (mm)	0.64 ± 0.07	0.63 ± 0.08 [b]	0.71 ± 0.11 [c]
Coronary artery calcification (CAC)			
Total calcium score §‡	0 (0-8.2)	0 (0-1.8)	1 (0-37.4)
Coronary artery calcification (CAC) (n[%])	22 (22.5)	13 (13.1) [b]	31 (31.6)

Mean ± SD; § Median (interquartile range); ‡ Kruskal-wallis for significance test, $p<0.05$; BMI=body mass index; LDLc=low density lipoprotein cholesterol; BP=blood pressure; HDLc=high density lipoprotein cholesterol; Marine n-3 fatty acids include eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3); n-3 fatty acids indicate the sum of α-linolenic acid (18:3n-3), eicosatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3); CAC is defined as those with coronary calcium score ≥10;

Significance test was based on ANOVA followed by Bonferroni test if the overall ANOVA was significant.

a. Under Bonferroni test, significant difference between Caucasian men and Japanese, $p<0.01$

b. Under Bonferroni test, significant difference between Japanese and Japanese-American, $p<0.01$

c. Under Bonferroni test, significant difference between Caucasian men and Japanese-American, $p<0.01$

3.4.2 Comparisons of risk factors between vitamin D deficient and sufficient groups according to three study populations

Table 17 presents the comparisons among the vitamin D deficient and sufficient groups over the three populations. The Caucasian men in the deficient vitamin D group were elderly and had significantly higher systolic blood pressure levels than those men in the sufficient vitamin D group. No other significant differences were observed, but those men with vitamin D deficient levels tended to have higher levels of risk factors than those with vitamin D sufficient levels.

Table 17. Characteristics between vitamin D deficiency and sufficient groups (n=295)

	Caucasian (n=98)		Japanese (n=99)		Japanese-American (n=98)	
	<20 ng/mL (n=11)	≥20 ng/mL (n=87)	<20 ng/mL (n=43)	≥20 ng/mL (n=56)	<20 ng/mL (n=9)	≥20 ng/mL (n=89)
Age (year)	47.3 ± 2.2*	45.3 ± 2.8	45.5 ± 2.9	44.6 ± 2.9	46.9 ± 3.1	46.3 ± 2.6
BMI (kg/m^2)	28.8 ± 4.2	27.3 ± 4.5	24.3 ± 3.2	23.1 ± 2.8	26.3 ± 5.0	28.4 ± 4.0
Systolic BP (mmHg)	129.3 ± 12.8*	121.9 ±10.1	125.8 ±16.4	123.9 ±17.7	131.6 ±13.6	129.2 ±14.2
Diastolic BP (mmHg)	77.7 ± 8.3	72.7 ± 7.9	76.4 ±11.0	74.2 ±12.3	77.4 ± 7.7	79.1 ±10.5
Hypertension (n[%])	3 (27.3)	10 (11.5)	9 (20.9)	13 (23.2)	4 (44.4)	37 (41.6)
LDL-C (mg/dL)	132.9 ±33.5	133.5 ±34.7	135.3 ±34.0	133.1 ±35.0	111.3 ±19.8	117.2 ±28.8
Triglycerides (mg/dL) §‡	127 (97-184)	118 (86-163)	147 (111-224)	127 (96-162)	121 (82-252)	168 (102-286)
HDL-C (mg/dL)	46.2 ± 7.9	47.9 ±13.9	52.6 ±12.9	54.1 ± 9.0	54.9 ±19.0	51.0 ±13.4
Total-C (mg/dL)	209.7 ± 29.9	207.6 ±38.6	218.9 ±37.5	214.2 ±41.2	202.0 ±18.3	207.4 ±32.1
Diabetes (n[%])	0 (0)	3 (3.5)	2 (4.7)	4 (7.1)	2 (22.2)	15 (16.9)
Current smoker (n[%])	1 (9.1)	4 (4.6)	24 (55.8)	31 (55.4)	1 (11.1)	15 (16.9)
Alcohol drinker (n[%])	6 (54.6)	36 (41.4)	30 (69.8)	37 (66.1)	4 (44.4)	32 (36.0)
n-3 fatty acids	4.6 ± 2.4	4.5 ± 1.9	9.6 ± 3.7	9.1 ± 2.6	4.5 ± 2.5	4.8 ± 1.6
Plant fatty acid (α-linolenic)	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.4	0.4 ± 0.4
Marine n-3 fatty acids	4.3 ± 2.4	4.5 ± 1.9	9.3 ± 3.6	8.9 ± 2.6	4.0 ± 2.5	4.3 ± 1.6
Serum vitamin D (25(OH)D) (ng/mL)						
(Mean, SD)	14.1 ± 3.1*	30.8 ± 6.9	14.9 ± 3.2*	25.3 ± 5.2	17.0 ± 1.9*	30.5 ± 7.4
(Median, interquartile-range)§‡	15.0 (12.0-16.0)*	29.0 (25.0-36.0)	16.0 (12.0-18.0)*	24.0 (21.0-27.5)	17.0 (17.0-18.0)*	29.0 (26.0-34.0)
Carotid intima-media thickness (IMT) (mm)						
Common IMT	0.69± 0.07*	0.64 ± 0.07	0.63 ± 0.08	0.62 ± 0.09	0.68 ± 0.14	0.71 ± 0.11
Coronary artery calcification (CAC)						
Total calcium score§‡	0 (0-26.7)	0 (0-8.2)	0 (0-3.1)	0 (0-1.0)	46.3 (6.4-57.8)	0 (0-28.8)
Coronary Calcium Score (n[%])	3 (27.3)	19 (21.8)	5 (11.6)	8 (14.3)	6 (66.7)	25 (28.1)

Mean ± SD; § Median (interquartile range); * Under t-test or Mann-Whitney U test for a continuous variable and under chi-square test for a categorical variable between vitamin D deficiency and normal, $p<0.05$; S.E.=standard error; BMI=body mass index; LDL-C=low density lipoprotein cholesterol; BP=blood pressure; HDL-C=low density lipoprotein cholesterol; CAC is defined as those with coronary calcium score ≥10; Marine n-3 fatty acids include eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3); n-3 fatty acids indicate the sum of α-linolenic acid (18:3n-3), eicosatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3

3.4.3 Univariate and multivariate models to estimate risks on intima-media thickness associated with vitamin D deficiency

Table 18 shows the univariate and multivariate associations of 25(OH)D levels on IMT. In a univariate model, 25(OH)D levels were a significantly and inversely associated with IMT only in Caucasian men (β=-0.23, p-value=0.022). However, in multivariate models adjusted for age, BMI, and other covariates (Models I, II, and III), the significant association between vitamin D deficiency and IMT disappeared. In Model III, marine n-3 fatty acid was included as a covariate, but the estimates and p-values rarely changed. Additionally, the present data did not show a significant correlation between 25(OH)D and marine n-3 fatty acids, even among the Japanese men (*Pearson* correlation coefficient [r]= -0.08, p-value=0.4624).

Table 18. Univariate and multivariate regression models with intima-media thickness (IMT) (n=295)

	Caucasian (n=98)			Japanese (n=99)			Japanese-American (n=98)		
Model	β	S.E.	*p*-value	β	S.E.	*p*-value	β	S.E.	*p*-value
25(OH)D	-0.23	0.022	0.022	-0.02	0.025	0.857	-0.07	0.042	0.497
Model I	-0.18	0.020	0.054	0.07	0.023	0.440	-0.08	0.040	0.422
Model II	-0.17	0.021	0.078	0.06	0.024	0.524	-0.09	0.041	0.388
Model III	-0.16	0.021	0.088	0.06	0.025	0.541	-0.09	0.042	0.401

β=Standardized parameter estimates; 25(OH)D was log-transformed; S.E.=standard error; Marine n-3 fatty acids were defined as eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3). In univariate model, 25(OH)D was only adjusted; In model I, age and BMI were adjusted; In model II, hypertension, diabetes, current smoking, alcohol, LDL-cholesterol and triglyceride were further adjusted; In model III, c-reactive protein and marine fatty acids were additionally adjusted

3.4.4 Odds ratios on coronary artery calcification (CAC) associated with vitamin D deficiency

Table 19 lists the associations between vitamin D deficiency and CAC. Unlikely the Japanese men and the Caucasian men, only the Japanese-American men showed significant associations of vitamin D deficiency on CAC. The Japanese-American men showed significantly increased odd ratios in an univariate model (OR=5.12, 1.19-22.07), even after adjusting for covariates (OR=8.02, 1.38-46.62).

Table 19. Odds ratios on coronary artery calcification (CAC) associated with vitamin D deficiency (n=295)

	Caucasian (n=98)		Japanese (n=99)		Japanese-American (n=98)	
Model	OR	95% CI	OR	95% CI	OR	95% CI
25(OH)D	1.34	0.32-5.56	0.79	0.24-2.61	5.12	1.19-22.07
Model I	0.77	0.17-3.53	0.40	0.10-1.60	6.05	1.23-29.81
Model II	0.92	0.19-4.37	0.42	0.10-1.78	8.02	1.38-46.62

OR=Odds Ratio; Reference groups are 25(OH)D ≥20ng/mL; CAC is defined as those with coronary calcium score ≥10; CI=Confidence Interval; In univariate model, only vitamin D deficiency (defined as <20 ng/mL) was adjusted; In the model I, age and BMI were adjusted; In the model II, hypertension, alcohol, LDL-cholesterol and triglyceride were further adjusted

3.5 DISCUSSION

In this population-based cross-sectional study, we found that the Japanese men do not have higher serum levels of vitamin D, despite their habitual fish intake which leads to high serum marine n-3 fatty acids, as compared to the Caucasian and Japanese-American men. We further found that vitamin D deficiency is not associated with subclinical atherosclerosis as measured by IMT and CAC, except for a few significant associations: 1) the Caucasian men had a significant inverse association of vitamin D on IMT only in a univariate model; and 2) the Japanese-American men showed significant associations of vitamin D deficiency with CAC in both the univariate and multivariate models.

Our findings of no association between vitamin D deficiency and subclinical atherosclerosis as measured by IMT or CAC are consistent with previous results. Michos and colleagues found no association with serum 25(OH)D levels on IMT of the common carotid artery (cIMT) and CAC in 650 Amish adults (cIMT p_{trend}=0.52, and CAC, p_{trend}=0.80).[92] Pilz and colleagues demonstrated that serum 25(OH)D levels did not have a significant association with cIMT in 614 men and women (β= -0.011, p=0.794).[93] In a Women's Health Initiative (WHI), that a treatment group of receiving calcium plus vitaminD3 for seven years showed no difference on CAC, as compared to a placebo group (p=0.74).[149]

In contrast, several studies are inconsistent with the present study. Targher *et al.* observed a strong inverse association on IMT associated with 25(OH)D among 390 type 2 diabetes patients (p<0.0001).[150] A Multi-Ethnic Study of Atherosclerosis (MESA) by de Boer *et al.*, which included 397 participants with chronic kidney disease and 976 without the disease,

showed that incident CAC (23% risk increase per 10 ng/mL of 25(OH)D decrease), but not prevalent CAC, was associated with low 25(OH)D for three years of follow-up.[97] These inconsistent findings mostly shown in clinical patients with diabetes or chronic kidney disease.

The lack of association between vitamin D deficiency and subclinical atherosclerosis may be attributable to the following potential confounding factors related to sunshine exposure: seasonal change, skin melatonin, physical activity, and job-related factors. Generally, dietary sources such as fish or fish oil (e.g. in salmon, tuna, mackerel), eggs, and fortified food products (e.g. milk, orange juice) contribute about 10-20% of the total vitamin D level; sunshine accounts for the remaining 80-90%.[88, 133, 148] Latitudes of the Japanese study site (Kusatsu, Japan) and the U.S. study site (Pittsburgh, PA) are similar at 35°01'N and 40°26'N, respectively. Unexpectedly, the Japanese-American men, who live closer to the equator (Honolulu, Hawaii, 21°18'N) with year-round sunshine, showed higher subclinical atherosclerosis than the other two populations, and had a significant association with CAC but not with IMT.

Availability to vitamin D fortified food products, as well as multi-vitamin supplementation practice in the Western culture, may explain the difference on serum vitamin D levels among the three populations. For instance, 14 (14.14%) Japanese men, 48 (48.98%) Caucasian men, and 61 (62.24%) Japanese-American men responded as "Yes, fairly regularly" to the question "Do you take vitamins regularly?" However, these numbers increased up to 37 (37.37%) Japanese in Japan, 53 (54.08%) Caucasian, and 66 (67.34%) Japanese-American men, when occasional users who answered "Yes, but not regularly" were included.

To take into account the different sources of vitamin D over three populations, we adjusted for marine n-3 fatty acids in the model. Additionally, we tested a possible link of marine n-3 fatty acids to 25(OH)D (correlation coefficients [r]= -0.3, p-value= <0.0001: specifically, in

the Japanese r= -0.1, p=0.46; in Caucasians r= -0.1, p=0.55; in Japanese-American r= 0.2, p=0.11), because fish is the primary food source of vitamin D, especially for the Japanese men.[90] We also tested and found that the three categories of 25(OH)D levels (<15, 15-30, ≥30ng/mL) had no association with IMT and CAC. The sample sizes for categories were too small for this examination. We further assessed whether IMT and CAC associated with vitamin D deficiency varied by hypertensive status or obese categories across three populations, but found that these variables did not modify the effects.

A possible mechanism between vitamin D deficiency and CHD may be linked to blood pressure,[146] not to subclinical atherosclerosis. Previous studies showed cross-sectional associations with blood pressure[94] and hypertension[95] in NHANES data, while prospective studies showed an increased risk on incident hypertension associated with 25(OH)D deficiency[100, 101]. In our data, systolic blood pressure was significantly associated with 25(OH)D deficiency in the Caucasian men (p-value =0.0283).

Strengths include that the present study is a population-based sample, and the study questions were examined and explored in three different populations, including the Japanese men who have habitual fish intake, the Caucasian men who live in similar latitudes as the Japanese men and follow similar dietary and multivitamin supplement practices as the Japanese-American men, the Japanese-American men who live in Hawaii with year-round sunshine. However, this study has several limitations. The study participants were all men aged 40-49 years, which limits the generalizability to other populations (e.g. older or women). Second, the cross-sectional study design could not establish causality, which could limits the inference of study results. It may lead to reverse causality or residual confounding. Third, seasonality and physical activity

measurements were not available for this present study. Fourth, the sample size was relatively small.

In conclusion, vitamin D deficiency did not appear to be associated with subclinical atherosclerosis as measured by IMT and CAC. This finding may suggest an association between vitamin D levels and non-atherosclerotic cardiovascular disease risk, such as hypertension. Therefore, this present study provides a better understanding of vitamin D deficiency related to subclinical atherosclerosis. Future studies to examine causal relationships are warranted.

3.6 REFERENCE

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4.0 MANUSCRIPT III: SERUM MARINE N-3 FATTY ACIDS ARE ASSOCIATED WITH CORONARY ARTERY CALCIFICATION (CAC) IN JAPANESE MEN IN JAPAN, BUT NOT IN CAUCASIAN MEN IN THE U.S.

A manuscript in preparation for publication

For the ERA-JUMP study investigators

4.1 INTRODUCTION

Coronary Heart Disease (CHD) mortality in Japan is much lower than that in Western countries. CHD mortality rates for men ages 35-74 in Japan (2004) and for men of the same ages in the U.S. (2005) were 50.4 and 169.4 (per 100,000 population), respectively.[10] To study the determinants of the difference in CHD mortality between the Japanese men and men in the U.S. may help establish preventive methods. In order to decrease CHD mortality in the U.S., both early identification and primary prevention are important, since half of the men who had sudden death from CHD showed no previous symptoms.[4] Coronary artery calcification (CAC), a non-invasive and reliable measurement of subclinical atherosclerotic development, has shown strong associations with cardiovascular events.[29] CAC also improves the CHD risk assessment over a traditional risk assessment (e.g. Framingham risk score), especially for individuals in the intermediate risk category.[32, 33]

Among the participants of 'Electron-Beam Tomography, Risk Factor Assessment among Japanese and U.S. Men in the Post-World War II Birth Cohort' (ERA-JUMP), Japanese men aged 40-49 years who adopted the Westernized lifestyle showed a similar or unfavorable profile with many traditional risk factors, as compared to the Caucasian men. Compared with the Caucasian men, the Japanese men in Japan had significantly higher rates of hypertension and cigarette smoking, similar levels of low-density lipoprotein cholesterol (LDLc), and similar prevalence of diabetes. In contrast, the Japanese men in Japan had significantly higher high-density lipoprotein cholesterol (HDLc), significantly lower body mass index (BMI), and significantly lower C-reactive protein (CRP) than the Caucasian men. The ERA-JUMP study found that Japanese men in Japan have substantially lower prevalence rates of CAC, as

compared to Caucasian men.[5] The study also found that Japanese-American men have similar or higher subclinical atherosclerosis, as compared to Caucasian men.[6] These data suggest that the environmental risk factors play a substantial role more than genetic factors in contributing to this substantially low prevalence rates of subclinical atherosclerosis and low CHD mortality of the Japanese men in Japan. This different CHD mortality is not attributable to misclassification of death code.[151]

Our previously reported ERA-JUMP study found that: 1) the Japanese men in Japan aged 40-49 showed more than twice the serum marine n-3 fatty acids than the Caucasian men of the same age, and 2) after adjusting for marine n-3 fatty acids, the significant difference in CAC prevalence between the two populations disappeared. These observations indicate that marine n-3 fatty acids can explain the difference in subclinical atherosclerosis between the Japanese and the Caucasian men.[6]

The extremely high levels of marine n-3 fatty acids in the Japanese men in Japan provide a unique opportunity to examine the following: 1) whether the incidence or progression of CAC is lower in the Japanese men in Japan than in the Caucasian men in the U.S. and 2) whether marine n-3 fatty acids are inversely associated with the incidence or progression of CAC. To achieve this, we tested the ERA-JUMP follow-up data of CAC scores associated with serum marine n-3 fatty acids in middle-aged men. Our study is the first one to examine the association between very high marine n-3 fatty acids and CAC changes in two populations: Japanese and Caucasian men.

4.2 METHODS

4.2.1 Study population

The ERA-JUMP study is a population-based multicenter study to measure prevalence and determinants of subclinical atherosclerosis in middle-aged men. Detailed descriptions of the study participants were previously published.[5] All participants, ages 40-49 without clinical cardiovascular disease or cancer at enrollment, were randomly selected from three study centers between 2002 and 2006: 313 Japanese men from Kusatsu, Shiga, Japan; 310 Caucasian men from Allegheny County, Pennsylvania; and 303 Japanese-American men from Honolulu, Hawaii.

For the ERA-JUMP follow-up study from 2007 to 2010, all participants from the previous examinations (from 2002 to 2006) were re-invited by mail or phone; the follow-up rates were about 83.7% (262 Japanese men) and 79.7% (247 Caucasian men) (this study did not focus on the Japanese-American men). After excluding missing data of coronary calcium scores (CCS) or one stent case, a total of 495 participants (253 Japanese and 242 Caucasian men) had both baseline and follow-up examinations. Because high alcohol consumption has a strong association with marine n-3 fatty acids and is a risk factor for CAC, the men who consumed more than 69g/day of ethanol were excluded (23 Japanese men).[6, 152] A total of 472 participants (230 Japanese and 242 Caucasian men) were in the final analyses. All participants signed informed consent forms for both the baseline and the follow-up. Both the Institutional Review Boards of

the Shiga University of Medical Science, Japan, and the University of Pittsburgh, U.S., approved this study.

4.2.2 Measurements of subclinical atherosclerosis risk factors

Measurements at baseline from self-administered questionnaires were used to obtain information on demographic characteristics, drinking or smoking habits, and any medication usage. The detailed procedures were published elsewhere.[5] Briefly, a physical examination to measure body weight and height was performed for all participants dressed in light clothing without shoes. After the participant emptied his bladder and sat quietly for 5 minutes, two measurements of blood pressure were averaged to use for analysis. 'Alcohol drinking' was defined as consuming alcohol >2 days/week. Alcohol consumption (g/day) was calculated based on ethanol concentrations (e.g. 12% for wine, 16% for sake, 5% for beer, and 40% for liquor). 'Current smoking' was defined as smoking over the last month.

4.2.3 Serum fatty acids measurement

Using gas-capillary-liquid chromatography (PerkinElmer Clarus 500; PerkinElmer, Waltham, MA), serum fatty acids were measured at baseline in the percentage unit of total fatty acid.[6] The intra-assay coefficients of variation (CV) between runs were eicosapentaenoic acid (EPA, 20:5n-3) 2.5%, docosapentaenoic acid (DPA, 22:5n-3) 2.5%, and docosahexaenoic acid (DHA, 22:6n-3) 7.0%.[6] Marine n-3 fatty acids were defined as the sum of eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3).

4.2.4 CAC measurement

CAC was measured twice, both at the baseline (2002~2006) and at the follow-up (2007~2010), using either Electron Beam Computed Tomography (EBCT) or Multi-Detector Computed Tomography (MDCT). At baseline, both study sites used EBCT. As for the follow-up scans, the Japan site used MDCT, while the U.S. site used EBCT.

EBCT and MDCT scanning were performed with a standardized study protocol in each of the two study sites. A GE-Imatron C150 Electron Beam Computed Tomography scanner (EBCT) (GE Medical Systems, South San Francisco, US), as well as MDCT scans using a 16 multi-slice computed tomography (Toshiba Medical System Corporation, Tochigi, Japan), were utilized. At the Cardiovascular Institute in the University of Pittsburgh, a trained scan reader quantified CAC in the Agatston method, using a Digital-Imaging-and-Communications-in-Medicine (DICOM) workstation and software by Accu-Image (AccuImage Diagnostic Corporation, San Francisco, U.S.).[153]

4.2.5 Statistical analyses

Using two scans both at baseline and follow-up, CAC prevalence, incidence, and progression were defined. The prevalence rate of CAC was defined as CCS≥10.[6] The incidence of CAC was defined as participants with a CCS≥10 at follow-up among those with CCS=0 at baseline. The progression of CAC was defined as a higher CCS at follow-up than CCS at baseline among those participants with CCS>0 at baseline.

To compare the general distributions of risk factors between the Japanese and the Caucasian men, a t-test for a continuous variable and a chi-square test for a categorical variable

were used. Because of a skewed distribution with such variables as triglycerides, ethanol, and CRP, a Mann-Whitney *U* test was used. The correlation coefficients between CAC and risk factors were evaluated. To assess the association between marine n-3 fatty acids and CAC incidence/progression, odds ratios were estimated using logistic regression models in univariate and multivariate models. Logistic models were adjusted as follows: adjustment for such covariates as age, follow-up time in year, smoking, and ethanol (model 1); further adjustment for hypertension and diabetes (model 2); additional adjustment for LDLc, HDLc, and triglycerides (model 3); and further adjustment for lipid medication and CRP (model 4). The variable of 'ethanol consumption (g/day)' was utilized as a categorical variable with '0, 1-15, 16-30, 31-45, 46+'. To confirm the results, Poisson regression models with robust (i.e. negative binomial distribution) were conducted to estimate the association between CAC (i.e. continuous form of coronary calcium scores) and marine n-3 fatty acids. All reported p values were based on the significance of less than 0.05 with two-sided tests. SAS 9.2 for Windows (SAS Institute, Inc., Cary, North Carolina, U.S.) was utilized for all statistical analyses.

4.3 RESULTS

4.3.1 General characteristics

Table 20 lists the general characteristics of the study population. The average ages of the two populations were both 45 years old. The Japanese men in Japan showed significantly higher rates of hypertension and cigarette smoking, as well as similar levels of LDLc and diabetes, as compared to the Caucasian men. In contrast, the Japanese men in Japan had significantly higher high-density lipoprotein cholesterol (HDLc), significantly lower body mass index (BMI), and significantly lower C-reactive protein (CRP), than the Caucasian men in the U.S.

The Japanese men in Japan had significantly higher levels of serum marine n-3 fatty acids than the Caucasian men in the U.S. ($p<0.0001$) by 242.1%.

Table 20. General descriptions of the study population at baseline (n=472)

	Japanese men (n=230)	Caucasian men (n=242)	*p*-value
Age (yrs)	45.1 ± 2.8	45.0 ± 2.9	0.93
Body mass index (kg/m^2)	23.7 ± 3.1	27.8 ± 4.0	<0.0001
Systolic blood pressure (mm Hg)	124.7 ± 15.9	122.6 ± 10.9	0.09
Diastolic blood pressure (mm Hg)	76.0 ± 11.6	73.3 ± 8.3	0.004
Hypertension (%)	55 (23.9%)	34 (14.1%)	0.006
LDL cholesterol (mg/dl)	134.2 ± 35.1	135.5 ± 31.8	0.67
HDL cholesterol (mg/dl)	53.0 ± 13.2	47.1 ± 12.0	<0.0001
Triglycerides (mg/dl)*	139.0 (105.0-181.0)	130.0 (95.0-186.0)	0.27
Current smoker (%)	109 (47.4%)	17 (7.0%)	<0.0001
Alcohol drinking (%)	149 (64.8%)	109 (45.0%)	<0.0001
Ethanol consumption (g/day)*	14.0 (2.0-36.0)	4.8 (0.9-16.1)	<0.0001
Diabetes mellitus (%)	12 (5.2%)	9 (3.7%)	0.43
C-reactive protein (mg/l)*	0.3 (0.2-0.7)	1.0 (0.5-1.9)	<0.0001
Lipid-lowering medications (%)	6 (2.6%)	32 (13.2%)	<0.0001
Marine fatty acids [% of total fatty acids (mg/dl)]	9.2 ± 2.8	3.8 ± 1.7	<0.0001

*Median (interquartile range) with Mann-Whitney *U* test

4.3.2 Mean follow-up time

Among those who had scans both at baseline and follow-up, the mean follow-up years were 6.17 years (±0.33, median 6.26 years) in Japanese men in Japan and 4.64 years (±0.19, median 4.6 years) in Caucasian men in the U.S.

4.3.3 Prevalence of CAC

Table 21 presents the prevalence of CAC between the two study populations. The Japanese men in Japan showed significantly lower prevalence rates (defined as ≥10) of CAC at both baseline as well as follow-up, than the Caucasian men (at baseline 8.7% vs. 26.0%, *p*-value <0.0001; at follow-up 17.0% vs. 38.4%, *p*-value <0.0001).

Table 21. Prevalence of CAC among the study population (n=472)

	Japanese men (n=230)		Caucasian men (n=242)	
	baseline	follow-up	baseline	follow-up
Coronary artery calcification prevalence (%)				
(defined as CCS≥10)	20 (8.7%)	39 (17.0%)	63 (26.0%)	93 (38.4%)
(defined as CCS>0)	65 (28.3%)	61 (26.5%)	130 (53.7%)	149 (61.6%)
Coronary calcium score* [median (interquartile range)]	0 (0-1.0)	0 (0-1.5)	1.2 (0-12.3)	3.0 (0-34.0)

*Median with Mann-Whitney Test: <0.0001

4.3.4 Incident CAC and its risk factors

Table 22 shows the distribution of risk factors between incident CAC and non-incident cases as well as incidence rates of the two populations. Among the Japanese men in Japan, those incident CAC cases had significantly higher rates of hypertension than non-incident CAC. No other risk factors were significantly different in either population. The incidence rate for the Japanese men in Japan was 1.0% (10 out of 165) for 6.2 years of follow-up, whereas that for the Caucasian men in the U.S. was 2.9% (15 out of 112) for 4.6 years of follow-up.

Table 22. Comparisons of risk factors for incident CAC (n=277)

	Japanese men (n=165)			Caucasian men (n=112)		
	Incident (n=10)	Negative (n=155)	*p*-value	Incident (n=15)	Negative (n=97)	*p*-value
Age (yrs)	45.4 ± 3.6	44.8 ± 2.8	0.53	44.2 ± 2.9	44.7 ± 2.9	0.51
Body mass index (kg/m^2)	22.8 ± 1.7	23.2 ± 2.9	0.67	27.7 ± 3.1	26.1 ± 3.0	0.07
Systolic BP (mm Hg)	140.6 ± 24.0	122.6 ± 14.2	0.04	122.7 ± 9.6	121.4 ± 11.3	0.67
Diastolic BP (mm Hg)	84.7 ± 16.2	74.2 ± 10.8	0.07	73.6 ± 8.4	71.5 ± 7.9	0.35
Hypertension (%)	8 (80.0%)	26 (16.8%)	<0.0001	2 (13.3%)	11 (11.3%)	0.69**
LDLc (mg/dl)	120.8 ± 43.7	130.8 ± 35.6	0.40	125.7 ± 29.8	134.6 ± 31.5	0.31
HDLc (mg/dl)	54.7 ± 13.5	53.9 ± 13.6	0.86	43.7 ± 7.7	48.7 ± 11.5	0.11
Triglycerides (mg/dl)*	129.5 (120.0-143.0)	131.0 (96.0-174.0)	0.86	112.0 (96.0-169.0)	120.0 (89.0-162.0)	0.83
Current Smoker (%)	6 (60.0%)	72 (46.5%)	0.41	0 (0%)	9 (9.3%)	0.61**
Alcohol drinking (%)	8 (80.0%)	101 (65.2%)	0.34	8 (53.3%)	53 (54.6%)	0.92
Ethanol consumption (g/day)*	35.3 (6.0-42.9)	14.0 (2.1-34.0)	0.08	6.2 (0.8-27.8)	8.2 (1.6-17.3)	0.99
Diabetes (%)	1 (10.0%)	6 (3.9%)	0.36**	0 (0%)	2 (2.1%)	>0.99**
C-reactive protein (mg/l)*	0.2 (0.3-0.6)	0.2 (0.3-0.6)	0.76	1.0 (0.7-1.8)	0.8 (0.4-1.6)	0.57
Lipid medications (%)	0 (0%)	3 (1.9%)	>0.99**	0 (0%)	9 (9.3%)	0.61**
Marine fatty acids [% of total fatty acids (mg/dl)]	8.5 ± 2.6	9.1 ± 2.5	0.46	3.5 ± 1.8	3.9 ± 1.8	0.38
Calcium score at follow-up*	27.9 (13.9-70.2)	0 (0-0)	<0.0001	24.3 (12.2-33.0)	0 (0-1.0)	<0.0001
Incidence	10/165 (6.1%)			15/112 (13.4%)		
Incidence rate	10/(165 * 6.2) (1.0%)			15/(112 * 4.6) (2.9%)		

Incidence is defined as coronary calcium score ≥10 at follow-up among those zero calcium score at baseline

*Median (interquartile range) with Mann-Whitney Test; **Fisher's Exact test

BP, Blood Pressure; LDLc, LDL Cholesterol; HDLc, HDL cholesterol;

Table 23 lists the associations of marine n-3 fatty acids with incident CAC. In the univariate model, both the Japanese men and the Caucasian men showed reduced risks for the incident CAC associated with marine n-3 fatty acids. In model I with the adjustment of follow-up year, smoking, and ethanol, both populations remained in reduced risk but not significant on the incident CAC associated with marine n-3 fatty acids. After adjusting for all covariates, including age, follow-up year, smoking, ethanol, hypertension, diabetes, LDLc, HDLc, triglycerides, lipid medication, and CRP, the Japanese men in Japan showed 54% (0.23-0.91) significant risk reduction on incident CAC associated with marine n-3 fatty acids. The Caucasian men had non-significant 21% (0.5-1.22) risk reduction on incident CAC associated with marine n-3 fatty acids.

Table 23. Associations of Marine n-3 fatty acids with Incidence Coronary Artery Calcification (CAC) in the Japanese men and Caucasian men (n=277)

	Japanese men (n=165)	Caucasian men (n=112)
	OR (95% CI)	OR (95% CI)
Univariate (marine fatty acids)	0.90 (0.69-1.17)	0.85 (0.59-1.22)
Model I	0.76 (0.55-1.05)	0.80 (0.52-1.23)
Model II	0.68 (0.46-1.01)	0.79 (0.51-1.23)
Model III	0.56 (0.33-0.94)	0.78 (0.52-1.17)
Model IV	0.46 (0.23-0.91)	0.79 (0.51-1.22)

Univariate is adjusted for age;
Model I is additionally adjusted for follow up year, smoking, and ethanol;
Model II is further adjusted for hypertension and diabetes;
Model III is further adjusted for LDLc, HDLc, Triglycerides;
Model IV is additionally adjusted for lipid medication, and CRP.
Incidence CAC was defined as coronary calcium score ≥10 at follow-up from zero CAC at baseline.
Marine fatty acids are defined as the sum of the EPA, DHA, and DPA.

Table 24 shows that the Japanese men in Japan had significantly lower incident CAC than the Caucasian men (p=0.041) (n=277). As compared to the Japanese men, the significantly higher risk on the incidence of CAC in the Caucasian men remained in model I (with the adjustment of age, BMI, hypertension, and follow-up year), model II (with the further adjustment of LDLc, HDLc, triglycerides, ethanol, and lipid medication), and model III (with the additional adjustment of smoking, CRP, and diabetes). However, when further adjusting for marine n-3 fatty acids, this significant difference on the incident CAC between the Japanese men in Japan and the Caucasian men was attenuated and became non-significant.

Table 24. Differences in Incidence of Coronary Artery Calcification (CAC) between Japanese men and Caucasian men (n=277)

	Differences in the odds ratio between two groups OR (95% CI)	*p*-value
Univariate (race)	2.40 (1.04-5.55)	0.041
Model I	15.00 (1.12-201.86)	0.041
Model II	18.22 (1.16-285.41)	0.039
Model III	18.40 (1.11-305.59)	0.042
Model IV	9.20 (0.50-168.81)	0.135

Model I is adjusted for age, BMI, hypertension, and follow-up year;
Model II is continuously adjusted for LDLc, HDLc, Triglycerides, ethanol, and lipid medication;
Model III is further adjusted for smoking, CRP, and diabetes;
Model IV is additionally adjusted for marine n-3 fatty acids.

Table 25 and table 26 show associations with the continuous form of CCS. Table 25 shows that the rate ratios of marine n-3 fatty acids on CCS at follow-up among those with zero CCS at baseline. In a univariate model (adjusted for age and ethanol), the Japanese men showed significant risk reduction (p=0.016), whereas the Caucasian men did not show significance (p=0.180). In the model I, II, and III, significant risk reduction on the increase in CCS associated with marine n-3 fatty acids were observed in the Japanese men in Japan, but not in the Caucasian men in the U.S. However, in model IV, after adjusting for lipid medication and CRP, the Caucasian men had significant risk reduction on the increase in CCS associated with marine n-3 fatty acids.

Table 25. Risks for the continuous changes of CAC among those zero CAC (n=277)

	Japanese men (n=165)		Caucasian men (n=112)	
	RR (95% CI)	p-value	RR (95% CI)	p-value
Univariate (marine fatty acids)	0.62 (0.43-0.91)	0.016	0.75 (0.49-1.14)	0.180
Model I	0.63 (0.45-0.88)	0.007	0.74 (0.49-1.12)	0.154
Model II	0.65 (0.46-0.91)	0.011	0.73 (0.47-1.13)	0.153
Model III	0.68 (0.47-0.99)	0.042	0.67 (0.44-1.02)	0.065
Model IV	0.68 (0.46- 1.00)	0.049	0.65 (0.42-1.00)	0.049

Univariate is adjusted for age and ethanol;
Model I is continuously adjusted for smoking;
Model II is further adjusted for hypertension and diabetes;
Model III is additionally adjusted for LDLc and HDLc
Model IV is further adjusted for lipid medication and CRP

The Table 26 shows that the Japanese men in Japan had significantly lower CCS than the Caucasian men (p=0.003). As compared to the Japanese men in the Japan, the Caucasian men had the significant increase in CCS in the univariate (with the adjustment of hypertension), model I (with the adjustment of age and BMI), model II (with the further adjustment of LDLc, HDLc, triglycerides, ethanol, and lipid medication), and model III (with the additional adjustment of smoking, CRP, and diabetes). However, when additionally adjusting for marine n-3 fatty acids, this significant difference between the Japanese men in Japan and the Caucasian men disappeared.

Table 26. Continuous differences in Coronary Artery Calcium scores between Japanese men and Caucasian men (n=277)

	Differences between two groups RR (95% CI)	*p*-value
Univariate (race)	4.42 (1.69-11.56)	0.003
Model I	4.37 (1.08-17.71)	0.039
Model II	4.80 (1.05-22.06)	0.044
Model III	5.86 (0.97-35.34)	0.054
Model IV	1.18 (0.18-7.66)	0.863

Univariate is adjusted for hypertension;
Model I is adjusted for age, and BMI;
Model II is continuously adjusted for LDLc, HDLc, Triglycerides, ethanol, and lipid medication;
Model III is further adjusted for smoking, CRP, and diabetes;
Model IV is additionally adjusted for marine n-3 fatty acids.

4.3.5 Progressive CAC and its risk factors

Table 27 shows the distribution of risk factors between progressive CAC cases and non-progressive cases. Those Japanese men who had progressive CAC had significantly higher levels of triglycerides than the Japanese men without progressive cases (p=0.005). The Japanese men in Japan showed no other significant risk factors. The Caucasian men who had progressive CAC used significantly higher rates of lipid medication than the Caucasian men without progressive CAC (p=0.03). The annual progressive rate of CAC for the Japanese in Japan was 4.5% (18 out of 65) for 6.2 years of follow-up, whereas that for the Caucasian men in the U.S. was 7.9% (47 out of 130) for 4.6 years of follow-up.

Table 27. Comparisons of risk factors for progressive CAC (defined as advancement of CAC among those with CAC>0 at baseline) (n=195)

	Japanese men (n=65)			Caucasian men (n=130)		
	Progress (n=18)	Negative (n=47)	p-value	Progress (n=47)	Negative (n=83)	p-value
Age (yrs)	45.7 ± 2.5	45.5 ± 2.8	0.76	45.7 ± 2.7	45.2 ± 2.8	0.35
Body mass index (kg/m^2)	25.8 ± 2.8	24.9 ± 3.5	0.36	29.0 ± 3.7	29.1 ± 4.7	0.92
Systolic BP (mm Hg)	127.6 ± 16.2	126.9 ± 17.1	0.88	123.6 ± 10.3	123.2 ± 11.2	0.82
Diastolic BP (mm Hg)	80.2 ± 10.7	78.8 ± 12.2	0.71	74.9 ± 8.1	74.4 ± 8.7	0.72
Hypertension (%)	6 (33.3%)	15 (31.9%)	0.91	5 (10.6%)	16 (19.3%)	0.20
LDLc (mg/dl)	149.5 ± 30.1	142.4 ± 30.7	0.40	135.6 ± 30.8	138.3 ± 33.1	0.64
HDLc (mg/dl)	46.1 ± 11.9	52.1 ± 11.3	0.07	47.4 ± 13.0	45.6 ± 12.4	0.43
Triglycerides (mg/dl)*	215.0 (146.0-246.0)	140.0 (118.0-182.0)	0.005	142.0 (92.0-193.0)	147.0 (96.0-229.0)	0.39
Current smoker (%)	9 (50.0%)	22 (46.8%)	0.82	5 (10.6%)	3 (3.6%)	0.11
Alcohol drinking (%)	11 (61.1%)	29 (61.7%)	0.97	20 (42.6%)	28 (33.7%)	0.32
Ethanol (g/day)*	7.0 (1.6-40.5)	11.4 (1.5-37.0)	0.96	4.9 (1.5-18.5)	3.3 (0-8.2)	0.05
Diabetes (%)	0 (0%)	5 (10.6%)	0.31**	2 (4.3%)	5 (6.0%)	>0.99**
C-reactive protein (mg/l)*	0.4 (0.2-0.9)	0.3 (0.2-0.7)	0.29	1.0 (0.7-2.0)	1.2 (0.7-2.2)	0.62
Lipid medications (%)	1 (5.6%)	2 (4.3%)	>0.99**	13 (27.7%)	10 (12.1%)	0.03
Marine fatty acids (%)	10.1 ± 3.8	9.4 ± 3.3	0.49	3.7 ± 1.5	3.7 ± 1.7	0.97
Coronary calcium score at baseline*	8.2 (3.9-37.8)	4.1 (1.3-11.3)	0.05	25.3 (5.5-68.4)	6.1 (2.4-18.9)	0.0003
at follow-up*	68.6 (47.0-126.5)	0 (0-9.5)	<0.0001	125.0 (47.7-266.7)	5.5 (0.0-33.6)	<0.0001
Progression	18/65 (27.7%)			47/130 (36.2%)		
Progression rate	18/(65 * 6.2) (4.5%)			47/(130 * 4.6) (7.9%)		

*Median (interquartile range) with Wilcoxon-Mann-Whitney Test
** Fisher's Exact test

Table 28 lists associations of marine n-3 fatty acids with the progression of CAC (n=195). Marine n-3 fatty acids showed no significant associations in either population, even after adjusting for covariates.

Table 29 shows the Japanese men in Japan had significantly lower progression of CAC than the Caucasian men (p=0.022) (n=195). As compared to the Japanese men, the Caucasian men had significantly higher progression of CAC. The significant differences on the progression of CAC were shown in univariate (with the adjustment of follow-up year), model I (with the further adjustment of age, BMI, hypertension, and ethanol), model II (with the additional adjustment of LDLc, HDLc, triglycerides, and lipid medication), and model III (with the further adjustment of smoking, CRP, and diabetes). However, when further adjusting for marine n-3 fatty acids, this significant difference on the progression of CAC between the Japanese men in Japan and the Caucasian men was attenuated and became non-significant.

Table 28. Associations of Marine n-3 fatty acids with the progressive CAC in both the Japanese men and the Caucasian men (n=195)

	Japanese men (n=65)	Caucasian men (n=130)
	OR (95% CI)	OR (95% CI)
Univariate (marine fatty acids)	1.05 (0.90-1.23)	1.00 (0.80-1.25)
Model I	1.07 (0.89-1.29)	0.98 (0.77-1.25)
Model II	1.06 (0.88-1.27)	0.98 (0.77-1.26)
Model III	1.23 (0.98-1.54)	0.95 (0.73-1.25)
Model IV	1.24 (0.97-1.57)	0.96 (0.73-1.27)

Univariate is adjusted for age ;
Model I is further adjusted for follow up year, smoking, and ethanol;
Model II is continuously adjusted for hypertension, and diabetes;
Model III is further adjusted for LDLc, HDLc, and Triglycerides;
Model IV is additionally adjusted for lipid medication, and CRP.
Marine fatty acids are defined as the sum of the EPA, DHA, and DPA.

Table 29. Differences in Progression of Coronary Artery Calcification (CAC) between Japanese men and Caucasian men (n=195)

	Differences in the odds ratio between two groups OR (95% CI)	*p*-value
Univariate (race)	15.41 (1.47-161.36)	0.022
Model I	17.11 (1.49-196.13)	0.023
Model II	17.20 (1.37-216.58)	0.028
Model III	15.67 (1.18-207.65)	0.037
Model IV	15.40 (1.06-222.84)	0.045

Univariate is adjusted for follow-up year;
Model I is adjusted for age, BMI, hypertension, and ethanol;
Model II is continuously adjusted for LDLc, HDLc, Triglycerides, and lipid medication;
Model III is further adjusted for smoking, CRP, and diabetes;
Model IV is additionally adjusted for marine n-3 fatty acids.

Table 30 and table 31 show associations with the continuous form of CCS. Table 30 shows that the rate ratios of marine n-3 fatty acids on CCS at follow-up among those with a prevalent CCS (>0) at baseline (n=195). In a univariate model as well as multivariate adjusted models (I, II, III, and IV), no significant associations of marine n-3 fatty acids on the progression of CAC were examined in the Japanese men in Japan and the Caucasian men.

Table 30. Risks for the continuous changes of progressive CAC among those greater than zero CAC

	Japanese men (n=65)		Caucasian men (n=130)	
	RR (95% CI)	p-value	RR (95% CI)	p-value
Univariate (marine fatty acids)	1.02 (0.87-1.20)	0.832	0.93 (0.79-1.09)	0.375
Model I	1.02 (0.87-1.20)	0.782	0.93 (0.79-1.09)	0.376
Model II	1.02 (0.87-1.19)	0.845	0.98 (0.84-1.16)	0.853
Model III	1.07 (0.89-1.27)	0.482	0.98 (0.83-1.16)	0.845
Model IV	1.07 (0.87- 1.31)	0.523	0.99 (0.85-1.16)	0.929

Univariate is adjusted for age and ethanol;
Model I is continuously adjusted for smoking;
Model II is further adjusted for hypertension and diabetes;
Model III is additionally adjusted for LDLc and HDLc
Model IV is further adjusted for lipid medication and CRP
Progressive CAC was defined as any advance of CAC among those with coronary calcium score >0 at baseline.
Marine fatty acids are defined as the sum of the EPA, DHA, and DPA.

Table 31 lists that the difference of CCS at follow-up between the Japanese men in Japan and the Caucasian men among those with a prevalent CCS (>0) at baseline. The Japanese men in Japan had significantly lower CCS than the Caucasian men (p=0.014) in the univariate model (with the adjustment of hypertension). After adjusting for covariates, shown in model I (with the adjustment of age and BMI), model II (with the further adjustment of LDLc, HDLc, triglycerides, ethanol, and lipid medication), and model III (with the additional adjustment of smoking, CRP, and diabetes), the advancement of CCS were not significantly different between the Japanese men in Japan and the Caucasian men. When additionally adjusting for marine n-3 fatty acids, this non-significant difference between the Japanese men in Japan and the Caucasian men remained.

Table 31. Continuous differences in the progression of Coronary Artery Calcium scores between Japanese men and Caucasian men (n=195)

	Differences between two groups RR (95% CI)	*p*-value
Univariate (race)	1.96 (1.15-3.34)	0.014
Model I	1.64 (0.89-3.00)	0.110
Model II	1.76 (0.87-3.53)	0.113
Model III	1.82 (0.88-3.78)	0.108
Model IV	1.78 (0.65-4.88)	0.266

Univariate is adjusted for hypertension;
Model I is adjusted for age, and BMI;
Model II is continuously adjusted for LDLc, HDLc, Triglycerides, ethanol, and lipid medication;
Model III is further adjusted for smoking, CRP, and diabetes;
Model IV is additionally adjusted for marine n-3 fatty acids.

4.4 DISCUSSION

In this follow-up study of 472 men ages 40-49, we found that the Japanese men in Japan had a significantly lower prevalence, incidence, and progression of CAC than the Caucasian men. In the multivariate-adjusted model, the Japanese men in Japan showed 54% significant risk reduction on incident CAC associated with marine n-3 fatty acids, whereas the Caucasian men had 21% non-significant risk reduction on incident CAC associated with marine n-3 fatty acids. The significant lower incident CAC in the Japanese men, as compared to the Caucasian men, was explained by marine n-3 fatty acids but not by traditional cardiovascular risk factors. The Japanese men and the Caucasian men did not show significant associations on the progression of CAC with marine n-3 fatty acids. The significantly lower progression rates of CAC in the Japanese men, as compared to the Caucasian men, was not explained either by traditional cardiovascular risk factors or by marine n-3 fatty acids.

To the best of our knowledge, this present study is the first to examine the associations of very high marine n-3 fatty acids on the incidence or progression of CAC. Our findings of the beneficial effects of very high marine n-3 fatty acids on the incident CAC among those zero CAC at baseline suggested anti-atherogenic benefits. This beneficial effect is consistent with previous reports of two large Japanese studies that examined the associations between marine n-3 fatty acids and significant risk reduction on non-fatal cardiovascular events.[54, 55] These findings of the two studies with very high marine n-3 fatty acid for about 5 years of follow-up suggested anti-atherogenic effect.

Additionally, the Rotterdam study of 1,570 asymptomatic participants with an average age of 64 found an inverse but weak association between higher fish intake (>19g/day) and CAC prevalence (mild to intermediate) as compared to those individuals with no fish intake

(significant prevalence rate (PR)=0.87, 0.78-0.98 for mild and intermediate calcification [CCS 11-400], but non-significant PR=0.88, 0.74-1.04 for severe [>400]).[105] However, EPA and DHA intake did not show significant associations (PR 0.93 and 0.97, $p>0.05$, respectively) on CAC, which the Rotterdam study defined as CCS>10.[105] Also, the previous ERA-JUMP study that examined subclinical atherosclerotic burden associated with marine n-3 fatty acids demonstrated a significant difference in CAC prevalence (mean difference 10.7%, 2.9-18.4%) between the Japanese and Caucasian men, but no significant association between CAC (defined as ≥10) and marine n-3 fatty acids.[6] In contrast, a large Multi-Ethnic Study Atherosclerosis (MESA) study did not demonstrate any significant association of dietary marine n-3 PUFA intake (220 vs. 40 mg/d) with CAC (defined as >0) (1.14 [0.94-1.38]) among 6,814 participants ages 45-84 without clinical CVD.[106]

Previous studies reported various risk factors for incident and progressive CAC. We found that hypertension in the Japanese men in Japan is a significant risk factor for incidence of CAC in middle-aged men without cardiovascular disease at enrollment. Japanese men showed higher rates of hypertension (23.9%) than Caucasian men (14.1%) at baseline. Another MESA study in patients with chronic kidney disease demonstrated such risk factors as male gender and diabetes.[43] The observed significant risk factors for incident CAC included age, diabetes, and smoking, and for progressive CAC, diabetes, and smoking.[154] A previously reported ERA-JUMP study showed a J-shaped association of alcohol consumption with CAC and a strong association in men with greater than 69g/day ethanol.[152] Another study suggested pro-atherosclerotic relation to heavy alcohol consumers (>60g/day) via increasing platelet aggregation and promoting LDL oxidation.[155] Especially, alcohol consumption is known to be positively associated with n-3 polyunsaturated fatty acids, specifically those with CHD.[156] Our present data also appeared to

have a positive association of alcohol consumption (i.e. ethanol amount) with marine n-3 fatty acids in the Japanese but not in Caucasian men, which the Japanese men have much higher amount of alcohol consumption than Caucasian men.

Previous findings of the effectiveness of lipid-lowering treatments on the progressive CAC are inconsistent.[34-40] Lipid oxidation and subsequent inflammatory responses has been considered to be attributable to developing atherosclerosis. Stains, lipid-lowering treatment, had drawn many attentions and expectation to show regressed progression of atherosclerosis. However, current findings on whether statins could effectively reduce CAC progression are inconclusive. Hecht *et al.* claimed that CAC is a predictor but not a prognostic factor.[157] A meta-analysis found that no effective reduction on CAC progression although statins reduced LDL cholesterol levels.[158] Beckman and colleagues reported that those with acute coronary events had less calcium in stenoses than those with stable angina.[159] These vulnerable plaques with less calcium may be more involved in inflammatory response with lipid oxidation, which makes more vulnerable to rupture.[159] Since zero CAC does not mean zero risk of cardiovascular events, and CAC is known to be in the later stage of atherosclerosis,[160] more future studies are warranted.

Very lower CAC scores in the Japanese in Japan as compared to those of Caucasian men may be attributable to very high marine n-3 fatty acids. CAC presence and progression have been considered as a natural process of human aging. Min and his colleagues found that the mean duration of the CAC development was 4.1±0.9 years; the CAC occurrences, most prevalent in the fifth year, were in a skewed distribution.[154] Within 5 years, a repeat scan is not recommended due to no detectable coronary calcium by EBCT scan.[161] Our incident CAC data were measured at about and greater than 5 years of follow-up (6.2 years in the Japanese and 4.6 years in the

Caucasian men), indicating that those incident cases were valid. An annual CAC progression rate was reported as 25.1% for 4.1 years[154].

Possible underlying mechanism of beneficial marine n-3 fatty acids on incidence of CAC can be suggested that the beneficial effect of n-3 fatty acids on atherosclerosis may be attributable to stabilizing atherosclerotic plaques.[48] Also, marine n-3 fatty acids has recently been suggested significant correlations with plaque stability and plaque inflammation.[162] Previous studies showed the reduction of platelet-monocyte aggregation, suggesting anti-inflammatory effects of avoiding to form atheroma.[163]

Strengths of the present study are followings: 1) we have firstly examined the association between very high marine n-3 fatty acid and incident/progressive CAC in a population-based cohort; and 2) the restricted age from 40 to 49 years allows to control possible confounding factors. However, this study also has several limitations: 1) The study population was men aged 40-49 years in two study sites, which would limit the generalizability of the results to women and to other age groups. 2) MDCT measurements of the follow-up study in the Japanese data might have affected to lower CAC. However, the association of marine n-3 fatty acids with incident CAC was substantially stronger than that of the Caucasian men. A MESA study to examine dual scans between EBCT and MDCT showed very high concordance rates (96%, k=0.92).[164] 3) low CAC (1-10) was found to increase risk for mortality[165], but it is not yet clear and future studies need to confirm whether incident CAC links to increase the CHD events.

In conclusion, very high marine n-3 fatty acids are associated with reducing incident CAC. This present study may suggest anti-atherogenic effect of very high marine n-3 fatty acids. Future studies to examine the underlying mechanism of very high marine n-3 fatty acids on anti-atherosclerosis are warranted.

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5.0 DISCUSSION

5.1 SUMMARY OF FINDINGS

The major findings of three studies were: 1) in a population-based cross-sectional study of 915 men aged 40-49, serum total n-6 fatty acids, including LA and AA, were inversely and significantly associated with plasma PAI-1, but not associated with plasma fibrinogen; 2) in a population-based cross-sectional sample of 295 middle-aged men, the Japanese men showed lower levels of serum vitamin D, despite high fish intake which is a major dietary source of vitamin D, than the Caucasian men or the Japanese-American men. In addition, vitamin D deficiency was not associated with subclinical atherosclerosis as measured by IMT and CAC, except for significant associations on IMT in an univariate model among the Caucasian men, as well as on CAC in both univariate and multivariate models among the Japanese-American men; and 3) in the ERA-JUMP follow-up study of 472 men, the Japanese men had a significantly lower prevalence, incidence, and progression of CAC than the Caucasian men. In the multivariate-adjusted model, the Japanese men showed 54% significant risk reduction on incident CAC associated with marine n-3 fatty acids, whereas the Caucasian men had 21% non-significant risk reduction on incident CAC associated with marine n-3 fatty acids. The significant lower incident CAC in the Japanese men, as compared to the Caucasian men, was explained by marine n-3 fatty acids but not by traditional cardiovascular risk factors. On the other hand, the

Japanese men and the Caucasian men showed no significant associations on the progression of CAC with marine n-3 fatty acids. The significantly lower progression of CAC in the Japanese men, as compared to the Caucasian men, was not explained either by traditional cardiovascular risk factors or by marine n-3 fatty acids. These findings may contribute to extend our understanding on subclinical cardiovascular disease associated with n-3 and n-6 fatty acids as well as vitamin D.

5.2 STRENGTHS AND LIMITATIONS

The ERA-JUMP study has strengths as followings: 1) it has a population-based sample who were randomly selected from general population of three study centers; 2) three different study populations comprised of Japanese, Caucasian, and Japanese-American men allow us to compare environmental risk factors taken into account genetic differences; and 3) the study participants were restricted within men aged 40-49 years, which help avoid confounding factors.
However, this study also has several limitations: 1) the first and second studies were conducted in a cross-sectional study, which could not assess a causal relationship; 2) the study population was all men aged 40-49, which may limit the generalizability into other populations such as women or other age groups; and 3) in the third study, CAC measurements among the Japanese men were examined in different CT technique between the baseline and the follow-up. However, the Japanese men showed substantially stronger association between marine n-3 fatty acids and CAC measurements than that of the Caucasian men. Also, a previous MESA study assessed the concordance rates of dual scans between EBCT and MDCT and showed very high concordance rates (96%, k=0.92).[164]

5.3 PUBLIC HEALTH SIGNIFICANCE AND FUTURE RESEARCH

The present findings of three sub-topics have public health implications. The findings of the significant inverse association between serum total n-6 fatty acids and PAI-1 and the no association between serum n-6 fatty acids and fibrinogen suggest the beneficial effect of n-6 fatty acids on fibrinolytic response as one of cardioprotective benefits. We have firstly examined the associations of n-6 fatty acids on PAI-1 and fibrinogen. It is of public health important, because n-6 fatty acids are the main component of cooking vegetable oils, as well as PAI-1 and fibrinogen are independent predictors for CHD. A future study to examine the causality between n-6 fatty acid and PAI-1 as well as fibrinogen is warranted.

Although many previous studies have demonstrated that vitamin D deficiency may increase CHD risk, few studies have examined the association between vitamin D deficiency and subclinical atherosclerosis over different populations. Because vitamin D is inexpensive, accessible, and safe, it is of public health importance if vitamin D could have a protective effect on subclinical atherosclerosis. Half of fatal events from CHD among men showed no previous symptoms.[4] It is important to establish preventive guidelines as well as early identification. Future longitudinal prospective studies with larger sample size to examine causal relationships are warranted.

The findings that high levels of serum marine n-3 fatty acids have a significant inverse association with the incidence of CAC, but not the progression of CAC, have an public health significance. The current findings suggest antiatherogenic effect of very high marine n-3 fatty acids. Future studies of a long-term clinical trial with high dose marine n-3 fatty acids on incident CAC in the U.S. general population are required.

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