SUPPLEMENTAL MATERIALS

Secular Trends in Cardiovascular Disease and Its Risk Factors in Japanese Half-Century Data From the Hisayama Study (1961-2009)

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Supplemental Methods

Definition of glucose intolerance in 1961

For participants with glycosuria at the baseline examination, researchers additionally performed either a full stomach test (with ≥2 bowls of rice plus a bar of sweet bean jelly) or 100-g oral glucose tolerance test (OGTT) at Kyushu University Hospital. For participants who underwent the full stomach test, glucose intolerance was defined as 2-hour or 3-hour postload blood glucose ≥7.8 mmol/L (140 mg/dL). For participants who underwent the 100-g OGTT, glucose intolerance was defined as 1-hour postload blood glucose ≥11.1 mmol/L (200 mg/dL) and 2-hour postload blood glucose ≥8.3 mmol/L (150 mg/dL).

Definition of glucose intolerance in 1974 and 1983

A single measurement of plasma glucose was performed at the baseline examination. Glucose intolerance was defined as either of the following criteria: (1) fasting plasma glucose ≥6.4 mmol/L (115 mg/dL), (2) 2-hour postprandial plasma glucose ≥7.8 mmol/L (140 mg/dL), (3) casual plasma glucose ≥11.1 mmol/L (200 mg/dL), or (4) known glucose intolerance in the earlier health examination or a medical history of diabetes.

Definition of glucose intolerance in 1993 and 2002

We performed 75-g OGTT for almost all participants aged 40-79 years, or a fasting blood glucose measurement for others. Glucose intolerance was defined as either impaired fasting glycemia, impaired glucose tolerance, diabetes mellitus (according to the 1998 WHO criteria), or use of oral hypoglycemic agents or insulin.

Legends to Supplementary Figures

Figure I: Comparison of age distributions in the town of Hisayama and in the country of Japan as a whole in 1960 and 2010 (National Census)

Proportions among the total population of Hisayama or Japan are shown.

Figure II: Comparison of occupational distribution in the town of Hisayama and in Japan as a whole in 1960 and 2010 (National Census)

Occupational distributions among the employed population aged ≥15 years, classified by the sectors of industry, are shown. Each sector was defined on the basis of the Japan Standard Industrial Classification (JSCI) by the Statistics Bureau, Ministry of Internal Affairs and Communications of Japan (http://www.stat.go.jp/english/index/seido/sangyo/san07-3.htm).

The primary sector includes (Division A) agriculture and forestry; and (B) fisheries. The secondary sector includes (C) mining and quarrying of stone and gravel; (D) construction; and (E) manufacturing. The tertiary sector includes (F) electricity, gas, heat supply and water; (G) information and communications; (H) transport and postal activities; (I) wholesale and retail trade; (J) finance and insurance; (K) real estate and goods rental and leasing; (L) scientific research, professional and technical services; (M) accommodations, eating and drinking services; (N) living-related and personal services and amusement services; (O) education learning support; (P) medical, health care and welfare; (Q) compound services; (R) services, not elsewhere classified; (S) government, except elsewhere classified; and (T) industries that could not be classified.

Figure III. Flow chart of cohorts for the present study

Participants in health examinations in 1961, 1974, 1983, 1993, and 2002 (all aged ≥40 years) were used to establish 5 cohorts in different decades (the 1960s, the 1970s, the 1980s, the 1990s, and the 2000s cohorts, respectively). Participants with a history of stroke or coronary heart disease (CHD) were excluded from each cohort. Participants who died during the examination period (except for the 2000s cohort), who moved out of the town during the examination period (for the 1960s and the 1990s cohorts), or who refused follow-up assessments (for the 2000s cohort) were also excluded. Consequently, the numbers of

participants were 1618 for the 1960s cohort, 2038 for the 1970s cohort, 2459 for the 1980s cohort, 1983 for the 1990s cohort, and 3108 for the 2000s cohort. Each cohort was followed up for 7 years (i.e., 1961-1968, 1974-1981, 1983-1990, 1993-2000, and 2002-2009, respectively).

Figure I

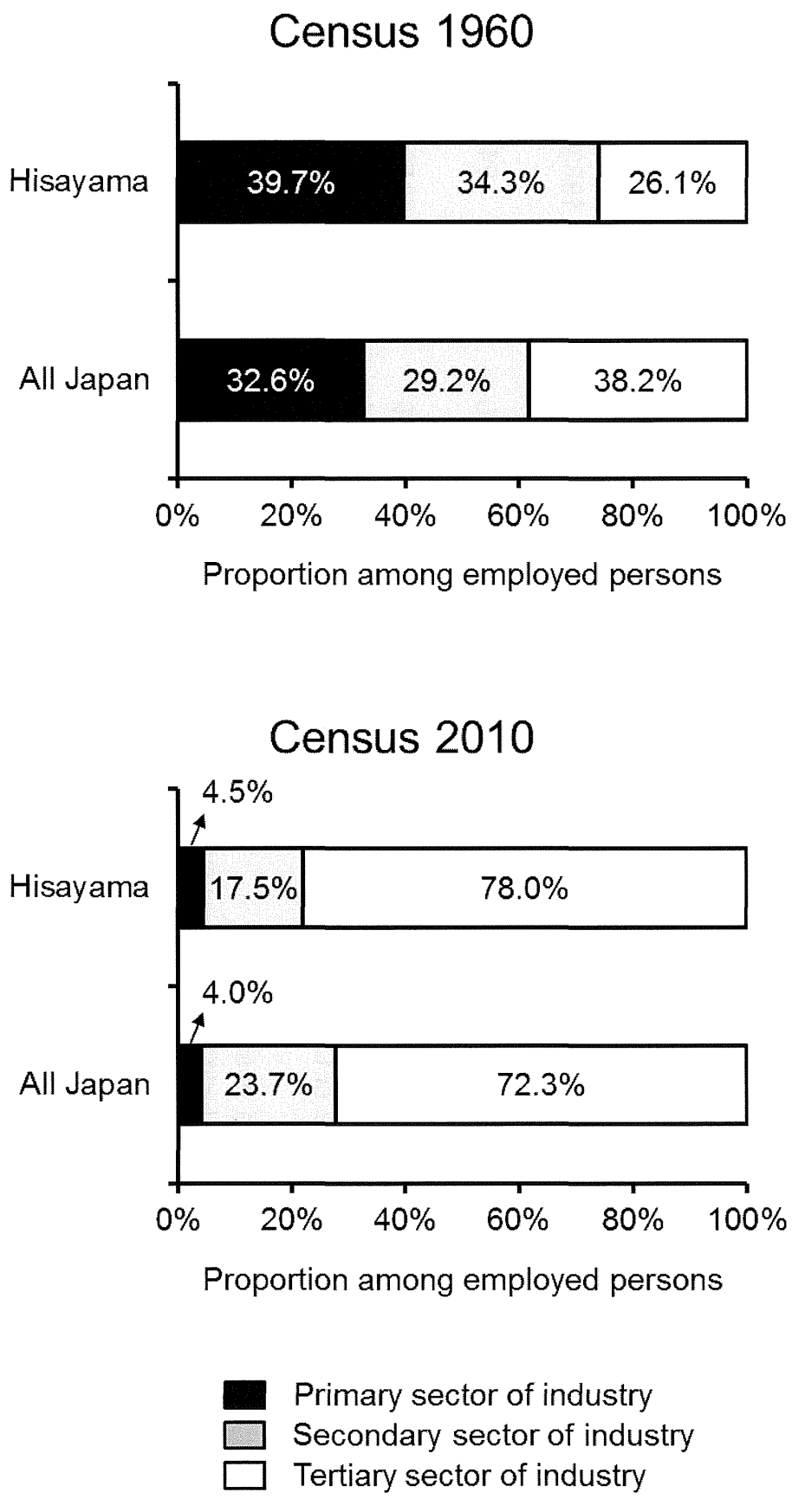
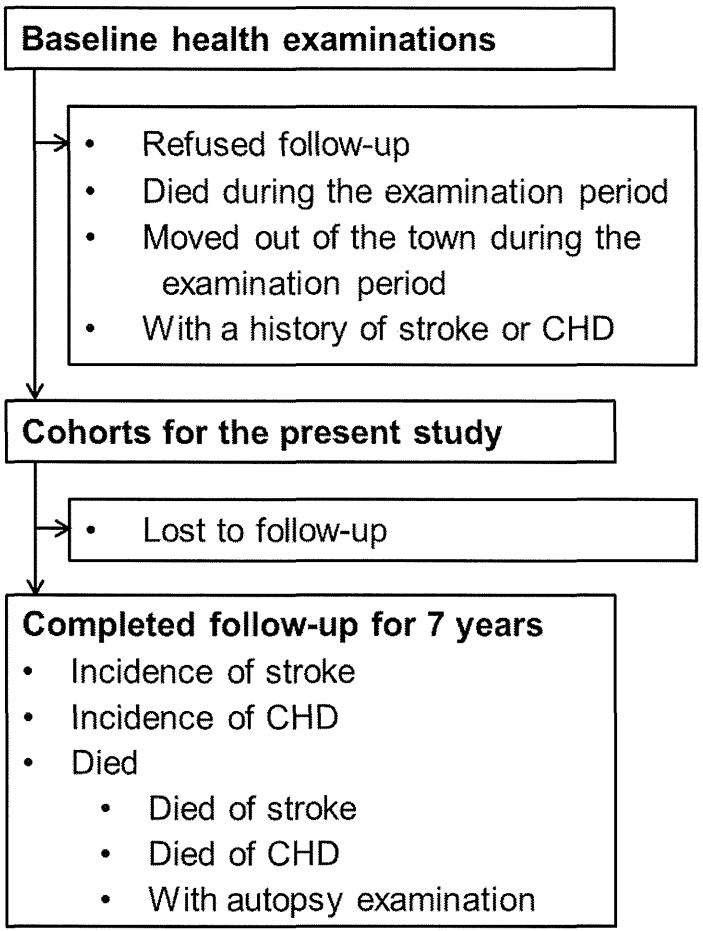


Figure II



	Number of participants				
	1960s	1970s	1980s	1990s	2000s
Baseline health examinations	**1658**	**2135**	**2551**	**2111**	**3328**
• Refused follow-up	-	-	-	-	30
• Died during the examination period	4	28	3	5	-
• Moved out of the town during the examination period	8	-	-	1	-
• With a history of stroke or CHD	28	69	89	122	190
Cohorts for the present study	**1618**	**2038**	**2459**	**1983**	**3108**
• Lost to follow-up	0	0	0	24	0
Completed follow-up for 7 years	**1618**	**2038**	**2459**	**1959**	**3108**
• Incidence of stroke	117	84	100	83	103
• Incidence of CHD	27	31	44	41	65
• Died	232	196	221	190	267
• Died of stroke	57	27	24	16	20
• Died of CHD	10	12	14	10	15
• With autopsy examination	181	165	185	156	170

Figure III

Table I. Estimated survival rate at 5 years and hazard ratio for death after the onset of stroke or acute myocardial infarction among 5 cohorts of the Hisayama Study

	Number of events*	Number of deaths†	Estimated survival rate at 5 years (%)‡	Hazard ratio for death (95% confidence interval) ‡	Unadjusted *P* value
Stroke					
1960s cohort (1961-1968)	117	71	22.2	1.00 (reference)	
1970s cohort (1974-1981)	84	41	38.5	0.64 (0.43-0.94)	0.02
1980s cohort (1983-1990)	100	35	55.3	0.39 (0.26-0.59)	<0.001§
1990s cohort (1993-2000)	83	30	58.7	0.36 (0.23-0.55)	<0.001§
2000s cohort (2002-2009)	103	36	63.0	0.31 (0.20-0.47)	<0.001§
P for trend				<0.001	
Acute myocardial infarction					
1960s cohort (1961-1968)	14	8	16.3	1.00 (reference)	
1970s cohort (1974-1981)	15	4	57.3	0.31 (0.09-1.05)	0.06
1980s cohort (1983-1990)	26	15	37.5	0.54 (0.22-1.34)	0.18
1990s cohort (1993-2000)	16	10	48.7	0.40 (0.14-1.11)	0.08
2000s cohort (2002-2009)	34	12	61.2	0.27 (0.10-0.70)	0.007§
P for trend				0.02	

Participants who developed stroke or acute myocardial infarction during the 7-year period were further followed up for the subsequent 5 years (or to the end of the follow-up period for each cohort).

* Number of stroke or acute myocardial infarction during 7 years.

† Number of death within 5 years after stroke or acute myocardial infarction.

‡ Age- and sex-adjusted.

§ $P<0.05$ after Bonferroni's correction for multiple comparisons.

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Association between ratio of serum eicosapentaenoic acid to arachidonic acid and risk of cardiovascular disease: The Hisayama Study

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ABSTRACT

Objective: We examined the association between the ratio of serum eicosapentaenoic acid to arachidonic acid (EPA/AA) or the docosahexaenoic acid (DHA)/AA and the development of cardiovascular disease in a general Japanese population.

Methods: A total of 3103 community-dwelling Japanese individuals aged ≥40 years were followed up for an average of 5.1 years. Serum EPA/AA ratios were categorized into quartiles. The risk estimates were computed using a Cox proportional hazards model.

Results: During the follow-up period, 127 subjects experienced cardiovascular events. Age- and sex-adjusted incidence rates of cardiovascular disease increased with lower serum EPA/AA ratios in individuals with high-sensitivity C-reactive protein (HS-CRP) of ≥1.0 mg/L (p for trend = 0.006), whereas no clear association was observed in those with HS-CRP of <1.0 mg/L (p for trend = 0.27). The multivariable-adjusted risk of cardiovascular disease increased significantly, by 1.52 times (95% confidence interval 1.12–2.04) per 0.20 decrement in serum EPA/AA ratio in subjects with HS-CRP of ≥1.0 mg/L. A lower serum EPA/AA ratio was significantly associated with an increased risk of coronary heart disease, but there was no evidence of an association with stroke. The magnitude of the influence of the serum EPA/AA ratio on the cardiovascular risk increased significantly with elevating HS-CRP levels taken as a continuous variable (p for heterogeneity = 0.007). However, no such association was observed for DHA/AA ratio.

Conclusion: Our findings suggest that a lower serum EPA/AA ratio is associated with a greater risk of cardiovascular disease, especially coronary heart disease, among subjects with higher HS-CRP levels in the general Japanese population.

1. Background

The influence of marine omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on cardiovascular disease has been attracting the attention of a health-conscious public. Marine omega-3 PUFAs are contained in fish or fish oil and must be taken through these foods; because they are not synthesized by the human body, they are called "essential fatty acids". In the 1970s, the Greenland Inuit, whose diet was high in marine omega-3 PUFAs, were found to have a substantially lower rate of coronary heart disease than Western people [1,2]. Growing epidemiological and clinical evidence suggests that the consumption of fish, fish oil, and marine omega-3 PUFAs reduces the risk of cardiovascular disease [3–10]. In addition, several clinical trials have demonstrated that intervention with marine omega-3 PUFA consumption decreased the risk of cardiovascular events [10–16].

Abbreviations: AA, arachidonic acid; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high-density lipoprotein; HR, hazard ratio; HS-CRP, high-sensitivity C-reactive protein; PUFA, polyunsaturated fatty acid.

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Arachidonic acid (AA), a chief source of omega-6 PUFAs, is also an "essential fatty acid" and is present in the phospholipids of cell membranes, as are EPA and DHA. It has been reported that AA is a source of mediators that cause inflammation in vessels and dysfunction of endothelium and platelets, whereas EPA and DHA have the opposite influence through competition with AA [17,18]. Thus, the balance between EPA or DHA and AA in the human body is likely to be important for regulating the production of the mediators and subsequent vascular function. Supportively, post-hoc analysis of the results of a clinical trial has demonstrated that a high serum ratio of EPA to AA (EPA/AA) levels may be a good biomarker for the risk of cardiovascular disease [19]. However, the association between serum EPA/AA ratio and the risk of cardiovascular disease has not been fully evaluated in general populations.

To elucidate this association, we performed a population-based prospective cohort study of cardiovascular disease and investigated whether subjects with lower serum EPA/AA ratio as well as lower serum DHA to AA (DHA/AA) ratio have an increased risk for the development of cardiovascular disease in a Japanese community.

2. Methods

2.1. Study population

The Hisayama Study is an ongoing, population-based prospective cohort study of cardiovascular disease and its risk factors in the town of Hisayama, which is a suburb in the Fukuoka metropolitan area on Kyushu Island, Japan. Hisayama's population is approximately 8000, and full community surveys of the residents have been repeated annually since 1961 [20]. In 2002 and 2003, a screening examination for the present study was performed in Hisayama. A detailed description of this examination was published previously [21]. Briefly, a total of 3328 residents aged 40 years or older (77.6% of the total population in this age group) underwent the examination. After excluding 30 subjects who did not consent to participate in the study, 190 subjects with a history of cardiovascular disease, and 5 subjects without available data on serum fatty acid levels, the remaining 3103 participants were enrolled in the study.

2.2. Follow-up survey

The subjects were followed up prospectively from the date of their comprehensive assessment to November 2007 by annual health examinations. For any subjects who did not undergo an annual examination in any year, or who moved out of town, their health status was checked by mail or telephone. We also established a daily monitoring system among the study team, local physicians, and the members of the town's health and welfare office. When a subject died, an autopsy was performed at the Department of Pathology of Kyushu University.

2.3. Measurements of serum fatty acid levels

Serum fatty acids levels were assayed by gas chromatography (SRL, Tokyo, Japan). Briefly, total lipids in plasma were extracted according to the Folch's procedure, followed by hydrolysis to free fatty acids. Free fatty acids were esterified with potassium methoxide/methanol and boron trifluoride–methanol. The methylated fatty acids were analyzed using GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) with omegawax-250 capillary column (SUPELCO, Sigma–Aldrich Japan, Tokyo, Japan). Reproducibility (i.e. the coefficient of variation) of the determination of serum EPA, DHA, and AA levels by this method was reported to be 4.4%, 2.3%, and 3.8%, respectively [22].

The information of the other risk factor measurements was described in the Supplementary methods.

Table 1
Baseline characteristics of the study population.

N of subjects	3103
Age, years	61.3 (12.5)
Men, %	42.0
Fatty acids	
Serum EPA, μg/mL	61.7 (42.1–89.1)
Serum DHA, μg/mL	138.0 (108.1–172.5)
Serum AA, μg/mL	148.3 (125.5–172.8)
Serum EPA/AA	0.41 (0.29–0.59)
Serum DHA/AA	0.93 (0.75–1.15)
Risk factors	
Systolic blood pressure, mmHg	131.8 (21.1)
Diastolic blood pressure, mmHg	78.4 (11.9)
Use of anti-hypertensive agents, %	22.3
Hypertension, %	42.6
Diabetes, %	16.9
Use of oral diabetes medications and/or insulin, %	4.8
Serum total cholesterol, mmol/L	5.28 (0.92)
Serum HDL cholesterol, mmol/L	1.62 (0.42)
Serum triglycerides, mmol/L	1.10 (0.78–1.63)
Use of lipid-modifying agents, %	9.8
Use of statins, %	7.7
Use of EPA drugs, %	0.6
Body mass index, kg/m^2	23.1 (3.4)
Serum HS-CRP, mg/L	0.47 (0.23–1.02)
HS-CRP ≥1.0 mg/L, %	25.6
Current smoking %	22.1
Current drinking, %	43.4
Regular exercise, %	10.3

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; HDL, high-density lipoprotein; HS-CRP, high-sensitivity C-reactive protein. Values are means (standard deviation), medians (interquartile range), or frequencies.

2.4. Definition of cardiovascular disease

The primary outcome of this study was cardiovascular disease, which was defined as a first-ever development of stroke and/or coronary heart disease. Stroke was defined as a sudden onset of non-convulsive and focal neurological deficit persisting for >24 h. Coronary heart disease was defined as acute myocardial infarction, silent myocardial infarction, sudden cardiac death within 1 h after the onset of acute illness, or coronary intervention (coronary artery bypass surgery or angioplasty) (Supplementary methods) [23]. All cardiovascular events were adjudicated on the basis of physical examination, a review of all available clinical data, including medical records and brain imaging, and autopsy findings by a panel of the study members, who remained blind to the information on each participant's serum fatty acid levels.

2.5. Statistical analysis

The serum EPA/AA ratios were divided into quartiles. The age- and sex-adjusted incidence rate of outcomes was calculated by the person-year method and adjusted for the age and sex distribution of the overall study population using the direct method. The Cox proportional hazards model was used to estimate the adjusted hazard ratios (HRs) with 95% confidence intervals (CIs) of the outcomes according to serum EPA/AA ratio. The assumption of the proportional hazards was checked graphically using the log cumulative hazard plots for the outcomes according to the serum EPA/AA ratio. The risk estimates per 0.20 decrement in the serum EPA/AA ratio were computed using the relevant Cox model including the serum EPA/AA ratio taken as a continuous variable. In the multivariable-adjusted model, the adjustment was made for clinically or biologically

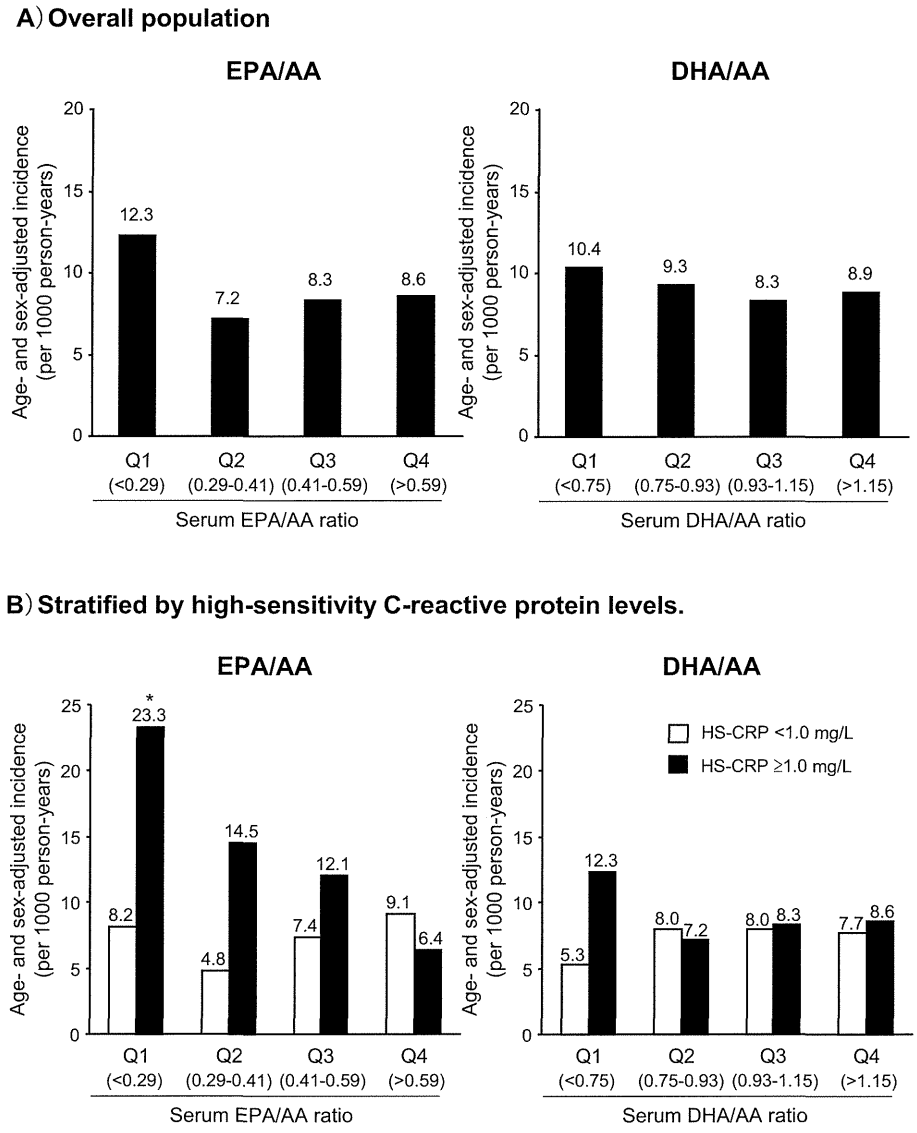

Fig. 1. Age- and sex-adjusted incidence rate of cardiovascular disease according to serum EPA/AA or DHA/AA ratio in the overall population (A) or in subgroups stratified by high-sensitivity C-reactive protein levels (B). *$p < 0.01$ vs. Q4.

plausible risk factors for the outcomes. The heterogeneities in the influence of the lower serum EPA/AA ratio on the outcomes according to serum high-sensitivity C-reactive protein (HS-CRP) levels were tested by adding a multiplicative interaction term between a continuous value of the serum EPA/AA ratio and the serum HS-CRP levels to be taken as a binary or continuous variable to the relevant model. The same analyses were conducted for the serum DHA/AA ratio. The SAS software package (SAS Institute, Cary, NC) was used to perform all statistical analyses. Two-sided values of $p < 0.05$ were considered statistically significant in all analyses.

2.6. Ethical considerations

This study was conducted with the approval of the Kyushu University Institutional Review Board for Clinical Research. Written informed consent was obtained from all participants.

3. Results

The baseline characteristics of the study population are shown in Table 1. The mean age was 61.3 years, and the proportion of men was 42.0%. The median value of serum EPA was 61.7 (interquartile range: 42.1–89.1) μg/mL, serum DHA 138.0 (108.1–172.5) μg/mL, and AA 148.3 (125.5–172.8) μg/mL. The median values of the serum EPA/AA and DHA/AA ratios were 0.41 (0.29–0.59) and 0.93 (0.75–1.15), respectively. The proportion of subjects with serum HS-CRP of 1.0 mg/L or over was 25.6%.

During the average 5.1-year follow-up period, 127 subjects experienced cardiovascular disease. These included 49 coronary heart disease events and 83 stroke events. First, we investigated the association between the serum EPA/AA ratio or the serum DHA/AA ratio and the age- and sex-adjusted incidence rate of cardiovascular disease among the overall individuals included in this study, but no clear association was detected (Fig. 1A).

Since low-grade systemic inflammation has been linked to atherosclerotic disease [24], and marine omega-3 PUFAs are reported to have anti-inflammatory effects [17,18], we hypothesized that the marine omega-3 PUFAs are likely to have stronger influence on the development of atherosclerotic disease in people with systemic inflammation than those without it. Therefore, we estimated the association between the serum EPA/AA ratio or DHA/AA ratio and the risk of cardiovascular disease according to serum HS-

CRP levels, in which high HS-CRP levels were defined as a serum HS-CRP level ≥1.0 mg/L, because we previously reported that subjects with HS-CRP of ≥1.0 mg/L had an increased risk of cardiovascular disease in our community [25]. Baseline characteristics according to the levels of serum EPA/AA or DHA/AA ratio and serum HS-CRP levels are shown in Supplementary Tables 1 and 2. The age- and sex-adjusted incidence rate of cardiovascular disease increased significantly with lower serum EPA/AA ratio among subjects with serum HS-CRP of ≥1.0 mg/L, but no clear association was observed in those with serum HS-CRP of <1.0 mg/L (Fig. 1B and Table 2). These associations remained unchanged after adjusting for potential confounding factors: age, sex, hypertension, diabetes, serum total cholesterol, serum high-density lipoprotein cholesterol, serum triglycerides, lipid-modifying agents, body mass index, current smoking, current drinking, and regular exercise (Table 2). With regard to serum DHA/AA ratio, there was no evidence of a significant association between the serum DHA/AA ratio and the risk of cardiovascular disease, regardless of serum HS-CRP levels (all p for trend >0.09), although the age- and sex-adjusted and multivariable-adjusted risk of cardiovascular disease tended toward increasing with a lower serum DHA/AA ratio in subjects with serum HS-CRP of ≥1.0 mg/L (Table 2).

As shown in Fig. 2, every 0.20 decrement in the serum EPA/AA ratio was associated with a 1.52-times (95%CI 1.12–2.04) increased risk of cardiovascular disease after adjusting for the above-mentioned confounding factors in subjects with serum HS-CRP of ≥1.0 mg/L. With regard to the subtypes of cardiovascular disease, the multivariable-adjusted risk of coronary heart disease increased significantly, by 2.23 times (95%CI 1.29–3.98) per 0.20 decrement in the serum EPA/AA ratio, whereas among subjects with serum HS-CRP of ≥1.0 mg/L the risk of stroke tended to be higher, but its association was not significant (HR 1.27, 95%CI 0.90–1.81). No clear associations between serum EPA/AA ratio and the risk of cardiovascular disease and its subtypes were found in those with serum HS-CRP of <1.0 mg/L. There were significant heterogeneities in the influence of the lower EPA/AA ratio on the risk of cardiovascular disease and coronary heart disease between subjects with serum HS-CRP below and above 1.0 mg/L (p for heterogeneity = 0.01 for cardiovascular disease; p for heterogeneity = 0.007 for coronary heart disease). To dismiss the possibility that these findings are generated by the play of chance in the subgroup analyses using the specific cut-off value, we performed the sensitivity analyses using a continuous variable of serum HS-CRP level. As shown in Fig. 3, the magnitude of the influence of every 0.20 decrement in the serum EPA/AA ratio on the risk of cardiovascular disease and coronary heart disease increased significantly with elevating serum HS-CRP levels taken as a continuous variable (both p for heterogeneity <0.01).

4. Discussion

The present study demonstrated that a lower serum EPA/AA ratio, which showed the balance of each PUFA concentration, was associated with an increased risk of the development of cardiovascular disease in individuals with higher serum HS-CRP levels. This association was not altered substantially even after adjusting for known cardiovascular risk factors. On the contrary, there was no evidence of a significant association between the serum DHA/AA

Table 2
Association of serum EPA/AA ratio or DHA/AA ratio with the development of cardiovascular disease.

Serum EPA/AA or DHA/AA levels	Median	N of events	N of subjects	Age- and sex-adjusted			Multivariable-adjusted[a]		
				HR (95%CI)	p	p for trend	HR (95%CI)	p	p for trend
Serum EPA/AA ratio									
Overall									
Q4 (>0.59)	0.74	37	776	1.00 (reference)		0.40	1.00 (reference)		0.35
Q3 (0.41–0.59)	0.50	29	776	0.97 (0.59–1.58)	0.90		0.97 (0.60–1.59)	0.91	
Q2 (0.29–0.41)	0.36	25	776	0.81 (0.49–1.36)	0.43		0.83 (0.49–1.39)	0.48	
Q1 (<0.29)	0.22	36	775	1.38 (0.87–2.18)	0.18		1.42 (0.88–2.29)	0.15	
HS-CRP < 1.0 mg/L									
Q4 (>0.59)	0.74	30	567	1.00 (reference)		0.27	1.00 (reference)		0.39
Q3 (0.41–0.59)	0.49	20	576	0.83 (0.47–1.47)	0.52		0.89 (0.50–1.59)	0.69	
Q2 (0.29–0.41)	0.35	13	585	0.54 (0.28–1.04)	0.07		0.58 (0.30–1.13)	0.11	
Q1 (<0.29)	0.22	17	581	0.87 (0.48–1.59)	0.66		0.92 (0.50–1.73)	0.81	
HS-CRP ≥ 1.0 mg/L									
Q4 (>0.59)	0.75	7	209	1.00 (reference)		0.006	1.00 (reference)		0.002
Q3 (0.41–0.59)	0.50	9	200	1.51 (0.56–4.07)	0.41		1.50 (0.55–4.07)	0.43	
Q2 (0.29–0.41)	0.36	12	191	1.91 (0.75–4.89)	0.18		2.20 (0.84–5.77)	0.11	
Q1 (<0.29)	0.22	19	194	3.31 (1.38–7.93)	0.007		3.84 (1.56–9.44)	0.003	
Serum DHA/AA ratio									
Overall									
Q4 (>1.15)	1.33	38	776	1.00 (reference)		0.48	1.00 (reference)		0.38
Q3 (0.93–1.15)	1.02	31	776	0.96 (0.60–1.55)	0.88		1.04 (0.64–1.68)	0.89	
Q2 (0.75–0.93)	0.84	31	776	1.05 (0.65–1.69)	0.84		1.14 (0.70–1.86)	0.61	
Q1 (<0.75)	0.65	27	775	1.22 (0.74–1.99)	0.44		1.26 (0.75–2.10)	0.38	
HS-CRP < 1.0 mg/L									
Q4 (>1.15)	1.33	25	543	1.00 (reference)		0.67	1.00 (reference)		0.99
Q3 (0.93–1.15)	1.02	24	577	1.07 (0.61–1.89)	0.80		1.14 (0.64–2.01)	0.66	
Q2 (0.75–0.93)	0.84	21	596	1.03 (0.57–1.83)	0.93		1.17(0.64–2.13)	0.61	
Q1 (<0.75)	0.65	10	593	0.78 (0.37–1.63)	0.50		0.86 (0.40–1.85)	0.71	
HS-CRP ≥ 1.0 mg/L									
Q4 (>1.15)	1.31	13	233	1.00 (reference)		0.10	1.00 (reference)		0.09
Q3 (0.93–1.15)	1.02	7	199	0.71 (0.28–1.79)	0.47		0.75 (0.29–1.92)	0.55	
Q2 (0.75–0.93)	0.84	10	180	1.14 (0.50–2.60)	0.76		1.20 (0.51–2.87)	0.68	
Q1 (<0.75)	0.64	17	182	1.86 (0.90–3.87)	0.10		1.93 (0.88–4.25)	0.10	

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; HS-CRP, high-sensitivity C-reactive protein.
[a] Adjusted for age, sex, hypertension, diabetes, serum total cholesterol, serum high-density lipoprotein cholesterol, serum triglycerides, lipid-modifying agents, body mass index, HS-CRP, current smoking, current drinking, and regular exercise. HS-CRP was removed from the relevant model in the subgroup analysis of HS-CRP.

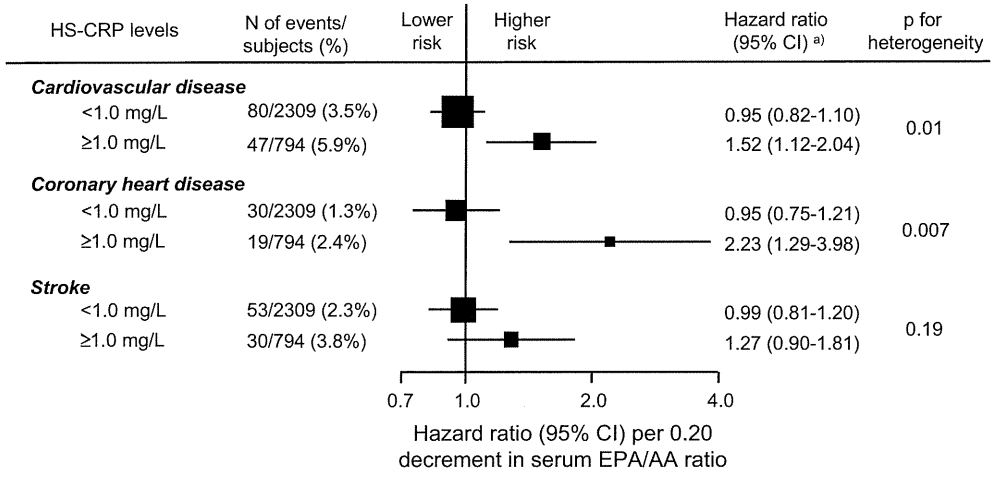

Fig. 2. The risk of incident cardiovascular disease and its subtypes per 0.20 decrement in the serum EPA/AA ratio in subjects with high-sensitivity C-reactive protein levels of less than 1.0 mg/L and of 1.0 mg/L or more. a) The risk estimates were adjusted for age, sex, hypertension, diabetes, serum total cholesterol, serum high-density lipoprotein cholesterol, serum triglycerides, use of lipid-modifying agents, body mass index, current smoking, current drinking, and regular exercise.

ratio and cardiovascular risk. These findings highlight the clinical importance of the serum EPA/AA ratio and serum HS-CRP levels as useful biomarkers of cardiovascular disease in general populations.

Inverse associations between dietary intake of fish as well as of marine omega-3 PUFAs and the risks of both cardiovascular disease and sudden cardiac death have been shown in several longitudinal observational studies [3–9]. These studies found an approximately 1.5- to 4.0-times increment in the risk of coronary heart disease or sudden cardiac death for the lowest compared with the highest quartile or quintile of dietary intake of fish or marine omega-3 PUFAs, which was estimated by responses to a food frequency questionnaire. In addition, several case–control or cohort studies have shown that lower serum or tissue levels of marine omega-3 PUFAs levels, which are a marker reflecting dietary intake of marine omega-3 PUFAs [26], were associated with a greater risk of coronary heart disease or sudden cardiac death [27–30]. The serum EPA/AA ratio is a possible biomarker for estimating the balance of EPA and AA in the human body, which reflects the cell membrane PUFA composition [31]. The results of a recent clinical trial conducted in Japanese people with hypercholesterolemia have demonstrated that the risk of coronary heart disease increased with lower serum EPA/AA ratio [19]. Likewise, the present study found that a lower serum EPA/AA ratio was significantly associated with increased risks of cardiovascular disease and coronary heart disease in individuals with higher HS-CRP levels. Taken together, these findings support the hypothesis that EPA has a preventive influence on cardiovascular events, and changes in cell membrane PUFA composition that affect the progression of atherosclerotic disease are likely to occur.

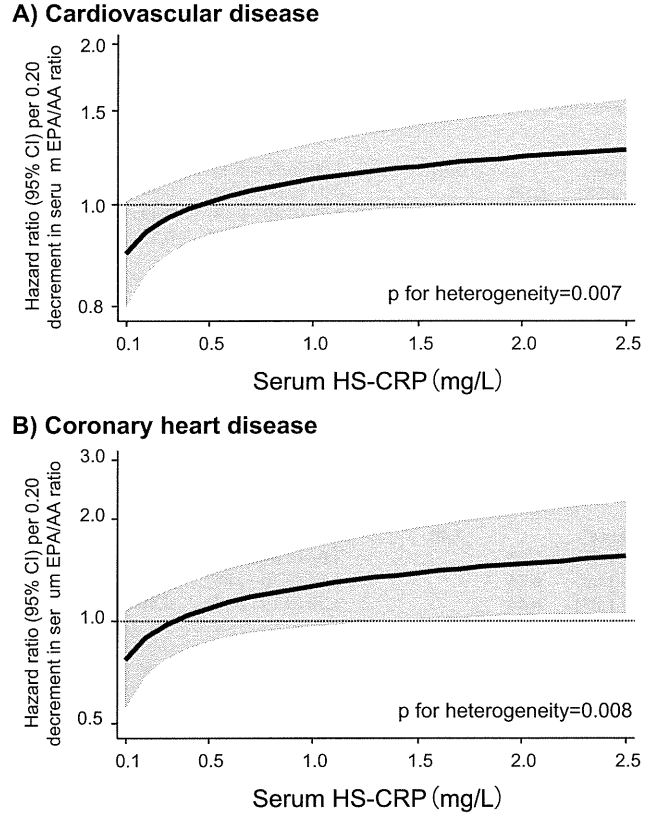

Fig. 3. The risk of incident cardiovascular disease (A) and coronary heart disease (B) per 0.20 decrement in serum EPA/AA ratio according to high-sensitivity C-reactive protein levels taken as a continuous variable. The risk estimates were adjusted for covariates shown in Fig. 2.

Meanwhile, the evidence from randomized control trials remains controversial. Two large open-labeled randomized trials demonstrated that supplementation with purified EPA or EPA + DHA significantly reduced cardiovascular risk [12,13]. Several randomized, double-blind, placebo-controlled trials also found marine omega-3 PUFA supplements had significant preventive effects against cardiovascular disease, while other trials showed no preventive effects [32–34]. These heterogeneous findings may arise from study design, the dose or method of intervention, or underlying intake of omega-3 and omega-6 PUFAs in the study population. A validity assessment of the intervention by marine omega-3 PUFA supplements using a serum marker of each PUFA (as blood pressure levels in the trials of anti-hypertensive agents) for each randomized allocation group may be useful for clarifying the heterogeneity of the treatment effect across trials, if such data are available.

The favorable effects of EPA on cardiovascular disease are mediated via several possible mechanisms [17,18]. EPA having 20 carbon chains in the structure acts as an inhibitor of AA-derived inflammatory eicosanoid mediators, such as leukotrienes, prostaglandins, and thromboxanes, by competing with AA for access to the cyclooxygenase and lipoxygenase enzymes [35]. The decreasing

production of AA-derived mediators induces an anti-inflammatory response, inhibition of monocyte adhesion and platelet aggregation, and enhancement of nitric-oxide mediated vasodilation [18,36,37]. In addition, EPA reduces the risk of plaque rupture in atherosclerotic vessels. An integrated backscatter intravascular ultrasound study has found that the amount of lipid-rich plaque increased significanlty with lower EPA levels, as well as lower DHA levels, in patients suspected of having coronary artery disease [38]. EPA decreases the production of mediators and enzymes from inflammatory cells, such as macrophages, which can thin and weaken the fibrous cap and subsequently make the plaque vulnerable and unstable [17]. Supportively, some randomized control trials conducted in patients awaiting carotid endarterectomy have shown that intervention by omega-3 PUFAs reduced the number of foam cells and T cells, reduced expression of mRNA for matrix metalloproteinases, interleukin-6, and intercellular adhesion molecule 1 in the endothelial cells, and increased plaque stability [39,40]. EPA was also reported to suppress the inflammatory response of macrophages activated by chronic inflammation itself [41]. Thus, individuals under the condition of chronic inflammation are likely to receive many benefits of EPA against atherosclerotic disease, as observed in this study.

When we investigated the subtypes of cardiovascular events, a low serum EPA/AA ratio was significantly related with an increased risk of coronary heart disease events, but not with the risk of stroke events. This discrepancy may be attributable to the fact that stroke events include several subtypes, such as lacunar infarction, atherothrombotic infarction, cardioembolic infarction, and hemorrhagic stroke. Given the etiology of each stroke subtype, the risk of atherothrombotic brain infarction may be expected to increase in subjects with lower serum EPA/AA ratios. Large-scale studies are required to elucidate the association between a lower serum EPA/AA ratio and the risk of stroke subtypes.

In the present study, serum EPA/AA ratio had a significant inverse association with the risk of cardiovascular events in individuals with higher HS-CRP levels, but the serum DHA/AA ratio had no such inverse association. The biological mechanism underlying the difference between EPA and DHA is unclear, but a prospective observational study conducted in patients with acute myocardial infarction showed that lower plasma levels of EPA, but not of DHA, were significantly associated with all-cause mortality [42]. Intriguingly, intervention by omega-3 PUFAs in patients awaiting carotid endarterectomy increased the proportion of EPA, but not of DHA, in carotid plaque phospholipids [40]. In light of these findings, EPA may have more preventive effects against the deterioration of atherosclerotic lesions than DHA among individuals at high cardiovascular risk. Further investigation is necessary to confirm this hypothesis.

Several limitations of the present study should be noted. First, only a single measurement of each serum PUFA level was obtained at the baseline examination. This may have caused the misclassification of study subjects into different categories. Such misclassification, if present, would weaken the association found in this study, biasing the results toward the null hypothesis. Second, we were unable to obtain information about medical treatment during the follow-up period. The lack of such information may have reduced the accuracy of our findings to some extent.

In conclusion, the present findings suggest that a lower serum EPA/AA ratio is associated with a greater risk of cardiovascular disease in subjects with higher serum HS-CRP levels, but no such association was observed in those with lower serum HS-CRP levels. These findings imply that the daily intakes of EPA-rich foods or the EPA medications are effective for reducing the risk of cardiovascular disease. The serum EPA/AA ratio was well correlated with the amount of fish intake estimated by using a diet history questionnaire in our subjects (Pearson's correlation coefficient = 0.18, $p < 0.001$). Importantly, serum EPA/AA ratio is more readily implemented in a routine clinical practice rather than the questionnaire of food intake. Therefore, these biomarkers may be applied more broadly for the optimal prevention of cardiovascular events in individuals who are at high risk of developing cardiovascular disease.

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Disclosures

Toshiharu Ninomiya and Yutaka Kiyohara received honoraria for lecture fees from Mochida Pharmaceutical Co., Ltd. Other authors declare that they have no competing interests.

Authors' contribution

Toshiharu Ninomiya contributed to the study concept and design, the data collection, the statistical analysis, the data interpretation, and the drafting of the manuscript. Masaharu Nagata contributed to the study concept, the data collection, and the data interpretation. Jun Hata, Daigo Yoshida, Tomoyuki Ohara, Hiro Kishimoto, Naoko Mukai, Masayo Fukuhara contributed to the data collection and the critical revision of the manuscript. Yoichiro Hirakawa contributed to the data collection and the data management. Mio Ozawa contributed to the data collection and the statistical analysis of the nutritional data. Takanari Kitazono contributed to the critical revision of the manuscript. Yutaka Kiyohara is a study coordinator and contributed to the obtainment of study funds, the study concept, the data interpretation, and the drafting of the manuscript. All authors approved the individual contributions for the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.09.023.

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SUPPLYMENTARY METHODS

Other risk factor measurements

A self-administered questionnaire concerning current use of antihypertensive agents, insulin, oral glucose-lowering agents, smoking habit, alcohol intake, and regular exercise was checked by trained interviewers at the screening. Each of these variables was classified as either habitual or not. The subjects who engaged in sports or other forms of exertion ≥3 times a week during their leisure time made up a regular exercise group. Body height and weight were measured in light clothing without shoes, and body mass index (kg/m^2) was calculated. Blood pressure was measured three times using an automated sphygmomanometer with the subject in the sitting position after at least 5 minutes of rest. The mean of the three measurements was used for the present analysis. Hypertension was defined as blood pressure ≥140/90 mmHg and/or current use of antihypertensive agents. Blood samples were collected from an antecubital vein. A portion of the serum was stored at -80°C. Plasma glucose levels were measured by the glucose oxidase method. Diabetes mellitus was defined as fasting plasma glucose levels of ≥7.0 mmol/L (126 mg/dL), 2-h post-loaded or casual glucose levels of ≥11.1 mmol/L (200 mg/dL), and/or current use of insulin or oral glucose-lowering agents. Serum total, high-density lipoprotein cholesterol, and triglyceride concentrations were determined enzymatically. Frozen samples were thawed in 2010 to measure the serum fatty acid levels and serum high-sensitivity C-reactive protein (HS-CRP) levels. Serum HS-CRP levels were measured using a modification of the Behring Latex-Enhanced CRP assay on a BN-100 Nephelometer (Behring Diagnostics, Westwood, MA).

Diagnostic criteria of myocardial infarction

Acute myocardial infarction was diagnosed when a subject met at least two of the following criteria: (1) typical symptoms, including prolonged severe anterior chest pain; (2) abnormal cardiac enzymes more than twice the upper limit of the normal range; (3) evolving diagnostic electrocardiographic changes; and (4) morphological changes, including local asynergy of cardiac wall motion on electrocardiography, persistent perfusion defect on cardiac scintigraphy, or myocardial necrosis or scars ≥1 cm long accompanied by coronary atherosclerosis at autopsy. Silent myocardial infarction was defined as myocardial scarring without any historical indication of clinical symptoms or abnormal cardiac enzyme changes.

Supplementary table 1: Baseline characteristics according to the levels of serum EPA/AA ratio and high-sensitivity C-reactive protein

Variables	Serum HS-CRP <1.0 mg/L		Serum HS-CRP ≥1.0 mg/L	
	Serum EPA/AA ratio <0.29 (Lowest quartile)	Serum EPA/AA ratio >0.59 (Highest quartile)	Serum EPA/AA ratio <0.29 (Lowest quartile)	Serum EPA/AA ratio >0.59 (Highest quartile)
N of subjects	581	567	194	209
Age, years	56.3 (12.8)	63.3 (10.7)**	64.6 (15.3)	65.0 (10.5)
Men, %	34.1	55.0**	43.3	56.5**
Systolic blood pressure, mmHg	126.4 (19.8)	134.5 (21.1)**	133.2 (22.3)	138.1 (23.1)*
Diastolic blood pressure, mmHg	75.8 (11.4)	80.0 (11.8)**	80.1 (13.0)	81.2 (13.1)
Use of anti-hypertensive agents, %	13.4	26.5**	20.6	31.1*
Hypertension, %	29.4	48.3**	44.3	59.8**
Diabetes, %	9	21.3**	21.1	32.1*
Use of oral diabetes medications and/or insulin, %	2.2	5.6**	6.7	8.6
Serum total cholesterol, mmol/L	5.21 (0.97)	5.27 (0.92)	5.16 (1.08)	5.30 (0.91)
Serum HDL cholesterol, mmol/L	1.65 (0.41)	1.66 (0.42)	1.44 (0.36)	1.51 (0.39)
Serum triglycerides, mmol/L	1.03 (0.77-1.54)	1.10 (0.77-1.63)	1.20 (0.88-1.70)	1.17 (0.85-1.73)
Use of lipid-modifying agents, %	5.9	10.1**	8.8	9.6
Body mass index, kg/m^2	22.3 (3.2)	23.2 (3.1)**	23.5 (4.2)	24.5 (3.5)**
Current smoking %	21.7	23.6	29.9	24.4
Current drinking, %	38.4	55.2**	32.0	50.7**
Regular exercise, %	9.3	14.3**	7.7	12.9

EPA, eicosapentaenoic acid; AA, arachidonic acid; HDL, high-density lipoprotein; HS-CRP, high-sensitivity C-reactive protein

Values are means (standard deviation), medians (interquartile range), or frequencies.

*p<0.05, **p<0.01 vs. Serum EPA/AA ratio <0.29

Supplementary table 2: Baseline characteristics according to the levels of serum DHA/AA ratio and high-sensitivity C-reactive protein

Variables	Serum HS-CRP <1.0 mg/L		Serum HS-CRP ≥1.0 mg/L	
	Serum DHA/AA ratio <0.75 (Lowest quartile)	Serum DHA/AA ratio >1.15 (Highest quartile)	Serum DHA/AA ratio <0.75 (Lowest quartile)	Serum DHA/AA ratio >1.15 (Highest quartile)
N of subjects	593	543**	182	233
Age, years	54.5 (11.7)	64.8 (10.6)	63.8 (15.2)	65.4 (10.6)
Men, %	34.9	51.6**	42.9	55.8**
Systolic blood pressure, mmHg	124.8 (20.3)	136.0 (20.7)**	132.1 (21.5)	141.0 (21.8)**
Diastolic blood pressure, mmHg	74.8 (12.0)	80.7 (11.5)**	79.6 (12.4)	82.9 (13.3)**
Use of anti-hypertensive agent, %	12.8	26.7**	22.5	34.3**
Hypertension, %	27	51.0**	42.3	62.2**
Diabetes, %	8.6	22.7**	18.7	34.8**
Use of oral diabetes medications and/or insulin, %	2.5	6.3**	6.0	7.7
Serum total cholesterol, mmol/L	5.20 (0.95)	5.29 (0.91)	5.19 (1.04)	5.29 (0.91)
Serum HDL cholesterol, mmol/L	1.74 (0.42)	1.55 (0.39)**	1.50 (0.39)	1.39 (0.37)**
Serum triglycerides, mmol/L	0.90 (0.68-1.25)	1.38 (0.91-2.01)**	1.12 (0.84-1.49)	1.58 (1.07-2.27)**
Use of lipid-modifying agents, %	7.6	8.8	9.3	10.7
Body mass index, kg/m^2	22.4 (3.1)	23.2 (3.2)**	23.1 (4.0)	24.7 (3.3)**
Current smoking %	22.6	24.1	30.8	24.9
Current drinking, %	44.4	49.5	30.2	50.2**
Regular exercise, %	8.2	16.8**	7.7	9.4

DHA, docosahexaenoic acid; AA, arachidonic acid; HDL, high-density lipoprotein; HS-CRP, high-sensitivity C-reactive protein

Values are means (standard deviation), medians (interquartile range), or frequencies.

*p<0.05, **p<0.01 vs. Serum DHA/AA ratio <0.75

DIABETICMedicine

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Research: Pathophysiology

Magnesium intake decreases Type 2 diabetes risk through the improvement of insulin resistance and inflammation: the Hisayama Study

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Abstract

Aims Early studies have shown that magnesium intake decreases the risk of Type 2 diabetes, but the results are still inconsistent. We prospectively examined the association between magnesium intake and incidence of Type 2 diabetes in a general Japanese population.

Methods A total of 1999 subjects without diabetes aged 40–79 years who underwent a 75-g oral glucose tolerance test were followed up prospectively for a mean of 15.6 years.

Results During the follow-up, 417 subjects developed Type 2 diabetes. The age- and sex-adjusted incidence of Type 2 diabetes significantly decreased with increasing magnesium intake quartile levels (≤ 148.5, 148.6–171.5, 171.6–195.5 and ≥ 195.6 mg/day, P for trend = 0.01). In multivariate analyses, after adjusting for comprehensive risk factors and other dietary factors, the hazard ratio of Type 2 diabetes was 0.67 (95% CI 0.49–0.92; $P = 0.01$) in the third quartile and 0.63 (95% CI 0.44–0.90; $P = 0.01$) in the highest quartile compared with the first quartile. In addition, the risk of Type 2 diabetes was 14% lower ($P = 0.04$) for a 1-SD increment of log-transformed magnesium intake in the multivariate-adjusted model. In stratified analysis, there were statistically significant interactions between magnesium intake and levels of homeostasis model assessment of insulin resistance, high-sensitivity C-reactive protein or alcohol intake on the risk of Type 2 diabetes (all $P < 0.05$).

Conclusions Our findings suggest that increased magnesium intake was a significant protective factor for the incidence of Type 2 diabetes in the general Japanese population, especially among subjects with insulin resistance, low-grade inflammation and a drinking habit.

Diabet. Med. 30: 1487–1494 (2013)

Introduction

Magnesium is an essential cofactor for multiple enzymes involved in glucose metabolism [1]. A large number of animal studies have shown that dietary-induced magnesium deficiency is associated with impaired post-receptor signalling of insulin [2] and increased insulin resistance [3]. Prior studies have observed an inverse association between magnesium intake and fasting insulin concentrations in blood [4,5]. These findings led to the hypothesis that magnesium itself plays a key role in regulating insulin action. Some prospective studies in Western peoples have shown that lower magnesium intake was a risk factor for developing diabetes [4,6,7], probably through increasing insulin resistance. However, it is not certain to what extent these findings apply to general Asian populations, which tend to have lower adiposity and lower insulin resistance. There have been three prospective studies which have investigated the association between magnesium intake and diabetes risk in Asian populations, but the findings were inconsistent; two studies from China and Japan have shown that the risk of developing diabetes decreased with elevating magnesium intake [8,9], but another study from Japan has found no significant association between them [10]. In addition, to the best of our

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What's new?

- A number of prospective cohort studies and meta-analyses have suggested that magnesium intake reduces the risk of diabetes, but the results are still inconsistent. In addition, most cohort studies have been derived from Caucasian populations, and thus the effect of magnesium intake on diabetes risk in Asians remains to be fully elucidated. In this paper, we demonstrate that higher intake of magnesium reduces the risk of Type 2 diabetes in Japanese.
- To the best of our knowledge, this is the first prospective study showing the effect modification by insulin resistance and low-grade inflammation on the association between magnesium intake and the development of Type 2 diabetes.

knowledge, no prospective studies have evaluated the interaction of magnesium intake with insulin resistance on incident Type 2 diabetes.

The aims of the present study were to investigate the association of magnesium intake with incident Type 2 diabetes in a general Japanese population, taking into account comprehensive risk factors, and to further compare the influence of magnesium intake between subjects with and without potential confounding factors including insulin resistance and its related states, such as low-grade inflammatory conditions.

Subjects and methods

Study population and follow-up survey

A population-based prospective study of cardiovascular disease and its risk factors has been underway since 1961 in the town of Hisayama, which is located in a suburb of the Fukuoka metropolitan area on Japan's Kyushu Island. In 1988, a screening examination with a dietary survey and a 75-g oral glucose tolerance test was performed in the town to establish a cohort for the present study. A detailed description of this examination has been published previously [11]. Briefly, 2587 residents aged 40–79 years (80.2% of this age group) underwent the screening examination. After exclusion of 82 subjects who had already eaten breakfast, 10 who were receiving insulin therapy and 15 who complained of nausea or general fatigue during the ingestion of glucose, a total of 2480 subjects completed the oral glucose tolerance test. Among these, 297 subjects with diabetes, two who died before the start of follow-up, and eight who had implausible total energy intake (< 800 or > 4200 kcal/day for men and < 500 or > 3500 kcal/day for women) [6] were excluded, and the remaining 2173 subjects (905 men and 1268 women) were enrolled in the baseline examination.

The baseline subjects were followed up prospectively from December 1988 to November 2009 by repeated annual health examinations. Of the baseline subjects, 1999 subjects (816 men and 1183 women) who underwent re-examinations were selected for the present study (follow-up rate 92.0%; mean follow-up period 15.6 years; mean frequency of follow-up examinations 10.5 times). One subject who developed overt Type 1 diabetes clinically during the follow-up period was censored at the time. During the follow-up, 417 subjects developed Type 2 diabetes (204 men and 213 women).

Nutritional survey

At baseline examination, a dietary survey was conducted using a 70-item Semi-Quantitative Food Frequency Questionnaire (SFFQ) concerning food intake [12]. The questionnaire was administered prior to initiation of this study and each subject was questioned by trained dieticians and nutritionists, who presented the subjects with food models of actual size during the examination. The average food intake per day was calculated from the frequency of meals per week and the amount of each food portion. Nutritional intake was calculated using the 4th revision of the Standard Tables of Food Composition in Japan [13] and its follow-up version for fatty acids, cholesterol and vitamin E [14]. Magnesium intake was calculated from a magnesium inclusion table that was developed by Shirota *et al.* [15]. The use of magnesium supplements was not considered in our study, because magnesium supplementation is not popular in Japan. All dietary nutrient variables were adjusted for total energy intake using the residual method [16]. The appropriateness of this questionnaire has been reported elsewhere [17]. In brief, the relative validity has been evaluated by comparison with 3-day dietary records and the correlations between means of daily intakes in the SFFQ and the dietary records were > 0.40 for most nutrients. In particular, the correlation coefficients were 0.53 for potassium and 0.42 for calcium, but magnesium was not validated because of the absence of a standardized table of food compositions for magnesium in Japan in 1988, the year that this study was initiated. However, the amount of magnesium ingestion is likely to be correlated with that of potassium or calcium because these elements tend to occur in similar foods [5].

Clinical evaluation and laboratory measurements

In the baseline and follow-up examinations, the study subjects underwent the oral glucose tolerance test between 8.00 and 10.30 AM after an overnight fast of at least 12 h. Plasma glucose levels were determined by a