

Serum Angiotensin-Like Protein 2 Is a Novel Risk Factor for Cardiovascular Disease in the Community

The Hisayama Study

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Objective—Angiotensin-like protein 2 (ANGPTL2), a proinflammatory mediator, has been reported to accelerate the development of insulin resistance, endothelial dysfunction, and atherosclerosis in mice. However, no cohort studies have examined the relationship between serum ANGPTL2 levels and the development of cardiovascular disease (CVD) in a general population.

Approach and Results—A total of 3005 community-dwelling Japanese aged ≥ 40 years without a history of CVD were divided into 4 groups according to the quartiles of serum ANGPTL2 concentrations (Q1, lowest and Q4, highest) and followed up for 10 years. The hazards ratios and their 95% confidence intervals for the development of CVD (coronary heart disease or stroke) were estimated using a Cox proportional hazards model. During the follow-up, 219 first-ever CVD events were observed. The risk of CVD increased significantly with elevating ANGPTL2 levels after adjustment for age, sex, serum total cholesterol, use of lipid-lowering agents, ECG abnormalities, smoking habits, alcohol intake, and regular exercise (hazards ratios [95% confidence interval], Q1, 1.00 [reference]; Q2, 1.27 [0.80–2.04]; Q3, 1.48 [0.95–2.32]; and Q4, 1.85 [1.20–2.85]; $P=0.003$ for trend). After additional adjustment for metabolic syndrome components and serum high-sensitivity C-reactive protein levels as an inflammatory marker, the association was attenuated but remained significant (hazards ratios [95% confidence interval], Q1, 1.00 [reference]; Q2, 1.21 [0.76–1.94]; Q3, 1.38 [0.87–2.17]; and Q4, 1.66 [1.05–2.60]; $P=0.02$ for trend).

Conclusions—Our findings suggest that elevated serum ANGPTL2 levels are a novel risk factor for the development of CVD in the general population. This association is partially mediated by metabolic disorders and inflammation. (*Arterioscler Thromb Vasc Biol.* 2016;36:1686-1691. DOI: 10.1161/ATVBAHA.116.307291.)

Key Words: angiotensin ■ cardiovascular disease ■ cohort study ■ inflammation ■ stroke

Angiotensin-like protein 2 (ANGPTL2) is a member of a protein family whose members are structurally similar to angiotensins.^{1,2} It has been reported that ANGPTL2 is a proinflammatory mediator, and it accelerates the development of insulin resistance, endothelial dysfunction, and atherosclerosis in mice.^{2–5} Histological studies of mice and humans have shown that ANGPTL2 is abundantly expressed in visceral adipose tissue, endothelial cells, and macrophages infiltrating atheromatous plaques.^{2–5} In healthy volunteers, an elevated concentration of serum ANGPTL2 was closely associated with obesity, insulin resistance, and increased levels of serum high-sensitivity C-reactive protein (hs-CRP), an inflammatory marker.³ Because these conditions are well known to be

risk factors for the development of cardiovascular disease (CVD),^{6–12} we hypothesized that increased concentrations of serum ANGPTL2 might be involved in the development of CVD through the accumulation of metabolic disorders and inflammation. However, no prospective epidemiological studies have examined the possible association between serum ANGPTL2 levels and the development of CVD or the possible roles of metabolic disorders and inflammation as mediators in such an association.

The aim of this study was to investigate these issues in a cohort study of a general population, with consideration for comprehensive cardiovascular risk factors including components of metabolic syndrome and serum levels of hs-CRP.

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Nonstandard Abbreviations and Acronyms

ANGPTL2	angiotensin-like protein 2
CVD	cardiovascular disease
HR	hazards ratio
hs-CRP	high-sensitivity C-reactive protein

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results

The baseline characteristics of the study population according to the quartiles of serum ANGPTL2 concentrations are shown in Table 1. The mean values of age, waist circumference, body mass index, systolic and diastolic blood pressures, fasting

plasma glucose, and serum levels of low-density lipoprotein cholesterol, triglycerides, and hs-CRP increased significantly with elevating serum ANGPTL2 levels. The subjects with higher serum ANGPTL2 levels had significantly higher frequencies of antihypertensive medication, antidiabetic medication, lipid-lowering medication, ECG abnormalities, regular exercise, metabolic syndrome, and its components. In contrast, the mean values of serum high-density lipoprotein cholesterol and frequencies of current smoking and drinking decreased significantly with higher serum ANGPTL2 levels. Serum ANGPTL2 levels did not show a clear association with sex and total cholesterol levels.

Figure I in the [online-only Data Supplement](#) shows the distributions of baseline serum ANGPTL2 concentrations in subjects with and without CVD events during the follow-up. The distribution of serum ANGPTL2 in subjects with CVD was significantly higher than in those without CVD ($P<0.001$).

Table 1. Baseline Characteristics by the Quartiles of Serum ANGPTL2 Concentrations, the Hisayama Study, 2002 to 2003

	Serum ANGPTL2 Levels				P for Trend
	Q1 (<2.25 ng/mL); n=751	Q2 (2.25–2.83 ng/mL); n=751	Q3 (2.84–3.61 ng/mL); n=752	Q4 (\geq 3.62 ng/mL); n=751	
Age, y	56 (11)	59 (12)	63 (12)	66 (12)	<0.001
Men, %	57.3	58.5	58.1	57.4	>0.99
Waist circumference (cm)	79.5 (8.4)	81.0 (9.1)	82.9 (9.5)	84.7 (9.5)	<0.001
Body mass index (kg/m ²)	22.4 (3.0)	22.8 (3.2)	23.4 (3.5)	23.8 (3.6)	<0.001
Systolic blood pressure (mm Hg)	126 (19)	129 (21)	134 (20)	137 (22)	<0.001
Diastolic blood pressure (mm Hg)	76 (11)	77 (12)	80 (11)	81 (13)	<0.001
Antihypertensive medication, %	13.8	17.8	24.9	31.2	<0.001
Fasting plasma glucose (mmol/L)	5.8 (0.9)	6.0 (1.1)	6.0 (1.2)	6.4 (1.6)	<0.001
Antidiabetic medication, %	2.3	2.7	4.5	8.9	<0.001
Serum total cholesterol (mmol/L)	5.25 (0.84)	5.33 (0.95)	5.33 (0.91)	5.26 (0.95)	0.79
Serum HDL cholesterol (mmol/L)	1.76 (0.43)	1.66 (0.42)	1.59 (0.39)	1.49 (0.39)	<0.001
Serum LDL cholesterol (mmol/L)	2.99 (0.78)	3.08 (0.86)	3.15 (0.82)	3.08 (0.84)	0.01
Serum triglycerides (mmol/L)	0.93 (0.68–1.31)	1.08 (0.78–1.58)	1.12 (0.79–1.65)	1.26 (0.93–1.87)	<0.001
Lipid-lowering medication, %	6.9	8.8	10.6	10.3	0.01
ECG abnormalities, %	12.5	15.6	16.6	18.9	<0.001
Current smoking, %	29.4	25.3	17.2	17.2	<0.001
Current drinking, %	51.9	46.7	41.4	36.4	<0.001
Regular exercise, %	9.3	10.5	12.4	12.3	0.04
Metabolic syndrome, %	17.7	28.8	34.7	49.9	<0.001
Metabolic syndrome components					
Elevated waist circumference, %	27.7	37.2	44.4	52.2	<0.001
High blood pressure, %	42.3	49.0	61.7	68.7	<0.001
High fasting plasma glucose, %	61.8	66.3	68.4	75.1	<0.001
Low HDL cholesterol, %	4.8	8.9	10.5	19.0	<0.001
High triglycerides, %	15.0	20.6	23.4	30.4	<0.001
Serum hs-CRP, mg/L	0.29 (0.15–0.58)	0.43 (0.21–0.86)	0.50 (0.26–1.12)	0.77 (0.36–1.74)	<0.001

Values are means (SD), percentages, or medians (interquartile ranges). ANGPTL2 indicates angiotensin-like protein 2; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; and LDL, low-density lipoprotein.

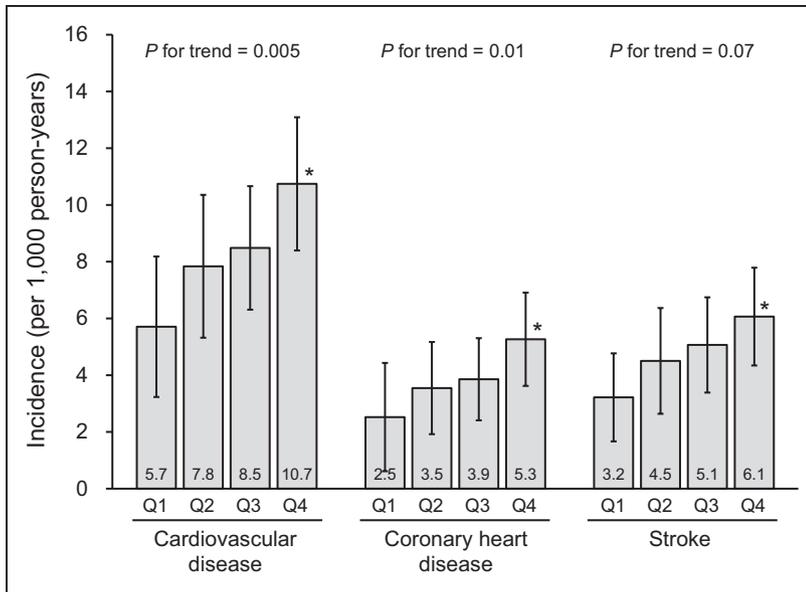


Figure. Age- and sex-adjusted incidence rates of cardiovascular disease and its subtypes according to the quartiles of serum angiopoietin-like protein 2 (ANGPTL2) concentrations, the Hisayama Study, 2002 to 2012. Quartiles of serum ANGPTL2 concentration: Q1, <2.25 ng/mL; Q2, 2.25 to 2.83 ng/mL; Q3, 2.84 to 3.61 ng/mL; and Q4, \geq 3.62 ng/mL. The bar indicates 95% confidence interval. * $P < 0.05$ vs Q1.

Figure demonstrates the age- and sex-adjusted incidence rates of CVD and its subtypes according to the serum ANGPTL2 levels. The incidence rates of total CVD and coronary heart disease increased significantly with elevating serum ANGPTL2 levels (P for trend=0.005 and 0.01, respectively). A similar but nonsignificant trend was observed for the incidence rate of stroke (P for trend=0.07).

Table 2 and Figure II in the [online-only Data Supplement](#) show the hazard ratios (HRs) for the development of CVD and its subtypes according to the serum ANGPTL2 levels. In model 1 with adjustment for age and sex, higher serum ANGPTL2 levels were significantly associated with an increased risk of total CVD (P for trend=0.005). This association remained significant after adjustment for conventional cardiovascular risk factors other than metabolic syndrome components (model 2, P for trend=0.003), and even after additional adjustment for metabolic syndrome components (model 3, P for trend=0.006) and serum hs-CRP (model 4, P for trend=0.02). A similar association was observed for coronary heart disease and stroke analyzed separately, although the associations with coronary heart disease in model 4 and with stroke in model 1 and model 4 did not reach the level of statistical significance.

Table 3 summarizes the HRs for the development of total CVD per 1 SD increment in log-transformed serum ANGPTL2 concentrations. In model 2, elevated ANGPTL2 was significantly associated with a higher risk of total CVD (HR, 1.19; 95% confidence interval, 1.03–1.38). The association diminished slightly when metabolic syndrome components were added into model 2 as a reference model (HR, 1.18; 95% confidence interval, 1.02–1.37; % reduction in log HR, 4.8%). The association attenuated moderately when serum hs-CRP was added into model 2 (HR, 1.15; 95% confidence interval, 0.99–1.33; % reduction in log HR, 22.0%) or when both metabolic syndrome components and serum hs-CRP were added into model 2 (HR, 1.14; 95% confidence interval, 0.98–1.34; % reduction in log HR, 22.7%).

Discussion

In this study, we demonstrated that the risk for the development of CVD increased significantly and linearly with elevating serum ANGPTL2 levels even after adjustment for conventional cardiovascular risk factors other than metabolic syndrome components. This association diminished but remained significant after additional adjustment for metabolic syndrome components and serum hs-CRP levels. These findings suggest that elevated serum ANGPTL2 levels are a novel risk factor for CVD, and the association is partially mediated by metabolic disorders and inflammation.

Some cross-sectional and case-control studies have shown the positive associations between serum ANGPTL2 levels and the presence of CVD and its surrogate outcomes, such as acute coronary syndrome,¹³ heart failure,¹⁴ carotid atherosclerosis,¹⁵ and albuminuria.¹⁶ In our recent cross-sectional and prospective cohort surveys of the Hisayama Study, elevated serum ANGPTL2 levels were significantly associated with a higher prevalence of chronic kidney disease¹⁷ and an increased risk for the development of type 2 diabetes mellitus.¹⁸ However, the longitudinal association of serum ANGPTL2 levels with the development of CVD in general populations remained unclear. To the best of our knowledge, this study is the first prospective cohort study to demonstrate that elevated serum ANGPTL2 concentrations were a significant risk factor for the development of CVD in the general population.

Several biological mechanisms could potentially explain the positive association between serum ANGPTL2 levels and the risk of CVD. ANGPTL2 is an adipokine secreted from adipose tissues,³ and it has been reported that serum ANGPTL2 concentrations were elevated in metabolic syndrome in mouse models and humans,^{3,19} suggesting that elevated serum ANGPTL2 concentrations may be a marker for the presence of metabolic syndrome. The latter, which is a clustering of metabolic disorders including abdominal obesity, dyslipidemia, hyperglycemia, and elevated blood pressure, has been recognized as one of the established risk factors for CVD.^{20,21} Thus, the association between serum ANGPTL2 levels and

Table 2. HRs for the Development of CVD and Its Subtypes According to the Quartiles of Serum ANGPTL2 Concentrations, the Hisayama Study, 2002 to 2012

Serum ANGPTL2 Levels, ng/mL	No. of Events/ at Risk	Model 1*		Model 2†		Model 3‡		Model 4§	
		HR (95% CI)	P Value						
CVD									
Q1 (<2.25)	29/751	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2 (2.25–2.83)	45/751	1.29 (0.81–2.06)	0.29	1.27 (0.80–2.04)	0.31	1.25 (0.78–2.01)	0.35	1.21 (0.76–1.94)	0.43
Q3 (2.84–3.61)	60/752	1.42 (0.91–2.22)	0.13	1.48 (0.95–2.32)	0.09	1.45 (0.92–2.28)	0.11	1.38 (0.87–2.17)	0.17
Q4 (≥3.62)	85/751	1.80 (1.17–2.79)	0.008	1.85 (1.20–2.85)	0.006	1.79 (1.15–2.79)	0.01	1.66 (1.05–2.60)	0.03
P for trend			0.005		0.003		0.006		0.02
CHD									
Q1 (<2.25)	11/751	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2 (2.25–2.83)	21/751	1.56 (0.75–3.24)	0.24	1.54 (0.74–3.20)	0.25	1.47 (0.71–3.07)	0.30	1.46 (0.70–3.04)	0.31
Q3 (2.84–3.61)	28/752	1.70 (0.84–3.45)	0.14	1.77 (0.87–3.58)	0.11	1.63 (0.80–3.31)	0.18	1.60 (0.78–3.27)	0.20
Q4 (≥3.62)	42/751	2.30 (1.17–4.54)	0.02	2.30 (1.17–4.54)	0.02	2.02 (1.01–4.05)	0.048	1.98 (0.98–3.99)	0.06
P for trend			0.01		0.01		0.04		0.05
Stroke									
Q1 (<2.25)	19/751	1.00 (reference)		1.00 (reference)		1.00 (reference)		1.00 (reference)	
Q2 (2.25–2.83)	27/751	1.16 (0.65–2.10)	0.61	1.15 (0.64–2.08)	0.64	1.16 (0.64–2.09)	0.63	1.10 (0.60–1.98)	0.77
Q3 (2.84–3.61)	36/752	1.29 (0.74–2.27)	0.37	1.35 (0.77–2.37)	0.30	1.38 (0.78–2.43)	0.27	1.26 (0.71–2.24)	0.42
Q4 (≥3.62)	50/751	1.59 (0.92–2.74)	0.10	1.64 (0.95–2.83)	0.07	1.69 (0.97–2.96)	0.07	1.49 (0.85–2.64)	0.17
P for trend			0.07		0.047		0.04		0.12

ANGPTL2 indicates angiotensin-like protein 2; CI, confidence interval; CHD, coronary heart disease; CVD, cardiovascular disease; and HR, hazard ratios.

*Model 1: adjusted for age and sex.

†Model 2: adjusted for the covariates in model 1+total cholesterol, lipid-lowering medication, ECG abnormalities, smoking habits, alcohol intake, and regular exercise.

‡Model 3: adjusted for the covariates in model 2+metabolic syndrome components (elevated waist circumference, high blood pressure, high fasting plasma glucose, low high-density lipoprotein cholesterol, and high triglycerides).

§Model 4: adjusted for the covariates in model 3+high-sensitivity C-reactive protein.

the future development of CVD might be attributable to the presence of metabolic syndrome. Experimental studies have shown that ANGPTL2 works as a proinflammatory mediator.²⁻⁵ Adipocyte-derived ANGPTL2 is reported to be a key mediator linking obesity to adipose tissue inflammation. An animal study showed that ANGPTL2 in adipose tissue induces chronic adipose tissue inflammation and systemic insulin resistance,^{2,3} resulting in an increased CVD risk. In addition, endothelium-derived ANGPTL2 promotes vascular

inflammation, most likely via the integrin $\alpha 5 \beta 1$ /Rac1/NF- κ B (nuclear factor-kappa B) pathway.² Because vascular injury accompanied by inflammation is considered to be an early feature of atherosclerosis, serum ANGPTL2 may be involved in the initiation and progression of atherosclerosis, resulting in an elevated risk of CVD. These facts suggest that the association between serum ANGPTL2 levels and the risk of CVD could be explained by the presence of ANGPTL2-induced inflammation in adipose tissue and arteries. In this study, the

Table 3. HRs for the Development of Cardiovascular Disease Per 1 SD Increment in Log-Transformed Serum ANGPTL2 Concentrations, the Hisayama Study, 2002 to 2012

	HR (95% CI)	Log HR	% Reduction in Log HR*
Model 2	1.19 (1.03–1.38)	0.174	(reference)
Model 2+metabolic syndrome components (model 3)	1.18 (1.02–1.37)	0.166	4.8
Model 2+hs-CRP	1.15 (0.99–1.33)	0.136	22.0
Model 2+metabolic syndrome components+hs-CRP (model 4)	1.14 (0.98–1.34)	0.135	22.7

Model 2: adjusted for age, sex, total cholesterol, lipid-lowering medication, ECG abnormalities, smoking habits, alcohol intake, and regular exercise. Metabolic syndrome components include elevated waist circumference, high blood pressure, high fasting plasma glucose, low high-density lipoprotein cholesterol, and high triglycerides. ANGPTL2 indicates angiotensin-like protein 2; CI, confidence interval; HR, hazard ratios; and hs-CRP, high-sensitivity C-reactive protein.

*% Reduction in log HR=[(log HR in model 2)–(log HR)]/[log HR in model 2]×100.

magnitude of the association of serum ANGPTL2 levels with the CVD risk was attenuated by 4.8% after adjustment for metabolic syndrome components and by 22.0% after adjustment for serum hs-CRP levels, indicating that the association can be partially explained by metabolic disorders and inflammation. Nevertheless, the association between ANGPTL2 quartiles and the CVD risk remained significant in the fully adjusted model, including metabolic syndrome components and serum hs-CRP levels (Table 2, model 4), suggesting that mechanisms other than metabolic disorders and inflammation may exist. However, although we controlled for a wide range of confounding factors in this analysis, the possibility of residual confounders may exist. In particular, serum hs-CRP levels may not entirely explain inflammation status because inflammation involves complex processes reflected in a variety of surrogate markers other than hs-CRP. ANGPTL2 is not the only determinant of hs-CRP; other inflammatory mediators, such as interleukins and tumor necrosis factors, are also well-known determinants of hs-CRP. Therefore, the influence of ANGPTL2-induced inflammation might not be fully adjusted by adding hs-CRP in model 4. Further investigations will be required to elucidate other possible mechanisms underlying the association between serum ANGPTL2 levels and CVD.

This study had several important merits, namely, the community-based prospective design, high participation rate in the baseline examination, and complete follow-up survey.²² However, some limitations should also be noted. First, ANGPTL2 concentrations were determined using serum samples frozen in long-term storage. Long-term storage time might result in the degradation of ANGPTL2 protein and possibly an exponential decrease in the concentrations of ANGPTL2 protein. However, because such a decrease in concentration was likely to occur equally among the samples, the possibility of misclassification in the ANGPTL2 quartiles may have been small and may have only minimally influenced the association observed in this study. Second, our findings were based on a single measurement of serum ANGPTL2 concentrations at baseline. The variability of serum ANGPTL2 concentrations during the follow-up was not taken into consideration. This limitation might weaken the association observed in this study, biasing the results toward the null hypothesis. Thus, the true association might be stronger than that observed. Finally, it remains unclear whether the conclusions of this study can be generalized to other ethnic populations with different genetic and lifestyle backgrounds because the prevalence of obesity and serum concentrations of hs-CRP are lower in Japanese than in Western people.²³

In conclusion, the findings of this study suggest that elevated serum concentrations of ANGPTL2 are a novel risk factor for the development of CVD, and that this association is partially mediated by metabolic disorders and inflammation. Further experimental, epidemiological, and clinical studies will be needed to reveal the precise role of ANGPTL2 in the development of CVD.

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Disclosures

None.

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Highlights

- The age- and sex-adjusted incidence rate of cardiovascular disease increased significantly and linearly with elevating serum angiotensin-like protein 2 concentrations.
- Elevating concentrations of serum angiotensin-like protein 2 were a significant risk factor for cardiovascular disease after adjustment for conventional risk factors other than the metabolic syndrome components.
- This association diminished but remained significant after additional adjustment for metabolic syndrome components and serum high-sensitivity C-reactive protein levels, suggesting that the association is partially mediated by metabolic disorders and inflammation.

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Serum Angiotensin-Like Protein 2 Is a Novel Risk Factor for Cardiovascular Disease in the Community: The Hisayama Study

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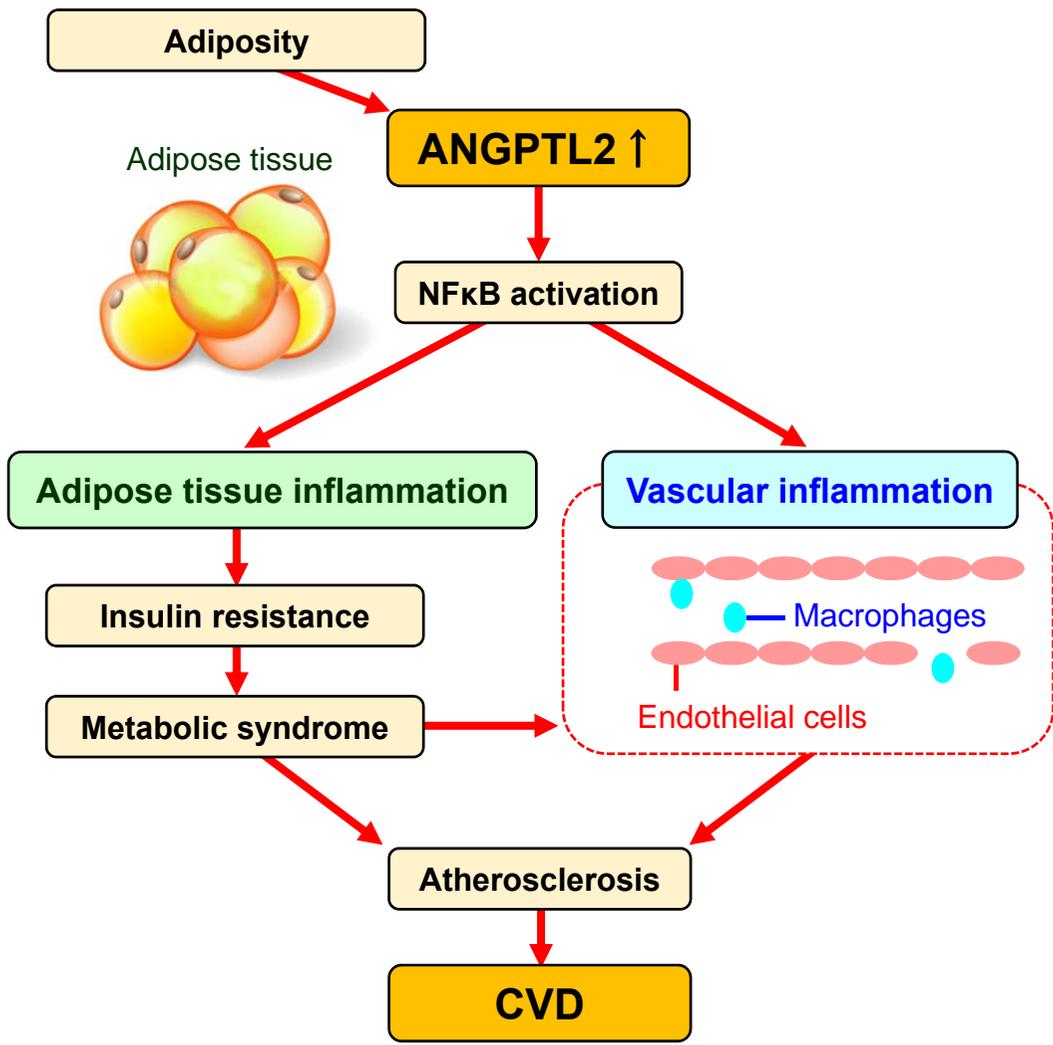
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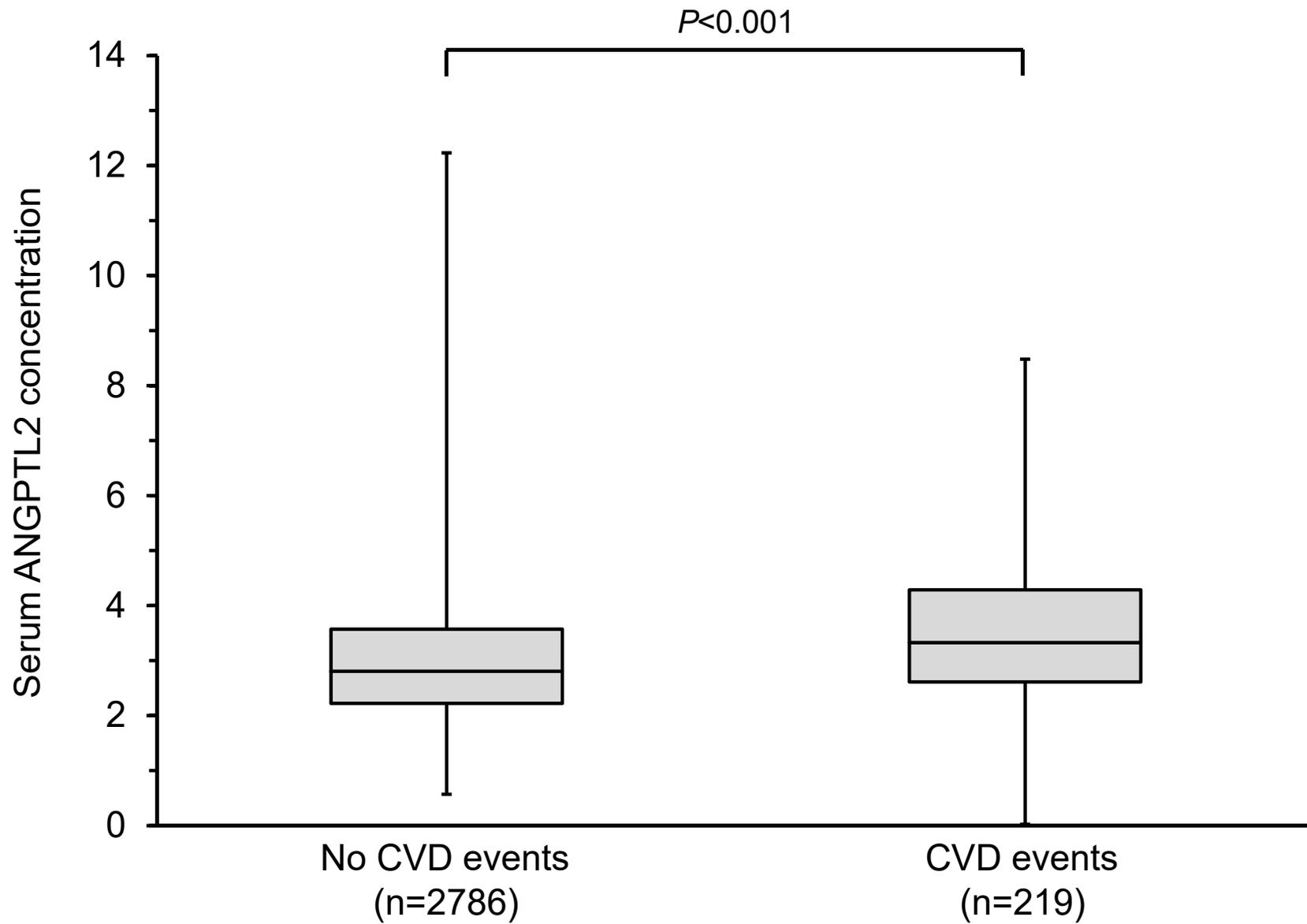


Figure I. Distribution of the baseline serum angiopoietin-like protein 2 (ANGPTL2) concentrations according to the presence or absence of cardiovascular disease (CVD) events during the follow-up period, the Hisayama Study, 2002-2012.

The line in the box and the edges of the box indicate the median and quartiles of serum ANGPTL2, respectively. Edges of the whiskers indicate the minimum and the maximum of serum ANGPTL2, respectively.

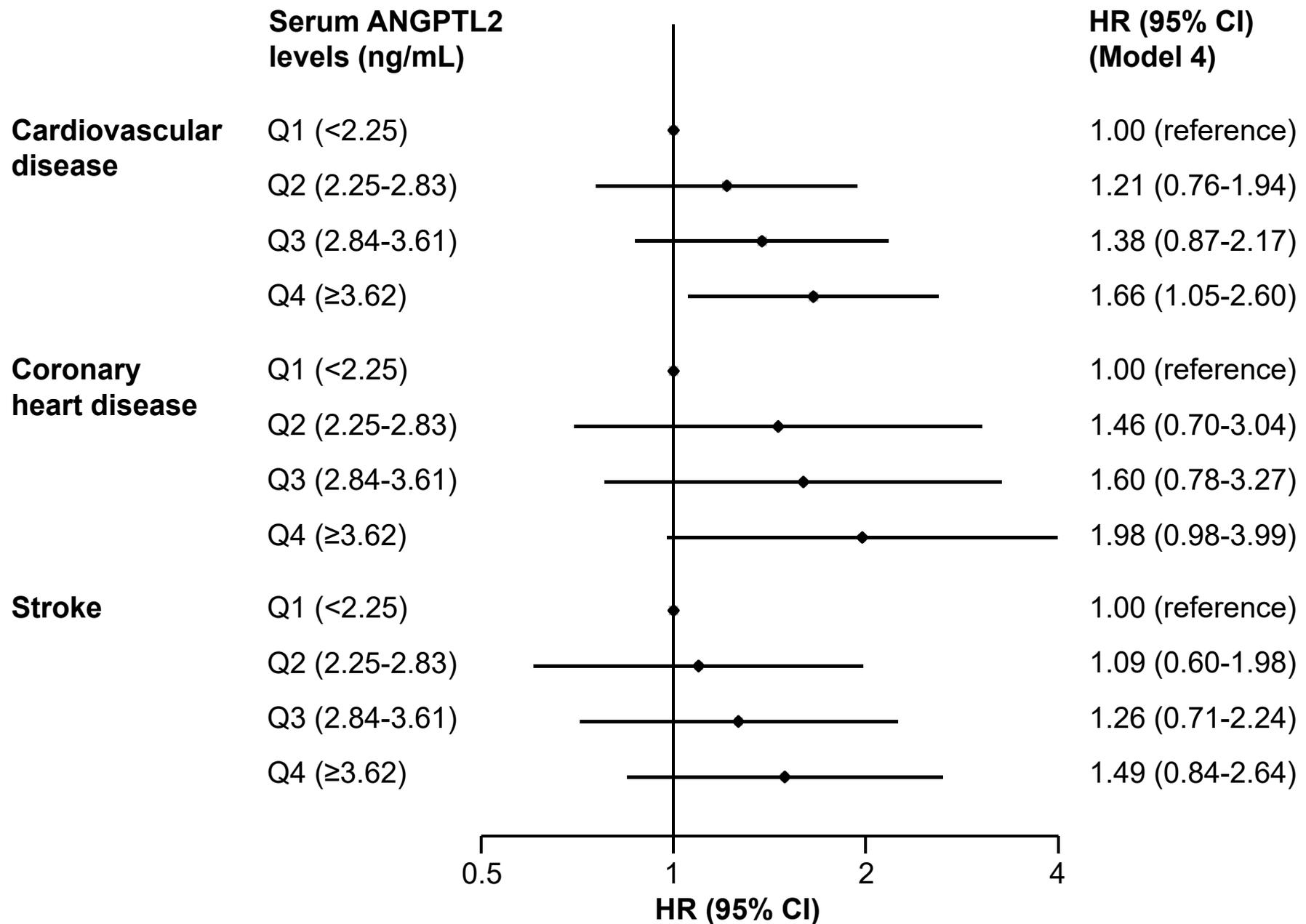


Figure II. Multivariable-adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for the development of cardiovascular disease and its subtypes according to the quartiles of serum ANGPTL2 concentrations, the Hisayama Study, 2002-2012.

Adjusted for age, sex, total cholesterol, lipid-lowering medication, electrocardiogram abnormalities, smoking habits, alcohol intake, regular exercise, metabolic syndrome components (elevated waist circumference, high blood pressure, high fasting plasma glucose, low high-density lipoprotein cholesterol, and high triglycerides), and high-sensitivity C-reactive protein (model 4).

Serum angiopoietin-like protein 2 (ANGPTL2) is a novel risk factor for cardiovascular disease in the community: the Hisayama Study

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Materials and Methods

Study population

The Hisayama Study is a prospective population-based cohort study of cardiovascular disease (CVD) established in 1961 in Hisayama, a town of approximately 8,400 people located in a suburb of the Fukuoka metropolitan area on Kyushu Island of Japan. In 2002-2003, a screening examination for the present study was performed in the town. A detailed description of this study was published previously.^{1,2} In brief, a total of 3,328 residents aged 40 or over (77.6% of the total population of this age range) participated in the comprehensive health examination. After exclusion of 30 individuals who refused to participate in the epidemiological research, 190 with a prior event of stroke or coronary heart disease (CHD), 71 for whom fasting blood samples were lacking, 18 with no measurement of serum ANGPTL2 concentration, and 14 with no measurement of waist circumference, the remaining 3,005 subjects (1,268 men and 1,737 women) were enrolled in the present study. The study was approved by the Kyushu University Institutional Review Board for Clinical Research, and written informed consent was obtained from all subjects.

Quantitation of ANGPTL2 protein

At the screening examination, portions of the serum specimens were stored at -80°C until used for the measurements of angiopoietin-like protein 2 (ANGPTL2) in 2011 and high-sensitivity C-reactive protein (hs-CRP) concentrations in 2004. Serum ANGPTL2 concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) at the Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan, using a Human ANGPTL2 Assay Kit (Code No. 27745: IBL, Fujioka, Japan), according to the manufacturer's instructions. The precoated plate, wash buffer, dilution buffer, labeled antibody, chromogen and stop solution were provided with the kit. Test serum samples were 10-fold diluted to 100 µl. The samples were incubated for 60 minutes at 37°C in a precoated plate and washed with wash buffer four times. Then, the labeled antibody solution was added into the wells of test samples and incubated for 30 minutes at 4°C. The precoated plate was washed five times and supplemented with chromogen. After incubating for 30 minutes at room temperature in the dark, stop solution was added and samples were measured at 450 nm by an iMark Microplate Absorbance Reader (BIO-RAD, Hercules, CA, USA). The subjects were divided into four groups according to the quartiles of serum ANGPTL2 levels (Q1, <2.25 ng/mL; Q2, 2.25-2.83 ng/mL; Q3, 2.84-3.61 ng/mL; Q4, ≥3.62 ng/mL).

Other risk factors

At baseline, fasting plasma glucose concentrations were measured by the hexokinase method. Serum concentrations of total and high-density lipoprotein (HDL) cholesterol and triglycerides were measured enzymatically. Serum concentrations of low-density lipoprotein (LDL) cholesterol were estimated using the Friedewald formula: [LDL cholesterol] = [Total cholesterol] - [HDL cholesterol] - [Triglycerides] / 5. For 46 subjects with excessively high triglyceride values (≥4.52 mmol/L), LDL cholesterol concentrations could not be estimated, because the Friedewald formula lost its validity in these cases.³ Serum hs-CRP concentrations were measured using a latex-enhanced nephelometric assay (Behring Diagnostics, Westwood, MA).

Waist circumference was measured at the umbilical level in a standing position by a

trained staff member. Body height and weight were measured in light clothing without shoes, and body mass index was calculated (kg/m^2). Blood pressure was measured 3 times in a sitting position using an automated sphygmomanometer, and the mean of the 3 measurements was used for the analysis. Electrocardiogram abnormalities were defined as left ventricular hypertrophy (Minnesota code, 3-1), ST depression (4-1, 2, 3), or atrial fibrillation/flutter (8-3).

Information on smoking habits, alcohol intake, physical activity, and medication for hypertension, diabetes, and dyslipidemia was obtained using a standardized questionnaire. Smoking and drinking habits were categorized as current use or not. Current smoking was defined when the subject smoked at least 1 cigarette per day. Current drinking was defined when the subject drank at least one alcoholic beverage per month. The subjects engaging in sports or other forms of exertion ≥ 3 times a week during their leisure time made up a regular exercise group.

Metabolic syndrome was defined according to the definition proposed in a joint interim statement by the relevant international organizations in 2009.⁴ According to this statement, the presence of any 3 of the following 5 components is required for the diagnosis: (1) elevated waist circumference (≥ 90 cm for men or ≥ 80 cm for women; these are the criteria for Asian populations); (2) high blood pressure ($\geq 130/85$ mmHg or use of an antihypertensive agent); (3) high fasting plasma glucose (≥ 5.6 mmol/L or use of an antidiabetic medication); (4) low HDL cholesterol (< 1.0 mmol/L for men; < 1.3 mmol/L for women); and (5) high triglycerides (≥ 1.7 mmol/L).

Follow-up survey

The subjects were followed up prospectively until November 2012 or their death (median follow-up period, 10.3 years) by annual health examinations or by mail or telephone for any subject who did not undergo the examination or who moved out of town. The development of CVD was also checked by a daily monitoring system organized by the study team, local physicians, and the town government. When a subject died, autopsy was performed at the Department of Pathology of Kyushu University, if consent for an autopsy was obtained. During the follow-up period, autopsy examination was performed for 221 (61.2%) of 361 deceased subjects. All available information about potential CVD events among the study population (medical records, physical, neurological, laboratory and radiological examinations, death certificates, and autopsy findings) was collected and reviewed by physician members of the study to determine the occurrence of CVD events under the standardized diagnostic criteria. There was no participant who could not be traced or contacted during the follow-up period to determine vital status and event information.

Endpoints

The endpoints of the present study were the development of CVD and its subtypes, namely, CHD and stroke. The diagnosis of CHD included acute and silent myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft surgery, and sudden cardiac death within 1 hour after the onset of acute illness. Acute myocardial infarction was diagnosed when a subject met at least 2 of the following 4 criteria: (1) typical symptoms including prolonged severe anterior chest pain; (2) evolving diagnostic electrocardiogram changes; (3) cardiac enzyme levels more than twice the upper limit of the normal range; (4) morphological changes (local asynergy of cardiac wall motion on echocardiography, persistent perfusion defect on cardiac scintigraphy, or myocardial necrosis or scars ≥ 1 cm long accompanied by coronary atherosclerosis at autopsy). Silent myocardial infarction was diagnosed for subjects without any historical indication of clinical symptoms or abnormal cardiac enzyme changes, using either of the following 2 criteria: (1) new onset of abnormal Q waves on electrocardiogram plus morphological myocardium changes (local asynergy on echocardiography or persistent perfusion defect on scintigraphy); (2) myocardial necrosis or scars ≥ 1 cm long accompanied by coronary atherosclerosis at autopsy. Stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit persisting for > 24 hours due to ischemia or hemorrhage of the brain. The diagnosis of stroke was based on

all available clinical data, including clinical history, neurological examination, brain imaging (computed tomography and magnetic resonance imaging), and autopsy findings. During the follow-up period, a total of 219 subjects had a first-ever CVD event. Among them, 102 subjects experienced a first-ever CHD event, and 132 had a first-ever stroke event.

Statistical analysis

The trends in the frequencies and means (standard deviations [SDs]) of risk factors across the serum ANGPTL2 quartiles were tested by logistic and linear regression analysis, respectively, using an ordinal variable (1, 2, 3, and 4 for the first (Q1), second (Q2), third (Q3), and fourth (Q4) quartiles, respectively). For serum triglycerides and hs-CRP, medians and interquartile ranges were determined, and log-transformed values were used in the statistical models due to the skewed distributions. The distributions of serum ANGPTL2 concentrations in 219 subjects with CVD events and in 2786 without CVD events during the follow-up period were compared by Wilcoxon rank-sum test. The incidence rate of CVD was calculated by the person-year method with adjustment for age and sex by the direct method. The Cox proportional hazards model was used to estimate the hazard ratio (HR) for the development of CVD and its 95% confidence intervals (CIs) among the serum ANGPTL2 quartiles (as a categorical variable) or per 1 SD increment in log-transformed serum ANGPTL2 concentrations (as a continuous variable). The trends in the incidence rates or HRs across the serum ANGPTL2 quartiles were tested by the Cox proportional hazards model using an ordinal variable. The adjustment was made by using covariates, namely, age, sex, total cholesterol, use of lipid-lowering agents, electrocardiogram abnormalities, smoking habits, alcohol intake, regular exercise, components of metabolic syndrome (elevated waist circumference, high blood pressure, high fasting plasma glucose, low HDL cholesterol, and high triglycerides), and log-transformed concentrations of serum hs-CRP. To examine the extent to which the association between serum ANGPTL2 levels and the CVD risk was explained by potential mediators (i.e., metabolic syndrome components and serum hs-CRP), the percentage reduction in the log HR after adding each potential mediator to the reference model was calculated by the following formula: [% reduction] = $([\log \text{HR in reference model}] - [\log \text{HR}]) / [\log \text{HR in reference model}] \times 100$, where log HR was the regression coefficient in the relevant Cox model. All statistical analyses were performed with SAS 9.3 (SAS Institute, Cary, NC). Two-sided values of $P < 0.05$ were considered statistically significant.

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