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Original article

Impact of combined elevations of homocysteine and asymmetric dimethylarginine on all-cause death – The Tanushimaru Study

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ABSTRACT

Background: Both homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) induce endothelial dysfunction. However, the impact of both elevations on all-cause death is not known. We investigated the association between elevations of Hcy or ADMA and all-cause death in a general population.

Methods: A total of 517 subjects (224 men, 293 women; mean age, 62.8 years) were recruited from a population-based survey in 1999 in Tanushimaru, and we measured fasting plasma Hcy and ADMA levels. We followed them up for over 20 years and examined the effect on mortality using Cox proportional hazard regression model.

Results: The mean follow-up years were 17.7 (1.8-20.8). In this period, 182 subjects have died (35.2%). The correlation between Hcy and ADMA was high (r=0.194; $p<0.001$). With Cox regression analysis after adjustments for age and sex, elevated log transformed Hcy levels were significantly associated with all-cause death ($p=0.028$). When Hcy and ADMA levels were divided into quintiles, the hierarchical model showed the synergistic effect of Hcy and ADMA on all-cause death.

Conclusions: This is the first report that we have measured Hcy and ADMA levels simultaneously in this community-dwelling Japanese, and we demonstrated that combined elevations of Hcy and ADMA had big impact on all-cause death in this epidemiological study.

Introduction

We have demonstrated that circulating homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) induce endothelial dysfunction [1–3]. However, the impact of both elevations on all-cause death is not known. Accumulating evidence in cohort studies has indicated that plasma Hcy increases the risk of mortality [4–9]. The meta-analysis reported by Fan et al. [10] suggested that Hcy levels are linearly and positively associated with risk of all-cause mortality.

Compared to Hcy, significant associations of ADMA with mortality are limited to subjects with end-stage renal disease [11], angiographic coronary artery disease (CAD) [12], acute myocardial infarction [13], after percutaneous coronary intervention [14], and patients aged over 65 years old [15]. Two cohort studies [16,17] from the USA showed contrasting results, in which the Framingham Offspring Study [16] indicated that ADMA was significantly associated with all-cause mortality, especially in non-diabetic individuals. However, The Dallas Heart Study [17] showed that symmetrical dimethylarginine (SDMA), but not ADMA was an independent predictor of all-cause death. The meta-analysis reported by Zhou et al. [18] suggested that circulating ADMA levels are positively associated with all-cause mortality, which are still controversial.

In the present study, we aimed to investigate the impact of both Hcy and ADMA on all-cause mortality during a long follow-up period in community-dwelling Japanese.

Methods

Study population

A periodic epidemiological survey was performed in 1999 in a rural farming community located in southwestern Japan (Tanushi-

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maru, a cohort of the Seven Countries Study [19]). Although the Seven Countries Study ended in 1989, we continued the epidemiologic study in the same district. As previously reported [20], the demographic backgrounds of the subjects in this area were similar to those of the Japanese general population. We examined 1,999 subjects aged over 40 years (794 males and 1,126 females) in the present study. Of these, we enrolled 517 subjects (224 males and 293 females: aged 40 to 89 years) without overt cerebrocardiovascular diseases and measured plasma levels of Hcy and ADMA in all subjects. Eventually, they were followed up and the period was 17.7±5.0 (1.8-20.8) years.

Data collection

The subjects' medical history as well as their use of alcohol and smoking status were ascertained by a questionnaire. They were classified as current habitual user or not. Height and weight were measured, and body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2) and was considered as an index of obesity. Blood pressure (BP) was measured in the right arm twice with a mercury sphygmomanometer after the subject had rested in the supine position for more than 5 min. The second BP was used for analysis. Blood was drawn from the antecubital vein for determination of fasting plasma glucose (FPG), glycosylated hemoglobin A_{1c} [HbA_{1c} (NGSP)], lipid profiles [total cholesterol, low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c), and triglycerides], immunoreactive insulin (IRI), blood urea nitrogen, creatinine, uric acid, Hcy, and ADMA levels in the morning after a 12-h fast. Estimated glomerular filtration rate was calculated using the Modification of Diet in Renal Disease study equation modified with a Japanese coefficient [21]. Fasting blood samples were centrifuged immediately after collection. Plasma concentrations of Hcy and ADMA levels were measured by the high-performance liquid chromatography (HPLC) method as previously described [2,22]. Serum total cholesterol, HDL-c, triglycerides, and creatinine were measured by the enzymatic assay method, and HbA_{1c} was measured by ion exchange HPLC. All blood chemistry analyses were performed at a commercial laboratory.

We have periodically followed them up over 2 years. Detailed information was collected from May 17th, 2018 to March 31st, 2020. Causes of death were determined based on a review of obituaries, medical records, death certificates, hospital charts, and interviews with their primary care physicians, families of the deceased, and other witnesses.

This study was approved by the Tanushimaru branch of the Japan Medical Association and the local mayor. All the participants gave informed consent. The Research Ethics Committee of Kurume University School of Medicine (Process numbers 9908/1999) approved the study, and the study conformed to the principles of the Declaration of Helsinki.

Statistical analysis

Because of skewed distributions, natural logarithmic transformation was performed for IRI, triglycerides, Hcy, and ADMA. Mean values, upper and lower 95% confidence limits, were exponentiated and presented geometric mean ± standard deviation (SD), where the SD in a 95% confidence interval was approximated as the difference between the exponentiated confidence limits divided by 3.92, the number of SD in a 95% confidence interval for normally distributed data. Student's t-test and chi-square tests were used to evaluate of continuous and categorical variables.

Because there are many subjects with the same value Hcy/ADMA on the boundaries in the tertile or quartile grouping, we selected quintile grouping. Survival curves of death from all causes for quintiles of Hcy levels were estimated by the Kaplan-Meier method and compared using the log-rank test. In order to investigate the impact of Hcy and ADMA levels on all-cause death, we created the hierarchical model by division of Hcy and ADMA into quintiles.

To obtain hazard ratios (HRs) for all-cause mortality, we finally performed Cox proportional hazards regression analysis after adjusting for age, sex, systolic BP, total cholesterol, FPG, and smoking status. Statistical significance was defined as $p<0.05$. All statistical analyses were performed using the SAS system (Release 9.4, SAS Institute, Cary, NC, USA).

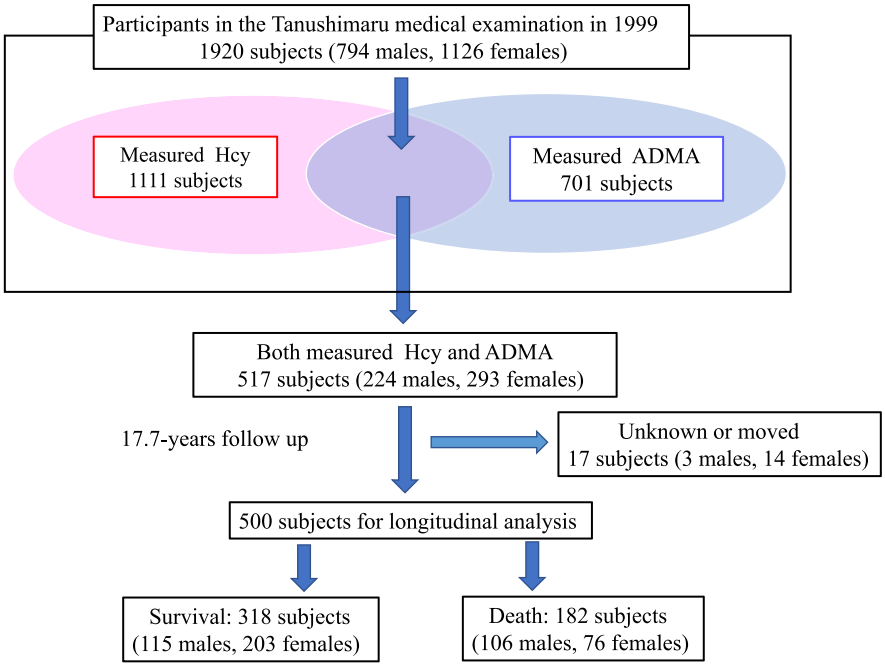


Fig. 1. The flow diagram of eligible subjects. Hcy, homocysteine; ADMA, asymmetric dimethylarginine.

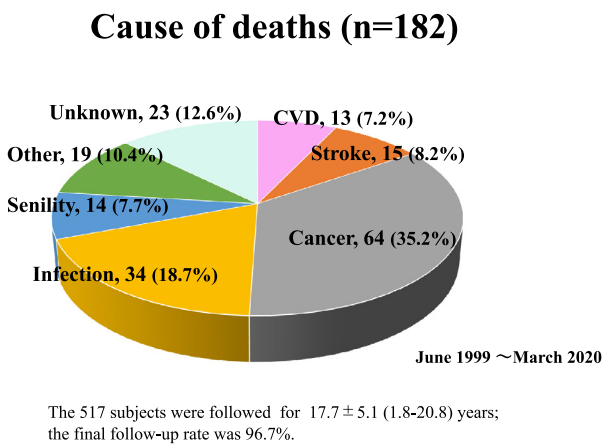


Fig. 2. Causes of death. CVD, cardiovascular disease.

Results

Study participants and outcomes

A total of 517 subjects were followed for 17.7±5.0 years. The flow diagram of eligible subjects is shown in Fig. 1. There were 182 deaths: the highest mortality was from cancer 64 (35.2%) (Fig. 2). Loss to follow-up was 17 subjects (3.3%); 13 subjects moved, and the details of 4 subjects were unknown. The final follow-up rate was 96.7% in this study.

Baseline demographics stratified Hcy and ADMA quintile

Demographics of participants at baseline stratified by Hcy and ADMA quintile are shown in Table 1a and b. At baseline, there were significant cross-sectional relationships between Hcy and several coronary risk factors and ADMA ($p<0.001$) (Table 1a). There were significant cross-sectional relationships between ADMA and several coronary risk factors and Hcy ($p<0.001$) (Table 1b).

Baseline characteristics stratified by vital status

Characteristics of participants at baseline stratified by vital status are shown in Table 2. Hcy ($p<0.001$) and ADMA ($p<0.001$) were significantly and positively associated with all-cause death.

Cox proportional hazards regression analysis of all-cause death

In the Cox proportional hazards regression analysis of all-cause death after adjusting for age and sex, BMI ($p<0.01$; inversely), systolic BP ($p<0.05$), LDL-c ($p<0.01$; inversely), total cholesterol ($p<0.01$; inversely), Hcy ($p<0.05$), and current smoking ($p<0.001$) were significantly associated with all-cause death. Although the correlation between Hcy and ADMA was significant (r=0.194; $p<0.001$; Fig. 3), the significance of ADMA disappeared after adjusting for age and sex (Table 3).

Kaplan-Meier curve

Fig. 4 shows the cumulative survival curves for all-cause death stratified by Hcy quintiles. Kaplan-Meier curves demonstrated that all-cause death was significantly higher in the highest of Hcy quintiles than in the lowest quintiles ($p=0.010$ by log-rank test). Although the significance disappears after adjustment for age and sex, the Kaplan-Meier curves demonstrated that all-cause death was significantly worse in the highest of ADMA quintiles than in the lowest quintiles ($p=0.03$ by log-rank test; data not shown). We finally demonstrated the impact of Hcy and ADMA levels on all-cause death, then, we created the hierarchical model by division of Hcy and ADMA into quintiles. This model showed the greater effect of combined Hcy and ADMA levels on all-cause death rather than Hcy or ADMA alone (Fig. 5).

HRs and 95% CI of all-cause death stratified by Hcy quintiles at baseline

In the Hcy quintiles, we calculated the HRs of all-cause death using the lowest quintile as the reference (Table 4). In the model 2 (adjusted for age and sex), a significant HR (1.791, 95% CI 1.027-3.124, $p=0.040$) for all-cause death was observed in the highest vs.

Table 1a
Demographics of participants at baseline stratified by homocysteine quintile.

Variables	Quintile 1(4.2-8.0)	Quintile 2(8.1-9.2)	Quintile 3(9.3-10.5)	Quintile 4(10.6-12.7)	Quintile 5(≥12.8)	*p*-value
Total n	105	103	102	104	103	
Homocysteine (μmol/L) †	7.0 (4.2-8.0)	8.7 (8.1-9.2)	9.9(9.3-10.5)	11.5 (10.6-12.7)	16.1 (12.8-49.1)	<0.001
Age (year)	59.1±10.1	60.9±8.7	60.7±10.1	65.6±9.8	67.7±10.6	<0.001
Sex (male), %	21 (20.0)	34 (33.0)	42 (41.2)	53 (51.0)	74 (71.8)	<0.001
Body mass index (kg/m^2)	23.0±3.0	22.8±3.5	23.4±3.3	23.4±2.8	22.7±3.0	0.368
Systolic blood pressure (mmHg)	129.2±17.5	133.2±18.2	134.5±20.2	138.1±18.6	140.0±21.3	0.001
Diastolic blood pressure (mmHg)	78.1±10.1	80.1±10.7	81.1±10.7	81.9±11.1	83.6±12.2	0.006
Total cholesterol (mg/dL)	199.2±31.8	201.0±31.2	205.1±37.1	196.5±32.5	189.3±39.4	0.019
LDL-cholesterol (mg/dL)	122.7±30.0	122.2±28.4	123.7±34.2	118.6±29.1	112.8±35.2	0.086
HDL-cholesterol (mg/dL)	55.9±14.2	57.7±11.5	57.3±14.9	55.4±14.2	52.2±13.9	0.039
Triglycerides (mg/dL) †	89.9(35-317)	95.6(37-385)	102.5(36-963)	101.1(39-692)	105.7(39-953)	0.150
Fasting plasma glucose (mg/dL)	95.7±21.1	98.8±24.0	99.2±16.3	96.5±15.7	13.0±4.6	0.549
HbA_{1c} (%) (NGSP)	5.2±0.8	5.3±0.9	5.3±0.7	5. 1±0.7	5.1±0.6	0.184
Immunoreactive insulin (μU/mL) †	4.58(1.0-21.0)	4.20(1.0-38.0)	4.43(1.0-14.0)	4.54(1.0-21.0)	4.23(1.0-33.0)	0.790
Blood urea nitrogen (mg/dL)	15.4±3.4	15.2±3.8	15.4±4.2	16.5±3.6	17.3±5.3	<0.001
Creatinine (mg/dL)	0.73±0.11	0.80±0.13	0.83±0.14	0.89±0.14	1.00±0.20	<0.001
eGFR (ml/min/1.73m^2)	68.3±10.9	64.1±9.2	63.3±10.4	59.1±8.8	55.5±12.3	<0.001
Uric acid (mg/dL)	4.4±1.2	4.6±1.1	4.9±1.3	5.3±1.4	5.9±1.7	<0.001
Intima-media thickness (mm)	0.65±0.19	0.64±0.16	0.67±0.17	0.72±0.17	0.79±0.22	<0.001
Asymmetric dimethylarginine (μmol/L)†	0.47(0.24-0.84)	0.44(0.25-0.82)	0.46(0.24-0.98)	0.50(0.20-0.94)	0.52(0.30-1.18)	<0.001
Smoking (yes), %	11 (10.5)	10 (9.7)	16 (15.7)	17 (16.4)	27 (26.2)	0.086
Alcohol intake (yes), %	17 (16.2)	21 (20.4)	22 (21.6)	31 (29.8)	40 (38.8)	0.001
Hypertensive medication (yes), %	13 (12.4)	14 (13.6)	21 (20.8)	33 (31.7)	33 (32.0)	<0.001

Data are mean±SD or range, unless otherwise indicated.
† Logarithm-transformed values were used in analyses.

Table 1b
Demographics of participants at baseline stratified by ADMA quintile.

Variables	Quintile 1(0.20-0.38)	Quintile 2(0.39-0.45)	Quintile 3(0.46-0.50)	Quintile 4(0.51-0.58)	Quintile 5(≥0.59)	*p*-value
Total n	101	107	97	106	106	
Asymmetric dimethylarginine (μmol/L) †	0.32(0.20-0.38)	0.42(0.39-0.45)	0.48(0.46-0.50)	0.54(0.51-0.58)	0.68(0.59-1.18)	<0.001
Age (year)	59.9±8.8	62.2±11.2	62.1±10.2	64.1±9.9	65.5±11.0	0.002
Sex (male), %	41 (40.6)	37 (34.6)	40 (41.2)	54 (50.9)	52 (49.1)	0.102
Body mass index (kg/m^2)	23.2±3.2	23.1±3.1	23.3±3.6	22.9±2.9	23.0±2.9	0.876
Systolic blood pressure (mmHg)	131.1±17.8	133.2±20.7	136.3±17.8	134.2±21.7	139.9±18.1	0.015
Diastolic blood pressure (mmHg)	80.8±9.5	79.9±11.2	82.5±11.0	79.9±11.9	81.8±11.5	0.362
Total cholesterol (mg/dL)	194.2±33.5	198.3±33.8	202.1±35.4	196.0±36.9	200.5±34.3	0.497
LDL-cholesterol (mg/dL)	117.5±31.3	121.6±31.0	124.6±32.3	116.4±32.4	120.2±30.9	0.360
HDL-cholesterol (mg/dL)	55.7±12.5	53.8±13.1	57.0±14.3	56.3±15.4	56.0±14.0	0.547
Triglycerides (mg/dL) †	94.2(38-328)	101.4(37-963)	90.2(35-420)	102.5(36-953)	105.4(40-692)	0.151
Fasting plasma glucose (mg/dL)	97.2±14.8	94.6±10.8	101.0±24.5	98.8±23.2	95.1±15.1	0.075
HbA_{1c} (%) (NGSP)	5.2±0.6	5.2±0.5	5.3±0.8	5.3±1.1	5.1±0.6	0.581
Immunoreactive insulin (μU/mL) †	4.59(2.0-38.0)	4.49(1.0-20.0)	4.24(2.0-33.0)	4.36(1.0-33.0)	4.31(1.0-16.0)	0.873
Blood urea nitrogen (mg/dL)	14.8±3.4	15.7±3.8	15.4±3.5	16.3±4.9	17.5±4.5	<0.001
Creatinine (mg/dL)	0.84±0.16	0.83±0.16	0.83±0.16	0.88±0.24	0.88±0.18	0.060
eGFR (ml/min/1.73m²)	63.1±9.9	62.3±10.0	62.8±11.0	61.7±12.6	60.5±12.4	0.516
Uric acid (mg/dL)	4.9±1.3	4.9±1.3	4.8±1.4	5.2±1.6	5.2±1.7	0.311
Intima-media thickness (mm)	0.62±0.13	0.69±0.18	0.69±0.17	0.71±0.19	0.76±0.23	<0.001
Homocysteine (μmol/L) †	9.5(5.5-26.6)	9.9(4.2-25.1)	10.2(4.9-22.8)	10.7(5.6-43.9)	10.9(5.4-49.1)	0.003
Smoking (yes), %	17 (16.8)	17 (15.9)	15 (15.5)	19 (17.9)	13 (12.3)	0.836
Alcohol intake (yes), %	26 (25.7)	20 (18.7)	28 (28.9)	27 (25.5)	30 (28.3)	0.457
Hypertensive medication (yes), %	16 (15.8)	27 (25.2)	20 (20.6)	23 (21.7)	28 (26.4)	0.399

Data are mean±SD or range, unless otherwise indicated.
† Logarithm-transformed values were used in analyses.eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 2
Characteristics of participants at baseline stratified by vital status.

Variables	Total(n=500)	Survival(n=318)	Death(n=182)	*p*-value
Age (year)	62.8±10.5	58.6±9.2	70.1±8.4	<0.001
Sex (male), %	221 (44.2)	115 (36.2)	106 (58.2)	<0.001
Body mass index (kg/m^2)	23.1±3.2	23.4±3.1	22.5±3.2	0.002
Systolic blood pressure (mmHg)	134.7±19.4	131.8±17.8	139.9±20.9	<0.001
Diastolic blood pressure (mmHg)	80.9±11.1	80.6±10.7	81.3±11.8	0.537
Total cholesterol (mg/dL)	197.8±34.8	201.6±33.7	191.3±35.7	0.002
LDL-cholesterol (mg/dL)	119.8±31.6	122.7±29.6	114.7±34.3	0.009
HDL-cholesterol (mg/dL)	55.6±13.9	56.4±13.9	54.4±13.8	0.120
Triglycerides† (mg/dL)	98.5 (35-963)	98.7 (35-692)	98.0 (36-963)	0.879
Fasting plasma glucose (mg/dL)	97.1±18.5	96.3±17.1	98.5±20.8	0.227
HbA_{1c} (%) (NGSP)	5.2±0.8	5.2±0.7	5.2±0.9	0.533
Immunoreactive insulin (μU/mL) †	4.37 (1-38)	4.44 (1-33)	4.27 (1-38)	0.475
Blood urea nitrogen (mg/dL)	16.0±4.2	15.5±3.5	16.9±5.1	<0.001
Creatinine (mg/dL)	0.85±0.18	0.82±0.15	0.91±0.22	<0.001
eGFR (ml/min/1.73m²)	61.9±11.3	63.7±10.8	58.8±11.4	<0.001
Uric acid (mg/dL)	5.0±1.5	4.9±1.4	5.3±1.6	0.001
Intima-media thickness (mm)	0.70±0.19	0.64±0.16	0.79±0.20	<0.001
Homocysteine (μmol/L) †	10.2 (4.2-49.1)	9.6 (4.2-49.1)	11.4 (4.9-26.1)	<0.001
Asymmetric dimethylarginine (μmol/L) †	0.48 (0.2-1.18)	0.46 (0.2-1.18)	0.50 (0.29-0.95)	<0.001
Smoking (yes), %	80 (16.0)	41 (12.9)	39 (21.4)	0.018
Alcohol intake (yes), %	128 (25.6)	68 (21.4)	60 (33)	0.006
Hypertensive medication (yes), %	110 (22.0)	53 (16.7)	57 (31.5)	<0.001

Data are mean±SD or range, unless otherwise indicated.
† Logarithm-transformed values were used in analyses.*p*-value: Survival vs. DeatheGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

the lowest quintiles of Hcy. In the final model 3 (adjusted for 4 major risk factors for CAD in addition to age and sex), a significant HR (1.845, 95% CI 1.064-3.201, p=0.029) for all-cause death was observed in the highest vs. the lowest quintiles of Hcy.

Discussion

Although much information has become available on the ability of plasma levels of Hcy and ADMA to predict mortality in healthy subjects [6–8,15,17] and patients with cardiovascular diseases [4,9,12–14,16], diabetes [5], and renal diseases [11], this is the first report demonstrating that combined elevations of Hcy and ADMA have a big impact on all-cause death in a general population.

As previously described, in this cohort, we have measured Hcy and ADMA simultaneously. In addition to the unique measurements to the study subjects, we have followed-up them for a long time (17.7±5.0 years). In that period, 182 subjects died. In this cohort, the most common causes of death were cancer (35%) and infection (19%) followed by cerebro-cardiovascular disease (15%). These death rates are compatible with those of the general Japanese population [23]; in Japan one third of all mortality is from cancer, one fifth from cerebro-cardiovascular disease, and the rest is due to various other causes, suggesting that this was not a biased local cohort and that our follow-up was appropriately performed.

The enrolled subjects were free from apparent cardiovascular diseases and cancers at baseline, and their Hcy level was

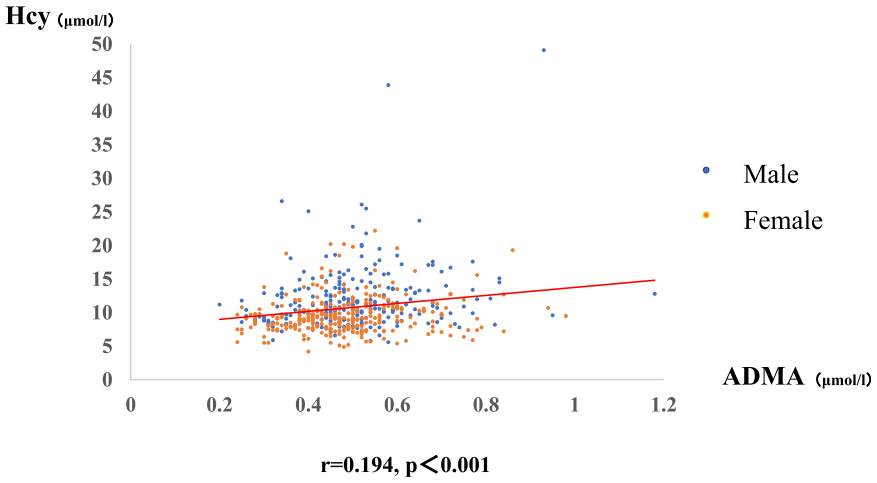


Fig. 3. The correlation between Hcy and ADMA. Hcy, homocysteine; ADMA, asymmetric dimethylarginine.

Table 3
Multivariate Cox proportional hazards regression analysis of all-cause death adjusted for age and sex.

Variables	β	SE	HR (95% CI)	p-value
Body mass index (kg/m^2)	-0.069	0.027	0.933 (0.885-0.983)	0.010
Systolic blood pressure (mmHg)	0.009	0.004	1.009 (1.001-1.017)	0.028
Diastolic blood pressure (mmHg)	0.006	0.007	1.006 (0.992-1.020)	0.426
Total cholesterol (mg/dL)	-0.006	0.002	0.994 (0.989-0.999)	0.011
LDL-cholesterol (mg/dL)	-0.006	0.003	0.994 (0.989-0.999)	0.022
HDL-cholesterol (mg/dL)	-0.005	0.005	0.995 (0.984-1.005)	0.343
Triglycerides† (mg/dL)	-0.090	0.167	0.914 (0.659-1.268)	0.591
Fasting plasma glucose (mg/dL)	0.004	0.004	1.004 (0.997-1.012)	0.277
HbA_{1c} (%) (NGSP)	0.033	0.103	1.034 (0.845-1.264)	0.747
Immunoreactive insulin (μU/mL) †	0.124	0.125	1.132 (0.885-1.447)	0.323
Blood urea nitrogen (mg/dL)	0.007	0.018	1.007 (0.972-1.042)	0.705
Creatinine (mg/dL)	0.636	0.486	1.889 (0.729-4.895)	0.191
eGFR (ml/min/1.73m^2)	-0.007	0.007	0.993 (0.979-1.007)	0.298
Uric acid (mg/dL)	-0.005	0.056	0.952 (0.853-1.062)	0.375
Intima-media thickness (mm)	0.555	0.437	1.742 (0.740-4.102)	0.204
Homocysteine (μmol/L) †	0.606	0.276	1.832 (1.066-3.150)	0.028
Asymmetric dimethylarginine(μmol/L) †	0.328	0.311	1.389 (0.755-2.553)	0.291
Smoking status (0, no; 1, yes)	0.637	0.206	1.890 (1.263-2.830)	0.002
Alcohol intake (0, no; 1, yes)	0.246	0.200	1.279 (0.865-1.892)	0.218
Hypertensive medication (0, no; 1, yes)	0.227	0.165	1.255 (0.907-1.735)	0.171

† Logarithm-transformed values were used in analyses.eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 4
HRs and 95% CIs of all-cause death stratified by homocysteine quintiles at baseline.

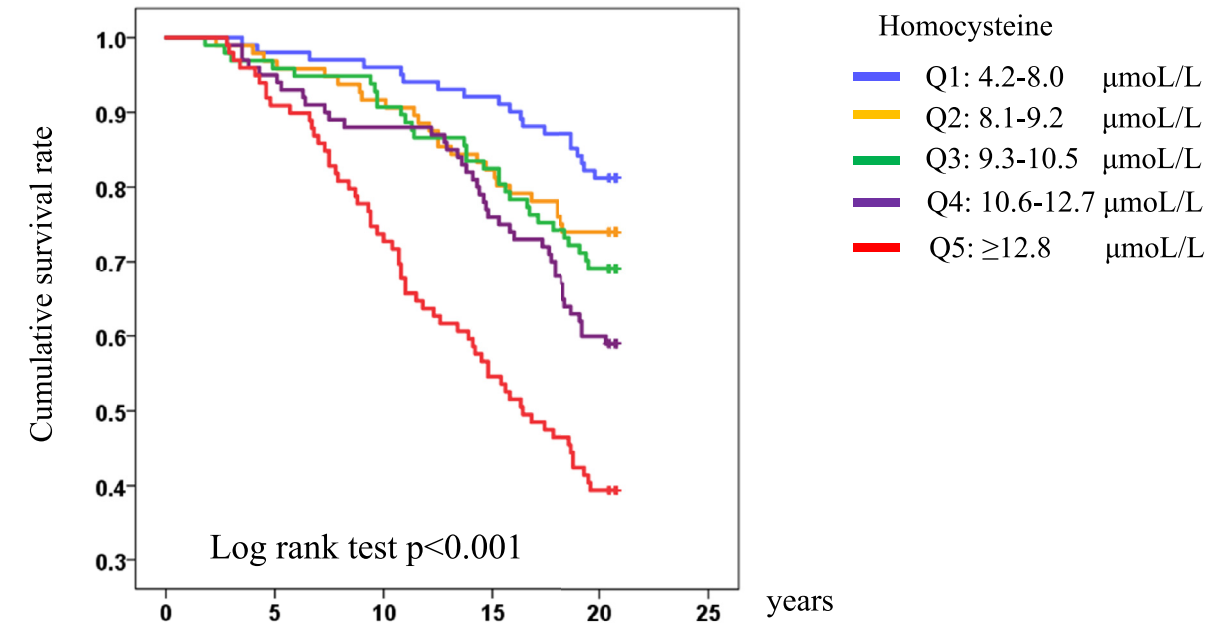
Models	Homocysteine (μmol/L) Quintile 1 (4.2-8.0)	Quintile 2 (8.1-9.2)	Quintile 3 (9.3-10.5)	Quintile 4 (10.6-12.7)	Quintile 5 (≥12.8)
Total no.	101	96	97	100	99
No. of deaths	19	25	30	41	60
Model 1	Reference	1.500 (0.826-2.723)	1.799 (1.012-3.195)*	2.514 (1.459-4.331)***	4.701 (2.803-7.884)***
Model 2	Reference	1.267 (0.694-2.310)	1.474 (0.824-2.635)	1.292 (0.741-2.253)	1.791 (1.027-3.124)*
Model 3	Reference	1.456 (0.794-2.667)	1.707 (0.950-3.067)	1.260 (0.722-2.201)	1.845 (1.064-3.201)*

Model 1: not adjusted.
Model 2: adjusted for age and sex.
Model 3: adjusted for age, sex, systolic blood pressure, total cholesterol, fasting plasma glucose, and smoking status.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

within normal range. Nonetheless, subjects in quintile 5 (Hcy≥12.8 μmol/L) had higher overall mortality, showing how such small variation of Hcy levels within the normal range could affect the all-cause mortality. The most likely explanation is that although cancer (35%), infection (19%), and cerebro-cardiovascular diseases (15%), were the main causes of death, the total number of these deaths was too small to demonstrate statistical significance.

It is interesting to note that there was a positive correlation between Hcy and ADMA at baseline. Nevertheless, our data showed that each impact of these novel risk factors on mortality was different, but the mechanism was not known, however; Gore et al. [17] showed that SDMA, but not ADMA was an independent predictor of all-cause death.

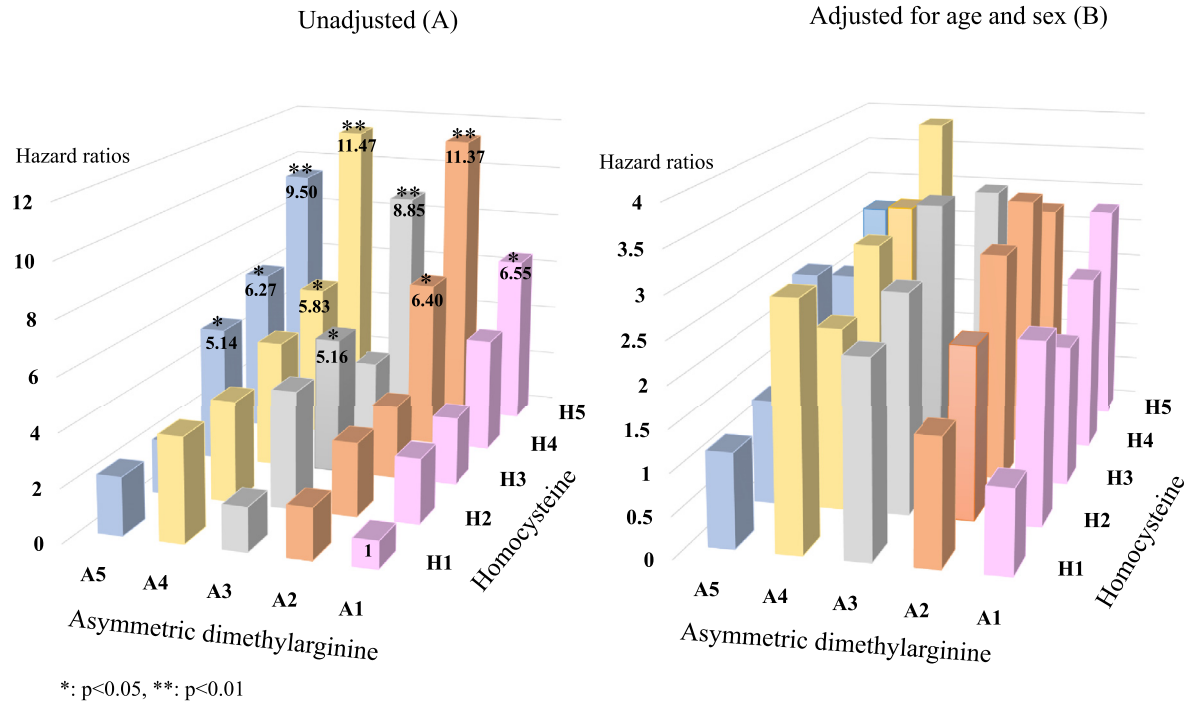
Because total cholesterol was inversely associated with all-cause death, it may need further consideration. In Japanese epidemiological studies, total cholesterol has never been a significant risk factor [20,24,25]. We have no clear explanation for the difference from



No. at Risk

Q1	101	99	97	83	82
Q2	96	93	88	79	71
Q3	97	93	88	80	67
Q4	100	95	88	78	60
Q5	99	90	73	54	39

Fig. 4. The cumulative survival curves for all-cause death stratified by homocysteine quintiles.



*: p<0.05, **: p<0.01

Fig. 5. The hierarchical model by division of homocysteine and asymmetric dimethylarginine into quintiles for all-cause death: (A) unadjusted (B) adjusted for age and sex.

Western countries where high cholesterol is a significant risk [26], but it might be genetic differences.

This study had several limitations. First, we have no information about medication for vitamins and folate at baseline. Second, some asymptomatic subjects with cerebro-cardiovascular diseases were potentially included. Similarly, we were not able to exclude subjects with subclinical cancers. Third, the total number of deaths from cancer or cerebro-cardiovascular death was small and limited the statistical power for these outcomes, which is causatively more relevant. Fourth, because male/female ratio was highly different among the Hcy quintile groups, the effect of Hcy on all-cause death was strongly observed only in the female group. Finally, the under-

lying pathophysiological mechanism for the association of high Hcy level with all-cause death was not revealed from our observational study.

The clinical implication of the study is that ADMA may be a good marker for mortality by the atherosclerotic and cardiorenal diseases [27,28]. All-cause mortality including cardiovascular mortality should be always examined in a cohort study with a medium- or long-term follow-up [29,30].

In conclusion, the present study demonstrated that combined elevations of Hcy and ADMA had a big impact on all-cause death rather than Hcy or ADMA alone in this epidemiological study.

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

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