

Plasma Cystathione and Risk of Incident Stroke in Patients With Suspected Stable Angina Pectoris

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Background—Cystathione is an intermediate product in the transsulfuration pathway and formed during the B6-dependent conversion of methionine to cysteine. Elevated plasma cystathione has been related to atherosclerosis, which is a major etiological factor for ischemic stroke. However, the role of cystathione in stroke development is unknown. Therefore, we prospectively assessed the association of circulating levels of cystathione with risk of total and ischemic stroke.

Methods and Results—Two-thousand thirty-six patients (64% men; median age, 62 years) undergoing coronary angiography for suspected stable angina pectoris were included. Stroke cases were identified by linkage to the CVDNOR (Cardiovascular Disease in Norway) project. Hazard ratios with confidence intervals (95% confidence interval) were estimated by using Cox-regression analyses. During 7.3 years of median follow-up, 124 (6.1%) incident strokes were ascertained, which comprised 100 cases of ischemic stroke. There was a positive association of plasma cystathione with risk of total stroke and ischemic stroke. Comparing the fourth versus the first cystathione quartiles, age- and sex-adjusted hazard ratios (95% confidence interval) were 2.11 (1.19–3.75) and 2.56 (1.31–4.99) for total and ischemic stroke, respectively. Additional adjustment for major stroke risk factors only slightly attenuated the associations, which tended to be stronger in patients without previous or existing atrial fibrillation at baseline (hazard ratio [95% confidence interval], 2.43 [1.27–4.65] and 2.88 [1.39–5.98] for total and ischemic stroke, respectively).

Conclusions—In patients with suspected stable angina pectoris, plasma cystathione was independently related to increased risk of total stroke and, in particular, ischemic stroke.

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Stroke is a leading cause of mortality and long-term disability in the world and remains a massive public health burden.¹ This highlights a pressing need to identify novel risk associations for stroke and improve our current understanding of its underlying pathophysiology.

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Cystathione is a sulphur-containing amino acid produced from homocysteine (Hcy) during the conversion of methionine (Met) to cysteine (Cys) by the pyridoxal 5'-phosphate-dependent enzymes, cystathione β -synthase and cystathione γ -lyase.² In rats, high dietary intake of Met has been shown to induce hypercholesterolemia,³ in addition to impairing endothelial morphology and promoting arteriosclerosis.⁴ Increased flux through cystathione β -synthase has also been suggested to worsen outcome of ischemic stroke in experimental studies.⁵ Notably, elevated circulating cystathione concentrations were observed in patients with cardiovascular disease (CVD),⁶ and increased levels were associated with oxidative damage⁷ and endothelial dysfunction.^{4,8} Both these processes play a critical role in the pathogenesis of atherosclerotic disease⁹ and hence to clinical manifestations such as ischemic stroke.^{1,10} Moreover, increased expression of cystathione metabolizing enzymes have recently been found in human atherosclerotic lesions and are shown to exacerbate angiogenesis, thereby increasing

Clinical Perspective

What Is New?

- Higher plasma cystathione levels are associated with increased risk of total stroke and ischemic stroke in patients with suspected stable angina pectoris.
- Risk associations tended to be stronger in patients without previous atrial fibrillation at baseline.

What Are the Clinical Implications?

- The findings of our study are hypothesis generating and should be confirmed by additional prospective studies with alternative designs.

the risk of plaque instability,¹¹ often associated with atherothrombotic events.¹² However, the role of plasma cystathione in relation to stroke risk has not previously been reported.

We conducted a prospective study among patients with suspected stable angina pectoris to examine the association of circulating levels of cystathione with subsequent risk of total and ischemic stroke.

Methods

Data, analytical methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Study Cohort

A detailed description of the study population has been published previously.¹³ In brief, 4164 patients who underwent coronary angiography for suspected stable angina pectoris at Haukeland and Stavanger University Hospitals in Western Norway during 2000–2004 were included. Of these, 2573 (61.8%) were subsequently enrolled in the WENBIT (Western Norway B-vitamin Intervention Trial; NCT00354081) and received either (1) folic acid, vitamin B12 and vitamin B6 (n=642), (2) folic acid and vitamin B12 (n=642), (3) vitamin B6 (n=643), or (4) placebo (n=646).¹⁴ Because B-vitamin supplementation has been reported to lower the risk of stroke in some studies,^{15,16} we excluded patients who received supplementation with B-vitamins in the WENBIT. In addition, patients with missing baseline data on cystathione and 1 patient with an extremely high cystathione level (20 µmol/L) were excluded, leaving 2036 patients eligible for the current analyses (Figure 1).

The study was carried out according to the principles of the Declaration of Helsinki and was approved by the regional

ethics committee (approval number 2010/1880) and the Norwegian Data Protection Authority. All study patients signed a consent form.

Baseline Data

Information on patients' lifestyle and medical history were obtained through a self-administered questionnaire and was validated against hospital records, as previously reported.¹⁴ Obesity was identified as having body mass index ≥ 30 kg/m². Current smokers were defined as those who reported currently smoking, those who quit smoking within <1 month preceding examination, or having serum cotinine levels ≥ 85 nmol/L at baseline.¹³ Diabetes mellitus included types 1 and 2 and was classified according to previous diagnosis or by having plasma fasting glucose > 7 mmol/L, nonfasting glucose > 11.1 mmol/L, or a single measurement of glycated hemoglobin $> 6.5\%.$ ¹⁷ Hypertension was defined according to existing diagnosis. Left ventricular ejection fraction was obtained either by echocardiography or ventriculography during cardiac catheterization. Angiographic extent of coronary artery disease (CAD) was graded as 0 to 3 according to the number of significantly stenotic coronary arteries.

Follow-up and Study End Points

Patients were followed from angiography until the onset of stroke or through December 31, 2009 (end of follow-up). The primary end point was total stroke, including hospitalization or death attributed to stroke according to the *International Statistical Evaluation of Disease, Tenth Revision (ICD-10)* (I60–I64 except I63.6), whereas the secondary end point was ischemic stroke (I63 except I63.6). Information on clinical outcomes was obtained from the CVDNOR (Cardiovascular Disease in Norway) project (<https://cvdnor.buib.no/>), recording all CVD discharge diagnosis from the patient administrative systems at Norwegian public hospitals during 1994–2009.¹⁸ Fatal stroke events were ascertained by record linkage to the Cause of Death Registry at Statistics Norway (<http://www.ssb.no/en>).

Biochemical Analyses

Details on the collection of blood samples and biochemical analyses for relevant clinical indices have been described previously.¹³ Among patients recruited at Haukeland University Hospital, blood samples were drawn 1 to 3 days before coronary angiography and immediately stored at -80°C , whereas such samples from patients at Stavanger University Hospital were drawn after the angiographic procedure and subsequently transported within 48 hours to the core laboratory, until separated and frozen at -80°C . All study-specific analyses were carried out at the laboratory of Bevital AS (Bergen,

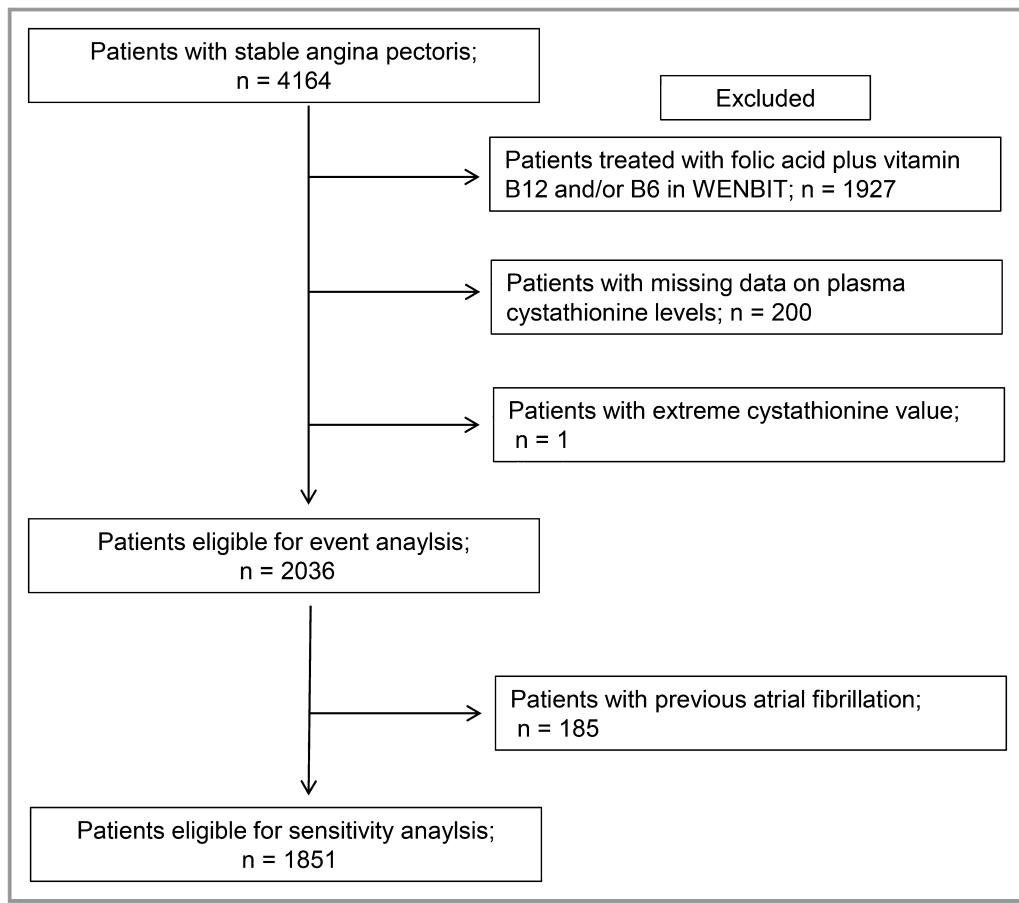


Figure 1. Flow diagram showing patient selection eligible for the study. WENBIT indicates Western Norway B-Vitamin Intervention Trial.

Norway; www.bevital.no) by laboratory staff blinded to the clinical outcomes of the patients. Plasma cystathione, Met, Hcy, and Cys concentration were determined with gas chromatography/tandem mass spectrometry.¹⁹ Plasma cystathione had within-day coefficient of variation of $\leq 1.8\%$ and between-day coefficient of variation of $\leq 3.6\%$. Serum apolipoprotein A1, apolipoprotein B, and C-reactive protein (CRP) were measured as previously described.¹³ Low-density lipoprotein cholesterol and estimated glomerular filtration rate (eGFR) were calculated using the Friedewald and Chronic Kidney Disease Epidemiology Collaboration formula, respectively.

Statistical Analysis

Baseline continuous variables are reported as median (interquartile range), whereas categorical variables are presented as counts (percentages). Differences in baseline characteristics across plasma cystathione quartiles were tested with logistic regression for categorical variables and linear median regression for continuous and ordinal data. Associations between cystathione and related 1-carbon metabolites were evaluated by Pearson's correlation, adjusted for age and sex.

Kaplan-Meier curves for stroke events were constructed to evaluate survival across plasma cystathione quartiles and difference tested by the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) for incident stroke were estimated using Cox regression models and reported according to quartiles of plasma cystathione, with the lowest quartile as the reference category and per 1-SD increment in log-transformed plasma cystathione. Additionally, HR and 95% CI for trends across increasing quartiles were assessed to determine whether their associations with stroke followed a linear pattern. These trend tests were performed by including the quartile-specific median cystathione value as a continuous variable in the Cox proportional hazards model and assessing significance with the Wald test. The numeric value of HR greater than 1 was considered a trend toward increased risk (positive trend) across the lowest to highest quartile of plasma cystathione. Proportionality assumptions were assessed by inspecting log minus log plots and calculating Schoenfeld residuals. Potential nonlinear relationships between cystathione and risk of incident strokes were visualized by generalized additive regression plots for multivariate models. Patients who did not experience any stroke event during follow-

Table 1. Baseline Characteristics According to Quartiles of Plasma Cystathione

	Quartiles of Plasma Cystathione					P_{trend}^*	P_{trend}^{\dagger}
	Q1	Q2	Q3	Q4			
Age, y	58 (14)	61 (15)	64 (16)	67 (14)	<0.001	...	
Male sex, n (%)	303 (59.5)	321 (63.1)	322 (63.3)	356 (69.9)	0.001	...	
Coronary risk factor, n (%)							
Obesity	77 (15.2)	100 (19.7)	107 (21.0)	119 (23.4)	0.001	<0.001	
Hypertension	189 (37.1)	230 (45.2)	253 (49.7)	276 (54.2)	<0.001	<0.001	
Diabetes mellitus	211 (41.9)	210 (41.7)	222 (43.6)	267 (52.5)	0.002	0.003	
Current smoking	191 (37.6)	160 (31.9)	150 (29.6)	134 (26.5)	<0.001	0.16	
eGFR, mL/min/1.73 m ²	95 (16)	90 (20)	87 (23)	78 (29)	<0.001	<0.001	
Serum CRP, mg/L	1.58 (2.50)	1.80 (3.0)	1.89 (2.97)	2.12 (3.44)	<0.001	<0.001	
Plasma ADMA, μmol/L	0.53 (0.15)	0.54 (0.13)	0.58 (0.16)	0.59 (0.17)	<0.001	<0.001	
Plasma 1-carbon metabolites, μmol/L							
Met	25.6 (7.2)	27.4 (8.1)	29.9 (11.3)	31.4 (12.7)	<0.001	<0.001	
tHcy	9.27 (3.13)	10.5 (3.69)	10.9 (4.27)	12.4 (5.32)	<0.001	<0.001	
Cys	283 (45.2)	294 (49.2)	298 (50.1)	306 (53)	<0.001	<0.001	
Plasma B-vitamin status							
Folate, nmol/L	11.9 (9.8)	9.81 (7.27)	9.75 (6.51)	9.89 (7.01)	<0.001	<0.001	
Cobalamin (B12), pmol/L	390 (213)	393 (193)	391 (210)	391 (231)	0.75	0.44	
PLP, nmol/L	45.6 (43.6)	42.3 (32.4)	41.9 (31.2)	40.2 (31.9)	<0.001	<0.001	
Serum lipids							
Triglycerides, mmol/L	1.28 (0.86)	1.45 (1.02)	1.52 (1.18)	1.60 (1.11)	<0.001	<0.001	
ApoB, g/L	0.91 (0.31)	0.89 (0.35)	0.87 (0.30)	0.86 (0.31)	0.002	0.02	
Apo A1, g/L	1.36 (0.39)	1.34 (0.36)	1.35 (0.36)	1.28 (0.31)	<0.001	<0.001	
LDL-C	3.1 (1.38)	3.0 (1.45)	2.9 (1.40)	2.8 (1.28)	<0.001	<0.001	
Previous CVD, n (%)							
AMI	166 (32.6)	166 (32.6)	189 (37.1)	228 (44.8)	<0.001	0.02	
PAD	38 (7.5)	38 (7.5)	49 (9.6)	64 (12.6)	0.002	0.17	
AF	35 (6.9)	42 (8.3)	48 (9.4)	60 (11.8)	0.005	0.34	
Cerebrovascular disease [†]	26 (5.1)	35 (6.9)	33 (6.5)	54 (10.6)	0.002	0.18	
Extent of CAD, n (%)							
No significant stenosis	225 (44.2)	210 (41.3)	192 (37.7)	141 (27.7)			
1-vessel disease	102 (20.0)	83 (16.3)	97 (19.1)	94 (18.5)			
2-vessel disease	84 (16.5)	102 (20.0)	99 (19.4)	92 (18.1)			
3-vessel disease	98 (19.3)	114 (22.4)	121 (23.8)	182 (35.8)			
LVEF (%)	70 (10)	70 (10)	66 (10)	65 (11)	<0.001	<0.001	
Medications before angiography, n (%)							
Aspirin	393 (77.2)	405 (79.6)	395 (77.6)	386 (75.8)	0.46	0.25	
Warfarin	18 (3.5)	24 (4.7)	25 (4.9)	47 (9.2)	<0.001	0.01	
Statins	318 (62.5)	339 (66.6)	348 (68.4)	363 (71.3)	0.002	0.06	
β-blocker	333 (65.4)	354 (69.5)	376 (73.9)	388 (76.2)	<0.001	0.002	
ACEIs	74 (14.5)	84 (16.5)	102 (20.0)	146 (28.7)	<0.001	<0.001	

Continued

Table 1. Continued

	Quartiles of Plasma Cystathione					P_{trend}^*	$P_{\text{trend}}^{\dagger}$
	Q1	Q2	Q3	Q4			
Medications after angiography, n (%)							
Aspirin	362 (71.1)	382 (75.0)	381 (74.9)	398 (78.2)	0.02	0.81	
Warfarin	17 (3.3)	26 (5.1)	23 (4.5)	43 (8.4)	0.001	0.02	
Statins	349 (68.6)	371 (72.9)	375 (73.7)	397 (78.0)	0.001	0.22	
β -blocker	315 (61.9)	330 (64.8)	352 (69.2)	382 (75.0)	<0.001	0.004	
ACEIs	78 (15.3)	85 (16.7)	106 (20.8)	144 (28.3)	<0.001	<0.001	

Continuous variables are presented as medians (interquartile range), and categorical variables are reported as counts (%). ACEIs indicates angiotensin-converting-enzyme inhibitors; ADMA, asymmetric dimethylarginine; AF, atrial fibrillation; AMI, acute myocardial infarction; apoA1, apolipoprotein A1; apoB, apolipoprotein B; BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; CVD, cardiovascular disease; Cys, cysteine; eGFR, estimated glomerular filtration rate; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; Met, methionine; PAD, peripheral artery disease; PLP, pyridoxal 5'-phosphate; tHcy, total homocysteine.

*Unadjusted.

[†]Adjusted for age and sex.

[‡]Composite of stroke, transient ischemic attack, or carotid stenosis.

up were censored. Model 1 included age (years) and sex, and the multivariate model 2 additionally included the categorical variables, obesity, diabetes mellitus, hypertension, smoking, previous acute myocardial infarction, and atrial fibrillation. Because B-vitamins are crucial in the metabolism of cystathione^{20,21} and systemic B-vitamin levels have been associated with CVD risk,^{22,23} we did not include these variables in the main multivariate model. However, to assess their potential influence on any cystathione-stroke relationship, these variables were additionally included in an extended model, as was eGFR, because elevated circulating cystathione concentrations have been reported in renal patients.²¹

To investigate effect modifications, survival analyses were explored according to the median values of continuous variable and subgroups of categorical variables, and possible interactions tested by including the interaction products term in Cox model 2. Moreover, the possibility of unmeasured confounding was investigated by performing additional sensitivity analysis and applying E-value formula to the multivariate model, according to the recent recommendations for observational studies.²⁴

Given the hypothesis-testing nature of our analyses, we did not adjust for multiple comparisons and a $P < 0.05$ was considered statistically significant.²⁵ Statistical analyses were performed using SPSS (Sample Power, Version 23; SPSS IBM, Armonk, NY) and R software (version 3.1.2; The R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline Characteristics

Baseline characteristics according to plasma cystathione quartiles are presented in Table 1. The study population

consisted of 64.0% men, and median (interquartile range) age was 62 (16) years, whereas plasma concentrations of cystathione were 0.27 (0.20) $\mu\text{mol/L}$. Cutoffs for serum plasma cystathione in quartiles were <0.20, 0.20 to 0.27, 0.27 to 0.39, and >0.39 $\mu\text{mol/L}$ in the first, second, third, and fourth quartiles, respectively. SD was 0.48 for plasma cystathione and 0.61 for log-transformed plasma cystathione. Patients in the upper quartiles of plasma cystathione more often were older, male, and obese and had hypertension, diabetes mellitus, and established CVD. Accordingly,

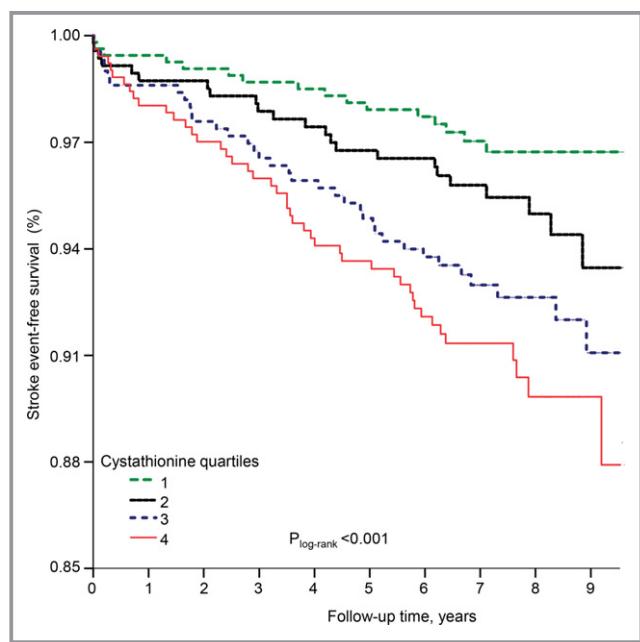


Figure 2. Stroke-event-free survival. Kaplan-Meier plot showing crude stroke-free survival according to the quartiles of plasma cystathione (designated as quartiles 1, 2, 3, and 4). Difference between quartiles was compared by the log-rank test.

Table 2. Risk Association Between Plasma Cystathione and Stroke

Plasma Cystathione							
		Unadjusted		Age and Sex Adjusted		Multivariate*	
		HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
Total Stroke							
Events	124						
Q1		Reference		Reference		Reference	
Q2		1.53 (0.82–2.87)	0.18	1.33 (0.70–2.49)	0.37	1.30 (0.69–2.45)	0.42
Q3		2.47 (1.39–4.38)	0.002	1.87 (1.04–3.33)	0.04	1.78 (0.99–3.19)	0.06
Q4		3.12 (1.78–5.44)	<0.001	2.11 (1.19–3.75)	0.01	2.01 (1.14–3.61)	0.02
Trend		1.46 (1.23–1.72)	<0.001	1.28 (1.08–1.52)	0.004	1.26 (1.06–1.50)	0.01
Per 1-SD [†]		1.41 (1.22–1.63)	<0.001	1.26 (1.07–1.47)	0.005	1.23 (1.05–1.44)	0.01
Ischemic Stroke							
Events	100						
Q1		Reference		Reference		Reference	
Q2		1.82 (0.88–3.75)	0.11	1.57 (0.76–3.25)	0.22	1.53 (0.74–3.16)	0.26
Q3		2.98 (1.52–5.82)	0.001	2.28 (1.16–4.48)	0.02	2.16 (1.09–4.26)	0.03
Q4		3.69 (1.92–7.08)	<0.001	2.56 (1.31–4.99)	0.01	2.39 (1.23–4.72)	0.01
Trend		1.51 (1.25–1.82)	<0.001	1.34 (1.11–1.62)	0.003	1.32 (1.09–1.59)	0.005
Per 1-SD [†]		1.46 (1.25–1.71)	<0.001	1.31 (1.11–1.56)	0.002	1.29 (1.09–1.54)	0.004

CI indicates confidence interval; HR, hazard ratio; Q1, first quartile; Q4, fourth quartile.

*Adjusted for age, sex, obesity, diabetes mellitus, hypertension, smoking, previous acute myocardial infarction, and atrial fibrillation.

[†]Log-transformed.

plasma cystathione showed an inverse association with left ventricular ejection fraction and a strong positive relationship with more-extensive CAD at angiography, also reflected by more-frequent use of CVD medications among subjects in the upper cystathione quartile. Furthermore, we observed positive associations of cystathione with serum CRP and plasma asymmetric dimethylarginine, whereas there were inverse associations with eGFR.

As anticipated, plasma cystathione was positively associated with the 1-carbon metabolites, plasma Met, total Hcy, and total Cyst ($r=0.24$, 0.11 , and 0.12 , respectively; $P<0.001$) and negatively to serum folate and plasma pyridoxal 5'-phosphate, whereas no relationship was observed with serum cobalamin. Patients in the higher cystathione quartiles also had higher levels of serum triglycerides and lower serum apolipoprotein A1, apolipoprotein B, and low-density lipoprotein cholesterol.

Plasma Cystathione and Risk of Stroke

During a median (interquartile range) follow-up of 7.3 (2.4) years, an incident stroke event occurred in 124 patients (6.1%), which comprised 100 (4.9%) cases of ischemic stroke. As illustrated in Figure 2, there was higher incidences of stroke

throughout higher plasma cystathione quartiles ($P<0.001$ for log-rank test).

Accordingly, we found an approximately linear positive trend between plasma cystathione and subsequent risk of stroke (Figure 3). In an unadjusted model, those in the highest plasma cystathione quartile compared with the lowest had increased risk of total stroke (HR, 3.12; 95% CI, 1.78–5.44). This association was particularly strong for ischemic strokes (HR, 3.69; 95% CI, 1.92–7.08). Corresponding HRs (95% CIs) were 2.11 (1.19–3.75) and 2.56 (1.31–4.99) in age- and sex-adjusted analyses and essentially similar in the multivariate models (Table 2).

Adjusting for previous CVD, extent of CAD, CRP, low-density lipoprotein cholesterol, or medications at baseline had no significant effect on the risk associations between plasma cystathione and either end point (data not shown).

Met, Hcy, and Cys are important metabolites of 1-carbon cycle and are closely associated with cystathione metabolism.^{2,7} Hence, we also studied the potential influence by adjusting for these parameters on the cystathione-stroke risk associations. In the multivariate model, neither plasma Met nor Cys were significantly associated with stroke risk, whereas plasma total Hcy was only borderline positively related to the risk before controlling for cystathione

Table 3. Hazard Ratios for Incident Stroke According to Plasma Levels of Methionine, Homocysteine, and Cysteine

	Total Stroke				Ischemic Stroke			
	Q4 vs Q1	P Value	Per 1-SD*	P Value	Q4 vs Q1	P Value	Per 1-SD*	P Value
Plasma Met								
Unadjusted	0.92 (0.54–1.57)	0.77	0.88 (0.74–1.06)	0.19	0.93 (0.51–1.67)	0.81	0.89 (0.73–1.09)	0.89
Model 1 [†]	1.04 (0.61–1.78)	0.88	0.92 (0.76–1.09)	0.35	1.05 (0.58–1.89)	0.88	0.93 (0.76–1.13)	0.44
Model 2 [‡]	1.02 (0.60–1.75)	0.93	0.91 (0.76–1.08)	0.29	1.01 (0.55–1.83)	0.98	0.91 (0.75–1.11)	0.37
Model 3 [§]	0.91 (0.51–1.62)	0.75	0.87 (0.73–1.06)	0.18	0.90 (0.48–1.75)	0.79	0.88 (0.71–1.08)	0.23
Plasma tHcy								
Unadjusted	2.63 (1.55–4.44)	<0.001	1.40 (1.21–1.61)	<0.001	2.74 (1.55–4.83)	<0.001	1.44 (1.23–1.69)	<0.001
Model 1 [†]	1.76 (1.01–3.04)	0.05	1.27 (1.08–1.50)	0.003	1.87 (1.03–3.38)	0.04	1.32 (1.11–1.59)	0.002
Model 2 [‡]	1.49 (0.85–2.60)	0.16	1.21 (1.02–1.45)	0.04	1.57 (0.86–2.87)	0.14	1.24 (1.03–1.54)	0.03
Model 3 [§]	1.27 (0.72–2.28)	0.39	1.14 (0.95–1.39)	0.12	1.30 (0.71–2.43)	0.40	1.16 (0.97–1.44)	0.10
Plasma tCys								
Unadjusted	3.38 (1.91–5.97)	<0.001	1.49 (1.26–1.76)	<0.001	2.88 (1.51–5.47)	<0.001	1.37 (1.14–1.65)	0.001
Model 1 [†]	1.80 (0.99–3.29)	0.54	1.16 (0.96–1.41)	0.10	1.51 (0.77–2.97)	0.23	1.06 (0.87–1.31)	0.56
Model 2 [‡]	1.75 (0.95–3.21)	0.07	1.13 (0.93–1.36)	0.20	1.44 (0.7–2.85)	0.29	1.02 (0.83–1.26)	0.84
Model 3 [§]	1.63 (0.89–3.01)	0.12	1.10 (0.91–1.33)	0.31	1.31 (0.66–2.61)	0.44	0.98 (0.81–1.22)	0.96

Met indicates methionine; Q1, first quartile; Q4, fourth quartile; tCyst, total cysteine; tHcy, total homocysteine.

*Log-transformed.

[†]Adjusted for age and sex.

[‡]Adjusted for variables in model 1 plus obesity, diabetes mellitus, hypertension, smoking, previous myocardial infarction, and atrial fibrillation.

[§]Adjusted for variables in model 2 plus plasma cystathione.

(Table 3). However, additional adjustment for these 1-carbon metabolites or plasma markers of B-vitamins status in the multivariate model did not appreciably alter the associations (Table 4), whereas including eGFR slightly attenuated the risk estimates (HR [95% CI] were 1.88 [1.04–3.41] for total stroke and 2.19 [1.09–4.41] for ischemic stroke, when comparing cystathione quartiles 4–1).

Subgroup Analyses

Figure 4 and Table 5 depict the risk associations between plasma cystathione and stroke according to several traditional risk factors and plasma B-vitamin status, respectively. Notably, we observed a stronger risk estimate of plasma cystathione among patients without than those with previous atrial fibrillation at baseline ($P_{\text{interaction}}=0.03$). The risk

Table 4. Risk Association Between Plasma Cystathione and Incident Stroke, When Separately Adjusted for Related 1-Carbon Metabolites and Markers of B-Vitamin Status

	Total Stroke				Ischemic Stroke			
	Q4 vs Q1	P Value	Per 1-SD*	P Value	Q4 vs Q1	P Value	Per 1-SD*	P Value
Plasma cystathione								
Model 2+Met [†]	2.10 (1.16–3.85)	0.01	1.23 (1.05–1.45)	0.01	2.50 (1.26–5.08)	0.01	1.30 (1.09–1.55)	0.004
Model 2+tHcy [†]	1.92 (1.05–3.57)	0.04	1.20 (1.03–1.43)	0.04	2.26 (1.10–4.63)	0.03	1.24 (1.03–1.50)	0.03
Model 2+tCys [†]	1.96 (1.08–3.49)	0.03	1.22 (1.04–1.42)	0.03	2.34 (1.18–4.62)	0.01	1.28 (1.07–1.53)	0.01
Model 2+folate [†]	2.09 (1.17–3.76)	0.01	1.24 (1.05–1.45)	0.01	2.42 (1.23–4.81)	0.01	1.29 (1.09–1.54)	0.004
Model 2+PLP [†]	1.95 (1.06–3.59)	0.03	1.21 (1.03–1.44)	0.03	2.27 (1.13–4.64)	0.02	1.26 (1.06–1.53)	0.01
Model 2+Cob [†]	2.05 (1.04–4.06)	0.04	1.26 (1.05–1.52)	0.02	2.52 (1.12–5.66)	0.02	1.33 (1.09–1.64)	0.01

Cob indicates cobalamin (B12); Met, methionine; PLP, pyridoxal 5'-phosphate; Q1, first quartile; Q4, fourth quartile; tCyst, total cysteine; tHcy, total homocysteine.

*Log-transformed.

[†]Adjusted for age, sex, obesity, diabetes mellitus, hypertension, smoking, previous myocardial infarction, and atrial fibrillation.

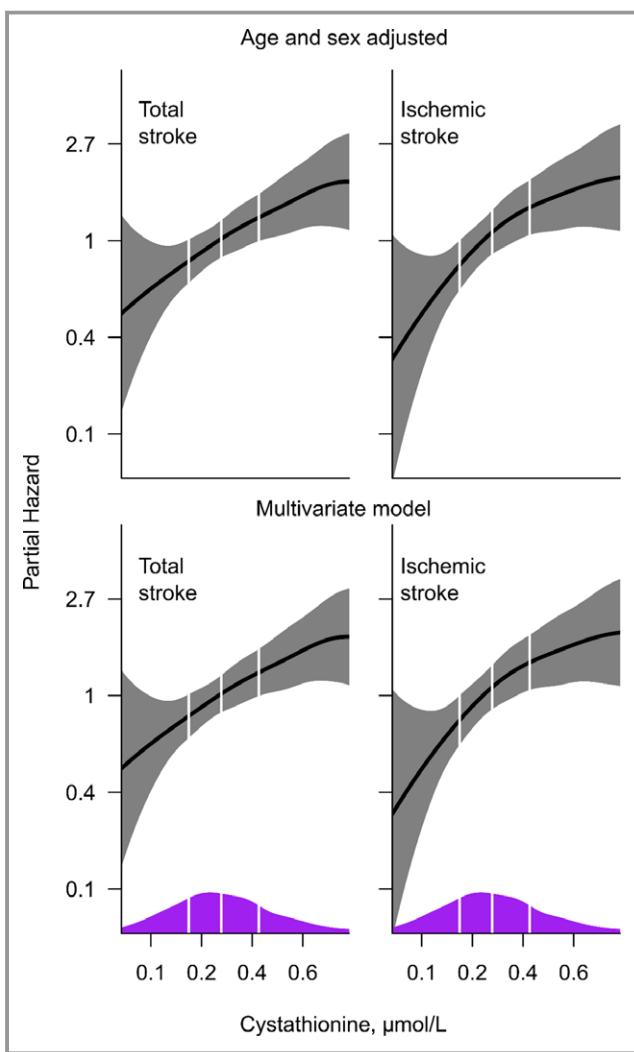


Figure 3. Association of log-transformed plasma cystathione with stroke and ischemic stroke. Multivariable models include age, sex, obesity, diabetes mellitus (yes/no), hypertension (yes/no), smoking (yes/no), previous acute myocardial infarction (yes/no), and atrial fibrillation (yes/no). Kernel density plots are superimposed along the x-axis, to display distribution of plasma cystathione. Vertical lines depict the 25th, 50th, and 75th percentile of the population.

relationship did not differ significantly according to other subgroup parameters.

Sensitivity Analysis

To elucidate the influence of previous atrial fibrillation further, we performed additional sensitivity analysis after excluding patients with previous atrial fibrillation (Figure 1). Notably, we obtained numerically stronger associations of cystathione with risk in the remaining 1851 patients without atrial fibrillation. In the multivariate model, HR (95% CI) were 2.43 (1.27–4.65; $P<0.005$) for total stroke and 2.88 (1.39–5.98;

$P<0.004$) for ischemic stroke, when comparing cystathione quartiles 4 to 1.

In addition, application of E-value formula to the multivariate model, also including eGFR, revealed high sensitivity of the observed association between cystathione and total stroke, with an E-value of 3.19 and 1.28 each, for the estimate and lower reported CI, respectively. Corresponding E-values were 3.80 and 1.40 for the ischemic stroke event analysis.

Discussion

Principal Findings

In a large cohort of patients undergoing elective coronary angiography for stable angina pectoris, elevated concentrations of plasma cystathione were strongly associated with increased risk of total stroke and ischemic stroke during follow-up. The risk relationship was independent of several established stroke risk factor and potential confounders, and tended to be stronger in patients without previous atrial fibrillation.

Strengths and Limitations

The major strengths of this study include the large sample size, detailed clinical information on patient characteristics, and its prospective design. Although the influence of residual confounding cannot be ruled out in an observational study, the risk estimates were consistent also after the adjustment for major stroke risk factors. Notably, the high E-value validates the strength and robustness of the observed findings to the presence of an unmeasured cofounder.²⁴

There are several additional aspects of the present study that merit consideration. First, because of lack of data, we were unable to perform separate analyses on the subtypes of stroke. Second, the current study is based on the measurement of blood parameters at only 1 time point, thus preventing assessment of the possible variation of cystathione during follow-up. However, the within-subject reproducibility has been shown to be fair to good for plasma cystathione,²⁶ which allows 1-exposure assessment of plasma cystathione status, with reduced risk of regression dilution bias. Third, we performed multiple subgroup comparisons, and it is possible that the significant interaction between cystathione and previous atrial fibrillation on stroke occurrence is attributed to chance (type I error). This is, however, not immediately supported by our observation of stronger risk estimates after excluding patients with atrial fibrillation at baseline. Fourth, the accuracy of self-reported information on use of medications, disease conditions, and other health issues pose a concern, despite validation of the

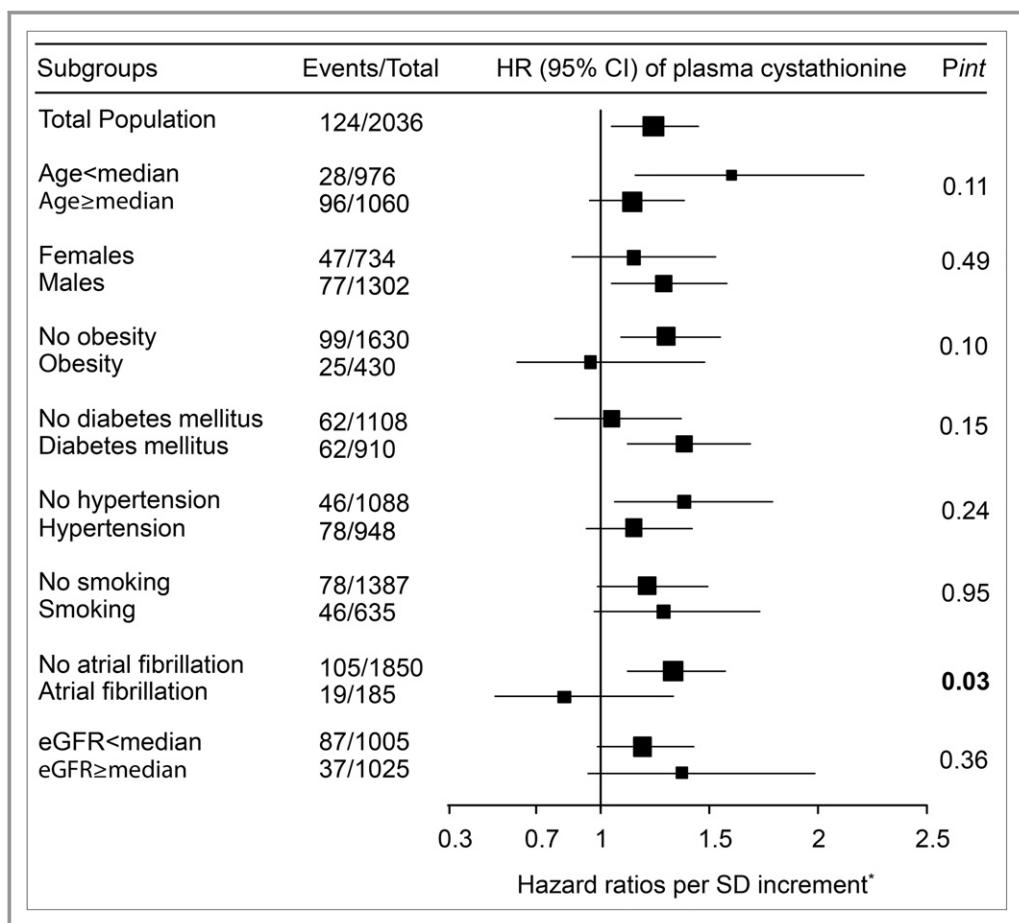


Figure 4. Forest plot depicting hazard ratios between plasma cystathione and total stroke according to several traditional risk factors. Box areas illustrate sample sizes, and horizontal lines depict 95% confidence intervals (CIs). eGFR indicates estimated glomerular filtration rate; HR, hazard ratio. *Adjusted for age, sex, obesity, diabetes mellitus, hypertension, smoking, previous myocardial infarction, and atrial fibrillation.

questionnaires. Fifth, blood samples from Stavanger University hospital were transported to Bevital AS, thus exposing the samples to room temperature for ≈ 3 days. This is unlikely to have introduced a bias, however, given that plasma cystathione concentrations are reported to be fairly stable during short-term storage under such conditions.²⁷ Furthermore, the potential of analytical bias was reduced by using 1 central laboratory for analyzing the plasma samples. Sixth, the long follow-up time and prospective design minimizes the potential risk of reverse causation. Yet, we cannot exclude that patients with established CVD at baseline may also have had lifestyle modifications or other interventions performed, which could somehow influence plasma cystathione status. Seventh, information on incident stroke end points were ascertained from the patient administrative database only, which indicates that cases with asymptomatic stroke without hospitalization might not have been reported. However, we do not suspect that such misclassification differs according to levels of plasma cystathione. Furthermore, the possibility of detection bias is unlikely, because each patient's unique

national identification number was linked to health registries with almost 100% coverage of all endpoints. Eighth, we studied patients treated with various medications for CVD, and thus our results may not be applicable to the general healthy population. Finally, our results are hypothesis generating and should be confirmed by additional prospective studies with alternative designs.

Cystathione and CVD in Other Epidemiological Studies

Data on the relationship between cystathione status and CVD are scant. In a small study among 14 healthy participants, plasma cystathione was related to impaired vascular function following Met loading.⁸ Another study reported high plasma cystathione levels among patients with CVD compared with those without CVD.⁶ To the best of our knowledge, this is the first large-scale investigation to demonstrate a significant relationship between plasma cystathione and subsequent risk of both total stroke and

Table 5. Association Between Plasma Cystathione Per SD (Log-Transformed) and Total Stroke According to Plasma Markers of B-Vitamin Status

Subgroups	Events/Total	HR (95% CI)*	P Value	<i>P</i> _{int}
Folate				
<Median	55/1015	1.20 (0.92–1.55)	0.027	0.56
≥Median	69/1015	1.21 (0.96–1.51)	0.09	
PLP				
<Median	80/1011	1.26 (1.03–1.55)	0.02	0.58
≥Median	44/1014	1.13 (0.85–1.49)	0.40	
Cob				
<Median	46/800	1.39 (1.05–1.84)	0.02	0.44
≥Median	41/800	1.15 (0.86–1.51)	0.34	

CI indicates confidence interval; Cob, cobalamin (B12); HR, hazard ratio; PLP, pyridoxal 5'-phosphate.

*Adjusted for age, sex, obesity, diabetes mellitus, hypertension, smoking, previous myocardial infarction, and atrial fibrillation.

ischemic stroke. However, the magnitude of risk association was stronger for ischemic, as compared with total stroke, and particularly stronger in patients without a history of atrial fibrillation, suggesting that elevated cystathione is associated with the atherosclerotic processes leading to stroke events.

Possible Mechanisms

Plasma cystathione, lipids, and CAD

Elevation of plasma cystathione has been related to an unfavorable lipid profile.⁶ Dyslipidemia is a less-well-known risk factor for stroke²⁸; however, it is pivotal in atherosclerosis and thereby may be linked to stroke.⁹ Interestingly, although higher plasma cystathione was related to an overall adverse CVD risk profile, including extent of CAD and previous CVD, we somewhat unexpectedly observed an inverse association between plasma cystathione and low-density lipoprotein cholesterol. However, this may be attributed to statin therapy, which was received by a majority of the patients. Nevertheless, adjusting for these parameters did not attenuate the risk estimates, implying that factors other than those traditionally predisposing to atherosclerosis are likely to play a role in the relationship between cystathione and stroke development.

Plasma cystathione and inflammation

Previous studies have reported increased cystathione concentrations in patients with asthma, a condition known to be related to systemic inflammation.²⁹ Accordingly, in our study, plasma cystathione concentration was directly associated with CRP, a marker of inflammation.³⁰ Accumulating

data indicate that inflammation may play a key role in the pathophysiology of atherothrombosis.³⁰ However, adjusting for CRP did not influence the relationship between cystathione and stroke risk, indicating that the associations are not mediated solely by CRP-related inflammation.

Plasma cystathione, endothelial dysfunction, and oxidative stress

Both experimental and clinical data have shown that increased systemic cystathione levels are related to alterations in endothelial function.^{4,8} Endothelial dysfunction, characterized by reduced nitric oxide-mediated vasodilatation of arteries, is a crucial step in the early formation of atherosclerotic lesions⁹ and is associated with plaque progression and subsequent ischemic stroke events.¹⁰ It is therefore interesting that plasma cystathione demonstrated a positive association with asymmetric dimethylarginine in our study, given that asymmetric dimethylarginine may induce vascular dysfunction through suppression of endothelial nitric oxide synthase.³¹ In addition, systemic cystathione elevation has previously been related to impaired downstream catabolism to cysteine, rather than increased production per se.^{7,32} Reduced cystathione-transsulfuration may impair glutathione production and thereby lead to oxidative stress.⁷ Oxidative stress can, in turn, further impair endothelial morphology by inactivating nitric oxide,⁹ in addition to mediating several other biological processes such as lipid peroxidation and induction of vascular lesions.¹ Notably, a previous study from a subsample of the current cohort showed that higher plasma cystathione concentration are related to low levels of reduced glutathione,³³ indicating impaired antioxidant defence.⁷

Plasma status of cystathione

Elevated cystathione may simply be a marker of high Met intake, which enhances flux through cystathione β-synthase.^{2,4} This could explain a strong association between plasma cystathione and Cys at baseline. However, a prospective study in 26 556 male Finnish smokers found no significant association between Met intake and risk of stroke.³⁴ Accordingly, we observed no significant relationship between either plasma Met or Cys with stroke risk, making them unlikely as confounders. Likewise, cystathione remained a significant predictor of stroke risk even after the adjustment for Hcy. Although we cannot exclude the influence of this metabolic precursor on our results, it is, however, unlikely that the observed associations of cystathione with stroke in the current study are confounded by Hcy status. Alternatively, high plasma cystathione may reflect suboptimal nutritional status.^{20,21} In this regard, previous studies showed that low serum concentrations of B-vitamins, in particular vitamin B6, were strongly associated with high

cystathione levels.^{21,29} However, any influence by poor nutrition status seems unlikely, because plasma levels of B-vitamins neither attenuated the association between plasma cystathione and stroke events nor introduced any significant effect modification. Conceivably, renal mechanism may be considered given that elevated circulating cystathione has been observed in patients with renal disease, possibly attributed to the combination of reduced urinary excretion and increased production.²¹ This could explain our observation of negative correlation of cystathione with eGFR. Notably, renal dysfunction is a major risk factor for ischemic stroke.³⁵ However, including eGFR had a minor influence on our estimates, suggesting other mechanisms than renal impairment being responsible for the current findings.

Conclusions

In conclusion, our observational study suggests that elevated plasma cystathione is associated with increased risk of total stroke and ischemic stroke in patients with stable angina pectoris, particularly in those without a history of atrial fibrillation. Our results motivate future studies to elucidate the role of cystathione in stroke development.

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Author Contributions

Nygård and Dhar designed research; Dhar analyzed the data, interpreted findings and wrote the manuscript; Dhar, Svingen, Ueland, Tell, and Nygård conducted research; Dhar and Lysne performed statistical analysis; Dhar, Svingen, Ueland, Lysne, Svenningsson, Tell, and Nygård critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Disclosures

None.

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