Elevated plasma cystathionine is associated with increased risk of mortality among patients with suspected or established coronary heart disease

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Running head: Cystathionine and mortality

Abbreviation list:

AMI, acute myocardial infarction

CAD, coronary artery disease

CBS, cystathionine β,synthase

CHD, coronary heart disease

CRP, C-reactive protein

CVD, cardiovascular disease

eGFR, estimated glomerular filtration rate

Met, methionine

PLP, pyridoxal,5´,phosphate

NORVIT, Norwegian Vitamin Trial

SAP, stable angina pectoris

tHcy, total homocysteine

WECAC, Western Norway Coronary Angiography Cohort.

WENBIT, Western Norway B,Vitamin Intervention Trial

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ABSTRACT

Background: Elevated circulating cystathionine levels are related to atherosclerotic cardiovascular disease, a leading cause of death globally.

Objective: We investigated whether plasma cystathionine was associated with mortality in patients with suspected or established coronary heart disease (CHD).

Design: Data from two independent cohorts of patients with suspected stable angina pectoris (SAP) (3033 patients; median 10.7 years follow-up; 648 deaths) or acute myocardial infarction (AMI) (3670 patients; median 7.0 years follow-up; 758 deaths) were included. Hazard ratios (HRs) with 95% confidence intervals (CIs) per 1-SD increment of log-transformed cystathionine were calculated using Cox-regression modelling. Endpoint data was obtained from National Health Registries.

Results: Among patients with SAP, there was a positive association between plasma cystathionine and death (age and sex adjusted HR [95% CI] per SD: 1.23 [1.14, 1.32], 1.29 [1.16, 1.44] and 1.17 [1.05, 1.29] for total, cardiovascular and non-cardiovascular mortality, respectively). Corresponding risk estimates were 1.28 (1.19, 1.37) for all-cause, 1.33 (1.22, 1.45) for cardiovascular and 1.19 (1.06, 1.34) for non-cardiovascular death among AMI patients. In both cohorts, estimates were slightly attenuated after multivariate adjustments for established CHD risk factors. Subgroup analyses showed that the relationship between cystathionine and all-cause mortality in SAP patients was stronger among non-smokers, and those with lower plasma concentration of pyridoxal-5-phosphate (P-interaction≤0.03 for both).

Conclusions: Elevated plasma cystathionine is associated with both cardiovascular and non-cardiovascular morality among patients with suspected or established CHD. The joint adverse

effects of high cystathionine with lifestyle factors and impaired vitamin B-6 status on mortality need further investigation.

Key Words: B-vitamins, coronary heart disease, cystathionine, mortality/survival, risk factors

INTRODUCTION

Atherosclerosis, characterized by the deposition of plaques inside the arterial wall is the major cause of cardiovascular disease (CVD) and CVD death (1-3). Cystathionine is a metabolite of the trans-sulfuration pathway formed during the pyridoxal 5´-phosphate -dependent (PLP) conversion of methionine (Met) to cysteine (4) and has been linked to oxidative damage (5,6), and impaired endothelial function (4,6), which are key players in the development of early atherosclerotic lesions (1). Interestingly, circulating cystathionine has been positively related with several factors involved in atherogenesis, including higher age (7,8), impaired kidney function (9), body mass index (BMI) (8) and unfavorable lipid profile (8), as well as with various pathological conditions, especially CVD (8). Moreover, elevated plasma cystathionine levels were predictive of acute myocardial infarction risk (10) and stroke events (6) among patients with coronary heart disease (CHD) in the same cohorts or a subsample of cohorts as those currently investigated.

Taken together, these observations suggest that cystathionine is associated with atherosclerotic CVD, and thus may affect survival. Indeed, the hepatic activity of cystathionine biosynthesizing enzyme, cystathionine β-synthase (CBS) is reported to be significantly lower in long-lived naked mole-rat than normal mouse (11). Others found that the deficiency of cystathionine γ-lyase (CSE), the principal enzyme involved in cystathionine catabolism (5) was associated with increased mortality in mice subjected to ischemia/reperfusion injury (12). Further, Met restriction, which is well known for life-extending effects (13), has been shown to decrease the level of CBS protein (14). However, these observations are based on measurements of gene expressions or enzymatic activities, and only one small study in humans (with sepsis) found that systemic concentrations of cystathionine could be predictive of poor survival (15).

We investigated the association between plasma cystathionine and the risk of all-cause, cardiovascular and non-cardiovascular mortality using data from two independent cohorts of patients with either suspected or verified CHD.

METHODS

Study cohorts

The present study consisted of patients from two large independent cohorts (**Supplemental Figure 1**) : the Western Norway Coronary Angiography Cohort (WECAC) with 4164 patients who were undergoing coronary angiography due to suspected stable angina (SAP) at Haukeland (n=3413) or Stavanger (n=751) University Hospitals, Norway in the period 2000-2004, and have been described in detail elsewhere (16). Approximately 2/3 of these patients were enrolled in the Western Norway B-vitamin Intervention Trial (WENBIT, NCT00354081) and randomly received treatments with folic acid plus vitamin B12 and/or vitamin B6, or placebo (17). The Norwegian Vitamin Trial (NORVIT, NCT00266487) included 3749 patients hospitalized for acute myocardial infarction (AMI) (18), who were randomized to identical interventions with B-vitamins as the patients in WENBIT. In the current study, we excluded patients without valid measurements on plasma cystathionine, leaving a total of 3033 and 3670 patients with SAP and AMI, respectively, eligible for the final analyses. The study met the mandate of the Helsinki Declaration, and was approved by the regional ethics committee and the Norwegian Data Inspectorate. All study participants provided written informed consent.

Baseline data and biochemical analyses

The collection of baseline information and biochemical analyses, including handling and storage of blood samples before analysis, have been reported earlier (16,18). Briefly, information about patients' lifestyle and medical history, including cardiovascular disease risk factors and medications, were obtained from self-administered questionnaires, and were validated against hospital records when available. Hypertension was defined by pre,existing diagnosis. Current smokers included those with self-reported current smoking, those having quit within the last month, or having plasma cotinine ≥85 nmol/L. The estimated glomerular

filtration rate (eGFR/1.73m^2) was calculated by the Chronic Kidney Disease Epidemiology Collaboration formula (19).

Plasma concentrations of cystathionine, Met, and total homocysteine (tHcy) were measured with the use of gas chromatography-tandem mass spectrometry, whereas plasma PLP, asymmetric dimethylarginine (ADMA), and serum cotinine were determined by liquid chromatography-tandem mass spectrometry. These analyses were carried out at Bevital AS laboratory, Bergen, Norway (www.bevital.no). In addition, among patients with SAP, serum C-reactive protein (CRP) concentrations were measured by an ultrasensitive immunoassay (Behring nephelometer II system N Latex CRP mono; Behring Diagnostics). Among AMI patients, we did not have information on CRP.

Follow-up and study end points

The study subjects were followed-up from enrollment until December 2012 (SAP patients) or December 2007 (AMI patients). Information on death was obtained from the Cause of Death Registry at Statistics Norway (www.ssb.no/en). The primary endpoint of interest was all-cause mortality, whereas secondary endpoints were death due to cardiovascular or non-cardiovascular causes. Cardiovascular mortality (ICD-10, I00-I99 or R96) and deaths due to cancer (ICD-10, C00-C97) were classified according to the 10th Revision of the International Classification of Disease.

Statistical analyses

Associations of plasma cystathionine with baseline categorical variables were visualized by bar charts. Correlation analyses between continuous variables were performed using Spearman rank correlations.

Cox proportional hazard regression models were used to estimate the association between plasma cystathionine and subsequent risk of death during follow up. The hazard ratios (HRs) and 95% confidence intervals (CI) were reported across quartiles of plasma cystathionine and per 1 standard deviation (SD) increment of log-transformed plasma cystathionine. The simple model included age and sex, and the multivariate model also included current smoking (yes/no), hypertension (yes/no), diabetes mellitus (yes/no), previous AMI (yes/no), BMI, serum total cholesterol (both continuous) and treatment with folic acid (yes/no) or vitamin B6 (yes/no). The proportionality of hazards was verified by inspection of survival plots and calculating Schoenfeld residuals. Potential non-linear associations between cystathionine and risk of all cause, cardiovascular and non-cardiovascular mortality were analyzed by generalized additive regression plots, adjusted for age and sex.

Subgroup analyses in both cohorts were performed according to traditional CHD risk factors and medications at discharge. We previously reported the association between cystathionine and AMI risk to be particularly strong among patients with low plasma PLP concentrations (10). Hence, we also examined whether the associations of cystathionine with mortality was modified by B-vitamin status, including serum folate and cobalamin, and plasma PLP, as well as according to the study treatment allocation among WENBIT and NORVIT participants. We also explored the combined influence of vitamin treatment and baseline vitamin status on risk associations in WENBIT or NORVIT. Tests for effect modifications were performed by entering interaction product terms to the Cox model, adjusted for age and sex. Moreover, sensitivity towards unobserved confounding was quantified by calculating E-values from the multivariate Cox regression model, according to the recent recommendations for observational studies (20).

All of the computations were performed using software SPSS for Windows (version 23; SPSS IBM, NY, USA) and R (R Development Core Team, version 3.2.1). Probability values were 2 sided and a $P<0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

The baseline associations of plasma cystathionine with selected continuous and categorical variables for both SAP and AMI populations are shown in **Figure 1** and **Supplemental Figure 2**, respectively and across quartiles of cystathionine in **Supplemental Table 1** (WENBIT patients) and **Supplemental Table 2** (NORVIT patients) (10). In both study populations (Figure 1), plasma cystathionine was positively correlated with age (r= 0.24 and 0.31 for SAP and AMI patients respectively), ADMA (r= 0.16 and 0.07) and inversely with eGFR levels (r= -0.36 and -0.29). As expected, cystathionine concentrations correlated positively with concentrations of both plasma Met, and tHcy and negatively with serum folate and plasma PLP. Moreover, among patients with SAP, plasma cystathionine was related to higher levels of serum triglycerides and CRP but lower low-density lipoprotein cholesterol and high-density lipoprotein cholesterol

In addition, in both study cohorts, those with hypertension, diabetes as well as those who had experienced previous AMI or used angiotensin-converting enzyme inhibitors had higher, while current smokers had lower plasma cystathionine levels (Supplemental Figure 2).

Follow-up and outcomes

The median (interquartile range) follow-up time was 10.7 (2.6) years and 7.0 (1.9) years for SAP and AMI-patients, respectively. Among patients with SAP, 648 (21.4%) died, of whom 301 and 347 due to cardiovascular and non-cardiovascular causes, respectively. Among patients with AMI, there were a total of 758 deaths (20.7%): 463 were caused by CVD and 295 by non-CVD causes.

Among patients with SAP, after adjusting for age and sex, higher plasma cystathionine was associated with increased risk of all-cause mortality (HR [95% CI] per SD: 1.23

[1.14,1.32]; $P < 0.001$). Multivariate adjustments left the risk associations essentially unaltered (**Table 1**). Further, the HRs (95% CI) per SD of plasma cystathionine were 1.23 (1.09, 1.40; P=0.001) for cardiovascular and 1.16 (1.03, 1.31; P= 0.01) for non-cardiovascular death in multivariate model (Table 1).

Met derived homocysteine is the only precursor of cystathionine (4), and elevated plasma tHcy has been positively associated with mortality risk in coronary artery disease (CAD) patients (21). Systemic cystathionine concentrations are also found to be elevated in pathological conditions, including inflammatory (15, 22) and renal disease (9); hence we additionally included the cystathionine precursors, as well as CRP and eGFR one at a time in the multivariate model. Including plasma Met plus tHcy, or serum CRP in the model only slightly attenuated the risk estimates, whereas controlling for eGFR moderately weakened the relationship between plasma cystathionine and the endpoints (**Supplemental Table 3**).

Among AMI patients, in age and sex adjusted analysis, HRs (95% CI) per SD increment of cystathionine were 1.28 (1.19, 1.37; P< 0.001) for all-cause death, 1.33 (1.22, 1.45; P< 0.001) for cardiovascular death and 1.19 (1.06, 1.34; P= 0.003) for non-cardiovascular causes of death (**Table 2**). In these patients, multivariable adjustments (Table 2) or controlling for metabolic precursors or eGFR only slightly altered the risk associations (Supplemental Table 3).

We found approximately linear relationship between plasma cystathionine concentrations and all-cause or CVD mortality in both cohorts, while some deviation from linearity was observed for the association with non-CVD mortality, especially among patients with SAP (**Figure 2**).

We next examined the relationship between plasma cystathionine and cancer related mortality in both study cohorts **(Table 3)**. Among patients with SAP, 187 (53.9%) of 347 non-CVD deaths had cancer as an underlying cause. In patients with AMI, 158 (53.6%) of

295 non-CVD deaths were due to cancer. Plasma cystathionine was associated with increased cancer mortality risk in an unadjusted model, but not after adjustment for age and sex (HR [95% CI] per SD: 1.05 [0.91, 1.22; P=0.51] and 1.06 [0.89, 1.25; P=0.50] in patients with SAP and AMI, respectively (Table 3). On the other hand, cystathionine showed stronger risk association with non-CVD mortality in the heterogeneous group of 160 deaths (SAP patients) and 137 deaths (AMI patients) in whom cancer was not underlying cause of death (multivariate-adjusted HRs [95% CI] per SD: 1.24 [1.05,1.48; P=0.01] and 1.23 [1.02, 1.47; P=0.03], respectively)

Subgroup analyses

Subgroup analyses according to traditional CHD risk factors and systemic B-vitamin status are presented in **Table 4** and according to WENBIT or NORVIT study treatment allocation and medications at discharge **in Supplemental Table 4 and 5,** respectively.

Among patients with SAP, the association between cystathionine and all-cause mortality was stronger in non-smokers and subjects who had PLP below the median (Table 4) (P for interactions = 0.001 and 0.01, respectively). The interaction remained significant after multivariate adjustment (data not shown)

In neither cohort did we observe any effect modifications according to other subgroup parameter (P for interactions > 0.05).

In addition, when exploring PLP subgroups according to folic acid or vitamin B6 treatment, we observed a similar trend towards increased risk with low PLP levels, regardless of study intervention (**Supplemental Table 6**).

Sensitivity analyses

To reduce any chance of potential reverse causation, we performed additional sensitivity analysis by excluding the first 365 days of follow-up (comprising 50 and 183 patients in the

SAP and AMI cohorts, respectively). The risk associations were slightly attenuated in the remaining data set. In patients with SAP, cystathionine provided multivariate-adjusted HRs (95% CI) per SD of 1.20 (1.10, 1.31; $P< 0.001$), 1.23 (1.07, 1.40; $P=0.003$) and 1.17 (1.04, 1.32; $P=0.01$) for total, cardiovascular and non-cardiovascular mortality, respectively. Corresponding risk estimates were 1.16 (1.06, 1.27; $P= 0.001$), 1.18 (1.04, 1.34; $P=0.01$) and 1.13 (95% CI: 0.99, 1.30; $P=0.06$) for AMI patients.

Furthermore, application of E-formula revealed high sensitivity of the observed association between cystathionine and endpoints across both study cohorts, as reflected by high E-value for the total effect estimate as well as for lower reported CI (**Supplemental Table 7**)

DISCUSSION

Principal findings

Using two independent, large cohorts of patients with SAP and AMI, we demonstrate that high plasma cystathionine levels were associated with an increased risk of mortality during follow-up independent of traditional risk factors, as well as potential confounders. Among SAP patients, the associations of cystathionine with all-cause mortality tended to be stronger among non-smokers and those with low plasma PLP levels.

Strengths and limitations

The major strengths of the current study are its long-term prospective design, large sample sizes, detailed characterization of patients in two independent populations together with information on outcomes obtained from public national registries. Furthermore, the sensitivity analyses indicated that the observed results are robust to unobserved cofounding, and therefore are expected to be reproducible by others with new data (20).

The current study has, however, some limitations. First, high plasma cystathionine may simply reflect Met and homocysteine surplus (4,22). However, controlling for plasma Met and tHcy had minimally attenuating effects on our estimates, indicating that the current findings are largely independent of these metabolic precursors. Second, our results are also unlikely to be explained by any bias from reverse causality because the estimates were only slightly attenuated after excluding the first year of follow-up. Third, we were unable to examine the subtypes of non-cardiovascular causes of death beyond those related to cancer. This, however, does not detract from our findings on plasma cystathionine and risk of overall mortality. Fourth, the majority of patients in the current study received study supplementation with folic acid and/or other B-vitamins, which can affect plasma cystathionine levels (23). Further, folate has been suggested to regulate tHcy and cystathionine status by inhibiting the enzyme glycine-N-methyl-transferase (GNMT) (24,25), a key regulator of the methylation

status in the cell and linked to regulation of cholesterol transport (26) as well as immune activation (27). However, B-vitamin intervention neither appreciably altered risk estimates of cystathionine when included in the multivariable model nor introduced any significant effect-modifications in subgroup analyses, indicating that supplementation with B-vitamin is unlikely to explain the observed risk association. Finally, we mainly studied subjects, in whom the majority being treated with several medications at discharge, and our results thus may not be applicable to a healthy patient cohort.

Plasma cystathionine and mortality in other epidemiological studies

Data on circulating cystathionine and poor prognosis are sparse. A study among 35 critically –ill patients reported higher plasma cystathionine levels in non-survivor group at certain time points during 28-days follow-up (15). However, to our knowledge, the present investigation is the first large-scale patient-based cohort study to reveal such an association. Notably, the findings were validated in a second patient cohort.

Possible mechanisms

Cystathionine, inflammation and plasma PLP status

High dietary intake of the cystathionine precursor Met in rats has been shown to increase the levels of the inflammatory marker, C-reactive protein (28), which is associated with an increased risk of mortality in patients with acute coronary syndrome (29) and in apparently healthy subjects (30). However, adjusting for CRP in SAP patients did not seem to attenuate the risk estimates, although cystathionine and CRP were positively correlated at baseline. Notably, high plasma concentration of CRP has been consistently related to low PLP status in several studies (31, 32) including patients with SAP (31). Inadequate vitamin B-6 status also results in decreased cystathionine γ-lyase activity, causing elevation of cystathionine in plasma (22). We previously showed that the positive association between cystathionine and AMI was significantly stronger among patients with low plasma PLP (10). In the present

study, a similar trend was seen in relation to mortality risk in PLP subgroup alone or also those jointly evaluated with B-vitamins treatment. Thus, the low B-6 status, rather than inflammation, could represent a possible link between CRP and cystathionine metabolism.

Cystathionine, endothelial dysfunction, oxidative stress and smoking status

Plasma cystathionine has been related to endothelial dysfunction and oxidative stress in previous report (6), which could at least partly account for the adverse prognosis observed in the current study, particularly regarding cardiovascular mortality. Accordingly, across both patients with SAP and AMI, plasma cystathionine showed positive associations with ADMA, an endogenous inhibitor of NO synthase (33). Similar observations have previously been made in a subset of the current SAP population (6). Interestingly, despite the occurrence of high oxidative stress in smokers (34), among SAP patients, elevated plasma cystathionine concentrations appeared to increase the risk of mortality particularly in non-smokers; however, putative associations may be masked among smokers, as smoking is the predominant risk factor for mortality (34). Another possibility is that patients with SAP at high risk of CVD may have quitted smoking before enrollment.

Cystathionine and renal function

In line with our findings, plasma cystathionine levels are elevated in patients with renal dysfunction (9), which is a major risk factor of mortality (35). Renal function could thus serve as potential confounder in our study, as suggested by the attenuation of risk estimates by adjusting for eGFR in SAP patients. However, a previous study in patients with end-stage kidney disease found no significant association between plasma cystathionine and adverse cardiovascular events (36). Further, our cohorts mainly consisted of patients without signs of severely impaired renal function, as reflected by eGFR levels. Therefore, it is not likely that our findings are explained solely by renal impairment.

Plasma cystathionine and non- CVD death

An unexpected finding from our study was the positive association between plasma cystathionine levels and non-CVD mortality across both cohorts. A nested case-control study found that high levels of serum cystathionine may be an independent predictor of early biochemical recurrence and aggressiveness of prostate cancer (37). However, we did not observe any increased cancer mortality risk according to elevated plasma cystathionine in either cohort, indicating that cancer is unlikely to have contributed to adverse non-cardiovascular prognosis. On the other hand, cystathionine has been associated with cognitive decline (38), liver dysfunction (39), asthma (22) as well as sepsis (15). In addition, experimental studies have demonstrated a link between cystathionine metabolism and diabetes mellitus (40). It has been suggested that aberrant fluxes through transsulfuration enzymes may be attributable to the metabolic consequences of some of these diseases (22, 39, 40). It is therefore interesting that in our recent observation in a subset of SAP patients, plasma cystathionine associated positively with lanthionine (41), which is an indirect marker of increased CBS flux and negatively with glutathione (41), indicating impaired CSE induction (5,6). However, our explanations concerning the strong association of cystathionine with non-cancer non-cardiovascular related prognosis are speculative and more research is certainly required to pinpoint the exact underlying mechanisms.

Conclusions

Elevated plasma cystathionine is a predictor of death among patients with either suspected or verified coronary heart disease. Our data should motivate further research on the trans-sulfuration pathway in relation to major lifestyle disease and mortality.

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Table 1. HRs (95% CIs) for mortality by quartiles of plasma cystathionine among patients with stable angina pectoris[1]

	Quartiles of plasma cystathionine								
	1	2		3		4		Per 1-SD[2]	
n	765	789		733		746			
Total death									
Incidence rate[3]	12.2	19.4		22.2		32.5			
Unadjusted	1	1.60 (1.25, 2.06)	<0.001	1.83 1.43,2.35)	<0.001	2.77 (2.18, 3.51)	<0.001	1.43 (1.34,1.53)	<0.001
Model 1	1	1.34 (1.04,1.73)	0.02	1.32 (1.03,1.70)	0.03	1.74 (1.36, 2.21)	<0.001	1.23 (1.14,1.32)	<0.001
Model 2	1	1.49 (1.12,2.01)	0.01	1.34 (1.01,1.79)	0.05	1.69 (1.28, 2.24)	<0.001	1.20 (1.10,1.31)	<0.001
Cardiovascular death									
Incidence rate[3]	4.4	8.8		10.4		16.6			
Unadjusted	1	2.00 (1.34,2.99)	0.001	2.36 (1.59, 3.50)	<0.001	3.87 (2.67,5.63)	<0.001	1.52 (1.38,1.68)	<0.001
Model 1	1	1.65 (1.10, 2.46)	0.02	1.64 (1.10, 2.44)	0.02	2.30 (1.57,3.37)	<0.001	1.29 (1.16,1.44)	<0.001
Model 2	1	1.97 (1.24, 3.13)	0.004	1.65 (1.03, 2.64)	0.04	2.15 (1.37, 3.36)	0.001	1.23 (1.09,1.40)	0.001

Non-cardiovascular death

Incidence rate[3]	7.8	10.5		11.8		15.9			
Unadjusted	1	1.38 (0.99,1.90)	0.06	1.54 (1.11, 2.12)	0.01	2.14 (1.57, 2.91)	<0.001	1.35 (1.22,1.48)	<0.001
Model 1	1	1.17 (0.85,1.63)	0.34	1.14 (0.82,1.58)	0.43	1.40 (1.02,1.92)	0.04	1.17 (1.05,1.29)	0.004
Model 2	1	1.24 (0.85,1.80)	0.26	1.18 (0.81,1.71)	0.39	1.42 (0.99, 2.05)	0.06	1.16 (1.03,1.31)	0.01

[1]HRs and 95% CIs were estimated by Cox regression. Model 1 was adjusted for age and sex. Model 2 was adjusted for model 1 and for body mass index, hypertension, diabetes, smoking, previous acute myocardial infarction, serum total cholesterol, and treatment with folic acid or vitamin B6

[2]Log-transformed.

[3]Presented as events per1000 patient years

Table 2. HRs (95% CIs) for mortality by quartiles of plasma cystathionine among patients with acute myocardial infarction[1]

	Quartiles of plasma cystathionine								
	1	2		3		4		Per 1-SD[2]	
n	874	974		921		901			
Total death									
Incidence rate[3]	15.7	23.5		33.7		57.1			
Unadjusted	1	1.51 (1.17, 1.94)	<0.001	2.16 (1.69, 2.75)	<0.001	3.65 (2.90, 4.59)	<0.001	1.57 (1.48,1.68)	<0.001
Model 1	1	1.27 (0.98,1.63)	0.07	1.34 (1.05, 1.71)	0.02	1.88 (1.49, 2.38)	<0.001	1.28 (1.19,1.37)	<0.001
Model 2	1	1.19 (0.91,1.55)	0.19	1.24 (0.96,1.60)	0.10	1.57 (1.23, 2.02)	<0.001	1.19 (1.10,1.29)	<0.001
Cardiovascular death									
Incidence rate[3]	9.2	13.3		20.1		37.2			
Unadjusted	1	1.44 (1.03, 2.00)	0.03	2.16 (1.57, 2.96)	<0.001	3.94 (2.93, 5.29)	<0.001	1.64 (1.52, 1.77)	<0.001
Model 1	1	1.20 (0.86, 1.67)	0.29	1.32 (0.96, 1.82)	0.09	1.97 (1.46, 2.67)	<0.001	1.33 (1.22, 1.45)	<0.001
Model 2[5]	1	1.12 (0.79, 1.59)	0.53	1.20 (0.86, 1.68)	0.27	1.60 (1.16, 2.20)	0.004	1.23 (1.12, 1.36)	<0.001

Non-cardiovascular death

Incidence rate[3]	6.5	10.3		13.6		19.8			
Unadjusted	1	1.61 (1.09, 2.38)	0.02	2.15 (1.48, 3.14)	<0.001	3.21 (2.23, 4.62)	<0.001	1.46 (1.32, 1.63)	<0.001
Model 1	1	1.37 (0.93, 2.03)	0.11	1.36 (0.93, 1.99)	0.12	1.73 (1.19, 2.51)	0.004	1.19 (1.06, 1.34)	0.003
Model 2	1	1.28 (0.86, 1.93)	0.22	1.29 (0.86, 1.92)	0.21	1.53 (1.03, 2.26)	0.04	1.13 (0.99, 1.28)	0.06

[1]HRs and 95% CIs were estimated by Cox regression. Model 1 was adjusted for age and sex. Model 2 was adjusted for model 1 and for body mass index, hypertension, diabetes, smoking, previous acute myocardial infarction, serum total cholesterol, and treatment with folic acid or vitamin B6

[2]Log-transformed.

[3]Presented as events per 1000 patient years

Table 3. HRs (95% CIs) for cancer and other non-cardiovascular mortality by quartiles of plasma cystathionine among patients with stable angina pectoris and acute myocardial infarction[1]

	Quartiles of plasma cystathionine								
	1	2		3		4		Per 1-SD[2]	
Patients with SAP									
n	765	789		733		746			
Cancer death									
Incidence rate[3]	4.3	6.3		6.6		7.4			
Unadjusted	1	1.48 (0.96, 2.72)	0.08	1.55 (1.01,2.39)	0.05	1.75 (1.14, 2.69)	0.01	1.22 (1.07, 1.40)	0.004
Model 1	1	1.26 (0.82, 1.94)	0.29	1.16 (0.75, 1.80)	0.49	1.16 (0.75, 1.79)	0.51	1.05 (0.91, 1.22)	0.51
Model 2	1	1.24 (0.76,2.03)	0.39	1.23 (0.75, 2.03)	0.41	1.25 (0.76, 2.06)	0.38	1.07 (0.91, 1.27)	0.39
Other non-cardiovascular death									
Incidence rate[3]	3.4	4.2		5.1		8.5			
Unadjusted	1	1.24 (0.75, 2.05)	0.39	1.51 (0.93, 2.46)	0.09	2.64 (1.68, 4.14)	<0.001	1.49 (1.31, 1.71)	<0.001
Model 1	1	1.06 (0.64, 1.75)	0.83	1.11 (0.68, 1.82)	0.68	1.71 (1.08, 2.71)	0.02	1.30 (1.13, 1.51)	<0.001

Model 2	1	1.21 (0.69, 2.14)	0.50	1.10 (0.62, 1..95)	0.74	1.61 (0.94, 2.74)	0.08	1.24 (1.05, 1.48)	0.01
Patients with AMI									
n	874	974		921		901			
Cancer death									
Incidence rate[3]	4.2	5.6		7.6		9.3			
Unadjusted	1	1.35 (0.82, 2.23)	0.24	1.86 (1.15, 3.00)	0.01	2.29 (1.15, 3.70)	0.001	1.29 (1.11, 1.51)	0.001
Model 1	1	1.16 (0.70, 1.92)	0.55	1.22 (0.75, 1.98)	0.44	1.31 (0.80, 2.36)	0.28	1.06 (0.89, 1.25)	0.50
Model 2	1	1.12 (0.67, 1.88)	0.67	1.14 (0.69, 1.89)	0.61	1.29 (0.78, 2.15)	0.32	1.05 (0.88, 1.25)	0.62
Other non-cardiovascular death									
Incidence rate[3]	2.3	4.7		6.0		10.6			
Unadjusted	1	2.10 (1.12, 3.95)	0.02	2.71 (1.46, 5.02)	0.002	4.91 (2.73, 8.81)	<0.001	1.67 (1.44, 1.93)	<0.001
Model 1	1	1.76 (0.93, 3.31)	0.08	1.63 (0.87, 3.05)	0.13	2.46 (1.35, 4.47)	0.003	1.35 (1.15, 1.59)	<0.001
Model 2	1	1.62 (0.83, 3.13)	0.25	1.58 (0.82, 3.06)	0.17	1.96 (1.03, 3.72)	0.04	1.23 (1.02, 1.47)	0.03

[1]HRs and 95% CIs were estimated by Cox regression. Model 1 was adjusted for age and sex. Model 2 was adjusted for model 1 and for body mass index, hypertension, diabetes, smoking, previous acute myocardial infarction, serum total cholesterol, and treatment with folic acid or vitamin B6. AMI, acute myocardial infarction; SAP, stable angina pectoris

[2]Log-transformed.

[3]Presented as events per1000 patient-years

Table 4. The association between plasma cystathionine per SD (log transformed) and all-cause mortality according to subgroups of traditional risk factors and plasma B-vitamin status[1]

	Patients with SAP					Patients with AMI				
Medications	n	Incidence rate[2]	HR (95% CI)	*P*-value	P_{int}	n	Incidence rate[2]	HR (95% CI)	*P*- value	P_{int}
Age										
< median	1463	10.2	1.15 (0.99, 1.35)	0.08	0.26	1750	9.8	1.28 (1.06, 1.54)	0.01	0.11
≥ median	1570	32.4	1.24 (1.14, 1.35)	<0.001		1920	54.5	1.27 (1.18, 1.37)	<0.001	
Sex										
Females	699	16.8	1.37 (1.15, 1.62)	<0.001	0.25	962	35.9	1.30 (1.16, 1.46)	<0.001	0.48
Males	2334	22.5	1.20 (1.10, 1.30)	<0.001		2708	29.9	1.27 (1.16, 1.38)	<0.001	
BMI										
No	1367	23.0	1.29 (1.16, 1.43)	<0.001	0.51	1824	35.8	1.29 (1.18, 1.42)	<0.001	0.62
Yes	1666	19.6	1.17 (1.06, 1.30)	0.003		1836	26.7	1.23 (1.10, 1.36)	<0.001	
Diabetes										
No	2027	19.4	1.17 (1.06, 1.29)	0.001	0.14	3282	28.9	1.25 (1.16, 1.35)	<0.001	0.42

Yes	978	24.9	1.28 (1.14, 1.45)	<0.001		359	58.2	1.35 (1.14, 1.60)	<0.001	
Hypertension										
No	1591	17.5	1.17 (1.04, 1.31)	0.01	0.53	2581	26.4	1.32 (1.21, 1.45)	<0.001	0.21
Yes	1442	25.4	1.26 (1.14, 1.39)	<0.001		1050	43.9	1.19 (1.06, 1.34)	0.003	
Smoking										
No	2054	20.5	1.32 (1.21, 1.44)	<0.001	0.001	1794	37.3	1.30 (1.18, 1.42)	<0.001	0.25
Yes	978	22.7	1.06 (0.93, 1.21)	0.36		1876	26.1	1.24 (1.11, 1.39)	<0.001	
PLP										
<median	1511	25.6	1.32 (1.20, 1.45)	<0.001	0.01	1831	39.2	1.31 (1.20, 1.43)	<0.001	0.51
≥ median	1522	16.9	1.09 (0.97, 1.23)	0.12		1839	24.1	1.22 (1.09, 1.37)	<0.001	
Cobalamin										
<median	1270	21.7	1.21 (1.08, 1.36)	0.001	0.37	1802	33.9	1.23 (1.12, 1.36)	<0.001	0.43
≥ median	1273	18.1	1.26 (1.12, 1.43)	<0.001		1803	27.5	1.33 (1.20, 1.48)	<0.001	
Folate										
<median	1518	22.3	1.29 (1.17, 1.44)	<0.001	0.11	1813	34.6	1.24 (1.12, 1.36)	<0.001	0.65
≥ median	1515	19.9	1.15 (1.03, 1.28)	0.01		1816	27.1	1.26 (1.13, 1.41)	<0.001	

[1]HRs and 95% CI were reported for per SD increment of plasma cystathionine and estimated by Cox hazards model, adjusted for age, and sex. AMI, acute myocardial infarction; BMI, body mass index; PLP, pyridoxal phosphate; SAP, stable angina pectoris;

[2]Presented as events per1000 patient-years

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Legends for figures

Figure 1. Associations of plasma cystathionine with baseline clinical relevant covariates. Spearman's rho of ranked values of the plasma cystathionine concentrations with important continuous covariates at baseline are reported for SAP (n=3033) and AMI (n=3670) patients, respectively. ADMA indicates asymmetric dimethylarginine; AMI, acute myocardial infarction; BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; SAP, stable angina pectoris; TG, triglycerides. *P<0.05; **P<0.01; ***P<0.001.

Figure 2. Dose–response relationship between log-transformed plasma cystathionine and the partial hazard of all-cause, cardiovascular and non-cardiovascular mortality. Generalized additive regression models are adjusted for age and sex in patients with SAP (n=3033) and AMI (n=3670). The shaded area surrounding solid lines show 95% confidence intervals. Density plots show the distributions of plasma cystathionine and vertical lines denote the 25th, 50th, and 75th, 90th percentiles, respectively. AMI, acute myocardial infarction; CVD, cardiovascular disease; SAP, stable angina pectoris.