

Hyperhomocysteinemia predicts total and cardiovascular mortality in high-risk women

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Objective The impact of homocysteine on cardiovascular disease can be more detrimental in women than in men, but it is unknown whether this applies to high-risk women. We therefore investigated the association of hyperhomocysteinemia with coronary artery disease (CAD) and cardiovascular mortality in high-risk women referred for CAD, both in the total population and in the hypertensive and normotensive cohorts.

Design A prospective study cohort.

Setting A tertiary centre.

Patients Inclusion criteria: 262 consecutive Caucasian postmenopausal women referred for coronary angiography. Exclusion criteria: acute myocardial infarction and vitamin supplementation.

Main outcome measure(s) We assessed total plasma homocysteine (tHcy), folate levels, and the MTHFR677C→T polymorphism. CAD was defined as a modified Duke Index score greater than 0; hyperhomocysteinemia as tHcy levels of 15 µmol/l or greater. The primary study outcome was cardiovascular mortality at follow-up.

Results Mild/moderate and severe hyperhomocysteinemia was found in 15.1 and 1.6% of women, respectively, without differences between CAD and non-CAD women. By the ATPIII criteria, 92.2% of the women were in the highest risk class and 55% had CAD; however, no association of

tHcy with the CAD score was found. After a median follow-up of 3.6 years, 23 women (9.1%) had died, 15 (6%) of cardiovascular causes. Women with high tHcy levels showed the worst all-cause and cardiovascular death-free survival at Kaplan–Meier and Cox regression analysis. Moreover, in the hypertensive cohort only women with hyperhomocysteinemia showed increased cardiovascular mortality.

Conclusion Hyperhomocysteinemia is common in high-risk women and adversely affects their prognosis, although it is unrelated to the CAD atherosclerotic burden. *J Hypertens* 24:851–859 © 2006 Lippincott Williams & Wilkins.

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Introduction

Mild/moderate hyperhomocysteinemia (15–30 µmol/l) can derive from folate or vitamin B₁₂ or B₆ deficiency, or a genetic predisposition as the common thermolabile variant (677C→T) of the methylene-tetrahydrofolate-reductase (MTHFR) gene [1]. Mild/moderate hyperhomocysteinemia would imply an excess risk of coronary artery disease (CAD), acute coronary events, and mortality [2–6], and a graded relationship with angiographically assessed CAD [2,7–13]. An association of hyperhomocysteinemia with total and cardiovascular mortality has also been described in population studies [14–16], and a link between hyperhomocysteinemia and cardiovascular events was suggested [17]. However, evidence derived mostly from studies in men [18], and scarce

attention was devoted to women; in most studies women formed only a small part of the population, or were excluded. In a recent meta-analysis, information on the association of hyperhomocysteinemia with CAD was confined to only 186 women that could be pooled together from 30 studies [19]. Furthermore, in the majority of studies that included women, the hyperhomocysteinemia-associated risk was estimated for the sexes combined, or after multivariate adjustment for sex and other risk factors, thus precluding the attainment of information on the relative risk of women [18]. This paucity of data is surprising because mechanistic data support the view that hyperhomocysteinemia might be more detrimental in women than in men [20,21]. Moreover, CAD is also a leading cause of death in women [22],

there is an excess risk of cardiovascular events with increasing total plasma homocysteine levels (tHcy) in healthy postmenopausal women [14], the odds ratio for CAD of a 5 $\mu\text{mol/l}$ tHcy increment might be higher in women than in men [23], and the association of tHcy with blood pressure can be stronger in women than in men [24].

A large prospective cohort, 24-year, follow-up study of middle-aged women, who were free of previous acute myocardial infarction (AMI) at baseline, recently partly filled this gap [16]. However, the observed increased risk was confined to women in the highest tHcy quintile, who had a median tHcy of 16.45 $\mu\text{mol/l}$, and became apparent only after 15 years of follow-up. This finding indicates that in low-risk women long-term prospective studies are necessary to show the effects of homocysteine levels on AMI morbidity and mortality, probably because the detrimental effects of mild hyperhomocysteinemia require a long time to become apparent in such populations. By contrast, there is no information on the impact of hyperhomocysteinemia and of the 677C→T MTHFR gene polymorphism on cardiovascular disease in high-risk women. We thus sought to determine prospectively the prevalence and determinants of hyperhomocysteinemia, its association with folate levels, the 677C→T of MTHFR genotype, CAD severity, and its impact on total and cardiovascular mortality at follow-up, in consecutive white women undergoing coronary angiography.

Methods

The inclusion criteria in the Genetic and Environmental Factors in Coronary Atherosclerosis (GENICA) study were previously described in detail [25,26]. In brief, we enrolled consecutive Caucasian patients of both sexes referred for coronary angiography for the investigation of chest pain or suspected CAD between 1999 and 2001. For the present study, the exclusion criteria comprised a refusal to participate in the study, male sex, and conditions that might affect tHcy levels, such as AMI, and folic acid, vitamin B₁₂ or B₆ supplements. The study protocol was approved by the Ethics Committee and written informed consent was obtained from each participant. The normal tHcy range was previously determined in a group of 101 consecutive healthy Caucasian normotensive blood donors, enrolled during the same period from the local blood bank, who had identical ethnic backgrounds.

Demographic and laboratory measurements

A history of cardiovascular events and information on smoking habits, presence/absence of hypertension, diabetes mellitus, hypercholesterolemia, hypertriglyceridemia, and all current medications were gathered with a standard questionnaire that was administered by the study staff. Women classified as current cigarette smokers, non-smokers and ex-smokers, were as described were as

described elsewhere [25,26]. Types I and II diabetes were defined according to published guidelines; hypercholesterolemia and hypertriglyceridemia were defined as described [27]. Body mass index (BMI) was calculated as weight/height² (kg/m²). Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase V for diastolic. Hypertension was defined as systolic pressure of 140 mmHg or greater or diastolic pressure of 90 mmHg or greater, or both, or the use of antihypertensive drugs.

Women were studied between 0830 and 1200 h; blood samples were taken before coronary angiography, and immediately put on ice and centrifuged. Total and high-density lipoprotein cholesterol, triglycerides, glucose, sodium, potassium, blood urea nitrogen, and creatinine levels were measured using conventional methods. Plasma folate levels were determined using a chemiluminescent method (ADVIA Centauri; Bayer, Milan, Italy) and tHcy levels (free plus protein-bound) were determined by high-performance liquid chromatography [28]. The accuracy and precision of tHcy measurements in our laboratory are validated by ERNDM (European quality control programme of laboratory measurements within the field of study of inborn errors of metabolism). Hyperhomocysteinemia was defined as a tHcy value above the 90th percentile of the distribution in a sample of healthy individuals from the same geographical area; mild/moderate and severe hyperhomocysteinemia were defined as tHcy values from 15 to 30 $\mu\text{mol/l}$, and 30 $\mu\text{mol/l}$, or greater, respectively.

DNA was extracted using standard methods; genotyping at the MTHFR 677C→T polymorphism was performed by LightCycler (Roche, Milan, Italy) using melting curve analysis from an allele-specific fluorescence resonance energy transfer probe. This method was preferred to the restriction fragment length polymorphism analysis (which showed inconsistent cleavage), because it was found to be 100% accurate when compared with sequencing.

Coronary angiography

Angiography was carried out by experienced invasive cardiologists; the left ventricular ejection fraction (LVEF) was determined according to a standard method. The severity of CAD was graded independently on an ordinal scale, where 0% corresponded to no stenosis and 100% to vessel occlusion assessed by two observers (M.Z. and L.P.), who were blinded to the patient's tHcy, folate levels and MTHFR genotype. The percentage of stenosis was derived by means of visual estimates if the between-estimate difference was less than 20%; greater inter-observer disagreement in the grading of stenosis was resolved by consensus. The burden of CAD present was summarized using the Duke Prognostic Index modified according to Mark *et al.* [29], in order to account for the impact of left main trunk stenosis (see Table 1). This index, which accurately predicted the 5-year

Table 1 Classification of the coronary artery disease burden according to a modified Duke Index Prognostic Score and distribution of women in the different score groups

| Extent of CAD | Prognostic weight (0–100) | Women (%) |
|---|---------------------------|-----------|
| No CAD \geq 50% | 0 | 45.4 |
| 1-Vessel disease 50–74% | 19 | 7.7 |
| > 1-Vessel disease 50–74% or | | |
| 1-Vessel disease 75% | 23 | 13.3 |
| 1-Vessel disease \geq 95% | 32 | 6.8 |
| 2-Vessel disease | 37 | 7.4 |
| 2-Vessel disease, both \geq 95% | 42 | 0.3 |
| 1-Vessel disease \geq 95% proximal LAD or | | |
| 2-Vessel disease \geq 95% LAD | 48 | 6.5 |
| 2-Vessel disease \geq 95% proximal LAD or | | |
| 3-Vessel disease | 56 | 4.9 |
| 3-Vessel disease \geq 95% in at least one | 63 | 2.2 |
| 3-Vessel disease 75% proximal LAD | 67 | 4.3 |
| 3-Vessel disease \geq 95% proximal LAD | 74 | 0.6 |
| Left main, 75% | 82 | 0.3 |
| Left main, \geq 95% | 100 | 0.3 |

CAD, Coronary artery disease; LAD, left anterior descending coronary artery.

mortality of medically treated patients [30], was used to divide women into those with mostly normal coronary arteries (non-CAD), namely with CAD score 0, and those with CAD, namely those with a score of CAD greater than 0.

Longitudinal study

Follow-up was assessed by a committee that was blinded to the tHcy values, based on all available information (hospital records, physician records, death certificates), and through direct contact with the patients or their family doctor and relatives, whenever necessary. According to the Syst-Eur Trial [31], deaths were defined as cardiovascular if sudden, caused by congestive heart failure, AMI or stroke.

Statistical analysis

One-way analysis of variance followed by Bonferroni's test was used to compare quantitative variables between groups; the distribution of categorical variables, including MTHFR genotypes and the agreement of genotype frequencies with the Hardy–Weinberg equilibrium, were tested by chi-squared analysis. A comparison of tHcy and folate values between groups was undertaken after natural logarithm (ln) transformation. To identify the determinants of tHcy, a stepwise regression analysis (inclusion and exclusion probability cutoff 0.05 and 0.10, respectively) was performed; the model with the significant determinants was then used for the adjustment of tHcy values. To determine the independent risk factors of CAD and a low (<40%) LVEF, logistic regression analysis was used. All-cause (total) and cardiovascular death rates and comparisons of survival curves were estimated using Kaplan–Meier analysis and the log-rank test, respectively, as a function of the tertile of tHcy. Cox stepwise (backward, Wald) regression analysis was also used to determine the relationships

between those clinical variables, among age, LVEF, serum creatinine, tHcy, hypertension, diabetes mellitus, cigarette smoking, hypercholesterolemia, BMI, Duke Prognostic Index, and ongoing medical treatment, independent predictors of events, and total and cardiovascular deaths at follow-up. Finally, we compared the all-cause death survival curve using Kaplan–Meier analysis and the log-rank test after dividing the women into a hypertensive and a normotensive cohort. Significance was set at $P < 0.05$.

Results

Demographic characteristics and coronary artery disease findings

Table 1 shows the distribution of the CAD score index. The main clinical and biochemical features of the women with and without CAD are depicted in Table 2. The distribution of the 677C→T of MTHFR genotypes was consistent with the Hardy–Weinberg equilibrium. The proportion of women with arterial hypertension, hyperlipidemia, and type II diabetes increased along with the quartile of CAD score, thus indicating clear-cut differences of major risk factors across groups with a different CAD atherosclerotic burden (Table 3).

Plasma levels of homocysteine and folate and association with coronary artery disease

Plasma tHcy and folate levels were measured in all 262 eligible women of the GENICA study. The tHcy values distribution was positively skewed (skewness index 2.4 ± 0.1) (Fig. 1a); therefore, ln transformation was necessary to attain a normal distribution (Fig. 1b) and

Table 2 Demographic and clinical features of the whole population of women, classified based on the absence or presence of angiographically assessed coronary artery disease (CAD)

| | Non-CAD | <i>P</i> | CAD |
|-----------------------------------|------------------|----------|------------------|
| Age (years) | 63 \pm 11 | < 0.0001 | 69 \pm 8 |
| BMI (kg/m ²) | 26.4 \pm 4.4 | NS | 26.7 \pm 4.6 |
| Systolic BP (mmHg) | 131 \pm 18 | 0.029 | 137 \pm 19 |
| Diastolic BP (mmHg) | 78 \pm 9 | NS | 76 \pm 9 |
| Pulse pressure (mmHg) | 58 \pm 17 | NS | 58 \pm 19 |
| Heart rate (beats/min) | 69 \pm 11 | NS | 67 \pm 10 |
| LVEF (%) | 66 \pm 15 | NS | 62 \pm 14 |
| Total cholesterol (mmol/l) | 37.0 \pm 7.2 | NS | 38.3 \pm 7.4 |
| HDL-cholesterol (mmol/l) | 9.9 \pm 2.9 | 0.001 | 8.6 \pm 2.2 |
| LDL-cholesterol (mmol/l) | 23.0 \pm 5.9 | 0.048 | 24.8 \pm 6.3 |
| Triglycerides (mmol/l) | 9.2 \pm 5.1 | 0.029 | 10.6 \pm 5.2 |
| Glycemia (mmol/l) | 39.7 \pm 6.9 | 0.002 | 46.2 \pm 16.9 |
| BUN (mmol/l) | 10.2 \pm 3.2 | NS | 11.2 \pm 5.2 |
| Creatinine (μ mol/l) | 81.3 \pm 20.3 | NS | 83.1 \pm 30.1 |
| Raw tHcy (μ mol/l) | 11.31 \pm 5.51 | NS | 11.85 \pm 7.19 |
| Adjusted tHcy (μ mol/l) | 11.3 \pm 2.6 | 0.056 | 12.1 \pm 2.8 |
| ln (tHcy) | 2.31 \pm 0.48 | NS | 2.36 \pm 0.46 |
| Plasma folate (nmol/l) | 11.0 \pm 5.0 | NS | 12.2 \pm 6.9 |
| MTHFR T ⁶⁷⁷ C genotype | 17/33/23 | NS | 29/53/42 |
| TT/CT/CC, <i>n</i> (%) | (23/45/32) | | (23/43/34) |

BMI, Body mass index; BP, blood pressure; BUN, blood urea nitrogen; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ln, natural logarithm; LVEF, left ventricular ejection fraction; Non-CAD, normal coronary arteries; tHcy, total plasma homocysteine. Data are expressed as mean \pm SD. Adjusted tHcy for serum creatinine, folate, MTHFR T⁶⁷⁷C genotype, LVEF and age.

Table 3 Prevalence of cardiovascular risk factors in postmenopausal women classified by quartiles of coronary artery disease score

| Risk factor (%) | Quartile of CAD score | | | | P |
|---|-----------------------|--------|-------|--------|----------|
| | First | Second | Third | Fourth | |
| Arterial hypertension | 53 | 68 | 78 | 73 | < 0.0001 |
| Hypercholesterolemia | 79 | 87 | 82 | 90 | NS |
| Hypertriglyceridemia | 30 | 37 | 43 | 52 | 0.013 |
| Cigarette smoking | 14 | 24 | 21 | 17 | NS |
| Overweight/obesity | 51 | 50 | 54 | 45 | NS |
| Type II diabetes mellitus | 17 | 20 | 23 | 38 | 0.011 |
| Hyperhomocysteinemia (defined by raw tHcy) | 21.8 | 15.8 | 9.1 | 17 | NS |
| Hyperhomocysteinemia (defined by adjusted tHcy) | 1.6 | 0 | 2.5 | 3.8 | NS |

CAD, Coronary artery disease; tHcy, total plasma homocysteine. Differences in the distribution across groups were tested by χ^2 analysis.

comparison between groups was undertaken on ln tHcy values. Mild/moderate and severe hyperhomocysteinemia, was defined as previously reported [32], and was found in 15.1 and in 1.6%, respectively, of the women. However, neither tHcy nor the rate of hyperhomocys-

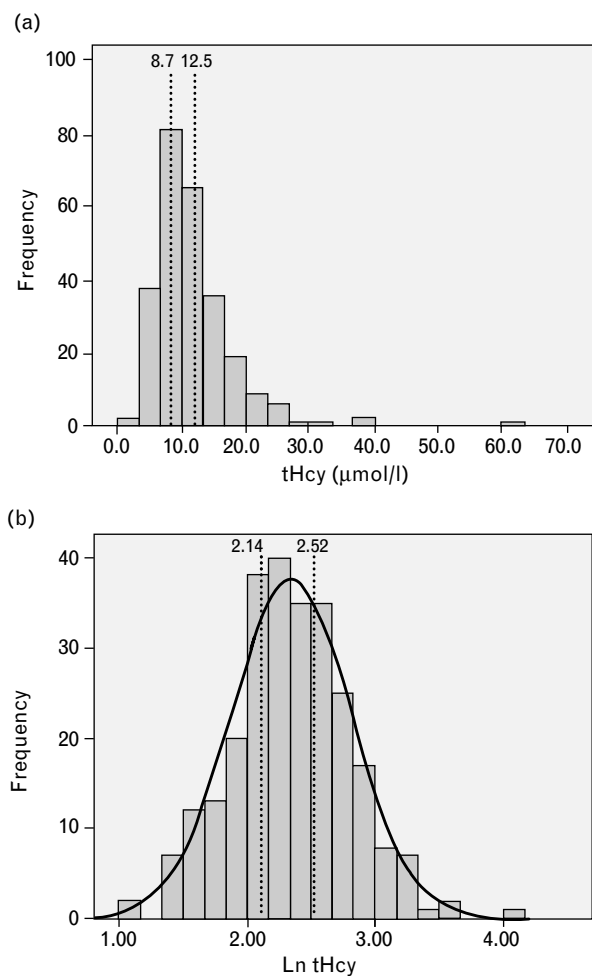
teinemia differed significantly across quartiles of CAD atherosclerotic burden (Fig. 2). Likewise, there were no differences in prevalence of the major cardiovascular risk factors across tertiles of tHcy (Table 4).

At regression analysis, serum creatinine levels ($\beta = 0.26$, $P < 0.0001$), LVEF ($\beta = -0.20$, $P = 0.001$), plasma folate levels ($\beta = -0.13$, $P < 0.028$), 677C→T MTHFR thermolabile variant ($\beta = -0.13$, $P < 0.0001$), and age ($\beta = 0.14$, $P = 0.023$) significantly predicted tHcy, and overall accounted for 13% of its variance. When calculated with tHcy adjusted for these covariates, the prevalence rate of hyperhomocysteinemia fell to 3.8%, but no association of hyperhomocysteinemia with the score of CAD emerged at the logistic regression analysis.

Cardiovascular history and treatment and association with hyperhomocysteinemia

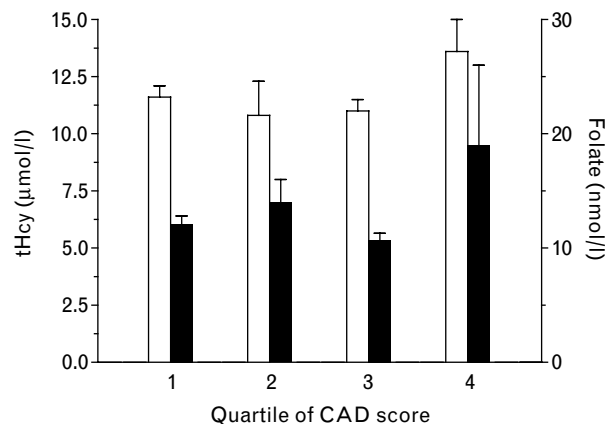
The women with hyperhomocysteinemia were similar to those without hyperhomocysteinemia in terms of the

Fig. 1



The histogram shows the distribution of raw total plasma homocysteine (tHcy) (a) and natural logarithm transformed (b) tHcy values. The cutoff values for defining tertiles are identified by the vertical lines.

Fig. 2



The histogram (mean \pm SEM) depicts total plasma homocysteine (tHcy; open bars) and plasma folate (black bars) values in the women classified by quartile of coronary angiography score. Analysis of variance followed by Bonferroni's test showed no significant differences of either tHcy or plasma folate across quartiles.

Table 4 Prevalence of cardiovascular risk factors in postmenopausal women classified by tertiles of total plasma homocysteine

| Risk factor (%) | Tertile of tHcy | | | P |
|---------------------------|-----------------|-----------------|----------------|----|
| | First (n = 83) | Second (n = 81) | Third (n = 88) | |
| Arterial hypertension | 31 | 33 | 36 | NS |
| Hypercholesterolemia | 33 | 33 | 34 | NS |
| Hypertriglyceridemia | 35 | 28 | 37 | NS |
| Cigarette smoking | 40 | 33 | 27 | NS |
| Overweight/obesity | 33 | 30 | 37 | NS |
| Type II diabetes mellitus | 34 | 37 | 29 | NS |

Cut-off values for defining total plasma homocysteine (tHcy) tertiles were: 8.7 and 12.5 $\mu\text{mol/l}$. Differences in the distribution across groups were tested by χ^2 analysis.

National Cholesterol Education Program class of risk [33], a history of transient ischemic attack, stroke, percutaneous transluminal coronary angioplasty, coronary artery bypass graft and peripheral vascular disease (all non-significant). However, they more commonly had suffered from angina ($P = 0.003$), myocardial infarction ($P = 0.035$), renal insufficiency ($P < 0.001$), and vascular surgery ($P = 0.049$).

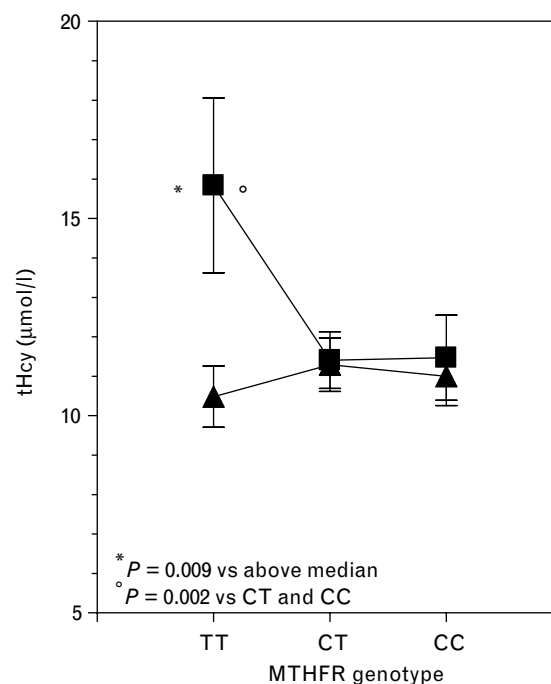
No women were on hormone replacement therapy; the rate of those on calcium antagonists, α_1 -blockers, angiotensin-converting enzyme inhibitors, nitrates, statins, fibrates, antiplatelet agents, heparin, and warfarin were similar between groups (all non-significant). However, treatment with β -blockers ($P < 0.001$), diuretics ($P < 0.001$), angiotensin type 1 receptor antagonists ($P = 0.047$), and digoxin ($P = 0.001$) were more common in women with hyperhomocysteinemia.

Impact of folate and the 677C→T MTHFR genotype on total plasma homocysteine and coronary artery disease

Figure 3 shows the tHcy values of the women classified by MTHFR genotypes and split into those below (squares) and above (triangles) the overall sample plasma folate median value, which was 10.31 nmol/l. The TT women with plasma folate levels below this cutoff had higher tHcy levels, compared with both the women with the other MTHFR genotypes and the TT women above the median of plasma folate. However, no significant impact of tHcy on the CAD atherosclerotic burden was found when the potential impact of significant predictors of tHcy, including MTHFR genotype and folate, was taken into consideration at logistic regression.

Effect of hyperhomocysteinemia and MTHFR genotypes on survival

Ninety-six per cent of the women had complete follow-up information, as only 10 women were lost at the follow-up, which ranged between 28 and 1778 days (median 1329). There were no differences in clinical, demographic, cardiovascular history, and treatment variables at baseline, between the women with and without follow-up data at logistic regression analysis.

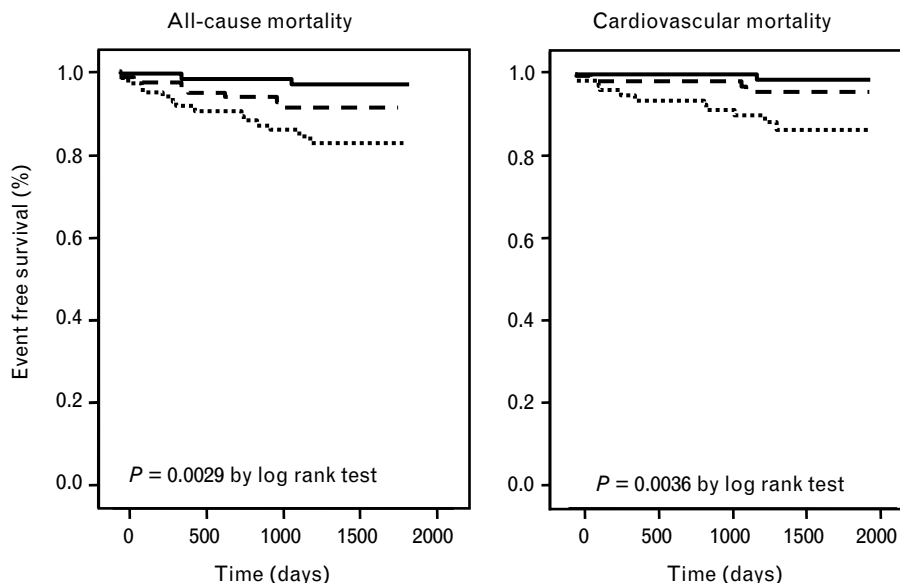
Fig. 3

The graph shows the raw total plasma homocysteine (tHcy; mean \pm SEM) values of all women classified by MTHFR genotypes and divided into those above (triangles) and below (squares) the overall median values of plasma folate for women. The tHcy levels were significantly higher in the TT homozygous women, who had plasma folate levels below the median value, compared with both those above this cutoff and the other genotypes. No significant differences in tHcy levels across MTHFR genotypes were seen in the women with plasma folate levels above the median value.

A total of 23 deaths, which entailed a high total (9.1%) and cardiovascular (6.0%) mortality rate, were recorded (Table 5). For the Kaplan–Meier analysis used to assess the effect of tHcy on mortality the women were divided into tertiles of tHcy, because the cutoff value for defining hyperhomocysteinemia was derived from a mixed sex group of healthy individuals. Cutoff values for defining tertiles were 8.7 and 12.5 $\mu\text{mol/l}$ (Fig. 1). Women in the highest tHcy tertile showed a significantly higher all-cause ($P = 0.003$) and cardiovascular mortality ($P = 0.004$) than those in the lower tHcy tertiles (Fig. 4).

The predictors of all-cause and cardiovascular mortality were further investigated with Cox regression using several models that entailed two covariates along with the tHcy tertile. This was because the number of deaths observed did not permit the inclusion of more than three variables in each model. The predictors of all-cause death identified by this approach are listed in Table 6. Importantly, the tertile of tHcy was a significant predictor in every model with a P value ranging from 0.050 to 0.007 and an Exp(B), which estimates the predicted change in the hazard between the last and the first two tertiles of tHcy, ranging from 2.9 to 4.0.

Fig. 4



All-cause (total, left panel) and cardiovascular deaths (right panel) at Kaplan–Mayer analysis in the high-risk women divided by the tertile of total plasma homocysteine (tHcy). The women in the highest tHcy tertile had significantly worse total ($P = 0.0029$) and cardiovascular death-free ($P = 0.0036$) survival by log rank test compared with the other tertiles. - - - - First tertile tHcy < 8.7 $\mu\text{mol/l}$. ——— Second tertile tHcy ≥ 8.7 , < 12.5 $\mu\text{mol/l}$. ····· Third tertile tHcy ≥ 12.5 $\mu\text{mol/l}$.

Table 5 Number of deaths observed at follow-up in women by tertile of total plasma homocysteine

| Deaths | Tertile of tHcy | | | Total |
|---------------------------|-----------------|-----------------|----------------|-------|
| | First (n = 83) | Second (n = 81) | Third (n = 88) | |
| Cardiovascular deaths | 3 (3.6%) | 1 (1.3%) | 11 (12.6%) | 15 |
| Non-cardiovascular deaths | 3 (3.7%) | 1 (1.3%) | 3 (3.4%) | 7 |
| Undefined deaths | 0 | 0 | 1 | 1 |
| Total deaths | 6 (7.2%) | 2 (2.5%) | 15 (17.0%) | 23 |

Cut-off values for defining total plasma homocysteine (tHcy) tertiles were: 8.7 and 12.5 $\mu\text{mol/l}$.

Table 6 Predictors of deaths at Cox regression analysis

| | P |
|---------------------------------|-------------------------|
| All-cause death predictors | |
| History of vascular surgery | < 0.0001 |
| Serum creatinine | 0.001 |
| Antiplatelet treatment | 0.002 |
| Age | 0.014 |
| Hypertension | 0.041 |
| tHcy tertile | 0.007–0.05 ^a |
| Cardiovascular death predictors | |
| Serum creatinine | < 0.0001 |
| Antiplatelet treatment | 0.007 |
| Age | 0.026 |
| CAD score | 0.035 |
| LVEF | 0.026 |
| tHcy tertile | 0.01–0.05 ^a |

CAD, Coronary artery disease; LVEF, left ventricular ejection fraction; tHcy, total plasma homocysteine. ^aDepending on the Cox regression model used.

The predictors of cardiovascular death are also listed in Table 6. The tertile of tHcy remained in each model tested, with P values ranging from 0.012 to 0.059 and Exp(B) values for the hazard between the highest and the lower two tHcy tertiles ranging from 4.2 to 6.0, thus confirming the results observed for total deaths.

By contrast with the significant effect of tHcy on total and cardiovascular deaths, no evidence for an impact of the 677C→T MTHFR on survival was found (not shown).

The significant impact of tHcy on all-cause and cardiovascular deaths was also found in the hypertensive subgroup ($n = 166$) where 21 deaths, of which 13 were cardiovascular, were observed, but not in the normotensive cohort. In the hypertensive women, those in the highest tertile of tHcy had a higher all-cause ($P = 0.02$) and cardiovascular ($P = 0.02$) mortality than those in the other tertiles.

Discussion

We recently showed an inverse relationship of tHcy with the LVEF and a significant impact of hyperhomocysteinemia on cardiovascular death in a mixed sex high-risk population of the GENICA study [32]. Considering the fact that the cardiovascular detrimental effect of hyperhomocysteinemia can be more prominent in women than in men [13], and also that information on the prognostic impact of hyperhomocysteinemia was confined to

low-risk women [14–16,34,35], or to all-cause (total) mortality [4], we examined the impact on cardiovascular death in the women's cohort. By the National Cholesterol Education Program criteria [33] 92% of these women were at high cardiovascular risk, as confirmed by the high mortality and cardiovascular event rate observed at follow-up. The relatively high number of deaths, along with the accuracy of the phenotyping and genotyping, and the completeness of the follow-up data, provided power to furnish novel information on the relationship of hyperhomocysteinemia with CAD and total and cardiovascular mortality in high-risk women.

Hyperhomocysteinemia and coronary artery disease

The CAD women exhibited the expected constellation of cardiovascular risk factors (Table 2) and a stepwise increase in their prevalence with the increasing CAD atherosclerotic burden (Table 3). By contrast, no such relationship of tHcy (Fig. 2), or the hyperhomocysteinemia rate (Table 3) with CAD extent could be found. Therefore, no tight or graded relationship of tHcy with the CAD extent existed in these high-risk women, in accordance with several [4,7,13,36–39], albeit not all [40,41], available data. Environmental and dietary factors [42], along with genetic differences, can explain these conflicting findings. Furthermore, in previous studies women were not enrolled consecutively or their number was quite small, thus exposing to a selection bias. Moreover, the atherosclerotic burden in the coronary arteries was never assessed by the Duke Prognostic Index score, which is one of the best systems available for this purpose [29,30,43]. It has to be acknowledged that our study was not affected by such biases. Nonetheless, even despite these strengths, it might have been underpowered to highlight a graded relationship between tHcy, the T⁶⁷⁷ MTHFR allele and CAD. We estimated that with a two-group *t*-test at a 0.05 two-sided significance level we had an 85% power to detect a difference in tHcy means of approximately 2.0 $\mu\text{mol/l}$ between non-CAD and CAD women, given this sample size and the observed spread of tHcy values. As the observed between-groups difference was smaller (0.35 $\mu\text{mol/l}$), it is likely that a type 2 (β) error occurred. It should also be noted, however, that differences between non-CAD and CAD women came out just as expected for most well-known risk factors, such as the lipid parameters, arterial hypertension, and diabetes mellitus (Table 3), indicating that their association with the coronary atherosclerotic burden is by no means stronger than that of tHcy.

Association of total plasma homocysteine with left ventricular ejection fraction and the 677C→T MTHFR genotype

Besides the known effect of serum creatinine, plasma folate, and age, we found an inverse relationship of LVEF with tHcy, which accords well with previous results in the mixed sex population [32]. The 677C→T MTHFR

genotype had a significant effect on tHcy and interacted with the folate levels. By splitting women according to the median plasma folate value, we discovered that those with lower folates, who were TT homozygous, had the highest tHcy levels, whereas those with higher plasma folate values showed no genotype differences (Fig. 3). Under low folate intake the TT homozygosity thus implies higher tHcy, and possibly an increased risk of a depressed LVEF and cardiovascular death [32]. Therefore, differences entailing the 677C→T MTHFR and maybe other gene polymorphisms and gene–environment interactions probably contribute to explaining the aforementioned controversial results.

Effect of hyperhomocysteinemia and MTHFR genotypes on survival

At follow-up we recorded a high total and cardiovascular mortality (Table 5). Furthermore, women in the highest tHcy tertile had a worse total and cardiovascular death-free survival compared with those with normal tHcy levels (Fig. 4), even despite showing no differences of prevalence in the major cardiovascular risk factors (Table 4) and being more intensively treated. The number of events limited our possibility of analysing multiple covariates in the same Cox regression model. However, when two potentially relevant variables were considered along with the ln (tHcy), the predictive role of high tHcy on all-cause and cardiovascular death was confirmed. These findings thus extend to cardiovascular mortality and previous evidence in high-risk women of an impact of hyperhomocysteinemia on total mortality in a mixed sex [4,32] and low-risk women populations [14–16,34,35]. They contrast with results of a negative Finnish retrospective study of 131 women who had either myocardial infarction or stroke after a 9-year follow-up [44]. The higher cardiovascular risk of our cohort, the prospective nature of our study and the inclusion of a large subgroup of hypertensive women probably explain this contrasting result. It is noteworthy that as in the mixed sex population of the GENICA study [32] hyperhomocysteinemia affected all-cause and cardiovascular deaths only in the hypertensive cohort.

The lack of effect of the 677C→T MTHFR *per se* on all-cause and cardiovascular death-free survival is also in keeping with previous results [32,45]. We would therefore like to propose that the impact of this polymorphism can be mediated by hyperhomocysteinemia probably through its interaction with a low folate intake, as shown in Fig. 3.

Impact of hyperhomocysteinemia on survival despite lack of association of total plasma homocysteine with coronary artery disease

The prevailing view on the pathogenesis of cardiovascular events involves a slow progression of coronary atherosclerosis, followed by plaque destabilization,

leading to acute coronary syndromes, sudden death, or stroke. The lack of association of hyperhomocysteinemia with CAD despite its impact on mortality, underscores, in our view, that the determinants of plaque growth, that is of CAD atherosclerotic burden, and of cardiovascular events, for example plaque destabilization, differ. Functional factors associated with hyperhomocysteinemia, including blunted nitric oxide bioactivity, an enhanced generation of asymmetric dimethylarginine [46] and of reactive oxygen species, leading to endothelial dysfunction [47] can play an important role in triggering events. As endothelial dysfunction predicts cardiovascular events in postmenopausal women [48], these are therefore probable putative mechanisms of the impact of homocysteine on survival.

It was recently shown that arteriole remodeling predicts cardiovascular events [49], and that mild hyperhomocysteinemia-induced increased collagen deposition leads to stiffer smaller arteries [50]. An influence of hyperhomocysteinemia on the activity of the matrix metalloproteinase that degrades the extracellular matrix and thereby affects plaque stability can also be important, inasmuch as reactive oxygen species activate matrix metalloproteinase 9 [51]. Therefore, functional and structural factors, which are not accurately reflected in the epicardial CAD atherosclerotic burden but affect coronary microcirculation or trigger plaque instability, can explain the impact of hyperhomocysteinemia on cardiovascular death.

In conclusion, we found that hyperhomocysteinemia predicts total and cardiovascular death at follow-up independently of major risk factors and ongoing medical treatment. As we found no association of tHcy with a score of coronary atherosclerotic burden, further investigation of the mechanisms underlying the impact of hyperhomocysteinemia on plaque stability and cardiovascular outcomes in high-risk women is warranted.

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