Serum Homocysteine in Relation to Mortality and Morbidity From Coronary Heart Disease

A 24-Year Follow-Up of the Population Study of Women in Gothenburg

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Background—Elevated serum total homocysteine (tHcy) is an established risk factor for cardiovascular disease (CVD), especially in men. However, there are few prospective population studies on female cohorts, and none of these has been longer than 13 years.

Methods and Results—The Population Study of Women in Gothenburg began in 1968/1969, at which time a representative population-based cohort of women aged 38, 46, 50, 54, and 60 years was recruited. The present cohort is a prospective follow-up of 1368 women in the original cohort for whom blood samples were stored and who were free of previous acute myocardial infarction (AMI) at the 1968/1969 baseline. Homocysteine was analyzed in 2001 with frozen serum from the baseline study and related to AMI incidence and mortality during 24 years of follow-up. Cox regression analyses were used with adjustment for age, traditional risk factors, and tHcy modifiers. For the fifth tHcy quintile, relative risk was 1.86 (95% CI 1.06 to 3.26) for AMI and 5.14 (95% CI 2.22 to 11.92) for death due to AMI. Age-standardized Kaplan-Meier plots for the fifth tHcy quintile versus others showed significant differences both for AMI and for death due to AMI that were apparent after 15 years of follow-up.

Conclusions—Homocysteine in middle-aged women is an independent risk factor for myocardial infarction and in particular mortality due to myocardial infarction. The study illustrates that long-term prospective studies might be necessary to show effects of homocysteine levels on AMI morbidity and mortality in women. (Circulation. 2004;109:601-606.)

Key Words: homocysteine • women • myocardial infarction

Serum total homocysteine (tHcy) is a well-known risk factor for coronary heart disease in men, whereas relatively fewer studies have demonstrated such associations prospectively in women. Alfthan et al1 followed a female Finnish cohort for 9 years and showed no association between tHcy and either acute myocardial infarction (AMI) or stroke, nor did Folsom et al2 after 3.3 years of follow-up. In contrast, Ridker et al3 in a nested case-control study in the United States, demonstrated that tHcy was a risk factor for cardiovascular disease (CVD) among postmenopausal women after 3 years of follow-up. Morris et al4 found a significant relation between tHcy and heart attack or stroke, especially in postmenopausal women, in their (NHANES III) study. Knekt et al5 with the longest reported follow-up of 13 years, found a significant relation between tHcy and coronary artery disease in women with prevalent heart disease at baseline but not among women free of heart disease at baseline. Recent systematic reviews indicate that most previous research has been based on relatively short-term follow-up, from 1.4 to 13 years.6,7 There has been some indication that elevated tHcy levels are more predictive of fatal than nonfatal vascular events8,9 and more predictive of hospitalization in older versus younger populations.10 although this is not yet clearly shown for women.

Homocysteine is an amino acid involved in the metabolism of methionine, in which folate and vitamins B12, B6, and B2 play a key role.11 However, the extent to which tHcy is an independent risk factor as opposed to a marker of other (eg, nutritional) risk factors for disease is not yet totally understood. The purpose of the present study was to test the hypothesis that tHcy is an independent risk factor for fatal and nonfatal AMI in a population-based sample of women followed up for 24 years.

Methods

Materials

In 1968, a representative sample of women in Gothenburg, Sweden, aged 38, 46, 50, 54, and 60 years were invited to participate in a...
Blood Samples
The original survey included fasting blood sampling aimed both for immediate and future analyses. Approximately 120 mL of blood was drawn from each study subject. A small part of the blood at the baseline examination was used for the initial analyses, and the remaining portion was allowed to clot at room temperature for 2 to 3 hours. After centrifugation, the serum was stored at −20°C in 2.5-mL covered polystyrene cups enclosed together in small batches in firmly tied plastic bags. The original frozen samples in the present study were stored for 28 years at −20°C, thawed once for other analyses, and stored again for 2 years at −80°C. Previous study has shown that the original samples were minimally evaporated and suitable for other analyses. It is possible that the methods for blood sample processing may have systematically affected the mean tHcy levels. However, other investigators have found that it is feasible to use stored, frozen plasma samples for tHcy analyses.

Biochemical Assay
All the available 1368 frozen samples were analyzed at Ullevål University Hospital, University of Oslo. For 6% of the original cohort, there were no blood samples available. The samples were transported to the laboratory uninterruptedly frozen. Serum tHcy was determined by the Abbott IMX homocysteine assay (Abbot Laboratories). S-creatinine, S-triglycerides, and vitamin B12 were among the blood tests originally performed at the baseline examination in 1968/1969. Thirty-one double samples were compared with and without the thaw and found to be in high agreement (R=0.99; mean difference 0.04 μmol/L, P=NS).

Statistical Procedures
Cox proportional hazard regression analyses were performed. Pearson’s or Spearman’s correlation was used for bivariate correlation calculations. Kaplan-Meier methods were used for survival plots, in which correction for age was performed with residuals on age generated from general linear model regression analysis. The fifth quintile was compared with the other 4 combined, later assessed as strata arms, for the Kaplan-Meier and proportional hazard regression analyses. The plots shown in Figure 2 illustrate the dose response between tHcy quintiles and relative risk for AMI and relative risk for death due to AMI by the spline method. Cox proportional hazards analyses were performed in 3 steps. The first step was performed with age as the sole correction; in the second step, established risk factors for AMI were added to the model; and finally, influential factors for homocysteine levels were included. SAS statistical software version 8.01 was used.

Results
During 24 years of follow-up, 88 cases of AMI, including 42 deaths due to AMI, occurred (Table 1). Two prevalent cases with a history of AMI before study initiation in 1968 were not included in the analysis. tHcy levels were in the expected range, from 3.05 to 79.87 μmol/L (median 11.15, mean 11.79, SD 4.58, 95% CI 11.54 to 12.03 μmol/L). S-homocysteine levels correlated positively to age (r=0.194 [Spearman correlation], P<0.001), negatively to vitamin B12 (r=−0.199 [Pearson correlation], P<0.001), and positively to creatinine (r=0.179 [Spearman correlation], P<0.001). Dietary vitamin correlations (Pearson’s) to tHcy were as follows: folate r=−0.084, P=0.005; vitamin B12 r=−0.069, P=0.02; and vitamin B6, r=−0.032, P=0.29. Selected sample characteristics are given in Table 1. Because of the high correlation between tHcy and age, all results presented here are age adjusted.

The Kaplan-Meier survival plot for death due to AMI is graded in days because the dates for death were recorded exactly, whereas time for AMI was recorded as the year in which it occurred. The separation of both strata arms occurred

| TABLE 1. Selected Sample Characteristics in the Prospective Study of Women in Gothenburg in the 1968/1969 Examination and 24-Year Mortality Figures in AMI in the Different Age Groups |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cohorts (Age [y] in 1968)      | No. of available samples | Mean tHcy (SD, μmol/L) | Mean waist/hip ratio (SD) | Mean B12 level (SD, pmol/L) | Systolic blood pressure (SD, mm Hg) | Diastolic blood pressure (SD, mm Hg) | Heart rate at rest, bpm (SD) | S-cholesterol (SD, mmol/L) | S-triglycerides (SD, mmol/L) | Smokers, % | Mean creatinine (SD, μmol/L) | AMI/24 years, n (%) | Deaths due to AMI, n (%) |
|--------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 38 (n=372)                      | 345             | 10.80 (4.15)    | 0.726 (0.050)   | 407 (131)       | 124 (14.5)      | 80 (10.7)       | 82 (12.9)       | 6.3 (0.91)      | 1.1 (0.44)      | 161 (46.8)     | 73.4 (12.4)     | 11 (3.2)        | 3 (0.8)        |
| 46 (n=431)                      | 408             | 11.68 (4.10)    | 0.733 (0.019)   | 390 (134)       | 133 (21.2)      | 84 (10.2)       | 80 (12.8)       | 6.8 (1.4)       | 1.2 (0.65)      | 181 (44.4)     | 75.1 (11.5)     | 26 (6.4)       | 8 (1.9)        |
| 50 (n=398)                      | 369             | 11.86 (5.19)    | 0.749 (0.051)   | 394 (140)       | 140 (22.1)      | 87 (10.7)       | 79 (12.7)       | 7.2 (1.1)       | 1.3 (0.58)      | 131 (35.5)     | 76.0 (36.2)     | 22 (6.0)       | 12 (3)         |
| 54 (n=180)                      | 169             | 13.44 (4.75)    | 0.754 (0.056)   | 412 (176)       | 145 (23.8)      | 88 (11.5)       | 81 (12.9)       | 7.4 (1.1)       | 1.4 (0.69)      | 67 (39.6)      | 77.8 (15.9)     | 20 (11.8)      | 14 (7.8)       |
| 60 (n=81)                       | 77              | 12.82 (4.15)    | 0.774 (0.059)   | 383 (105)       | 158 (27.3)      | 92 (12.3)       | 80 (12.8)       | 6.9 (1.2)       | 1.2 (0.59)      | 16 (20.8)      | 77.8 (13.3)     | 9 (11.7)       | 5 (6.2)        |
at 15 years in the total AMI and fatal AMI plots (Figure 1). Similar results were seen with a standard Kaplan-Meier plot, without age adjustment (not shown).

The Cox regression analyses for proportional hazards were conducted in 3 steps (Table 2). In model 1, only correction for age, a strong correlate of tHcy, was performed. In model 2, established risk factors for CVD were added, and finally, in model 3, the more specific, influential factors on homocysteine levels were added (vitamin B12 levels, coffee intake, dietary folate level, and creatinine level). As shown in Table 2, subjects in the highest quintile for tHcy had 2.5-fold excess risk of subsequent AMI mortality compared with the other 4 groups combined after adjustment for age. Additional adjustment for CVD risk factors yielded a similar relative risk (2.8), whereas further adjustment for tHcy correlates increased the point estimate to 5.1. Results for incident AMI, fatal and nonfatal, were between 1.8 and 1.9 and were significant in all 3 models. The relative risk per 1-μmol/L increment of tHcy was between 1.04 and 1.07 for fatal AMI, which was statistically significant in all 3 models. For total (fatal and nonfatal) AMI, the relative risk was 1.02 to 1.04, which was significant in model 2. Analysis of nonfatal AMI only (not shown) revealed no associations.

In addition to Kaplan-Meier plots and to Table 2, which compared the fifth quintile to all others, we also plotted the risk across all quintile ranges (Figure 2). The results demonstrated a nonlinear association with no apparent excess risk until the fifth quintile.

Discussion

The present study demonstrated that tHcy was an independent risk factor for fatal and, to a lesser extent, nonfatal myocardial infarction in women during long-term follow-up. The available tHcy correlates and classic CVD risk factors included in multivariate models did not explain the observed effects of tHcy. Kaplan-Meier analyses showed detectable associations after 15 years of follow-up both for death due to AMI and for AMI. However, when a similar analysis was performed with nonfatal AMI as the end point, the association vanished, thus emphasizing the specific impact of elevated tHcy levels on the fatal end points.

When one reviews the literature, it is apparent that men have been studied more extensively than women. In a recent meta-analysis,16 38 studies regarding coronary heart disease were included, 2 of which were designed as cohort studies, and both of which included men only. Six of 10 nested case-control prospective studies included only men. 3 studies included both genders pooled, and only 1 study analyzed men and women separately. Another aspect that distinguishes existing prospective studies is length of follow-up. Focusing on prospective coronary heart disease studies in which data regarding women separately are extractable, the follow-up range is 3 to 13 years in 4 studies.2,3,5,17 Ridker et al3 showed a 2-fold relative risk of any CVD event in women with high tHcy after 3 years of follow-up. Knekt et al5 found a significant relative risk among 45- to 64-year-old women with preexisting risk factors at baseline after 13 years of follow-up, but not for the whole group of women. Folsom et al2 found that after correction for confounders, only vitamin B6 was an independent protective factor against CVD during an average follow-up of 3.3 years. In a recent case-cohort study, de Bree et al17 did not find tHcy to be a risk factor for cardiovascular mortality either in men or in women initially aged 20 to 59 years with mean follow-up time of 10.3 years. This shortage of prospective studies on women and varying follow-up may explain in part some of the uncertainty in the literature. Particularly in middle-aged women, an extended follow-up time may be needed.

The main question when any association between a possible risk factor and a defined end point is assessed is to determine whether it represents a causal mechanism or is confounded by another factor or chance finding. The magnitude and statistical significance of the association in the present material make a chance finding less likely. The
determination of whether the association represents a causal mechanism also depends on the plausibility of the finding and how it fits with current knowledge, as recently discussed by Pasterkamp et al.\textsuperscript{18} and Christensen et al.\textsuperscript{19} The possibility that tHcy is not a causal factor for AMI but indicates an increased risk of complications might be supported by the weakened association after the first few years in other prospective studies.\textsuperscript{20–22} This could also be consistent with the present finding of a stronger association with fatal end points. However, this is not consistent with the fact that 15 years was needed in the present study to demonstrate the separation in the strata arms of the Kaplan-Meier plot.

To further investigate whether we were observing an effect of uncontrolled confounding by factors that are associated with both Hcy and AMI, we used a sequential modeling approach. The only exclusion criterion in this analysis was prior AMI. We recognize that a number of other medical conditions measured in the present study (eg, high blood pressure) may have affected the results. However, when controlling for age, traditional CVD risk factors, and tHcy-related variables, including dietary folate and s-creatinine, the results were stable throughout all 3 steps, which indicates that the tHcy association was not biased by these potential confounders. Although it remains plausible that Hcy exerts its strongest negative effects in subjects with preexisting atherosclerotic plaques, the present multivariate adjusted results do not support the view that elevated tHcy is simply a marker of preexisting CVD risk factors or renal dysfunction.

We were particularly interested in the possible mediative role of dietary Hcy determinants such as folate and whether this might explain the apparently adverse effect of Hcy, given the known correlation between dietary folate and laboratory tHcy measures in data from the present study and others.\textsuperscript{23,24} Neither serum B\textsubscript{12}, dietary folate, nor coffee consumption explained the association between tHcy and AMI. However, specific weaknesses of the dietary data must be noted. The dietary folate data were based on a single 24-hour recall method, which cannot possibly capture individuals’ usual

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Proportional hazard plot in relation to tHcy quintiles. Upper plot refers to AMI morbidity. Lower plot refers to mortality due to AMI. Dashed lines represent lower and upper 95\% CI. Gray line represents reference line corresponding to relative risk for first quintile (1). Both plots are drawn using full regression model (model 3) with correction for age, risk factors, and tHcy modifiers.}
\end{figure}
TABLE 2. Proportional Hazard Regression Analyses of Risk for Total and Fatal AMI Over 24 Years

<table>
<thead>
<tr>
<th>Variables Included</th>
<th>Quintile 5 vs Quintiles 1 Through 4*</th>
<th>Linear tHcy†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>RR for AMI (95% CI)</td>
<td>1.8 (1.1–2.8)</td>
</tr>
<tr>
<td></td>
<td>RR for death due to AMI (95% CI)</td>
<td>2.5 (1.3–4.8)</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, smoking, BMI, W/H ratio, triglyceride, cholesterol, blood pressure</td>
<td>RR for AMI (95% CI)</td>
<td>1.9 (1.2–3.1)</td>
</tr>
<tr>
<td></td>
<td>RR for death due to AMI (95% CI)</td>
<td>2.8 (1.4–5.6)</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, smoking, BMI, W/H ratio, triglyceride, cholesterol, blood pressure, B12, coffee, creatinine, dietary folate</td>
<td>RR for AMI (95% CI)</td>
<td>1.9 (1.1–3.3)</td>
</tr>
<tr>
<td></td>
<td>RR for death due to AMI (95% CI)</td>
<td>5.1 (2.2–11.9)</td>
</tr>
</tbody>
</table>

RR indicates relative risk; BMI, body mass index; and W/H, waist/hip.
Model 1 includes correction for age only; model 2, correction for age plus CVD risk factors; model 3, correction for age, risk factors, and known tHcy-level modifiers.

*The relative risk for study participants with tHcy values within the fifth quintile was compared to the rest, ie, participants with tHcy values within quintiles 1 through 4 with relative risk defined as 1. The lower limit for the fifth tHcy quintile was 14.18 μmol/L, and the median value within the fifth quintile was 16.75 μmol/L.
†Relative risk per 1-μmol/L s-homocysteine increase, tHcy analyzed as a continuous variable.

Inexpensive and safe tHcy-lowering treatment with combined vitamins B12, B6, and folate has shown promising potential of reducing secondary coronary events in a few recently published studies, which may have wider clinical application in the future. However, whether this is applicable to all vascular events remains to be shown. Interestingly, vitamin B12 deficiency, folate deficiency, and hyperhomocysteinemia have more recently been linked to risk for development of dementia, which may reflect its vascular etiology. In this context, tHcy analysis in everyday clinical practice appears preferable to both B12 and folate analyses because it captures possible deficiencies in either or both of the vitamins, as well as being a risk factor for CVD. In addition, such a strategy is less costly than analysis of levels of both vitamins.

Because risk for developing coronary heart disease is significantly lower for women than for men and does not reach levels comparable to those for males until very advanced ages, it is a special challenge to identify risk factors in free-living female populations. We are not aware of any other population-based, prospective studies of women that combined extended follow-up with analyses of frozen baseline serum samples that relate tHcy to health end points.

In conclusion, the present study found that a homocysteine level in excess of 14.2 μmol/L in middle-aged women is an independent risk factor for future myocardial infarction, particularly fatal events. The impact of slightly to moderately elevated homocysteine levels on myocardial infarction could be detected many years after the blood samples, subsequently analyzed for tHcy, were collected.

Acknowledgments

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