Hyperhomocysteinemia Is Inversely Related With Left Ventricular Ejection Fraction and Predicts Cardiovascular Mortality in High-Risk Coronary Artery Disease Hypertensives

Maurizio Cesari, Mario Zanchetta, Alberto Burlina, Luigi Pedon, Giuseppe Maiolino, Daniele Sticchi, Achille C. Pessina, Gian Paolo Rossi

Objective—The purpose of this study was to investigate the relationship of plasma homocysteine (tHcy) levels with coronary artery disease (CAD) and left ventricular ejection fraction (LVEF) in high-risk patients undergoing coronary angiography for suspected CAD.

Methods and Results—In 936 consecutive patients, we measured LVEF, tHcy, folate levels, and quantified CAD with a modified Duke Index score. We also genotyped patients at the methylene-tetrahydrofolate-reductase 677C→T polymorphism. Hyperhomocysteinemia (HHcy) was defined as tHcy levels ≥15.46 μmol/L; total and cardiovascular mortality was assessed at follow-up that lasted 43 months (median). CAD was confirmed in 75% of patients and ruled out in the rest (non-CAD group). No relationship of HHcy with either arterial hypertension or the CAD score was found. In contrast, there was a significant inverse relationship of tHcy with LVEF in arterial hypertensive but not in normotensive patients, regardless of previous myocardial infarction. At logistic regression, HHcy was the strongest predictor (P=0.001) of a low (<40%) LVEF, followed by type 2 diabetes mellitus and cigarette smoking. At follow-up, HHcy significantly predicted cardiovascular mortality but only in the arterial hypertension subgroup.

Conclusions—In arterial hypertensive but not in normotensive patients, HHcy predicts cardiovascular mortality and a low LVEF, independent of CAD and history of myocardial infarction. (Arterioscler Thromb Vasc Biol. 2005;25:115-121.)

Key Words: homocysteine ■ arterial hypertension ■ coronary artery disease ■ survival ■ MTHFR 677C→T polymorphism ■ left ventricular ejection fraction

Hyperhomocysteinemia (HHcy) might play a causative role in the development of coronary artery disease (CAD) and cardiovascular (CV) disease and originates from an interplay of environmental factors, such as folate and B₁₂ vitamin deficiency, and genetic factors, including the thermolabile variant (677C→T) of methylene-tetrahydrofolate-reductase (MTHFR) gene. On the basis of plasma homocysteine (tHcy) concentrations, HHcy has been classified as mild/moderate (15 to 30 μmol/L), intermediate (30 to 100 μmol/L), and severe (>100 μmol/L). Mild/moderate HHcy is common (5% to 7%) in the population and might increase the risk of CV disease, but its association with such phenotype is weaker than that of severe HHcy. Prospective studies in high-risk subjects suggested that HHcy can be a risk factor for overall mortality, recurrent CV events, and hospitalization, but studies of healthy subjects did not confirm this contention. Thus, it would appear that mild/moderate HHcy plays a minor role for CV disease and might attain a greater importance only in high-risk patients.

Theoretically, HHcy could increase blood pressure by decreasing the endothelial production of NO, inducing accumulation of the endogenous NO synthase inhibitor asymmetric dimethylarginine, or lowering NO bioactivity through induction of oxidative stress. Nonetheless, only a weak association between HHcy and arterial hypertension was found, and there are no data in high-risk patients. HHcy can exert nefarious effects on the heart by inducing coronary arteriolar remodeling, left ventricular (LV) hypertrophy, and interstitial fibrosis with concomitant diastolic dysfunction. In humans, tHcy has also been related to LV hypertrophy, albeit not consistently, and to the development of congestive heart failure. A significant relationship of tHcy with LV structure but not fractional shortening has also been reported. However, data on the impact of HHcy on LV function are limited and conflicting.

Thus, in the arterial hypertensive and normotensive patients of the Genetic and Environmental Factors in Coronary Atherosclerosis (GENICA) study, a prospective investiga-
tion of high-risk patients undergoing coronary artery angiography, we sought to clarify the relationship of HHcy with LV ejection fraction (LVEF) and the extent of CAD.

**Methods**

The general criteria for enrollment of patients and controls in the GENICA study, and the inclusion and exclusion criteria for this study are detailed in the online Methods (available at http://atvb.ahajournals.org). The study protocol was approved by the ethics committee, and written informed consent was obtained from each participant.

**Demographic and Clinical Measurements**

Information on history of CV events, current medications and concurrent conditions, and measurement of body mass index and blood pressure was obtained as described in detail (online Methods).

**Coronary Angiography**

Angiography and LVEF measurement were performed according to a standard method; the severity of CAD was graded with the Duke Prognostic Index modified to account for the impact of left main trunk stenosis (online Methods).

**Laboratory Measurements**

The laboratory techniques used are detailed in the online Methods.

**Longitudinal (Follow-Up) Study**

Follow-up was assessed by a committee that was unaware of the tHcy values on the basis of all available information (hospital records, physician records, death certificates, and direct contact with patients or their family doctor and relatives whenever necessary). Death was defined, according to the Syst-Eur Trial,29 as CV if sudden, resulting from congestive heart failure, acute myocardial infarction, or stroke.

**Statistical Analysis**

Quantitative variables were compared by one-way ANOVA followed by Bonferroni's test. The distribution of categorical variables and their agreement with the Hardy–Weinberg equilibrium were investigated by χ² analysis. Natural logarithm (ln) transformation of tHcy and folate values was undertaken for comparison of groups. To identify the determinants of tHcy, a stepwise (backward) regression analysis with inclusion and exclusion cutoffs of 0.09 and 0.10, respectively, was performed; the model with significant determinants was then used for adjustment of tHcy values. To determine the independent risk factors of CAD and a low (<40%) LVEF, logistic regression analysis with the backward stepwise selection (Wald criterion) for the effect of the 677C→T MTHFR variant and the other CV risk factors was used (details of the coding of the variables are available in the online Methods). Significance was set at P<0.05.

Comparison of receiver operator characteristic (ROC) curves investigating the usefulness of HHcy and the score of CAD as predictors of a low LVEF was performed with MedCalc (version 7.3.0.0; MedCalc Software). CV death rates and comparisons of survival curve were estimated by Kaplan–Meier and with the log-rank test. Cox stepwise (backward) regression analysis was also used to determine the relationships between clinical variables (gender, age, ejection factor, creatinine, and tHcy), MTHFR genotypes, and CV death at follow-up period.

**Results**

**Demographic Characteristics and CAD Findings**

In healthy subjects, the tHcy values distribution was positively skewed (skewness index 2.43±0.08), and the median (range) tHcy value was 9.90 μmol/L (6.41 to 26.50). Thus, the 90th percentile of the tHcy distribution (ie, 15.46 μmol/L) was used as cutoff to define HHcy. The skewed distribution was seen also in the patients; therefore, comparison of tHcy values between groups was undertaken after ln transformation. We investigated 936 patients originally recruited in the GENICA study. Of these, 25% had normal coronary arteries (eg, a CAD score of 0 [non-CAD]), and the rest showed significant CAD (Table I, available online at http://atvb.ahajournals.org). Overall arterial hypertension was present in 566 (60%) patients; the hypertensives entailed 63% of CAD versus 53% of non-CAD (χ²=6.80; P=0.009). Patients with arterial hypertension were receiving treatment with at least one antihypertensive agent of the following class: 59% angiotensin-converting enzyme inhibitors, 42% calcium entry blockers, 40% β-blockers, 38% diuretics, 7% sartans, and 6% α₁-blockers.

The Table shows the main features of patients divided according to the presence or absence of CAD and arterial hypertension. CAD patients were older than non-CAD and showed higher plasma levels of triglycerides, total/high-density lipoprotein (HDL)-cholesterol ratio, and glucose in the arterial hypertensive and normotensive cohorts. The prevalence of the CV risk factors in the patients classified according to quartiles of CAD score is shown in Table II (available online at http://atvb.ahajournals.org). Significant differences of gender, rates of arterial hypertension, hypertriglyceridemia, cigarette smoking, and type 2 diabetes mellitus were seen across quartiles of CAD. In contrast, no differences of MTHFR genotypes distribution were seen.

**tHcy Levels and Their Determinants**

No differences of raw tHcy and folate levels across groups were found (Table). Regression analysis allowed identification of serum creatinine (P<0.0001), the 677C→T MTHFR genotype (P<0.0001), plasma folate levels (P<0.0001), LVEF (P=0.001), and age (P=0.032) as the predictors of tHcy, overall accounting for 14.4% of tHcy variance. A model with these covariates was used to calculate adjusted tHcy values in individual patients. This adjustment allowed identification of a slight but significant (P<0.05) difference of tHcy between non-CAD and CAD arterial hypertensive patients (Table).

**Prevalence of HHcy and Association With Arterial Hypertension and CAD Severity**

Overall, 79% of patients had normal tHcy, 18% had mild/moderate HHcy, and 3% had intermediate HHcy, as defined previously. The prevalence of mild/moderate and severe HHcy was similar in arterial hypertensive and normotensive (22% versus 21%; NS) patients, even when they were divided according to the extent of CAD (Table II) or when adjusted tHcy values were used.

**Association of tHcy With LVEF**

A low (<40%) LVEF was found in 92 (9.8%) patients. LVEF was a significant predictor of tHcy at a stepwise regression analysis. Correlation analysis showed that LVEF was significantly and inversely related with tHcy in arterial hypertensive (r=−0.189; P<0.0001) but not in normotensive patients (r=−0.078; NS). This inverse relationship also remained significant in the arterial hypertensive patients when they
were split into those with and without previous myocardial infarction (Figure 1). HHcy was the strongest predictor of a low LVEF in the whole population (β=0.772; P=0.001; odds ratio [OR], 2.16 [1.36 to 3.44]), followed by type 2 diabetes mellitus (β=0.607; P=0.011; OR, 1.83 [1.15 to 2.92]) by logistic regression analysis. In contrast, smoking, CAD score, male gender, low HDL-cholesterol, arterial hypertension, being overweight, hypercholesterolemia, and age >60 years did not enter in the model (ie, they were not useful predictors of a low LVEF).

Knowledge of tHcy, smoking, and type 2 diabetes mellitus status overall allowed a correct classification of patients in the low or not low LVEF class in 89% of cases. At ROC curve analysis, the area under the HHcy ROC curve for identifying a low (<40%) LVEF in hypertensive patients (n=566) was 0.624±0.043 (95% CI, 0.582 to 0.665; ie, significantly greater not only than that under the identity line but also than that under the score of CAD; area under the curve [AUC]=0.513±0.042; 95% CI, 0.002 to 0.221; difference between AUC=0.111±0.056; P=0.05). The latter did not differ from that under the identity line. Therefore, in the arterial hypertension subgroup, knowledge of tHcy was useful for identifying patients with low LVEF but not those with CAD.

### MTHFR Genotype
The MTHFR 677C→T allele distribution was consistent with the Hardy–Weinberg equilibrium in arterial hypertensive and normotensive patients, with and without CAD. There were no differences of genotypes frequency between CAD/non-CAD, across quartiles of CAD score (Table II), and between the groups with and without arterial hypertension (Table). Analysis of covariance showed a significant effect MTHFR...
**Figure 2.** The graph shows the tHcy levels adjusted for significant covariates (see text for explanation) of the patients divided by MTHFR 677C→T genotype and into quartiles of LVEF. Analysis of covariance showed a significant effect of MTHFR 677C→T genotype, folate status, and LVEF (all \( P<0.001 \)) on tHcy. Patients were therefore split into those below (left) and above (right) the overall median of plasma folate. In both subgroups, the TT homozygous had higher tHcy than the other genotypes. There was a stepwise increase of tHcy with decreasing LVEF quartile that was particularly evident in the subgroup below the overall median of plasma folate.

677C→T genotype, folate status, and LVEF (all \( P<0.001 \)) on tHcy. Therefore, to further investigate whether folate affected the relationship between tHcy and LVEF, we split our patient population according to the overall median (10.31 nmol/L) of plasma folate values. We found that in all LVEF quartiles, tHcy values were higher (\( P<0.001 \)) in the TT homozygous compared with the other MTHFR genotypes and within each genotype in the patients whose plasma folate levels were below the median value compared with those above this cutoff. The stepwise increase of tHcy with decreasing LVEF quartile, which was particularly evident in the subgroup below the overall median of plasma folate, is shown in Figure 2.

**Effect of HHcy and MTHFR Genotypes on Survival**

The median duration of follow-up was 43 months, and complete information could be obtained in 888 (94.8%) patients. The CV mortality rate was high in the normotensive patients. The CV mortality rate was significant lower survival from CV death (Kaplan–Meier analysis, patients with HHcy had a significantly (7.9%) and in the arterial hypertensive (9.4%) patients. At follow-up, the CV mortality rate was high in the normotensive patients. The majority (89%) had a normal LVEF. We used state-of-the-art techniques for genotyping the patients for the common MTHFR 677C→T single-nucleotide polymorphism (SNP), measuring LVEF, tHcy, folate, and quantifying the coronary artery atherosclerotic burden. Thus, we could establish the prevalence of HHcy and the relationship of tHcy, folate, and the MTHFR 677C→T genotype with the CV phenotypes CAD and LVEF with unprecedented accuracy in high-risk patients.

**Prevalence of HHcy**

The GENICA study entailed mainly high-risk patients because 96% of them were in the highest class of risk according to the Adult Treatment Panel III criteria; however, the majority (89%) had a normal LVEF. We used state-of-the-art techniques for genotyping the patients for the common MTHFR 677C→T single-nucleotide polymorphism (SNP), measuring LVEF, tHcy, folate, and quantifying the coronary artery atherosclerotic burden. Thus, we could establish the prevalence of HHcy and the relationship of tHcy, folate, and the MTHFR 677C→T genotype with the CV phenotypes CAD and LVEF with unprecedented accuracy in high-risk patients.

**Discussion**

The GENICA study entailed mainly high-risk patients because 96% of them were in the highest class of risk according to the Adult Treatment Panel III criteria; however, the majority (89%) had a normal LVEF. We used state-of-the-art techniques for genotyping the patients for the common MTHFR 677C→T single-nucleotide polymorphism (SNP), measuring LVEF, tHcy, folate, and quantifying the coronary artery atherosclerotic burden. Thus, we could establish the prevalence of HHcy and the relationship of tHcy, folate, and the MTHFR 677C→T genotype with the CV phenotypes CAD and LVEF with unprecedented accuracy in high-risk patients.

**Prevalence of HHcy**

The A First important finding of this study is the recognition that HHcy is common in white patients referred for CAD because 18% and 3% of them had mild/moderate and intermediate HHcy, respectively. It is noteworthy that these prevalence rates fell sharply after tHcy adjustment for the covariates that clustered with HHcy (serum creatinine, the 677C→T MTHFR genotype, plasma folate levels, LVEF, and age). Unexpectedly, even despite the large sample size and high HHcy prevalence rate, we found no association of either HHcy or tHcy with CAD severity, in contrast with some studies but not all reports. Indeed, in the study that first described an association of tHcy with subsequent mortality, tHcy correlated only weakly with CAD extent in the cohort of patients who had coronary artery angiography, suggesting that: (1) tHcy can affect survival independent of development of CAD, and (2) the determinant of coronary atherosclerotic burden and of CV death differ, although they might have some overlap.

**Association of HHcy With Arterial Hypertension**

The Third National Health and Nutrition Examination Survey provided evidence for an independent positive association of blood pressure with tHcy. Studies also found a correlation of tHcy with blood pressure, only with systolic blood pressure, and no correlation whatever after adjustment for confounders. Thus, data are conflicting and mostly confined to low-risk patients. We found no differences of tHcy or...
HHcy rate between patients with and without arterial hypertension in the group as a whole and in CAD and non-CAD patients. Borderline significantly higher tHcy values were seen in CAD arterial hypertensive patients compared with CAD normotensive patients but only after adjustment of tHcy values for confounders. We also found no differences of MTHFR genotypes between non-CAD and CAD patients or between arterial hypertensive and normotensive patients. Accordingly, our results do not support the contention that a genetic susceptibility to chronic HHcy, which can impair endothelium-dependent vasodilatation, involves a higher risk of arterial hypertension.

**Relationship of HHcy With LV Ejection Fraction**

Previous data on the relationship of LVEF and tHcy were limited and conflicting: in elderly CAD patients, an inverse relation was reported, but a positive \( r=0.13; P<0.001 \) or no correlation was also described. Accordingly, our detection of an inverse relationship between tHcy and LVEF is novel and might explain the association of HHcy with CV mortality, inasmuch as LVEF is a powerful determinant of survival.

HHcy not only resulted to be inversely related with LVEF but also emerged as the strongest \( P=0.001 \) determinant of a low \(<40\%\) LVEF at logistic regression analysis. Indeed, comparative ROC curve analysis showed that HHcy but not the CAD score was useful for predicting a low LVEF in arterial hypertensive patients. It is noteworthy that in the arterial hypertensive but not normotensive patients, the inverse relation between LVEF and tHcy was seen, regardless of previous myocardial infarction (Figure 1), thus suggesting that arterial hypertension rather than loss of cardiomyocytes is crucial for this negative association. Thus, our finding fills a gap of information and suggests that arterial hypertension may act as a factor unmasking, or perhaps favoring, the onset of a low LVEF.

The relationship of tHcy with LVEF was by no means weak, suggesting that HHcy could be only a minor determinant of LV function. However, this might not be the case because: (1) individuals prone to HHcy (see below) can have a lifelong exposure to surges of HHcy with dietary methionine intake or with vitamin deficiency, and therefore the adverse impact of HHcy on LVEF may build up over the years; and (2) this correlation emerged from a cross-sectional type of study that is typically exposed to the risk of underestimating rather than overestimating the effect of relevant predictors because of regression dilution bias. The importance of HHcy as a determinant of LVEF was supported recently by the observation that it predicted the onset of congestive heart failure, CV mortality, and a low LV fractional shortening in a cohort of the Framingham Heart Study that was free of heart disease. Experimental evidence for a direct negative effect of HHcy on LV function exists. Furthermore, chronic HHcy was shown recently to induce LV hypertrophy, perivascular and interstitial collagen deposition, and impaired diastolic function, thus suggesting that HHcy can increase CV mortality via its adverse effects on the LV. Regrettably, we could measure neither LV mass nor LV collagen in our population and therefore could not rule out the hypothesis that the inverse relationship of HHcy with LVEF was attributable to the concomitance of LV hypertrophy or fibrosis. Furthermore, our results did not establish a mechanistic link between HHcy and LV dysfunction.

**MTHFR Genotypes, CAD, and LV Ejection Fraction**

The results obtained with the MTHFR 677C→T genotypes and folate measurement are of additional interest. While confirming the findings by Brilakis et al that this SNP did not affect either CAD per se, they would also suggest the lack of effect of the SNP on LVEF. However, we found that under low folate intake, the genetic predisposition to higher tHcy values translated into HHcy and was associated to a stepwise decrease of LVEF (Figure 2). Thus, these results document for the first time a MTHFR gene–environment interaction that might adversely impact on LV function in humans.

**Effect of HHcy and MTHFR Genotype on Survival**

We gathered follow-up information on all the patients and recorded a substantial number of CV deaths (eg, a 7.9\% CV mortality rate in the normotensive patients and 9.4\% in the arterial hypertensive patients). At Kaplan–Meier analysis, CV death-free survival was significantly associated with lower tHcy but not with MTHFR genotypes in the whole population \( P=0.0003 \) and in the arterial hypertensive patients \( P=0.0012 \) but not in the normotensive patients (NS; Figure 3). At Cox regression analysis with multivariate adjustment for major CV risk factors, the independent predictors of event-free survival were age \( P<0.0001 \), LVEF \( P<0.0001 \), CAD score \( P=0.005 \), and hypercholesterolemia \( P=0.034 \). Similar results were obtained when ongoing medical treatment was considered. Interestingly, tHcy became a significant, albeit weak \( P=0.06 \), independent predictor of event-free survival, along with age \( P<0.0001 \), CAD score \( P=0.005 \), and hypercholesterolemia \( P=0.046 \), only after forcing the LVEF out of the model. Thus, these results not only confirm that tHcy predicts overall mortality but, more importantly, indicate that it foretells CV mortality in a mixed-gender, high-risk population, in keeping with data in healthy women of the Population Study of Women of Gothenburg. Moreover, the adverse prognostic impact of HHcy was seen in arterial hypertensive patients and can be associated with a lowering of LVEF through mechanisms that deserve further investigation.

**Conclusions**

In consecutive high-risk patients referred for coronary artery angiography, HHcy was common but not associated with the coronary atherosclerotic burden or with arterial hypertension. Moreover, HHcy predicted CV mortality and a low LVEF in arterial hypertension but not in normotensive patients, regardless of CAD severity or history of myocardial infarction. Given the important prognostic role of LVEF, these results, along with experimental data, suggest that HHcy might nefariously affect CV outcome, at least in part, via its adverse effect on the left ventricle.
Acknowledgments

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HYPERHOMOCYSTEINEMIA IS INVERSELY RELATED WITH LEFT VENTRICULAR EJECTION FRACTION AND PREDICTS CARDIOVASCULAR MORTALITY IN HIGH-RISK CORONARY ARTERY DISEASE HYPERTENSIVES

Maurizio Cesari, MD; Mario Zanchetta, MD; Alberto Burlina, MD; Luigi Pedon, MD; Giuseppe Maiolino, MD; Daniele Sticchi, BScD; Achille C. Pessina, MD, PhD; Gian Paolo Rossi, MD, FACC, FAHA.

DATA SUPPLEMENTS

METHODS

The criteria for enrolment of patients and controls in the GENICA study were already reported and will be briefly summarized. Consecutive Caucasian patients of both genders referred for coronary artery angiography for investigation of chest pain and/or suspected CAD were enrolled. Conditions that might affect tHcy levels, such as recent onset renal insufficiency, acute myocardial infarction and folic acid, vitamin B<sub>12</sub> or B<sub>6</sub> supplements, as well as the refusal to participate in this study, represented exclusion criteria. The study protocol was approved by the Ethics Committee and a written informed consent was obtained from each participant. A group of 101 consecutive healthy Caucasian normotensive blood donors with the same ethnic background, enrolled during the same period from the blood bank of the same hospital, served for establishing the normal tHcy range. These subjects were selected with a negative family history of CAD, myocardial infarction, and stroke, non-smoking status, absence of hypercholesterolemia, hypertriglyceridemia and type II diabetes mellitus. For ethical reasons they did not undergo coronary artery angiography; however, based on epidemiological studies, a cohort fulfilling these inclusion criteria is expected to have a very low prevalence of asymptomatic CAD.

Demographic and clinical measurements
History of CV events and information on current medications, smoking habits, presence/absence of arterial hypertension, type II diabetes mellitus, hypercholesterolemia, and hypertriglyceridemia were gathered with a standard questionnaire that was administered by a study staff. The definitions of arterial hypertension, current smokers, non-smokers and ex-smokers; having type II diabetes mellitus and impaired glucose tolerance; hypercholesterolemia and hypertriglyceridemia and overweight or obesity were as described.\(^1\) Body mass index (BMI) was calculated as weight/height\(^2\) (Kg/m\(^2\)). Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase V for diastolic.\(^1\)

**Coronary angiography**

Angiography was carried out by experienced interventional cardiologists. LVEF was determined according to a standard method.\(^2\) The severity of CAD was graded independently by two observers (M.Z. and L.P.), who were blinded to patient’s tHcy, folate levels and MTHFR genotype, on an ordinal scale where 0% corresponded to no stenosis and 100% to total vessel occlusion (Table 1). The percentage of stenosis was derived by the mean of the two estimates if they differed by less than 20%; any between-observer disagreement exceeding this threshold was resolved by consensus. To score the atherosclerotic burden we used the Duke Prognostic Index,\(^3\) modified to account for the impact of left main trunk stenosis.\(^4\) This index was shown to accurately predict five years mortality of medically treated patients.\(^5\)

**Laboratory measurements**

Patients were studied between 8.30 and 12.00 a.m. Blood samples were taken before coronary angiography, immediately put on ice and centrifuged.\(^1\) Total and HDL-cholesterol, triglycerides, glucose, sodium, potassium, blood urea nitrogen (BUN), and creatinine levels were measured with conventional methods. Plasma folate levels were determined with a chemiluminescent method (Bayer–ADVIA Centauri\(^\text{TM}\), Milan, Italy) and total (free plus protein-bound) tHcy levels by high-performance liquid chromatography (HPLC).\(^6\) Accuracy and precision of tHcy measurement are validated by ERNDM (European quality control program of laboratory measurements within the
HHcy was defined as tHcy value above the 90th percentile of the distribution in healthy subjects and was classified as moderate (15 to 30 µmol/L), intermediate (30-100 µmol/L), and severe >100 as previously described. DNA was extracted with standard methods; genotyping at the MTHFR 677C→T polymorphism was performed with LightCycler™ (Roche, Milan) and melting curve analysis with allele-specific FRET (fluorescence energy transfer) probes. This method was 100% accurate when compared to sequencing. Primer and probes sequences are available from the corresponding author upon written request.

**Longitudinal (follow-up) study**

Follow-up was assessed by a committee (GPR, GM, DS), that was unaware of 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). Deaths was defined according to the Syst-Eur Trial, as CV if sudden, due to congestive heart failure, acute myocardial infarction or stroke.

**STATISTICAL ANALYSIS**

Quantitative variables were compared by one-way ANOVA followed by Bonferroni’s test. The distribution of categorical variables, including MTHFR genotypes and their agreement with the Hardy-Weinberg equilibrium were investigated by chi-square analysis. Natural logarithm (ln) transformation of tHcy and folate values was undertaken for comparison of groups. To identify the determinants of tHcy, a stepwise (backward) regression analysis with inclusion and exclusion cutoffs of 0.09 and 0.10, respectively, was performed; the model with significant determinants was then used for adjustment of tHcy values. To determine the independent risk factors of CAD and a low (<40%) LVEF, logistic regression analysis with the backward stepwise selection (Wald criterion), for the effect of the 677C→T MTHFR variant, and the other CV risk factors, was used. To this end independent variables were coded as follows: age: 0 for <60 years, 1 for ≥ 60 years; body mass index: 0 for <26 kg/m², 1 for ≥ 26 kg/m². Absence or presence of arterial hypertension, type II diabetes mellitus and LDL- cholesterol ≥100 mg/dl were coded as 0 and 1 respectively.
Cigarette smoking was coded as 0 for non-smoker and 1 for current or ex-smoker; 677C→T: 0 for TT, 1 for CT and TT. The dependent variable was coded as: 0 for a CAD score = 0; 1 for a CAD score ≥0; LVEF<40% = 0 and ≥40% = 1. Odds ratios were calculated as a measure of the association between the risk factors and the CAD or low LVEF phenotype, with the effect of the mutant T allele for the 677C→T MTHFR polymorphism assumed to be recessive (TT vs. CT and CC). Significance was set at \( p < 0.05 \). Comparison of Receiver Operator Characteristics (ROC) curves investigating the usefulness of HHcy and the score of CAD as predictors of a low LVEF was carried out with MedCalc (vers. 7.3.0.0., MedCalc Software, Mariakerke, Belgium). CV death rates and comparisons of survival curve were estimated by Kaplan-Meier and with the log-rank test. Cox stepwise (backward) regression analysis was also used to determine the relationships between clinical variables (gender, age, EF, creatinine, tHcy), MTHFR genotypes, and CV death at follow-up period.

References


Table I: Classification of the CAD burden according to a modified Duke Index Prognostic Score and distribution of patients in the different score groups.

<table>
<thead>
<tr>
<th>Extent of CAD</th>
<th>Prognostic Weight (0-100)</th>
<th>Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CAD ≥ 50%</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>1-vessel disease 50%-74%</td>
<td>19</td>
<td>6.7</td>
</tr>
<tr>
<td>&gt;1-vessel disease, 50% to 74% or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-vessel disease, (75%)</td>
<td>23</td>
<td>16.6</td>
</tr>
<tr>
<td>1-vessel disease, (≥ 95%)</td>
<td>32</td>
<td>9.2</td>
</tr>
<tr>
<td>2-vessel disease</td>
<td>37</td>
<td>13.9</td>
</tr>
<tr>
<td>2-vessel disease, (both ≥ 95%)</td>
<td>42</td>
<td>2.4</td>
</tr>
<tr>
<td>1-vessel disease, ≥ 95% proximal LAD or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-vessel disease, ≥ 95% LAD</td>
<td>48</td>
<td>8.4</td>
</tr>
<tr>
<td>2-vessel disease, ≥ 95% proximal LAD or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-vessel disease</td>
<td>56</td>
<td>4.1</td>
</tr>
<tr>
<td>3-vessel disease, ≥ 95% in at least one</td>
<td>63</td>
<td>4.8</td>
</tr>
<tr>
<td>3-vessel disease, 75% proximal LAD</td>
<td>67</td>
<td>3.6</td>
</tr>
<tr>
<td>3-vessel disease, ≥ 95% proximal LAD</td>
<td>74</td>
<td>2.1</td>
</tr>
<tr>
<td>Left main (75%)</td>
<td>82</td>
<td>2.8</td>
</tr>
<tr>
<td>Left main (≥ 95%)</td>
<td>100</td>
<td>0.4</td>
</tr>
</tbody>
</table>

CAD = coronary artery disease; LAD = left anterior descending coronary artery.
Table II: Prevalence of CV risk factors in the patients classified by quartiles of CAD score

<table>
<thead>
<tr>
<th></th>
<th>1 (n= 232)</th>
<th>2 (n= 213)</th>
<th>3 (n= 244)</th>
<th>4 (n= 247)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender (%)</td>
<td>51</td>
<td>77</td>
<td>86</td>
<td>83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Arterial hypertension (%)</td>
<td>53</td>
<td>63</td>
<td>59</td>
<td>67</td>
<td>=0.026</td>
</tr>
<tr>
<td>Hypercholesterolemia (%)</td>
<td>81</td>
<td>83</td>
<td>85</td>
<td>90</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertriglyceridemia (%)</td>
<td>30</td>
<td>45</td>
<td>47</td>
<td>44</td>
<td>=0.001</td>
</tr>
<tr>
<td>Cigarette Smoking (%)</td>
<td>37</td>
<td>63</td>
<td>72</td>
<td>62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Overweight (%)</td>
<td>54</td>
<td>55</td>
<td>57</td>
<td>54</td>
<td>NS</td>
</tr>
<tr>
<td>NIDDM (%)</td>
<td>16</td>
<td>20</td>
<td>27</td>
<td>34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HHcy (%) (moderate/intermediate)</td>
<td>22/2</td>
<td>15/4</td>
<td>18/3</td>
<td>20/2</td>
<td>NS</td>
</tr>
<tr>
<td>MTHFR 677C→T genotype (%) (TT/TC/CC)</td>
<td>22/44/34</td>
<td>20/44/36</td>
<td>22/44/34</td>
<td>19/45/36</td>
<td>NS</td>
</tr>
</tbody>
</table>

Differences in the distribution of the categorical variables was tested by χ² analysis. NIDDM= type II diabetes mellitus