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Hyperhomocystinemia in patients with coronary artery disease

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Abstract

Hyperhomocystinemia has been related to an increased risk of cardiovascular disease in several studies. The C677T polymorphism for the gene that encodes the methylenetetrahydrofolate reductase enzyme (MTHFR) and low plasma folate levels are common causes of hyperhomocystinemia. Due to differences in nutritional patterns and genetic background among different countries, we evaluated the role of hyperhomocystinemia as a coronary artery disease (CAD) risk factor in a Brazilian population. The relation between homocysteine (Hcy) and the extent of CAD, measured by an angiographic score, was determined. A total of 236 patients referred for coronary angiography for clinical reasons were included. CAD was found in 148 (62.7%) patients and 88 subjects had normal or near normal arteries. Patients with CAD had higher Hcy levels [mean (SD)] than those without disease (14 (6.8) *vs* 12.5 (4.0) μM; P = 0.04). Hyperhomocystinemia (Hcy >17.8 μM) prevalence was higher in the CAD group: 31.1 *vs* 12.2% (P = 0.01). After adjustment for major risk factors, we found an independent association between hyperhomocystinemia and CAD (OR = 2.48; 95% CI = 1.02-6.14). Patients with a more advanced coronary score had a higher frequency of hyperhomocystinemia and tended to have higher mean Hcy levels. An inverse relation between plasma folate and Hcy levels was found (r = -0.14; P = 0.04). Individuals with the MTHFR C677T polymorphism had a higher prevalence of hyperhomocystinemia than those without the mutated allele. We conclude that hyperhomocystinemia is independently associated with CAD, with a positive association between Hcy level and disease severity.

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- Folic acid deficiency

Introduction

Atherosclerosis is the leading cause of mortality in the Western world. In most patients, traditional risk factors such as dyslipidemia, hypertension, smoking, and diabetes can be identified. The reduction of cardiovascular events can be achieved by controlling these factors. Nevertheless, the prevention of disease progression and new events in patients with established coronary artery disease (CAD) and no "known" risk factors is still a great challenge. Therefore, there is a continuous search for new risk factors that

could explain the disease in these patients; hyperhomocystinemia is one of them.

Homocysteine (Hcy) is a sulfhydryl amino acid involved in methionine metabolism. The detrimental effect of severe hyperhomocystinemia on the cardiovascular system was first described by McCully (1) in 1969. He reported diffuse atherosclerotic lesions in a post-mortem study of two children with homocystinuria, an inborn error of Hcy metabolism in which extremely high plasma Hcy levels are found. Fatal events before the age of 30 occur in 25% of these patients (2).

The harmful effect of hyperhomocystinemia on the vascular system has been confirmed by experimental studies in animals (3). After the introduction of reliable routine methods for Hcy determination, clinical studies showed the association of mild to moderate hyperhomocystinemia not only with coronary disease (4-6), but also with stroke (7) and peripheral artery disease (8).

Plasma Hcy concentration is dependent on some nutritional and genetic factors. A low plasma folate level is a common cause of hyperhomocystinemia, but vitamin B_{12} deficiency may also play a role. Folate is a substrate for the remethylation cycle of Hcy, and vitamin B_{12} is a co-factor in its metabolism. Folate supplementation is an effective therapy for Hcy normalization (9).

A common polymorphism for the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is related to mild to moderate elevation of plasma Hcy levels. This enzyme converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, required for the conversion of Hcy to methionine. The C677T polymorphism results in an amino acid change from alanine to valine. This substitution causes thermolability and reduction of enzyme activity (10).

Since genetic and nutritional differences are present among different countries, it is reasonable to assume that Hcy may play a different role as a coronary risk factor according to the population studied. To the best of our knowledge, no study assessed the relation between Hcy and coronary disease in any Latin American country, a developing region susceptible to nutritional deficiencies (11). Although the prevalence of malnutrition is decreasing in Brazil, some diseases like anemia, highly dependent on iron, folate and vitamin B_{12} intake are now reaching epidemic levels (12).

The aim of the present cross-sectional study was to evaluate the role of hyperhomocystinemia as a coronary risk factor in a Brazilian population. Moreover, using an angiographic index, we evaluated the association between plasma Hcy level and the extent of coronary disease.

Patients and Methods

Patients

On the assumption that up to 25% of patients would have normal or near normal arteries upon an angiogram, sample size was calculated with $\alpha = 0.05$ and a power of 90% to detect a 16% difference in hyperhomocystinemia prevalence. So, 236 patients referred to the Heart Institute for a selective coronary angiogram were enrolled. All patients had been previously seen by their attending physician, and the angiography was requested based on the clinical judgment of the latter. Patients whose coronary angiogram was requested for a reason other than suspected coronary disease were also included. We excluded patients younger than 18 years, with acute coronary syndrome in the last 30 days, those who had taken any medication containing vitamins or folic acid in the 6 weeks before enrollment, and patients with creatinine above 1.5 mg/dL.

A questionnaire with clinical data was completed by all patients on the day of enrollment. Blood pressure, weight and height were recorded. All measurements were performed by the same research nurse, using

the same technique and always the same calibrated equipment. The study was approved by the institutional Ethics Committee and written informed consent was obtained from all patients.

Coronary angiography

Angiograms were evaluated by an experienced angiographer blind to the clinical data. CAD was considered to be present when any stenotic lesion ≥40% in any of major epicardial coronaries and their branches was observed. Subjects with normal or near-normal arteries (no lesion greater than 40%) formed a control group.

All angiograms were scored according to the Friesinger index (13). This index ranges from 0 (completely normal arteries) to 15. Each of the 3 arteries (right coronary, circumflex and anterior descending artery), with their major branches, was analyzed independently and scored from 0 to 5. The final score was the sum of the results for each artery. An artery with no wall irregularity was scored as 0. Score 1 was determined by parietal irregularities, less than 30%. If the artery had a single stenotic lesion causing a narrowing of less than 70%, the score was 2. The same degree of obstruction, but at more than one specific site of the artery, was scored as 3. An artery with any lesion greater than 70% was scored as 4. A score of 5 was assigned when complete occlusion of the proximal right coronary, circumflex or anterior descending artery was found. Lesions in the left main coronary were assessed using the same scoring system, but doubled (the lesion was considered in two arteries).

Measurement of plasma homocysteine

Blood samples were collected before angiography, after a 12-h fast. The samples were kept on ice and immediately centrifuged at 0ºC to obtain plasma. Plasma Hcy levels were quantified by high-performance liquid chromatography using a Shimadzu Class-Vp System. The technique described by Fiskerstrand et al. (14) was used.

Genetic analysis

Genotyping for the C677T polymorphism of MTHFR was performed by PCR amplification. The reaction primers have been described elsewhere (15). The resulting fragments were separated by 1.5% agarose gel electrophoresis.

Definitions of risk factors

Age was evaluated as a continuous and categorical variable. Hypertension was defined as a history of systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg, confirmed by measurement at enrollment, or antihypertensive therapy. Patients were considered to be smokers when regularly using tobacco for the last 6 months. Diabetes was defined as fasting glucose above 126 mg/dL or use of hypoglycemic drug therapy. Hyperlipidemia was present if total cholesterol >240 mg/dL, or LDL-cholesterol >160 mg/dL or if the patient was on lipid-lowering therapy.

Hyperhomocystinemia was defined according to values found in patients with normal or near-normal coronary arteries. We defined hyperhomocystinemia as any value above the 90th percentile for this group.

Statistical analysis

Continuous variables are reported as means ± SD. Categorical variables are presented as percentages. Log transformation was used for analysis of skewed data (Hcy). Univariate (2-sided *t*-tests and chi-square tests, Pearson's correlation) analyses were performed when appropriate. Odds ratios (OR) and 95% confidence interval (95% CI) were calculated by standard formulas. To determine whether the association of CAD

and hyperhomocystinemia was modulated by any other variable, we used multiple logistic regression analysis.

Results

A total of 236 patients had undergone angiography. Most were referred for a coronary angiogram for coronary disease investigation (83.9%). Heart failure was the reason for the angiographic study in 6.8%, valve disease in 6.3% and other reasons in 3% of cases. Only 13 patients (5.5%) had no risk factor for CAD. One risk factor was present in 72 patients (30.5%), while 86 (36.5%) had two risk factors, 56 (23.7%) had three, and 9 (9.8%) had all of them.

Coronary angiograms showed 88 subjects with no stenotic lesion >40%, a result considered to indicate normal or near-normal arteries. One hundred and forty-eight patients (62.7%) had coronary disease (at least one stenotic lesion >40% in a major epicardic coronary).

Table 1. Clinical characteristics of 236 patients with and without coronary artery disease (CAD).

Characteristics	CAD (N = 148)	Normal or near-normal arteries (N = 88)	P
Clinical			
Age (years)	58.6 (10.9)	54.7 (12)	0.01
Male sex	98 (66%)	37 (42%)	<0.01
Caucasian	101 (79%)	58 (66%)	0.02
BMI (kg/m^2)	28.4 (5)	27.5 (5)	ns
Normal	32 (22%)	27 (30.7%)	ns
Overweight	64 (43%)	21 (23.7%)	<0.01
Obese	43 (29%)	28 (31.8%)	ns
Hyperlipidemia	101 (68%)	33 (37.5%)	<0.001
Diabetes	53 (36%)	17 (19%)	0.01
Smoking	45 (30%)	17 (19%)	0.06
Hypertension	109 (74%)	73 (83%)	ns
Biochemical			
Cholesterol (mg/dL)	220.8 (50.7)	204.3 (36.1)	0.01
HDL (mg/dL)	36.0 (11)	42.9 (13.5)	<0.01
LDL (mg/dL)	150.9 (45.6)	132.2 (31.9)	<0.01
Triglycerides (mg/dL)	169.3 (89.9)	152.0 (88.1)	ns
Glucose (mg/dL)	124.4 (56.7)	107.1 (42.7)	0.01
Nutritional			
Vitamin B12 (pg/ml)	488.3 (399)	434 (287)	ns
Serum folate (ng/dL)	7.4 (3.3)	7.7 (3.6)	ns

Data are reported as means (SD) for continuous variables and numbers (%) for categorical variables, and were analyzed statistically by 2-sided *t*-test and chi-square test, respectively. ns = nonsignificant.

Characteristics of the patients

Patients with CAD were older than those without significant coronary abnormalities (58.6 *vs* 54.7 years; P = 0.01), and were predominantly males. Body mass index was similar in both groups, despite a higher prevalence of overweight subjects among CAD patients. The prevalence of traditional risk factors was different in the two groups; hyperlipidemia was found in 68% of patients with CAD and in 37.5% of those without CAD (P < 0.001). There was also a significant difference regarding diabetes (36 *vs* 19%; P = 0.01) and a trend toward a higher frequency of smoking among patients with CAD. No difference was observed regarding hypertension.

In relation to biochemical characteristics, plasma levels of total cholesterol and LDL-cholesterol were higher in patients with CAD, whereas the inverse relation was seen with HDL-cholesterol levels. Plasma glucose levels were also higher in patients with CAD. No statistically significant difference was found in vitamin B_{12} or plasma folate levels between groups. Clinical and biochemical features are listed in Table 1.

Homocysteine

Mean plasma Hcy level and prevalence of hyperhomocystinemia were higher in patients with CAD compared to those with normal or near-normal arteries: Hcy level [mean (SD)] was 14.4 (6.8) *vs* 12.5 (4.0) μM (P = 0.04) and hyperhomocystinemia was found in 31.1 *vs* 12.2% (P = 0.02).

By univariate analysis, the OR for coronary disease was 2.55 (95% CI = 1.15-5.67) in patients with hyperhomocystinemia relative to subjects with plasma Hcy level below

17.3 μM. In a stepwise multivariate regression analysis, where age, creatinine, gender, hyperlipidemia, diabetes, and tobacco use were included, an independent association between hyperhomocystinemia and CAD was found (OR = 2.48; 95% CI = 1.002-6.14). Age, male sex, hyperlipidemia, and diabetes were also independently associated with CAD (Table 2).

When plasma Hcy level was evaluated according to Friesinger score, we found a trend toward more advanced CAD in patients with hyperhomocystinemia. Patients with no arterial wall abnormalities (score 0) had the lowest mean Hcy level: 12 ± 3.6 μM. Those with a score between 1 and 5 had 13.8 ± 5.3 μM, and those with a score of 6 to 10 had 14.1 ± 7.4 μM. The highest mean plasma Hcy level was found in patients with the more severe CAD (scores 11 to 15): 14.5 ± 7.1 μM (P = ns by ANOVA). The difference between the two extreme groups was statistically significant (P = 0.04). The same trend was found regarding the prevalence of hyperhomocystinemia. Among patients with normal arteries (score 0), only 6% had hyperhomocystinemia. This proportion increased in patients with more extensive disease: 18% in patients with a score of 1 to 5, 21% in patients with a score of 6 to 10, and 23.5% in patients with a score of 11 to 15 (P = 0.19 between all groups; P = 0.03 between patients with score 0 and score 11-15).

Homocysteine, MTHFR polymorphism and folate status

The C677T MTHFR polymorphism was found in the heterozygous form (CT genotype) in 30.9% of the study population, while 9.1% had the homozygous form (TT genotype). The same proportion was found despite gender and race. We found no difference in TT genotype prevalence between patients with CAD and those without disease (data not shown).

Hcy level was similar regardless of MTHFR genotype. Mean concentration was 12.3 (4.6) μM in patients with the CC genotype, 15.6 (8.8) μM in patients with the CT genotype, and 12.8 (4.7) μM in patients with the TT genotype (P = ns). Nevertheless, patients with the mutant allele had a higher prevalence of hyperhomocystinemia: Hcy above 17.3 μM was present in 29% of homozygous individuals (TT), while this prevalence was 26% in patients with genotype CT and only 11% in the group with the wild genotype (genotype CC); P = 0.03.

Regarding nutritional factors, we found opposite results regarding vitamin B_{12} and folate. There was no significant correlation between Hcy and plasma vitamin B_{12} levels (r = -0.035; P = ns), but we found a negative and significant correlation with folate (r = -0.14; P = 0.04). Comparing patients according to folate quartiles, we found that those with the lowest folate levels (1st quartile, folate <4.9 ng/dL) had a higher prevalence of hyperhomocystinemia than those in the highest quartile (folate >9.4 ng/dL) (30.3 *vs* 9.2%; P < 0.01).

The correlation between Hcy and folate status differed according to MTHFR genotype (Table 3). In patients without the mutant allele, this correlation was not statistically significant (r = -0.11; P = ns). However, in patients with genotypes CT and TT combined, a statistically significant correlation was demonstrable (r = -0.25; P < 0.05). The wild genotype seems to exert a protec-

Table 2. Variables independently associated with coronary artery disease (CAD) after stepwise multiple regression analysis.

Variable	Univariate analysis OR (95% CI)	Multivariate analysis OR (95% CI)
Gender (male)	2.6 (1.5;4.6)	3.86 (1.97;7.56)
Hyperlipidemia	3.6 (2.1;6.2)	3.96 (2.08;7.55)
Hyperhomocystinemia	2.4 (1.1;5.4)	2.48 (1.002;6.14)
Age	-	1.04 (1.01;1.07)
Diabetes	2.3 (1.2;4.4)	2.14 (1.02;4.51)

Multivariate models adjusted for age, gender, diabetes, smoking, serum creatinine, and hyperlipidemia. OR = odds ratio; 95% CI = 95% confidence interval.

tive effect against hyperhomocystinemia even in patients in the lowest folate quartile. In the same way, patients in the top folate quartile had low Hcy levels even when they carried the mutant allele.

Additional factors related to homocysteine

Men have had higher Hcy levels than women (14.9 (6.6) *vs* 12.4 (5.6) μM; P < 0.001). Age was analyzed in two distinct ways, i.e., as a continuous and categorical variable (≤60 or >60 years old). There was no relation between Hcy level and age in neither way, although patients with hyperhomocystinemia were older than those with Hcy <17.3 μM (60 *vs* 56 years; P = 0.04).

Smoking, frequently related to high-plasma Hcy levels, was not a cause of hyperhomocystinemia in our population. Homocysteine was 13.7 (5.0) μM in smokers and 13.8 (6.7) μM in non-smokers. Despite the fact that no patient with creatinine above 1.5 mg/dL was included in the study, we found a consistent and positive correlation between creatinine and Hcy (r = 0.42; P < 0.001).

Discussion

This is the first study from a Latin American country to show an independent association between hyperhomocystinemia and CAD. Our results are in agreement with the meta-analysis by Christen et al. (16), in which 43 studies were analyzed. In another meta-analysis, Boushley et al. (17) evaluated 27 studies and showed that Hcy is an independent and gradual risk factor for CAD. The OR for CAD ranged from 1.6 for men to 1.8 for women, for each Hcy level elevation of 5 μM. The increase in coronary risk resulting from this increase is similar to an increase of 20 mg/dL in total cholesterol levels. The authors considered that 10% of the risk of the general population for the development of CAD can be attributed to Hcy.

Prospective studies reported conflicting results. Some identified Hcy as an independent risk factor (5,18), but others did not (19,20). Recently, Wald et al. (21) published a comprehensive meta-analysis of 16 prospective studies. Data from 144,936 patients with 3144 combined events were analyzed. The OR for a 5 μM increase in Hcy level was

Table 3. Comparison between patients with poor (bottom quartile: ≤4.9 ng/dL) and high (top quartile) folate status in terms of mean plasma homocysteine (Mean Hcy) levels and frequency of hyperhomocystinemia (Hyper Hcy) according to MTHFR genotype.

	Folate quartiles				P
	1st (N = 56)	2nd (N = 58)	3rd (N = 55)	4th (N = 54)	
All patients					
Mean Hcy (μM)	15.7 ± 7.5	14.2 ± 8.0	12.5 ± 3.7	12.5 ± 4.3	0.05
Hyper Hcy	30.3%	20.0%	9.1%	9.2%	<0.01
CC genotype (60% patients)					
Mean Hcy (μM)	13.4 ± 5.1	13.0 ± 4.7	11.2 ± 4.4	12.3 ± 4.1	ns
Hyper Hcy	18.5%	15.9%	14.8%	10.7%	ns
CT (30.9% patients) + TT (9.1% patients) genotype					
Mean Hcy (μM)	16.7 ± 7.7	17.4 ± 12.2	13.4 ± 3.1	11.8 ± 4.3	0.04
Hyper Hcy	35.7%	33.3%	10.5%	7.1%	ns

Data are reported as means ± SD in μM for Mean Hcy and as percent for Hyper Hcy. Folate quartiles are: 1st ≤4.9 ng/dL, 2nd 4.9-7.0 ng/dL, 3rd 7.0-9.4 ng/dL, 4th >9.4 ng/dL. P, Bottom *vs* top quartile. Mean Hcy level compared by *t*-test after log transformation; frequency (%) compared by chi-square test. ns = nonsignificant.

1.23 (95% CI = 1.14-1.32). Zylberstein et al. (22) published the results of a 24-year follow-up evaluating Hcy as a risk factor for coronary morbidity and mortality in 1368 women. For the fifth Hcy quintile, relative risk was 1.86 (95% CI = 1.06 to 3.26) for acute myocardial infarction and 5.14 (95% CI = 2.22 to 11.92) for death due to acute myocardial infarction.

Toole et al. (23) analyzed the effect of homocysteine-lowering therapy upon recurrent stroke. Despite persistent association between baseline Hcy levels and outcome, folate, vitamins B_{12} and B_6 alone or in combination did not prevent new vascular event after 2 years. However, this and other studies had their statistical power questioned (24). Also, the mandatory fortification of food with folate in certain countries has masked the effect of treatment on stroke risk (25).

Only a few studies have assessed the relationship between plasma Hcy levels and the extent of coronary disease. In 70 patients, Tsai et al. (26) demonstrated a positive correlation between Hcy levels and the extent of coronary disease, but clearly only in patients with a low CAD risk profile (less than 3 risk factors). Evaluating only the number of injured vessels, Chao et al. (27) showed that patients with progressively higher Hcy levels have more arteries injured. The Friesinger score that we used is not only accurate for evaluation of extension and severity of coronary disease, but also as a prognostic indicator. In the CASS study (28), its prognostic value was better than scores that considered only the number of injured vessels or coronaries with proximal stenotic lesions.

The mechanism(s) by which Hcy is atherogenic is still not completely understood. Endothelial dysfunction seems to play a major role. Hcy is a highly reactive amino acid that produces endothelial injury in both experimental animals and cell cultures (30).

Woo et al. (31) demonstrated impaired flow-mediated dilatation of the brachial artery in 17 healthy individuals who had no other risk factor except hyperhomocystinemia; furthermore, Hcy levels and endothelial function were corrected with folic acid supplementation. Since toxicity is dependent on the degree of hyperhomocystinemia, this could explain, at least in part, the graded effect we found between Hcy concentration and the extent of CAD.

The inverse and significant correlation of Hcy with folate, but not with vitamin B_{12}, are compatible with the metabolic pathway of Hcy. Folate is a substrate in the remethylation cycle, where Hcy is converted to methionine, in which it donates the methyl group for this reaction. On the other hand, vitamins B_{12}, B_2 and B_6 function only as enzyme cofactors. Although median folate level was similar and within the normal range in both patients and controls, a considerable proportion of patients had low-plasma folate levels. The lower limit proposed by the WHO is 6 ng/mL. Almost 30% of patients had folate below this limit, and their Hcy levels were higher than those of patients with folate within the normal range. Larger studies have previously shown this relation. In the Framinghan study (32), patients in the lowest decile of folate had mean Hcy 15.6 μM, while those in the superior decile had 11 μM.

The data regarding the prevalence of C677T MTHFR polymorphism showed that the TT genotype occurs in our population at a similar frequency as that observed in Caucasian populations (33). Arruda et al. (34), evaluated the C677T polymorphism in three distinct ethnic groups in Brazil. Genotype TT was found in a similar prevalence among Caucasian descent (10%). The prevalence was lower among Black (1.45%) and Indian populations (1.2%).

We also assessed the combined effect of low plasma folate levels in patients with MTHFR polymorphism. As previously mentioned, 40% of the population carries the mutant allele either in homozygosis (9.1%)

or heterozygosis (30.9%). This group is particularly susceptible to hyperhomocystinemia when exposed to low folate levels. Accordingly, countries such as the United States and Canada already enrich their grains with folic acid in an effort to reduce folate-neural tube defects. If lowering Hcy levels is proved to reduce cardiovascular risk, a high folate intake should be recommended for everyone. On the other hand, patients with the wild genotype (CC) seem to be less prone to increased Hcy levels, even in the presence of low folate levels.

Our findings are relevant since several studies have shown that Hcy levels differ among countries with similar nutritional habits. Thus, it may be useful to know the data for our population. Alfthan et al. (35) have demonstrated these inter-country differences among several European countries, Japan and Israel. Moreover, these investigators demonstrated a direct relationship between plasma Hcy concentration and mortality from all cardiovascular diseases according to WHO data.

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