

## Protein-bound homocyst(e)ine. A possible risk factor for coronary artery disease.

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### Research Article

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# Protein-bound Homocyst(e)ine

## A Possible Risk Factor for Coronary Artery Disease

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### Abstract

The development of atherosclerotic changes and thromboembolism are common features in homocystinurics. Hence, we postulate a positive correlation between the level of homocyst(e)ine in the blood and the occurrence of coronary artery disease. Homocysteine is found either as free homocystine, cysteine-homocysteine mixed disulfide, or protein-bound homocyst(e)ine. In nonhomocystinuric subjects, most homocysteine molecules are detectable in the protein-bound form. Thus, protein-bound homocyst(e)ine in stored plasma which reflected total plasma homocyst(e)ine was determined in 241 patients with coronary artery disease (173 males and 68 females). The mean  $\pm$  SD total plasma homocyst(e)ine was  $5.41 \pm 1.62$  nmol/ml in male patients,  $4.37 \pm 1.09$  nmol/ml in male controls,  $5.66 \pm 1.93$  nmol/ml in female patients, and  $4.16 \pm 1.62$  nmol/ml in female controls. The differences between the patients with coronary artery disease and the controls were statistically significant ( $P < 0.0005$ ).

### Introduction

Homocystinurias are inborn errors of methionine metabolism caused by a deficiency of cystathionine synthase or by defects in the remethylation of homocysteine<sup>1</sup> to methionine. McCully postulated that the accumulation of homocysteine may cause atherosclerosis (1). There are at least two lines of evidence which suggest that excessive homocystine may be associated with atherosclerotic coronary artery disease. First, patients with untreated homocystinuria have a greatly increased tendency to develop severe atherosclerosis and intravascular thromboembolism (1, 2). A second line of evidence is the experimental production of preatherosclerotic and atherosclerotic lesions in baboons by a continuous infusion of homocystine (3). Wilcken and Wilcken demonstrated an association of homocysteine accumulation with the development of coronary artery disease by the measurement of cysteine-homocysteine mixed disulfide after methionine loading. They found a significantly increased plasma level of cysteine-homocysteine mixed disulfide in patients with coronary artery disease compared with that in controls (4). However, recent studies showed no difference in the mean plasma concentrations of free homocystine and cysteine-homocysteine mixed

disulfide between patients with coronary artery disease and the controls (5, 6).

Recently, we have demonstrated the presence of protein-bound homocyst(e)ine in the plasma of both homocystinuric patients and normal subjects (7). In nonhomocystinuric subjects, homocysteine is predominantly found in the protein-bound form (7, 8). Furthermore, most free homocystine and cysteine-homocysteine mixed disulfide in the protein-free fraction escape detection when the plasma or serum is stored for more than a week (9), since they are spontaneously bound to protein molecules (7, 8). Thus the determination of protein-bound homocyst(e)ine in stored plasma is suitable for the quantitation of total homocyst(e)ine, which is the sum of protein-bound homocyst(e)ine in vivo, free homocystine, and its derivatives. Hence, the quantitation of plasma protein-bound homocyst(e)ine enables us to investigate the hypothesis that the concentration of total homocyst(e)ine in the plasma is positively associated with the occurrence of coronary artery disease.

The present study demonstrates the finding of significantly higher total plasma homocyst(e)ine in patients with angiographically proven coronary artery disease compared with subjects of similar age and sex but without angiographic abnormalities. In addition, there is a modest, but statistically significant correlation between total plasma homocyst(e)ine and serum creatinine, uric acid, and cholesterol.

### Methods

443 subjects under 69 yr of age with or without coronary artery disease were studied. They were patients whose blood samples were available when admitted to the Section of Cardiology, Rush-Presbyterian-St. Luke's Medical Center during the period between 1981 and 1983 for evaluation of potential cardiovascular disorders. All subjects had extensive evaluation including history, physical examination, blood chemistry, ECG, X-ray, and coronary angiography. In order to minimize the contribution of other risk factors, subjects with hyperlipidemia (total serum cholesterol above 375 mg/100 ml and total serum triglyceride above 250 mg/100 ml), diabetes mellitus, hypertension, hypercreatininemia (serum creatinine above 1.2 mg/100 ml in males and above 1.0 mg/100 ml in females), and hyperuricemia (serum uric acid above 8.5 mg/100 ml in males and above 7.5 mg/100 ml in females) were excluded.

Patients were defined as having coronary artery disease when angiographs demonstrated at least 70% obstruction of one or more major coronary arteries. These patients were subgrouped according to the number of involved vessels. The control subjects had angiographically proven normal coronary arteries and comprised two subgroups. The first subgroup consisted of subjects with cardiac diseases other than coronary artery disease, such as valvular disease or myocarditis, and no evidence of heart failure. The second subgroup consisted of subjects whose studies revealed no cardiovascular disease. Most subjects had similar medications. Patients with renal diseases were excluded.

After an overnight fast, a blood specimen was obtained for the determination of plasma protein-bound homocyst(e)ine. The plasma was separated within 4 h after sampling and stored at  $-22^{\circ}\text{C}$  for 4–8 wk. The method of analysis was described previously (10). The plasma samples

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1. Homocysteine is readily oxidized to homocystine that is detectable in the tissue fluid of homocystinurics.

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were precipitated with 4 vol of 3.75% sulfosalicylic acid in 0.3 M lithium citrate buffer, pH 2.0. The acid precipitates of the plasma were treated with 2-mercaptoethanol at 37°C for 120 min. Immediately after incubation, the mixture was treated with iodoacetic acid for S-carboxymethylation of the released sulfhydryl compounds, and the protein was then reprecipitated with sulfosalicylic acid. The supernatant was used for the determination of protein-bound homocyst(e)ine. In contrast, direct measurement of reduced homocysteine showed a lower value because of spontaneous oxidation during the analysis (7). The S-carboxymethylated sulfhydryl compounds were chemically stable. Accordingly, this treatment provided reproducible values in contrast to the direct measurement of the sulfhydryl compounds. There were only two peaks derived from protein-bound cyst(e)ine and homocyst(e)ine in the ion-exchange chromatography. The value of protein-bound homocyst(e)ine was calculated as nanomoles of homocystine (equivalent to  $2 \times$  homocysteine) per milliliter of plasma. The term "total plasma homocyst(e)ine" was used interchangeably with protein-bound homocyst(e)ine in stored plasma in this study. Both reflected the total of bound homocyst(e)ine in vivo and the free derivatives bound to protein during storage.

Other laboratory studies included blood cell counts, serum electrolytes, serum creatinine, fasting serum glucose, serum uric acid, serum total and HDL cholesterol, serum triglyceride, serum total protein, serum albumin, and urine analysis.

Differences between the patients with coronary artery disease and control subjects were evaluated using Student's *t* test for independent samples. Pearson correlation coefficient was used to measure the association between two variables. A multiple regression equation relating total plasma homocyst(e)ine to age, sex, and coronary artery disease was developed.

## Results

A total of 241 patients with coronary artery disease and 202 control subjects with angiographically normal coronary arteries were studied (Table I). The mean total plasma homocyst(e)ine values in the controls and the patients were 4.25 and 5.48 nmol/ml, respectively, and the difference was statistically significant ( $P < 0.0005$ ).

The frequency distributions of total plasma homocyst(e)ine values were similar in both the patients and the controls (Fig. 1). Values  $> 9$  nmol/ml were seen in four patients and three controls. The 90th, 95th, and 99th percentile values in the patients with coronary artery disease were 7.5, 8.5, and 11.5 nmol/ml, respectively. In contrast, the corresponding percentile values in the controls were 5.5, 6.5, and 9.5 nmol/ml. A comparison of the distributions of total plasma homocyst(e)ine levels indi-

cated that high total plasma homocyst(e)ine values were seen more commonly among patients with coronary artery disease.

The age and sex composition of the subjects and their total plasma homocyst(e)ine values grouped by decade are shown in Table II. In male subjects, the largest difference of mean total homocyst(e)ine values between the patients and controls was found in the age range between 60 and 69 yr, followed by the age range between 50 and 59 yr. In female subjects, the largest difference was also observed in the age range between 60 and 69 yr. There was a significant correlation between age and total plasma homocyst(e)ine levels for females ( $r = 0.30$ ,  $P < 0.0005$ ) but not for males. In the females, there was an abrupt increase of total plasma homocyst(e)ine after 50 years of age, suggesting that menopause had an influence on the plasma homocyst(e)ine level (Table II).

The total plasma homocyst(e)ine values were analyzed in relation to the severity of coronary atherosclerosis. Patients with coronary artery disease were subdivided according to the number of involved vessels. In both the male and the female patients, there was no apparent correlation between the levels of total plasma homocyst(e)ine and the number of involved vessels. In the control subjects, the levels of total plasma homocyst(e)ine were not affected by the presence of valvular disease. In addition, no positive correlation was observed between the total plasma homocyst(e)ine values and Gensini's severity scores of coronary artery disease (11).

Since several factors had been considered to have a positive association with the development of coronary artery disease, subjects with known hypertension, diabetes mellitus, hypercholesterolemia, hyperuricemia, and hypercreatininemia were excluded from the study. Nonetheless, serum creatinine and uric acid values showed a strong positive correlation with total plasma homocyst(e)ine values both in patients with coronary artery disease and controls (Table III). There was a tendency toward positive correlation between total serum cholesterol and total plasma homocyst(e)ine in the patients with coronary artery disease, but this relationship was not apparent in the controls (Table III). When total plasma homocyst(e)ine levels were adjusted for cholesterol levels using an analysis of covariance, there was still a significant difference in the adjusted levels between the patients and controls ( $P < 0.0005$ ). In contrast, body weight, systolic and diastolic blood pressure, serum protein, serum albumin, hemoglobin, and hematocrit showed no correlation with the total plasma homocyst(e)ine values.

Table I. Comparison of Plasma Protein-bound Homocyst(e)ine Concentrations (Mean $\pm$ SD) in Patients with Coronary Artery Disease and Controls by Decades

| Age   | Controls |                 | Patients |                 | Difference between means | Significance |
|-------|----------|-----------------|----------|-----------------|--------------------------|--------------|
|       | No.      | PBH*            | No.      | PBH*            |                          |              |
| yr    |          |                 |          |                 |                          | P‡           |
| <30   | 13       | 3.65 $\pm$ 0.90 | 1        | 2.96 $\pm$ 0.00 |                          |              |
| 30-39 | 22       | 4.16 $\pm$ 1.11 | 21       | 5.14 $\pm$ 2.01 | -0.98                    | <0.056       |
| 40-49 | 49       | 4.12 $\pm$ 1.15 | 67       | 5.30 $\pm$ 1.54 | -1.18                    | <0.0005      |
| 50-59 | 57       | 4.39 $\pm$ 1.54 | 80       | 5.68 $\pm$ 1.77 | -1.29                    | <0.0005      |
| 60-69 | 61       | 4.38 $\pm$ 1.61 | 72       | 5.56 $\pm$ 1.71 | -1.18                    | <0.0005      |
| Total | 202      | 4.25 $\pm$ 1.40 | 241      | 5.48 $\pm$ 1.72 | -1.23                    | <0.0005      |

\* Protein-bound homocyst(e)ine (PBH) of stored plasma in nanomoles per milliliter. ‡ P values were calculated using Student's *t* Test.

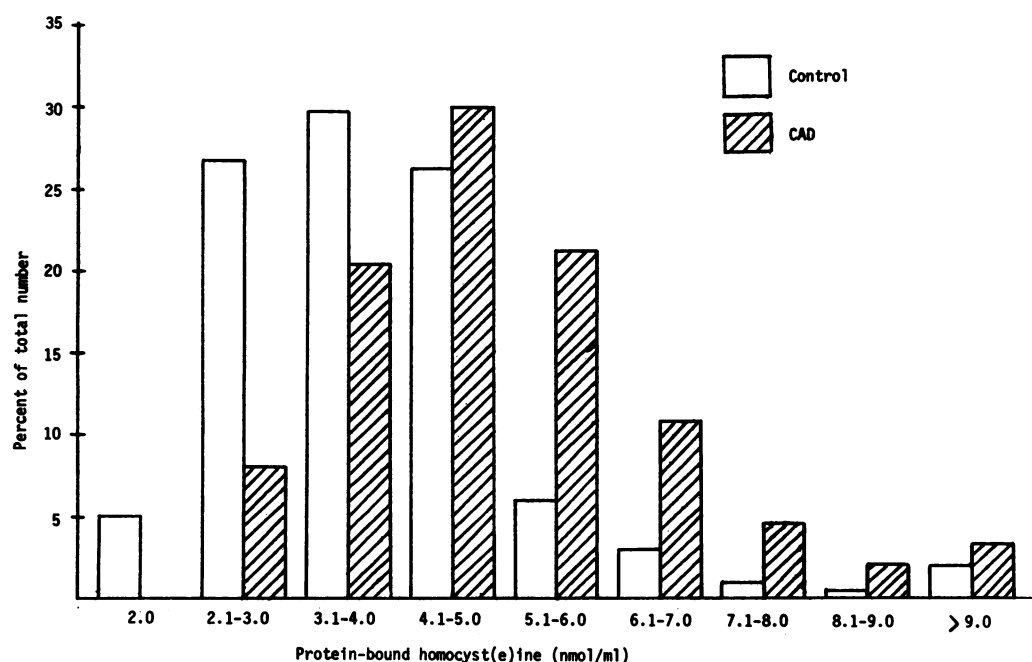


Figure 1. Distribution of protein-bound homocyst(e)ine concentrations in stored plasma from patients with coronary artery disease (CAD) and controls.

## Discussion

The association of a marked increase of plasma homocystine and the occurrence of atherosclerotic changes and thrombosis in homocystinurics has been well documented (1-3). In 1974, Sardharwalla et al. (12) studied plasma cysteine-homocysteine disulfide concentrations after oral methionine loading in heterozygotes for cystathionine synthase deficiency. They demonstrated an accumulation of this mixed disulfide among obligatory heterozygotes. Subsequently, Wilcken and Wilcken (4) reported significant differences in the plasma levels of cysteine-homocysteine mixed disulfide after methionine loading between 25 patients with coronary artery disease and 22 controls. It was postulated that heterozygotes for homocystinuria might be at an

increased risk of developing coronary heart disease (13). On the assumption that these heterozygotes had persistent mild homocystinemia with ordinary diets, Mudd et al. (14) compared the incidence of coronary heart disease and strokes in parents and grandparents of homocystinurics with that in parents and grandparents of other genetic disorders. Their study revealed no positive correlation between the incidence of coronary heart disease or strokes and the heterozygosity for homocystinuria (14). Recently, Wilcken et al. (5) reported that the concentrations of plasma cysteine-homocysteine disulfide following methionine loading in heterozygotes for homocystinuria showed considerable overlap with the concentrations in controls. More recently, Boers et al. (6) also reported no pathological postmethionine accumulation of homocystine and cysteine-homocysteine mixed di-

Table II. Comparison of Plasma Protein-bound Homocyst(e)ine Concentrations (Mean $\pm$ SD) in Patients with Coronary Artery Disease and Controls by Sex and Decades

| Sex    | Age<br>yr | Controls |                 | Patients |                 | Differences<br>between means | Significance<br><i>P</i> † |
|--------|-----------|----------|-----------------|----------|-----------------|------------------------------|----------------------------|
|        |           | No.      | PBH*            | No.      | PBH*            |                              |                            |
| Male   | <30       | 5        | 3.41 $\pm$ 0.64 | —        | —               | —                            | —                          |
|        | 30-39     | 14       | 4.46 $\pm$ 1.16 | 19       | 5.14 $\pm$ 2.10 | -0.68                        | 0.246                      |
|        | 40-49     | 25       | 4.72 $\pm$ 1.00 | 54       | 5.42 $\pm$ 1.56 | -0.70                        | 0.019                      |
|        | 50-59     | 26       | 4.42 $\pm$ 1.01 | 60       | 5.70 $\pm$ 1.57 | -1.28                        | <0.0005                    |
|        | 60-69     | 23       | 4.03 $\pm$ 1.16 | 40       | 5.70 $\pm$ 1.50 | -1.67                        | 0.003                      |
|        | Total     | 93       | 4.37 $\pm$ 1.09 | 173      | 5.41 $\pm$ 1.62 | -1.04                        | <0.0005                    |
| Female | <30       | 9        | 3.75 $\pm$ 1.01 | 1        | 2.96 $\pm$ 0.00 | —                            | —                          |
|        | 30-39     | 8        | 3.63 $\pm$ 0.82 | 2        | 5.23 $\pm$ 0.40 | -1.60                        | —                          |
|        | 40-49     | 24       | 3.50 $\pm$ 0.97 | 13       | 4.79 $\pm$ 0.40 | -1.29                        | 0.008                      |
|        | 50-59     | 30       | 4.41 $\pm$ 1.91 | 20       | 5.64 $\pm$ 2.29 | -1.23                        | 0.055                      |
|        | 60-69     | 38       | 4.60 $\pm$ 1.81 | 32       | 6.13 $\pm$ 1.80 | -1.53                        | 0.001                      |
|        | Total     | 109      | 4.16 $\pm$ 1.62 | 68       | 5.66 $\pm$ 1.93 | -1.50                        | <0.005                     |

\* Protein-bound homocyst(e)ine (PBH) of stored plasma in nanomoles per milliliter. † *P* values were calculated using Student's *t* Test.

Table III. Correlation of Plasma Protein-bound Homocyst(e)ine Concentrations with Other Factors

|             |          | Controls | Patients |
|-------------|----------|----------|----------|
| Age         | No.      | 202      | 241      |
|             | <i>r</i> | 0.115    | 0.086    |
| Creatinine  | No.      | 199      | 232      |
|             | <i>r</i> | 0.223*   | 0.171‡   |
| Uric acid   | No.      | 188      | 215      |
|             | <i>r</i> | 0.271*   | 0.159‡   |
| Cholesterol | No.      | 177      | 218      |
|             | <i>r</i> | 0.061    | 0.141§   |

\*  $P < 0.001$ .

‡  $P < 0.01$ .

§  $P < 0.05$ .

sulfide in 25 patients with premature occlusive coronary artery disease.

Our previous studies showed that homocyst(e)ine was found in a protein-bound form in the plasma of nonhomocystinuric subjects (7). When normal plasma was incubated with a final concentration of 40  $\mu$ M homocystine (which is  $\sim 10$  times the plasma protein-bound homocyst(e)ine value in normal subjects), 80% of the exogenous homocystine was recovered from the acid precipitates of plasma (7). The fact that a substantial fraction of homocyst(e)ine was bound to plasma proteins, albumin, and erythrocyte membranes precipitated in strong acid was also demonstrated by other investigators (8, 15–17). Therefore, the conventional method for the determination of homocystine after excluding the acid precipitates of plasma is rather inadequate for the evaluation of total homocyst(e)ine in tissue fluids. Although cysteine-homocysteine mixed disulfide is detectable in the plasma of normal subjects, the concentration of protein-bound homocyst(e)ine in stored plasma was approximately threefold greater than the concentration of the mixed disulfide. Furthermore, the mean value of protein-bound homocyst(e)ine in the stored plasma from the control subjects in this study was in agreement with the mean value of total homocysteine (free homocystine, cysteine-homocysteine mixed disulfide, and protein-bound homocyst(e)ine) in the normal plasma measured by a radioenzymatic method (8). Thus, the determination of protein-bound homocyst(e)ine in plasma, after all homocysteine and its derivatives are permitted to bind to proteins, provides a more sensitive means to study the potential relationship of increased homocyst(e)ine and the occurrence of coronary artery disease. The present study has demonstrated a significantly increased total plasma homocyst(e)ine in patients with coronary artery disease when compared with the controls.

Many factors may influence total plasma homocyst(e)ine in these nonhomocystinuric subjects. Significant decrease but not total absence of the enzymes involved in the synthesis of cystathionine from homocysteine and serine, the remethylation of homocysteine to methionine, and the metabolism of tetrahydrofolate, which is a methyl donor for homocysteine, may result in an increased homocyst(e)ine concentration. The decrease in these enzyme activities may be genetically determined or due to dietary variations in factors such as folic acid or pyridoxine. Our previous study demonstrated a 3–4-fold increase in protein-bound homocyst(e)ine in patients requiring chronic hemodialysis (18). It suggested that the kinetics of homocyst(e)ine binding,

transport, and release may influence the concentration of protein-bound homocyst(e)ine in the plasma. Hormonal influences in the female may be an important factor. In the present study there was an abrupt increase in total plasma homocyst(e)ine among females after 50 yr of age, suggesting a relationship with menopause. Boers et al. (19) observed that both fasting and postmethionine-loading cysteine-homocysteine disulfide levels were significantly lower in premenopausal women compared with the levels in men and postmenopausal women. During pregnancy, a substantial decrease in plasma homocyst(e)ine was observed in females (unpublished data). Finally, dietary protein intake may influence plasma homocyst(e)ine levels. In this study, no records of dietary habits were available for evaluation. It may be speculated that one or more mechanisms may be responsible for an increased total plasma homocyst(e)ine concentration in a given subject.

In some specific disorders the contribution of a single factor may be paramount in the development of atherosclerosis, such as homocystine in homocystinurias and cholesterol in familial hypercholesterolemia. In the present study, there appears to be a significant correlation between total plasma homocyst(e)ine and cholesterol in patients with coronary artery disease. Thus, it may be argued that a combination of factors such as relatively mild elevations of cholesterol and plasma homocyst(e)ine may increase the tendency toward the development of atherosclerosis. Future studies of the factors that may influence the level of plasma homocyst(e)ine are required. These should include genetic factors such as the enzymes involved in the metabolism of methionine and environmental factors such as protein and vitamin intake. An improved understanding of the mechanism of increased plasma homocyst(e)ine in nonhomocystinuric subjects may provide further insight into the risk factors predisposing patients to coronary artery disease.

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