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Fasting and Post-Methionine Load Plasma Homocysteine Levels in Patients With Angiographically Defined Coronary Artery Disease

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Abstract: Elevated plasma homocysteine concentration either in fasting state or after methionine loading is an independent risk factor for vascular disease. The aim of this study was to evaluate the relationship between fasting total homocysteine (tHcy) and post-methionine load tHcy in healthy control subjects and in patients with angiographically defined coronary artery disease (CAD). Methionine loading has been used to investigate impaired methionine metabolism, especially of the trans-sulphuration pathway. We investigated the effect of methionine excess on total plasma homocysteine in 65 control subjects (44 men and 21 women) who attended a polyclinic of Gülhane Military Medical Academy (GMMA) in Ankara. Forty-one patients (30 male, 11 female) who underwent cardiac catheterisation at GMMA formed the patient group. Plasma total homocysteine

concentrations were measured by using high pressure liquid chromatography with fluorescence detection. The expected increase in plasma homocysteine was found in both men and women in the control and CAD groups. In three of 21 women and three of 44 men with the methionine-loading test, plasma homocysteine levels were higher than 30 µmol/l. In conclusion, it is thought that methionine loading mainly stresses catabolism through homocysteine trans-sulphuration; therefore, oral methionine loading in CAD patients leads to a higher accumulation of homocysteine in plasma than in healthy subjects, indicating that abnormal tHcy response after methionine loading may be a sensitive test for the diagnosis of CAD and the genetic defects of the Hcy metabolism.

Key Words: Homocysteine, methionine, coronary artery disease.

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Introduction

Cardiovascular disease is the leading cause of mortality in most Western countries. Hypertension, diabetes, hyperlipidaemia, obesity and smoking are the main risk factors for atherosclerosis and its thrombotic complications. However, these factors alone cannot account for all of the deaths caused by vascular pathologies.

Homocysteine is a sulphur-containing amino acid derived from the metabolism of methionine, an essential amino acid. Total homocysteine can be metabolised via the sulphuration pathway to form methionine. Only a small amount of total homocysteine circulates freely, and most of it is protein bound. Hyperhomocystinaemia results from an inhibition of the remethylation pathway or from an inhibition or a saturation of the trans-sulphuration pathway in homocysteine metabolism. However, folate, vitamin B₁₂ and vitamin B₆ deficiency are believed to be the major determinants of the increased

risk of cardiovascular disease related with hyperhomocystinaemia (1).

Homocysteine concentrations rise with age in both sexes. Women in general have lower concentrations than men, though these rise with menopause, cigarette smoking, a high intake of caffeine or alcohol, a sedentary lifestyle, various cancers, end-stage renal disease, hypothyroidism, inflammatory bowel disease, following organ transplantation, and the use of inhibitors of folate such as methotrexate and carbamazepine, or antagonists of folate absorption such as colestipol and cholestyramine (1-2). The modification of dietary patterns can have also substantial effects on fasting levels of total serum homocysteine (3).

Many clinical and epidemiologic studies have shown a relation between total homocysteine levels and coronary artery disease, peripheral artery disease, stroke and venous thrombosis (4-12). The prevailing view of the pathogenesis of coronary atherosclerosis is that it is

followed by unstable angina, myocardial infarction or sudden death. The acute event is frequently due to the rupture or erosion of an atherosclerotic plaque associated with thrombus formation (13-14). There is increasing evidence that homocysteine may affect the coagulation system and the resistance of the endothelium to thrombosis (15), and that it may interfere with the vasodilator and antithrombotic functions of nitric oxide (16). Notably, the vascular complications reported in patients with homocystinuria are related to thrombosis rather than to atherosclerosis (17,18).

The methionine-loading test is used to reveal additional abnormalities of the homocysteine metabolism not detected by homocysteine concentration measurements obtained in the fasting state. An abnormal postmethionine-load homocysteine elevation above fasting levels must be taken into account in the general identification of patients bearing trans-sulphuration defects such as vitamin B6 deficiency subjects or heterozygotes for cystathionine β -synthase (CBS) deficiency.

Based on recent retrospective, prospective and experimental studies, mild to moderate elevations of fasting or post-methionine-load plasma homocysteine are considered independent risk factors for cardiovascular diseases in both men and women (19-21).

Our purpose was to evaluate the relationship between fasting total homocysteine (tHcy) and post-methionine load tHcy in healthy control subjects and in patients with angiographically defined coronary artery disease. We performed the methionine-loading test to also clarify the risk of vascular disease due to hyperhomocystinaemia in which a hidden risk of homocystinaemia caused by heterozygous error in an enzyme needed in this particular metabolic pathway can be made visible.

Materials and Methods

Subjects: From June 2000 through October 2001, 65 patients (44 men and 21 women) who attended a polyclinic of Gülhane Military Medical Academy (GMMA) in Ankara were enrolled as controls. All subjects underwent a comprehensive history and physical examination by a physician and completed a questionnaire. None of the subjects had uremic renal disease or used anticonvulsants. Control patients had no

prior history of coronary heart disease (CHD) or symptoms compatible with angina pectoris and had normal resting electrocardiograms.

Forty-one patients (30 male, 11 female) who underwent cardiac catheterisation at GMMA due to clinical suspicion of CHD and who had > 50% occlusion in at least one coronary artery were taken for this study. Those who had had any acute coronary syndrome in the previous 6 months were not included in this study.

Blood Collection: Blood samples were collected after 12-h fasting to perform routine tests. Samples were obtained on the day before the angiography in the patient group. Blood specimens containing EDTA for homocysteine assay were placed on ice within 10 min of collection and blood specimens containing no anticoagulant for vitamin B12 and folic acid and were allowed to clot for 30 min. Serum and plasma samples were subsequently centrifuged at 2000 g in a refrigerated centrifuge for 15 min and stored at -70°C until analysis.

***Methionine-Loading Test:** Methionine was administered at a dose of 0.1 g/kg body weight in 200 ml of orange juice after 12 h fasting. Blood was collected immediately before and 2 and 6 h after methionine intake. All subjects received a standardised diet containing only foods low in methionine and folate from the evening before until 24 h after methionine intake. EDTA blood samples for post-methionine load homocysteine determination were placed on ice after collection and processed within 30 min. All samples were stored at -70°C until analysis. The study protocol was approved by the committee for the protection of human subjects of GMMA.

Chromatographic Equipment and Chemicals. We used a Hewlett Packard model 1050 HPLC pump, HP 1100 fluorescence detector and HP 3396 pump integrator (Aromolale, PA, USA). Methionine was obtained from Ajinomoto (Japan). All of the chemicals were of analytical grade and obtained from Sigma (St Louis, USA) and Merck (Darmstadt, Germany)

Analysis of Plasma Homocysteine. Plasma total homocysteine was measured by a kit for homocysteine from Chromsystems (Germany).

The compounds were separated on a 4.6 x 150 mm Chromsystems ODS-2 reverse phase column with a 5 μm

spherisorb ODS-2 silica cartridge guard column (Alltech, Deerfield IL USA). Isocratic elution was performed at a flow rate of 1.7 ml/min with mobile phase.

A five-point calibration curve was obtained using five levels of standards. The correlation between the peak area and the concentration was $r: 0.9986$.

Retention times for the total homocysteine and internal standards were 2.3 and 4.1 min, respectively.

Statistical Methods. Differences in mean values (mean \pm standard error) between the patient and control groups were tested by Student's t test.

Results

The baseline characteristics and parameter levels of the study participants are shown in Table. Three of 21 healthy female subjects, and one of 44 healthy male subjects had higher fasting plasma homocysteine levels of $12 \mu\text{mol/l}$ (Figure 1A). However, in three of 21 women and three of 44 men who had standard methionine-loading test plasma homocysteine levels were higher than $30 \mu\text{mol/l}$. (Figure 1B) The peak post-methionine-loading serum levels of plasma homocysteine in controls and CAD patients are shown in Figures 2 and 3.

Table. Characteristics of healthy control and CAD subject patients.

	Healthy subjects	CAD subjects
Sex (M/F)	44/21	30/11
Age (Years)	33.4 ± 8.5	53.8 ± 12.9
Fasting Homocysteine ($\mu\text{mol/l}$)	7.17 ± 2.3	13.75 ± 4.5
Postload Homocysteine ($\mu\text{mol/l}$)	21.27 ± 6.2	35.14 ± 8.7
Vitamin B ₁₂ (pg/l)	397.52 ± 93.3	264.88 ± 76.8
Folic Acid (ng/l)	10.08 ± 2.2	5.77 ± 1.2
Mean Folate/Homocysteine Rate ($\times 10^3$)	1.40	0.42
Number with Hyperhomocystinaemia:		
Preload $> 12.0 \mu\text{mol/l}$	4	14
After load $> 30.0 \mu\text{mol/l}$	6	20

Discussion

This study set out to investigate the effect of methionine loading on plasma homocysteine levels in healthy control and CAD subjects. We used the methionine-loading test to reveal additional abnormalities of the homocysteine metabolism. Hyperhomocystinaemia is associated with an increased risk of atherosclerotic vascular disease and thromboembolism in both men and women. In 1976, Wilcken and Wilcken (22) published the first results

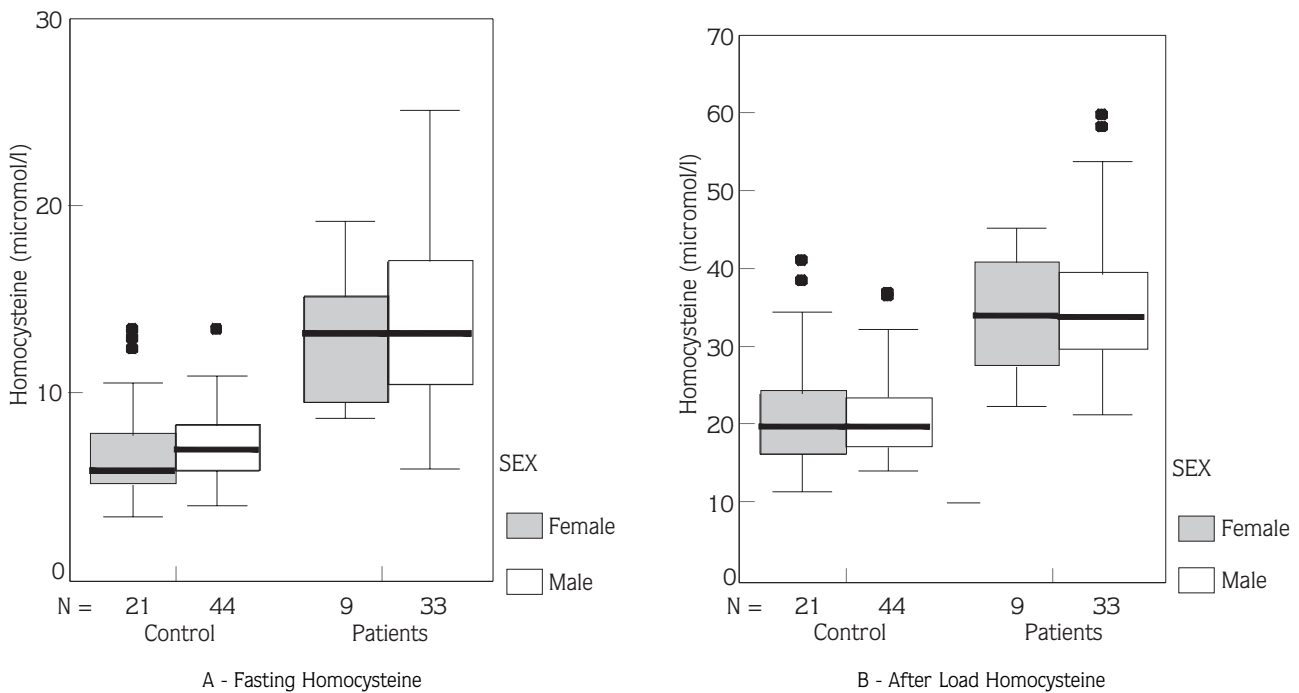


Figure 1. Plasma fasting and after load homocysteine levels in controls and in patients with CAD.

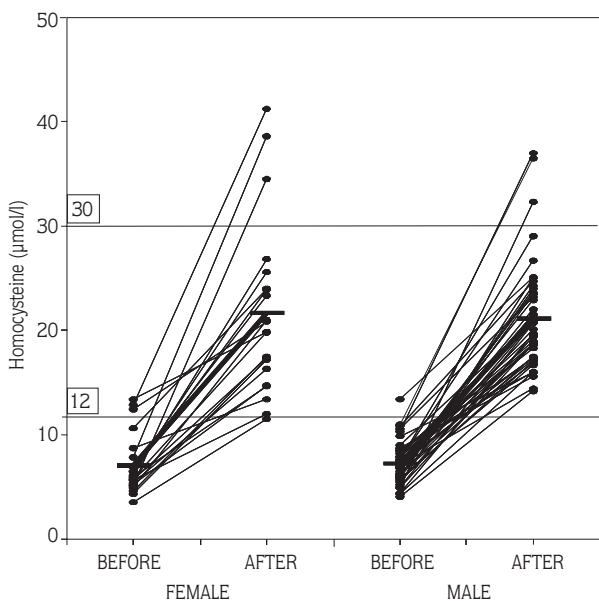


Figure 2. Plasma fasting and after load homocysteine levels in healthy controls.

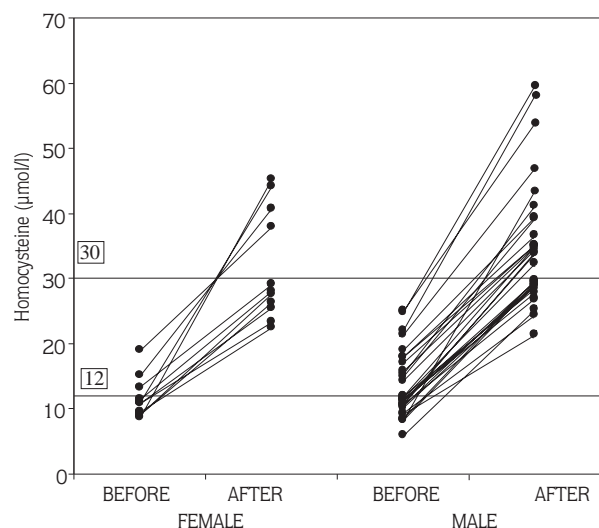


Figure 3. Plasma fasting and after load homocysteine levels in CAD patients.

suggesting that even mild hyperhomocysteinaemia could play a possible role in the pathogenesis of coronary artery disease. They showed that about 30% of young patients with angiographically proven coronary artery disease demonstrated mild hyperhomocysteinaemia after a methionine load. Murphy-Chutorian et al. (23) reported methionine intolerance in 16 of 99 patients with coronary heart disease.

Previous studies have suggested that post-methionine-load homocysteine in the absence of fasting hyperhomocystinaemia could account for over 40% of all hyperhomocystinaemic persons (24). This was only partially confirmed in our CAD patients, as suggested by the presence of six CAD patients (22%) who presented with impaired methionine tolerance and increased homocysteine after the load test. If we take into account how methionine intake could be higher than the metabolic capacity, impaired methionine tolerance could be a problem in CAD patients. Previous studies (25,26) have demonstrated that methionine-rich meals normally cause a slight increase in the plasma homocysteine concentration. Diets, particularly rich in eggs may contain a methionine intake (up to 2 g/day) that is higher than normal metabolic capacity (~1 g/day). This indicates a transient but constant increase in homocysteine concentrations.

Boers et al. (15), using a methionine-loading test, identified hyperhomocystinaemia in 14 of 50 patients (28%) with premature peripheral and cerebral arterial disease under 50 years of age. Brottstrom et al. (7) reported methionine intolerance in 26 of 72 patients (36%) with cerebrovascular disease who were under 55 years of age. Malinow et al. (15) detected higher fasting serum total homocysteine levels in 47 patients with occlusive peripheral vascular disease than in normal subjects. Araki et al. (27) found elevated serum homocysteine levels in 45 patients with cerebral infarction as compared with 45 normal subjects, 45 patients with hypertension and 20 patients with cerebral bleeding.

In our study, the proportion of hyperhomocystinaemia was higher in the angiographically defined CAD patient group. In contrast, hyperhomocystinaemia was rare in our control group, with only three of 21 normal female subjects and one of 44 normal male subjects having a fasting plasma homocysteine levels higher than 12 µmol per liter. The principal finding of this study is the observation of hyperhomocystinaemia in 34% of patients with angiographically defined CAD. The high rate of hyperhomocystinaemia occurrence in our patient group is similar to the findings of Boers et al. (19), who observed elevated homocysteine in 21% of coronary heart disease patients and 32% of venous thrombosis patients. Blacher et al. (20) also observed that a significant percentage

(15%) of their coronary heart disease patients were hyperhomocystinaemic. This contradicts the study published by Amundsen *et al.* (28), who found no difference in fasting homocysteine levels between controls and coronary heart disease patients.

An inverse relation between both serum folate levels and serum vitamin B₁₂ concentrations and hyperhomocystinaemia has been reported by several authors (29). In our study, low folate and vitamin B₁₂ status were defined as a concentration below 3 µg/l and 180 ng/l respectively and hyperhomocystinaemia correlated with reduced folate and B₁₂ levels. The mean folate and vitamin B₁₂ levels for CAD groups were significantly lower than those of the control group. This suggests that increases in homocysteine may cause a reduction in 5-methyltetrahydrofolate, which results from a disturbed remethylation pathway, preventing the normal conversion of 5-methyltetrahydrofolate to methionine. This must be considered in evaluating the relationship between folate and homocysteine in vascular disease.

The mean folate/homocysteine rates in the CAD group were substantially lower than those of the control group. This reflects an increased body burden of homocysteine per unit quantity of folate. Homocysteine in plasma reflects the status or activity in only one folate-dependent pathway. Elevated homocysteine levels can indicate a reduced regeneration of methionine; however, this can also be due to vitamin B₁₂ deficiency or to genetic defects

in the enzymes of the trans-sulphuration pathway, which can also contribute to hyperhomocystinaemia. These confounding factors make it difficult to prove conclusively that folate deficiency alone is the cause of homocystinaemia in a particular patient. The folate/homocysteine ratio may prove useful in determining a relative lack of folate in the absence of severe folate deficiency.

In conclusion, elevated plasma homocysteine concentration, either in the fasting state or after methionine loading, is an independent risk factor for vascular disease. Methionine loading may be used to investigate the impaired methionine metabolism, especially of the trans-sulphuration pathway, and to reveal additional abnormalities of the homocysteine metabolism.

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