Prevalence of Hyperhomocyst(e)inemia in Patients With Peripheral Arterial Occlusive Disease

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A micromethod adapted for automated determinations was used to measure basal plasma levels of homocyst(e)ine [H(e)]. These levels included the sum of free and bound forms of homocysteine, its disulfide oxidation product, homocystine, and the homocysteine-cysteine-mixed disulfide. Two groups of subjects were studied: apparently healthy individuals (n=103) and patients with peripheral arterial occlusive disease (PAOD) (n=47). Because age in PAOD patients was higher than in control subjects, the control subjects were subdivided into younger and older groups (aged 60 years or less and more than 60 years, respectively). The H(e) levels in the younger groups were 11.18±3.58 (mean±SD, expressed as homocysteine) and 8.58±2.82 nmol/ml in men and women, respectively; in the older groups, the levels were 10.74±2.16 and 9.04±2.16 nmol/ml in men and women, respectively. There was a positive correlation of H(e) levels with age in the younger control women (r=0.373; p<0.02); no significant correlations were present in the other three control groups. Levels of H(e) in PAOD patients (15.44±5.76 and 17.04±8.26 nmol/ml in men and women, respectively) were significantly higher than those indicated above in the older controls. Next, the PAOD patients were assigned to two subgroups: 1) those with normal levels of H(e) (within two standard deviations of the mean of the control values) and 2) those with elevated levels of H(e). Age, cholesterolemia, and the prevalence of smoking and diabetes were similar in both subgroups. These results suggest that elevated plasma H(e) is an independent risk factor for arterial occlusive disease. (Circulation 1989;79:1180–1188)

The positive correlation existing between hyperlipoproteinemia and atherosclerosis has been firmly established through data derived from numerous epidemiologic and experimental observations1 as well as from intervention trials.2,3 Although most of the clinical data have been obtained in relation to coronary heart disease (CHD),4 hyperlipoproteinemia is also observed in patients with peripheral arterial occlusive disease (PAOD).5 However, plasma lipid levels and the major risk factors combined do not account for all cases of clinical atherosclerosis.6 In a recent editorial,7 the possibility has been entertained that hyperhomocyst(e)inemia may be an additional, independent risk factor for occlusive arterial disease.

Homocysteine is a thiol-containing amino acid derived from the metabolism of methionine; under conditions prevailing in body fluids, homocysteine is readily oxidized to the disulfide, homocystine, and to homocysteine-cysteine–mixed disulfide.8 Moreover, homocysteine and the disulfides are spontaneously converted to protein-bound forms; all of these chemical species occurring normally in plasma will be collectively referred to here as homocyst(e)ine [H(e)].

Greatly elevated plasma levels of H(e) (20 times above normal) are found in subjects with homocystinuria. These high levels result from any of several inborn errors of metabolism; these patients may exhibit mental retardation, multiple mesenchymal and skeletal abnormalities, early arteriosclerosis, and arterial and venous thrombosis, including pre-

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mature acute myocardial infarction and stroke.\textsuperscript{8} Moderately elevated basal plasma levels of H(e) (two to five times normal) are observed in certain subjects with CHD who do not exhibit the systemic manifestations of homocystinuric patients.\textsuperscript{9} Moreover, patients with CHD or with PAOD exhibit higher plasma H(e) levels than control subjects after methionine loading.\textsuperscript{10–12}

In the present paper, using a micromethod adapted for automated determinations, we measured basal levels of H(e) in a group of apparently healthy individuals and in patients with PAOD.

**Methods**

**Subjects**

Four groups of individuals were studied. Group 1 consisted of 37 men and 35 women from an apparently healthy population from the staff of the Oregon Regional Primate Research Center; the presence of cigarette smoking, hypertension (blood pressure $>140/90$ mm Hg), diabetes, or hypercholesterolemia was not an excluding criterion. Informed consent was obtained in accordance with the Institutional Review Board of the Oregon Regional Primate Research Center. A group 2 consisted of 15 women and 16 men without clinical evidence of cerebral, coronary, or iliac femoral arterial disease and without the usual risk factors for atherosclerosis. This group was recruited from the Veterans Administration and Medical Center, Portland, Oregon and from the Oregon Health Sciences University; informed consent was obtained in accordance with the Institutional Review Boards of these institutions. The group had volunteered as age- and sex-matched control subjects in an ongoing study on symptomatic carotid disease (B. Coull, M.R. Malinow, and N. Beamer, unpublished data). Group 3 consisted of 21 men and 16 women who were referred to the Division of Vascular Surgery, The Oregon Health Sciences University, for symptoms or signs suggestive of the presence of PAOD and were evaluated by noninvasive or invasive techniques (see below). Group 4 consisted of 5 men and 5 women who were referred to two of us (T.I. and D.M.) for possible surgical intervention because of symptoms or signs suggesting PAOD; in these patients, the anatomical diagnosis was confirmed by contrast arteriography performed by transfemoral or transaxillary techniques. Homocysteine levels were determined in patients from groups 3 and 4 as part of the clinical workup. Results in groups 1 and 2, as well as groups 3 and 4, will be reported together; analysis of certain risk factors (see below) were performed only in groups 1, 3, and 4. Plasma cholesterol was measured by a colorimetric method\textsuperscript{13} or, in group 2, by an enzymatic method.\textsuperscript{14} Fifteen plasma samples kindly provided by Drs. V.C. Marquardt Jr. and J.R. Swanson, The Oregon Health Sciences University, were analyzed simultaneously by both methods; a factor derived from these determinations was used to express results by the colorimetric method in all subjects. The presence of cigarette smoking, hypertension, and diabetes was obtained by history. Because all cases were not evaluated by coronary angiography, the presence of CHD was not included in the study. In the combined groups 3 and 4 ($n=47$), there was 35 instances of occlusive cerebrovascular disease, 32 instances of lower extremity arterial disease, and 8 instances of abdominal aortic aneurysm (see Table 1); there were no cases of evolving stroke or peripheral gangrene.

**Noninvasive Diagnosis of Peripheral Arterial Occlusive Disease**

For cerebrovascular testing, patients underwent ultrasound and pulsed Doppler duplex scanning\textsuperscript{15} with a Diasonics DRF-400 (Milpitas, California) or Acuson 128 (Mountain View, California) scanner. The common carotid arteries, internal carotid arteries, and external carotid arteries were imaged. Doppler spectral analysis, systolic and diastolic frequency, and velocity data were recorded from proximal and distal sites in each artery as well as at specific sites of stenosis. By use of this information, carotid arteries were categorized into one of six degrees of percent diameter reduction stenosis with the criteria established by Strandness\textsuperscript{16}: grade 1, 0\% diameter reduction stenosis; grade 2, 1–16\%; grade 3, 17–49\%; grade 4, 50–79\%; grade 5, 80–99\%; and grade 6, occlusion. For purposes of this study, patients were identified as having occlusive cerebrovascular disease if there was arteriographic plaque identified on the B mode image that corresponded to at least grade 3 stenosis. The accuracy of testing in this form, when compared with angiography, has been established at greater than 85\% for all degrees of stenosis through blinded comparison of the results of 534 arteriograms and noninvasive examinations (L.M. Taylor Jr., L. Loboa, J.M. Porter, unpublished data).

For detection of lower extremity arterial disease, patients underwent recording of palpable lower extremity pulses, segmental analog Doppler waveforms, and four-level segmental Doppler pressures.\textsuperscript{17} Patients were diagnosed as having lower extremity arterial disease if the resting ankle pressure index (Doppler ankle pressure divided by highest brachial pressure) was less than 0.85. The accuracy of this method for detection of disease has been estab-

**Table 1. Distribution of Arterial Diseases in Patients**

<table>
<thead>
<tr>
<th>Location of disease</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid arteries</td>
<td>21 (Men) 14 (Women)</td>
</tr>
<tr>
<td>Iliac femoral arteries</td>
<td>16 (Men) 16 (Women)</td>
</tr>
<tr>
<td>Aortic aneurysm</td>
<td>7 (Men) 1 (Women)</td>
</tr>
<tr>
<td>Total*</td>
<td>26 (Men) 21 (Women)</td>
</tr>
</tbody>
</table>

*Numbers equal more than total patients because patients had more than one arterial territory affected.
lished at greater than 90% by blinded comparison of the results of 300 limb arteriograms with the vascular laboratory examinations (L.M. Taylor Jr., L. Loba, J.M. Porter, unpublished data).

Arteriography

Angiography was performed by transfemoral or transaxillary Seldinger technique. Angiograms from group 2 patients were examined by vascular surgeons or radiologists or both without knowledge of the results of noninvasive testing. Percent diameter reduction stenosis was determined by measuring the narrowest segment of appropriate artery compared with the closest adjacent apparently normal segment of the same artery. In arteriograms from group 3, the percent diameter reduction stenosis was estimated visually by vascular surgeons, and it was found to be greater than 70% in all cases.

Determination of Homocyst(e)ine

H(e) was determined by a modification of the method of Smolin and Schneider. All determinations, shown in the tables, were performed at the Beaverton Laboratory.

Chemicals. The following chemicals of the highest purity available were used: l-homocysteine (Fluka Chemical, Ronkonkoma, New York, and Sigma Chemical, St. Louis, Missouri); n-amyl alcohol (Mallinckrodt, St. Louis, Missouri); trichloroacetic acid (Aldrich Chemical, Milwaukee, Wisconsin); sodium hydroxide (Fluka Chemical). Sodium borohydride (NaBH₄), monochloroacetic acid, octysulfate sodium salt, and urea were purchased from Sigma Chemical.

Apparatus. High-pressure liquid chromatography (HPLC) was performed with a Bioanalytical System 400 LEC chromatograph (BAS, West Lafayette, Indiana) equipped with a single piston pump and a single gold and mercury electrode at a potential of +0.15 V. We used a Phase II 3 μm ODS column (BAS 6213) (100×3.2 mm) that was protected with an ODS guard 7 μm Phase II column (BAS 6206) (15×3.2 mm) and maintained at 27°C. Stainless steel tubing was used between the buffer reservoir and the detector to exclude oxygen. The system was equipped with an automatic sample injector (model LC 2000, Dynatech Precision Sampling, Baton Rouge, Louisiana) with capacity for 68 1-ml crimp-top style samples.

Chromatographic conditions. The mobile phase consisted of 0.1 M monochloroacetic acid and 1.8 mM octysulfate, pH 3.2, which were filtered through a Millipore glass apparatus equipped with 0.45 μm nylon filter (Schleicher and Schnell, Keene, New Hampshire). The buffer was kept at 37°C in a heating mantle, continuously purged with nitrogen, and maintained under positive pressure to remove dissolved oxygen. The solvent was circulated through the column at a flow of 0.8 ml/min. An injection volume of 20 μl was used in a 50 μl loop.

Sample preparation. About 5 ml fasting venous blood was drawn into a tube containing one drop of dipotassium ethylenediamine tetraacetate (13.8% wt/vol) (Sequester-Sol®, Cambridge Chemical Products, Fort Lauderdale, Florida); nonfasting samples were also obtained within 1 or 2 days from certain individuals. The plasma was separated by centrifugation and was stored frozen until use. For the determination of H(e), 200 μl plasma was diluted with 100 μl water and with 300 μl 9M urea, pH 9.0; 50 μl n-amyl alcohol was then added as an antifoaming agent. Reduction of disulfides and cleavage of the protein-bound sulfur-containing amino acids were performed by the addition of 50 μl of a solution of 10% (wt/vol) NaBH₄ in 0.1N NaOH. Samples were incubated in a water bath at 50°C for 30 minutes. The samples were cooled to room temperature, and the reaction was stopped by the addition of 500 μl 20% trichloroacetic acid. The proteins were separated by centrifugation for 4 minutes at 12,000g (Microfuge E, Beckman Instruments, Palo Alto, California), and the supernatant was passed through 0.45 μm filters (HV, Nihon Millipore Kogyo, K.K., Yonezawa, Japan). In some cases, plasma and serum were obtained; samples were prepared in identical manner. In all instances, duplicate samples were processed, and the results were averaged.

Preparation of standards and calculations. Standards were prepared with the same procedure as above, but 100 μl solution of l-homocysteine was added to pooled plasma replacing 100 μl water; the concentrations of added homocysteine (Fluka Chemical) were defined after all additions required for reduction and deproteinization. Solutions of l-homocysteine were prepared by M.R.M. (chemical obtained from Fluka Chemical) and S.S.K. (chemical obtained from Sigma Chemical) in their respective laboratories. The standard stock solutions were prepared by M.R.M. and kept up to 2 months at 4°C; dilutions contained 5–20 nmol/ml of homocysteine. They were heated at 50°C for 30 minutes and sonicated for 10–15 minutes before use. Standard curves of pooled plasma with added homocysteine were obtained by duplicate at the beginning, middle, and end of each daily run; if there were less than 15 samples, the standard curves were obtained at the beginning and end of each run. The concentration of H(e) was calculated from peak heights from pooled plasma containing homocysteine as external standard.

Statistics

Statistical evaluation concentrated on three main variables: basal plasma levels of H(e), age, and presence of PAOD. Variables such as plasma cholesterol, sex, cigarette smoking, hypertension, and diabetes were considered secondary variables not directly associated with the main thrust of the study.

Means, standard deviations, and standard errors were calculated for the continuous variables. Age and plasma cholesterol, as well as the natural log
(ln) transformation of H(e) levels, were normally distributed. Pearson's product-moment correlation coefficients were computed among H(e) and age. Student's two-sample t statistic was used to assess differences between the control and PAOD groups; because these two groups were significantly different with respect to age, the control subjects were subdivided into younger (≤60 years of age) and older (>60 years of age) subgroups. Levels of H(e) for the PAOD groups were contrasted only with the older control subjects. Moreover, H(e) levels were age-adjusted to the mean for the older control group (age 65.9 years) by linear regression, and one-way analysis of variance was used to assess the age-adjusted data. Statistical analyses were performed using the nontransformed and ln-transformed H(e) data; because results were similar, only the nontransformed H(e) data are presented in the tables.

Results

Chromatography of Plasma Homocyst(e)ine

Typical chromatographs of detector response for homocystine standard added to pooled plasma are shown in Figure 1. Homocysteine eluted at about 3.2 minutes, and samples could thus be run every 10 minutes. Figure 2 shows the linear response of the detector over the range of 0–80 pmol added homocystine; the tests were performed with standards prepared in the Beaverton and Chicago laboratories; results with both standards were identical. The range covers the H(e) concentrations usually found in human plasma. The within-assay precision of the method was determined by escalating the procedure to a 5 ml sample and dividing the sample into nine aliquots; Table 2 shows that the coefficient of variability (CV) was 1.1%. The between-assay precision of the method was estimated in several ways: 1) from replicate analyses performed within 2 hours (CV 2.1%) or through an 11-hour span (CV 6.6%) and 2) from replicate analyses of aliquots of a pooled sample performed periodically throughout 1.5 months (CV 11.4%) or again throughout 2.0 months (CV 9.1%). Recovery of different amounts of homocystine added to pooled plasma samples was 101.2±5.5% (mean±SD) (Table 2). Plasma values were slightly higher than in serum (8.72±2.86 nmol/}

![Figure 1. A: Chromatogram of plasma pool. B: Chromatogram of same sample with added homocystine (5 nmol/ml). H, homocysteine.](image)

![Figure 2. Graph of detector response to different amounts of homocystine added to plasma pools. Standards were prepared by M.R.M. in Beaverton (○) and by S.S.K. in Chicago (△). Each data point is the mean of three determinations. Intersection with the ordinate identifies the homocyst(e)ine level in the pooled plasma.](image)

![Table 2. Chromatography of Plasma Homocyst(e)ine](table)
ml [mean±SD] and 8.32±2.86 nmol/ml [n=7; p<0.001 by paired t test, not shown]; fasting and nonfasting plasma gave similar results (9.86±3.32 and 9.62±2.56 nmol/ml [n=13; p=NS by paired Student’s t test], not shown). Twenty-three samples were analyzed in Beaverton and Chicago; values determined by M.R.M. and B.U. (15.02±7.20 nmol/ml) were higher than those by S.S.K. and P.W.K.W. (12.14±5.08 nmol/ml) (p<0.01 by paired Student’s t test, not shown); the Pearson’s index of correlation between samples (r=0.931; p<0.01, not shown) demonstrated a high positive correlation between values determined in both laboratories, and it also suggested there was a small systematic difference between the two methods.

Concentration of Homocyst(e)ine in Plasma

The ages of the control and experimental groups were different (48.0±10.6 years in the apparently healthy individuals, not shown; and 70.1±10.6 years in the PAOD patients, Table 3). Consequently, the control groups were subdivided into two subgroups (≤60 years and >60 years). In the resulting older groups, ages were similar in control men and the PAOD men, but the PAOD women were slightly older than the corresponding control women.

The concentration of H(e) (Table 4) was 11.18±3.5 and 10.74±2.16 nmol/ml in the younger and older control men, respectively; the levels were 8.58±2.82 and 9.04±2.16 nmol/ml in the younger and older control women, respectively. In both age groups, men had higher levels than women (p<0.05 by Student’s t test). As stated, levels of H(e) in PAOD patients were compared only with those levels determined in the older control groups because ages were much closer. The H(e) values were significantly higher in the patients with PAOD, but there were no sex-related differences (15.54±5.76 and 17.04±8.26 nmol/ml in men and women, respectively). These differences remained after H(e) levels, with and without log transformation, were adjusted to the mean age in the control groups (not shown). The diagram in Figure 3 shows the distribution of H(e) levels in the older controls as well as in the PAOD patients; the diagram demonstrates that 22 out of the 47 PAOD patients had levels exceeding those observed in the 29 older controls (p<0.001, x²). Finally, when age was considered the independent variable, plasma H(e) was not significantly correlated with age in the control subjects (r=0.98, p=NS; not shown). However, H(e) correlated significantly with age in the younger control women (r=0.373, p<0.02; not shown); no significant correlations were present in older women or in men (not shown).

Prevalence of Risk Factors

Plasma cholesterol levels determined by the colorimetric method were 3.57% higher than by the enzymatic method (not shown); this factor was used

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**Table 3. Number and Age of Control Subjects and Patients With Peripheral Arterial Occlusive Disease**

<table>
<thead>
<tr>
<th></th>
<th>Subjects (n)</th>
<th></th>
<th>Age* (yr)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤60 yr</td>
<td>&gt;60 yr</td>
<td>PAOD</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>35</td>
<td>18</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>39</td>
<td>11</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>29</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤60 yr</td>
<td>&gt;60 yr</td>
<td>PAOD</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>41.8±10.4</td>
<td>66.2±4.8</td>
<td>69.0±10.2</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>40.3±9.0</td>
<td>65.5±2.1</td>
<td>71.6±11.2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>41.0±9.6</td>
<td>65.9±3.9</td>
<td>70.1±10.6</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean±SD.
PAOD, peripheral arterial occlusive disease.
Brackets indicate p<0.05 by Student’s t test.

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**Table 4. Plasma Cholesterol and Homocyst(e)ine Levels in Control Subjects and in Patients With Peripheral Arterial Occlusive Disease**

<table>
<thead>
<tr>
<th></th>
<th>Plasma cholesterol (mg/dl)</th>
<th></th>
<th>Plasma homocysteine (nmol/ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤60 yr</td>
<td>&gt;60 yr</td>
<td>PAOD</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>198±38</td>
<td>233±42</td>
<td>202±55b</td>
<td>11.18±3.58</td>
</tr>
<tr>
<td>Women</td>
<td>200±51</td>
<td>254±43</td>
<td>212±55</td>
<td>8.58±2.82</td>
</tr>
<tr>
<td>Total</td>
<td>200±51</td>
<td>241±43</td>
<td>207±55</td>
<td>9.80±3.44</td>
</tr>
</tbody>
</table>

Values are mean±SD.
PAOD, peripheral arterial occlusive disease.
Brackets indicate p<0.05 by Student’s t test.
for standardization of results in group 2 subjects (see "Methods"). Levels in the younger control subjects and the PAOD patients were similar; older controls had higher plasma cholesterol levels than younger control subjects or PAOD patients (Table 4); whether differences were present in high density lipoprotein levels was not determined. The number of control group 1 subjects that smoked (n=9) or had hypertension (n=3) or diabetes (n=1) was small (not shown), and the respective prevalences were lower than in the PAOD patients (p<0.001, $\chi^2$, for each contrast, not shown). Moreover, age, levels of plasma cholesterol, and the prevalence of smoking and diabetes were similar in PAOD patients with normal or elevated levels of H(e); in the last sub-

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Plot of plasma homocyst(e)ine concentrations in older control subjects and in patients with peripheral arterial occlusive disease (PAOD); number of subjects is indicated between parentheses. Values are expressed in nmol/ml homocysteine. Horizontal lines indicate mean±2 SD. In PAOD patients, only means are indicated. (See text.)

Table 5. Characteristics of Peripheral Arterial Occlusive Disease Patients With Normal or Elevated Plasma Levels of Homocyst(e)ine

<table>
<thead>
<tr>
<th>Plasma homocyst(e)ine</th>
<th>n</th>
<th>Men</th>
<th>Age (yr)</th>
<th>H(e) (nmol/ml)</th>
<th>Plasma cholesterol levels (mg/dl)</th>
<th>Smoking (%)</th>
<th>Hypertension (%)</th>
<th>Diabetes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25</td>
<td>15</td>
<td>69.2±23</td>
<td>11.68±1.76</td>
<td>207±110</td>
<td>36</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
<td>Elevated</td>
<td>22</td>
<td>12</td>
<td>71.2±19.28</td>
<td>21.46±7.44</td>
<td>205±112</td>
<td>18</td>
<td>77</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are mean±SD.

Normal, within 2 SD of mean from older controls (see "Methods"); Elevated, above 2 SD of mean from older controls (see "Methods").

None of the findings are significantly different in patients with normal or elevated plasma H(e) with the exception of H(e) levels (p<0.001) and hypertension (p<0.05).

Discussion

The measurement of blood thiol compounds by HPLC coupled with electrochemical detection 19 has definite advantages over methods requiring precolumn or postcolumn derivation 9,20-23 and has been used by several investigators. 18,24-27 The HPLC electrochemical method of Smolin and Schneider, 18 with the minor modifications we have introduced, required small aliquots of plasma and simple preparation of samples, demonstrated adequate sensitivity and specificity, and could be adapted to an automated injection procedure. In our experience, a relatively large number of duplicate determinations could be run daily; differences between plasma and serum were minimal, and values are similar in fasting and nonfasting samples.

Practical considerations prevented the evaluation of control subjects with the invasive and noninvasive methods used in the PAOD patients; however, all control subjects were active, and none had symptoms of PAOD. Thus, the control group is probably representative of a middle-aged working population in apparently good health. Our control values of plasma H(e) were similar to those reported by Brattstrom 28 and Stabler et al 23 but somewhat higher than those reported previously by Kang et al., 9,29 Refsum et al., 21 and Araki and Sako. 22 Such differences are not unusual when blood values are determined by different methods.

Basal plasma levels of H(e) were higher in men than in women; sex differences in the plasma concentration of these sulfur-containing amino acids have been observed previously. 9,29 Moreover, Boers et al 30 demonstrated that the serum levels of homocysteine–mixed disulfide, before and after methionine loading, were lower in premenopausal women than in older women. Similarly, Kang et al. 9 showed a positive correlation between basal H(e) levels and age in women. In our analysis of women 60 years of age or younger, which probably included premenopausal and postmenopausal subjects, there was also a significant positive correlation between basal H(e) levels and age; we observed no significant correlation in older women. These results accord with those reported by Boers et al 30 and Kang et al. 9 On the other hand, those authors did not find differ-
ences in the H(e) levels of younger or older men; we, too, did not detect significant correlations between H(e) levels and age in men. Basal plasma levels of H(e) were elevated in the PAOD patients when values were contrasted with either the younger or older control subjects. Furthermore, H(e) levels adjusted for age were significantly higher in PAOD patients than in control subjects.

The average cholesterolemia was similar in PAOD patients and in younger controls, but it was lower than in the older controls; the significance of this finding is not clear, but it could result from the selection process of subjects or other unidentified factors. Age and the prevalence of hypertension, smoking, and diabetes were higher in PAOD patients than in control subjects, as reported in numerous studies of patients with atherosclerosis. Finally, age, cholesterolemia, and the prevalence of smoking and diabetes were similar in PAOD patients with normal or with elevated levels of H(e), but hypertension was more frequent in patients with high levels of H(e); reasons for this disparity need further study. The overall findings suggest that hyperhomocyst(e)inemia is an independent risk factor for occlusive atherosclerosis; such a conclusion has been advanced by other investigators.

There are several lines of evidence suggesting that abnormalities of H(e) metabolism may be implicated in the pathogenesis of atherosclerosis and thrombosis. Homocystinuric patients have a variety of inborn metabolic errors in the remethylation or transulfuration pathways of H. These defects result in greatly elevated plasma levels of H(e), and the patients exhibit premature arteriosclerotic changes, as early as the age of 7.5 weeks; anatomical observations of several such cases confirmed the presence of thickened intima in the aorta and in other arteries causing luminal narrowing. The occurrence of premature coronary occlusion and thrombosis, which result in cerebral, renal, and pulmonary infarcts, has been reported in homocystinuric patients (see reference list in Reference 8). Our findings demonstrating moderate basal hyperhomocyst(e)inemia in patients with PAOD confirm similar observations after methionine loading in atherosclerotic patients and extend the results of Kang et al in CHD subjects. Mudd cautioned about methodological limitations in studies on basal H(e) levels. Our observations demonstrate the feasibility of performing determinations of basal H(e) levels and suggest that this test could be performed on a routine basis.

Multiple causes of hyperhomocyst(e)inemia have been recognized. Thus, accumulation of H(e) in body fluids may result from deficiencies of cystathionine β-synthase, which converts homocysteine to cystathionine; of methylenetetrahydrofolate (MTHF) reductase, which converts MTHF to methyltetrahydrofolate, which in turn is a substrate for the remethylation of homocysteine; and from defects in the metabolism of methylcobalamin, the coenzyme of methionine synthase in the remethylation of homocysteine. Furthermore, deficiency of pyridoxine, folic acid, or cobalamin may also elevate plasma levels of H(e).

An observation similar to ours was conducted previously; patients with PAOD showed higher levels of nonprotein-bound H(e) measured after methionine load; factors disturbing methionine metabolism, such as vitamins B6, B12, and folate deficiencies, and renal and hepatic impairments, were excluded. Heterozygosity for cystathionine β-synthase deficiency was also demonstrated in these patients by measuring levels of enzyme activities in cultured fibroblasts. Moreover, a thermostable variant of MTHF reductase was observed frequently in the lymphocytes of subjects with CHD. The pathogenesis of hyperhomocyst(e)inemia was not investigated in our patients and could, as indicated above, result from impairment of certain enzymes in the metabolism of methionine and homocysteine, nutritional deficiencies, or other factors; further studies are needed to clarify involved etiologies.

The mechanisms through which moderate hyperhomocyst(e)inemia may result in coronary and peripheral arterial disease are under investigation. Thus, homocysteine infused for 3 months in baboons caused patchy endothelial aortic denudation, accumulation of intimal proliferating smooth muscle cells, and increased platelet consumption. Direct chemical injury to vascular human endothelial cells by increased concentrations of homocysteine was demonstrated in vitro. Moreover, cultured endothelial umbilical cord cells from an obligate heterozygote for cystathionine β-synthase deficiency also exhibited dysfunction in the presence of methionine. This finding suggests that sulfur-containing amino acids may accumulate in enzyme-deficient cells and cause injury to them. Other in vitro evidence suggests that copper-catalyzed hydrogen peroxide generated from the oxidation of homocysteine may cause endothelial cell injury, and increased plasma copper concentration has been observed in patients with homocystinuria due to cystathionine β-synthase deficiency. Furthermore, treatment of cultured endothelial cells with homocysteine increased Factor V activity and prothrombin activation by Factor Xa. Finally, homocysteine oxidized low-density lipoproteins (LDL) in the presence of redox metals. This oxidation led to their recognition by the acetyl LDL receptor and to a loss of recognition by the LDL receptor; these mechanisms may be involved in the chemotaxis of monocytes and their transformation into foam cells (reviewed in Reference 52).

The present observations demonstrate the feasibility of using a relatively simple method for measuring basal plasma levels of H(e) and confirm that hyperhomocyst(e)inemia is a common occurrence in patients with PAOD. Moreover, the reports mentioned above suggest that multiple mechanisms may
be operative in hyperhomocyst(e)inemia and that this condition is likely to damage vascular endothelium, promote thrombosis, oxidize LDL, and thus influence atherogenesis. Elevated levels of H(e) often decrease toward normal values after the ingestion of small doses of folate or other similarly innocuous treatments. Whether normalization of hyperhomocyst(e)inemia would favorably alter the evolution of atherosclerosis needs to be established.

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**KEY WORDS** • atherosclerosis • thrombosis • genetics
Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease.
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