We measured plasma homocyst(e)ine [H(e)] and other coronary risk factors in 266 patients with early coronary artery disease from 170 families in which two or more siblings were affected and in 168 unmatched controls. The mean H(e) concentration adjusted for significant correlates (serum creatinine, uric acid, and low-density lipoprotein cholesterol) was 12.0 μmol/L in proband cases compared with 10.1 μmol/L in controls (P = 0.0001). Many (17.6%) of the proband cases had H(e) concentrations exceeding the 95th percentile for the controls (relative odds = 4.9, P < 0.001). H(e) among cases was bimodally distributed even after adjustment for concentrations of plasma vitamins. Concordant high H(e) was seen in at least 10 (12%) of 85 families with two or more affected siblings. We conclude that a substantial proportion of early familial coronary artery disease is probably related to production of high concentrations of H(e) by one or more major genes.

Indexing Terms: heritable disorders/creatinine/uric acid/low-density lipoproteins/cholesterol/myocardial infarction/hyperhomocyst(e)inemia/multivariate analysis

Homocysteine exists in plasma as protein-bound and so-called free forms. About 70% of the total plasma homocysteine is bound to albumin. The free forms include homocysteine (homocysteine disulfide) and homocysteine–cysteine mixed disulfide. Only trace amounts of reduced homocysteine are found in plasma (1). Total homocysteine in plasma, including protein-bound and free forms, has been referred to as homocyst(e)ine [H(e)]. Studies evaluating H(e) as a cardiovascular risk factor have consistently demonstrated—for peripheral vascular, cerebrovascular, and coronary artery disease (CAD)—more persons with high H(e) among cases than among controls. Increased concentrations of free forms of homocysteine have also generally been reported among patients with occlusive artery disease (2–5), with one notable exception of a negative finding in relation to CAD (6).

Important recent findings relating H(e) to increased atherosclerotic risk include thickened carotid artery walls, as assessed by high-resolution B-mode ultrasound imaging (7), in persons with high concentrations of plasma [H(e)] and evidence for increased risk of coronary disease associated with H(e), as assessed prospectively in the Physician’s Health Study (8). In most of these studies, H(e) was found to be positively related to serum uric acid and creatinine but unrelated to other cardiovascular risk factors (2).

Although nutritional factors (especially intake of vitamins B6, B12, and folate) (8–10) are known to influence plasma H(e) strongly, genetic factors are also thought to play an important role. Thus, Clarke et al. (3) found that 18 of 60 coronary cases after methionine loading had free H(e) concentrations in serum within the range of obligate heterozygotes for cystathionine β-synthase deficiency. A thermolabile genetic variant of methylenetetrahydrofolate reductase has been reported to be about twice as common in cases with early CAD as in controls and is associated with increased concentrations of H(e) (11). Genest et al. (12) examined H(e) in relatives of male CAD probands. Among the 71 probands, 28% had H(e) concentrations greater than the 90th percentile of normal controls, and 14% of the probands had at least one first-degree relative with H(e) concentrations similarly increased. Although significant spouse/spouse correlation in that study suggested that shared environment contributed to similar concentrations within families, segregation of high H(e) values was apparent in several families. However, no assessment of concurrent diet or plasma concentrations of vitamins was made in that study.

We have previously reported increased concentrations of H(e) among a small series of male and female patients with early familial CAD (13). The present study represents an analysis of a much larger group of early familial coronary cases and controls and provides more stable estimates of risk and multivariate evaluation of several factors that may influence H(e) concentrations. In addition, we measured the plasma concentrations of vitamins B6 and B12 as well as folate in cases and their affected siblings. Cases were ascertained on the basis of two or more siblings having early coronary disease within a family. This provided a unique resource and opportunity to study the occurrence of familial hyperhomocysteinemia.

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6 Nonstandard abbreviations: H(e), plasma homocyst(e)ine; CAD, coronary artery disease; MI, myocardial infarction; CABG, coronary artery bypass grafting; PTCA, percutaneous transluminal coronary angioplasty; ODS, octadeclylsyl; and LDL, HDL, and VLDL, low-, high-, and very-low-density lipoprotein, respectively.

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Materials and Methods

Study Participants

Familial coronary probands were ascertained from two sources. About 75% were identified on computerized discharge records from area hospitals. Men ≤55 years and women ≤65 years who were living in Utah and had been discharged with a diagnosis of myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), or coronary artery bypass surgery (CABG) were contacted by mail to determine whether they had another first-degree relative who had had a similarly early onset of CAD. Those who responded positively were probands with early familial CAD. After 2861 letters were sent, 61 patients were confirmed deceased and 223 had bad addresses and could not be contacted, leaving 2577 potential respondents. Of these, 1481 (57%) did not respond and no further contacts were made. Of the 1096 responders, 339 (31%) reported having an affected first-degree relative. The remainder either reported a negative family history (667) or could provide no information on their relatives. Of the 339 probands with a positive family history, the first 157 who could be scheduled were screened in the Cardiovascular Genetics Research clinics. We were able to recruit for screening >90% of those invited to our clinic. Other living, affected first-degree relatives were also screened. This study included only families with two or more siblings having early CAD (n = 128 families); 29 affected parent–offspring pairs were excluded. An additional 42 probands with early familial coronary disease were ascertained in a similar fashion from a population-based registry of family history forms, which included health information completed as part of the high school curriculum in 50 northern Utah high schools (14, 15). In all, 266 early coronary cases in 170 sibships were included in this study. In 85 sibships, two or more affected living siblings were screened in our clinic.

Given our high recruitment rate (>90%), the current sample is probably quite representative of available families. Although detailed characteristics of nonresponders to our initial letter are not available, we did compare demographic features of the participants with those of all others on the hospital discharge computer files who were sent initial letters. Age and sex distributions were not significantly different. However, significantly more nonparticipants were coded as having had acute MI (52%) as opposed to other ischemic heart diseases. Thus, the participants (36% with acute MI) probably represent individuals who had less severe CAD and survived long enough to participate in our study.

Control subjects were ascertained either from a random population sample of parents listed on the family history forms noted above (n = 58) or were spouses of hypertensive siblings who had participated in previous studies in our clinic (n = 108) (16). Tests for comparability of the two control groups showed no significant differences for any cardiovascular risk factor or H(e), except for a slightly higher plasma total cholesterol among the spouses of hypertensive siblings (P = 0.05).

Because use of a slightly higher serum cholesterol concentration would only provide a more conservative comparison with the early familial coronary cases, we combined the two control groups for all further analyses.

This study was approved by the Institutional Review Board of the University of Utah Medical Center. Subjects signed informed consent statements before participating.

We used the following clinical definitions to categorize subjects:

1. Early familial coronary disease was defined as the presence of two or more first-degree relatives with onset of CAD by age 55 in men and age 65 in women. CAD included prior diagnosis of MI, PTCA, or CABG.

2. A participant was considered to have hypertension if taking antihypertensive medication with a prior physician diagnosis of hypertension, or if the mean of two supine diastolic blood pressures taken with a Critikon Dynamap automated blood pressure machine (Critikon, Tampa, FL) was >95 mmHg.

3. Participants were considered diabetic if they had a prior physician diagnosis of diabetes or if the fasting serum glucose upon screening was >7.77 mmol/L (140 mg/dL).

4. Cigarette smoking was defined as smoking daily for ≥1 year.

Laboratory Methods

H(e) determinations. Most homocysteine in plasma is bound to proteins via disulfide bonds with thiol-containing residues. In addition, oxidized homocysteine (homocystine) and also homocysteine–cysteine mixed disulfide are present in plasma. Total homocysteine, H(e), was measured after reduction of disulfide bonds and detection of released homocysteine by HPLC.

We used the method of Malinow et al. (17) with minor modifications. Briefly, after an overnight fasting, blood samples were collected into glass tubes containing EDTA, mixed, and without delay placed on ice. Plasma was separated by centrifugation within 1 h and stored at −70°C until analyses. To measure H(e), we added to a 200-μL plasma specimen 300 μL of 9 mol/L urea, 50 μL of methanol, and 50 μL of NaBH₄ in 0.1 mol/L NaOH to liberate free homocysteine. After incubation protein was removed by trichloroacetic acid precipitation and centrifugation, and the supernate containing homocysteine was collected. For the HPLC we used a Hewlett-Packard (Palo Alto, CA) Model 1049L. The concentration of the free sulfhydryl group of homocysteine was then quantified by a 1049A electrochemical detector equipped with a gold working electrode and a solid-state reference electrode. The columns were from Bioanalytical Systems (West Lafayette, IN): MF626 Phase II octadecylsilyl (ODS) Hypersil 7-μm-particle guard cartridge and MF 6213 Phase II ODS Hypersil 3-μm-particle analytical column. The mobile phase, modified for better resolution between homocysteine and cysteine, consisted of 0.1 mol/L monochloroacetic acid, 1.0 mmol/L sodium dodecyl sulfate, and 1.0 mmol/L potassium chloride. L-Homocysteine dissolved in 0.1 mol/L...
HCl was used to prepare standard solutions. All H(e) determinations were performed in the laboratories of Associated Regional and University Pathologists (Salt Lake City, UT).

The assay was linear to at least 187 \( \mu \text{mol/L} \). The detection limit, determined as twice the baseline noise level, was 1 \( \mu \text{mol/L} \). Within-assay precision was calculated from results obtained for 10 separate HPLC aliquots from an extracted sample. The imprecision (CV) was 0.86% at a mean concentration (±SD) of 36.0 ± 0.31 \( \mu \text{mol/L} \). To test error introduced by the extraction step, we reextracted low- and high-concentration samples 10 times on the same day. Imprecision, including that of the extraction step, was 13.5% and 11.0% at mean H(e) concentrations of 6.5 and 55.5 \( \mu \text{mol/L} \), respectively. Between-day imprecision, including the imprecision of sample extraction, was calculated from results of 10 separate determinations obtained over a 2-month period; the CVs were 16.4% and 13.9% for mean H(e) concentrations of 6.75 and 45.8 \( \mu \text{mol/L} \), respectively. Although considerable variability appears to be introduced by the extraction step, the CVs are similar to those reported by others (when separate extractions were performed on the same plasma sample) (17). Increased randomness in H(e) measurements due to the extraction step would tend to obscure any real relationships that may exist. However, because the method was the same for both cases and controls, no bias is introduced.

We assessed the accuracy of our assay by comparing 29 samples analyzed in our laboratory with results for the same samples determined previously by the Oregon Regional Primate Research Center (courtesy of M.R. Malinow, Portland, OR). The correlation coefficient was 0.955.

Samples were assayed after supplementation with possible interfering substances. No interference was detected from methionine, glutathione, cystine, or penicillamine.

**Lipid determinations.** Blood samples were collected in the morning after 12–16 h of fasting and prepared according to guidelines of the Lipid Research Clinic's program Manual of Laboratory Operations (18). Lipids were measured by a microscale procedure developed in our laboratory (19). Briefly, high-density lipoprotein (HDL) was measured in the supernate after precipitation of apolipoprotein B-containing particles with dextran sulfate-MgCl\(_2\) and centrifugation in an Eppendorf microcentrifuge. Triglyceride-rich lipoproteins, primarily very-low-density lipoprotein (VLDL), were separated from low-density lipoprotein (LDL) and HDL by use of a Beckman (Fullerton, CA) TL100 ultracentrifuge. The value for VLDL cholesterol was taken as the measured cholesterol in the top fraction. We verified this value by comparison with the total cholesterol minus the cholesterol in the bottom fraction (containing LDL + HDL); the results were virtually identical. Cholesterol and triglycerides in total plasma and subfractions were measured with a Roche (Rutherford, NJ) Fara II automated analyzer. Our lipid laboratory participates in the standardization program of the Centers for Disease Control and Prevention, Atlanta, GA.

**Vitamin assays.** All assays were performed with plasma collected as described above from fasting subjects. Vitamin B\(_6\) (as pyridoxal 5'-phosphate) was determined by a radioenzymatic method. In the presence of vitamin B\(_6\), tyrosine decarboxylase apoenzyme converts tyrosine to tyramine. The amount of [\(^{3}H\)]tyramine formed from [\(^{3}H\)]tyrosine during a timed incubation is proportional to the vitamin B\(_6\) in the specimen (20). The reference range for this assay is 20–93 nmol/L.

The Ciba-Corning (Medfield, MA) ACS automated chemiluminescence system was used to quantify vitamin B\(_{12}\) and folate in plasma. The ACS utilizes a solid-phase assay in which vitamin B\(_{12}\) or folate in the sample competes with acridinium ester-labeled vitamins for limited binding sites on vitamin-binding proteins covalently coupled to magnetic particles (21). Vitamin B\(_{12}\) concentrations of 148–664 pmol/L are considered normal; values <66–74 pmol/L are evidence for frank deficiency. The reference range for folate is 6.8–36 nmol/L.

**Statistical Analysis**

The SAS Statistical Software Package was used for data analysis (SAS Institute, Cary, NC). Statistical analyses of data for triglycerides and H(e) were done after logarithmic transformation to normalize the distribution. Statistical tests included Student's t-test, \( \chi^2 \), Fisher's Exact Test, Pearson's correlation, and stepwise multiple linear regression with use of both backward and forward stepping procedures. Analysis of covariance was performed with use of the SAS GLM program to correct for potential confounding variables to obtain and compare corrected H(e) concentrations in cases and controls.

All of the above analyses were performed with only unrelated cases and controls because related cases would not provide independent observations. In families with multiple screened siblings, the unrelated cases were selected as the sibling with the earliest onset of CAD. To examine differences in H(e) between cases and controls, including all cases of siblings affected with early coronary disease, we used a general linear model incorporating a term for the intraclass correlation between dependent observations (22) (courtesy of Munoz, Rosner, Carey, and Vandenburgh from the Channing Laboratory, Harvard Medical School, Boston, MA). To determine whether the observed distribution was best described by a single, continuous distribution or a commingling of multiple distributions, we performed maximum likelihood analysis by the method of Day (23). Equal as well as unequal standard deviations in the two modes were tested.

**Results**

Clinical characteristics of unrelated early familial coronary cases and controls are shown in Tables 1 and 2. Compared with the controls, the cases were, on average, 9 years older and more often male. In addition, there
were marked differences in the prevalence of hypertension and diabetes. The cases had significantly higher body mass index, blood pressures, and concentrations of serum glucose, plasma total cholesterol, triglycerides, and VLDL cholesterol. Plasma HDL cholesterol was lower among the cases. H(e) was significantly higher in the cases than in the controls. Other potential determinants of H(e) also showed differences between cases and controls, most notably serum concentrations of creatinine, uric acid, and albumin.

Measured concentrations of plasma LDL cholesterol did not differ between the cases and the controls, probably because of treatment of 52% of the cases for high cholesterol (34% were taking drugs). Only 6% of the controls were on a diet and none was taking lipid-lowering medication.

Percentiles of H(e) among the control group are given in Table 3. Among the male controls from this study (n = 168), H(e) was 11.3 ± 2.96 µmol/L (mean ± SD; range 6–19 µmol/L). For female controls, H(e) was 8.8 ± 2.7 µmol/L (range 4–18 µmol/L). Mean H(e) concentrations were 3.3 µmol/L higher in cases than in controls (P = 0.0001). Student’s t-test was performed on the logarithmically transformed variable.

The distribution of unadjusted, nontransformed H(e) values in all cases (including all affected siblings) and controls is shown in Fig. 1. The cases have higher mean H(e) concentrations than do the controls, as well as many more individuals in the high range. Among unrelated cases, 30 (18%) of 170 had H(e) values above the controls’ sex-specific 95th percentile, compared with 7 (4.2%) of 168 controls (relative odds = 4.9, χ² = 15.7, P < 0.001). Logarithmic transformation of H(e) results in a gaussian distribution among controls, but the increased number of high values in cases remains apparent (not shown). H(e) concentrations remained significantly higher in cases when comparisons were made within age- and sex-specific groups (Fig. 2).

Despite the striking differences in H(e) between cases and controls, the question arises, would the differences remain after adjustment for potentially confounding covariates? We performed multiple stepwise regression with H(e) as the dependent variable and all the variables in Tables 1 and 2 as potential independent variables. Both forward and backward stepping models were tested with similar results. Table 4 shows the results of the forward multiple stepwise linear regression. In these analyses, all unrelated cases and controls were considered together, regardless of case status.
Creatinine was the strongest and most consistent correlate of H(e). Furthermore, creatinine explained sex differences within the case and control groups (Fig. 3). Other variables entered into both forward and backward stepping models included uric acid, lipid-lowering medication, measured LDL cholesterol, cigarette smoking, and age. Total serum protein was moderately correlated with log-transformed H(e) in univariate analysis ($r = 0.19, P = 0.014$); however, on stepwise regression, measured LDL entered first and markedly decreased the residual correlation between total protein and H(e).

Differences in H(e) between the cases and the controls were then tested by analysis of covariance. Lipid-lowering medication was not included in these analyses because none of the controls was taking these medications and, among cases, there was no difference in H(e) concentrations between those taking and those not taking drugs to lower lipid concentrations. The beginning

### Table 4. Independent variables correlated with plasma H(e) (log transformed) in multiple forward stepwise regression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient*</th>
<th>SE</th>
<th>Partial R²</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.791</td>
<td>0.262</td>
<td>0.214</td>
<td>9.1</td>
<td>0.0027</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.484</td>
<td>0.0791</td>
<td>0.052</td>
<td>5.4</td>
<td>0.021</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.0291</td>
<td>0.0125</td>
<td>0.028</td>
<td>11.8</td>
<td>0.0012</td>
</tr>
<tr>
<td>Lipid-lowering medication</td>
<td>0.144</td>
<td>0.0438</td>
<td>0.025</td>
<td>6.8</td>
<td>0.0087</td>
</tr>
<tr>
<td>Measured LDL cholesterol</td>
<td>0.00119</td>
<td>0.000459</td>
<td>0.0202</td>
<td>10.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>0.0747</td>
<td>0.0228</td>
<td>0.010</td>
<td>5.3</td>
<td>0.021</td>
</tr>
<tr>
<td>Age, years</td>
<td>0.00473</td>
<td>0.00205</td>
<td>0.007</td>
<td>3.2</td>
<td>0.075</td>
</tr>
<tr>
<td>Protein</td>
<td>0.0631</td>
<td>0.0353</td>
<td>0.007</td>
<td>3.2</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Model

0.36 24.8 0.0001

* For the final model with all listed covariates included.

Variables tested included: age, gender (0 = female, 1 = male), body mass index, cigarette smoking (never = 0, former = 1, current = 2), hypertension (no = 0, yes = 1), diabetes (no = 0, yes = 1), taking lipid-lowering medication (no = 0, yes = 1), systolic blood pressure, diastolic blood pressure, and plasma triglycerides, HDL cholesterol, measured LDL cholesterol, glucose, uric acid, creatinine, and total protein (all in mg/dL). Unrelated familial coronary cases (n = 170) and controls (n = 168) were included together.
model tested included serum concentrations of creatinine, uric acid, and measured LDL cholesterol, as well as cigarette smoking and age. These were the only variables that were selected at \( P < 0.05 \) in both the forward and backward stepping models. We also tested the interaction terms of these variables with case status, eliminating nonsignificant interaction terms stepwise on the basis of their \( F \) values. None of the interaction terms remained significant, suggesting that effects of the selected variables did not differ between the case and control groups. Age and cigarette smoking status were also not significantly related to log-transformed H(e) concentrations in the analysis of covariance. In the final model, besides case status, only serum creatinine, uric acid, and measured LDL cholesterol remained significant \( (P < 0.0001, P < 0.0001, \text{and } P = 0.004, \text{respectively}) \). The results of this analysis of covariance are shown in Fig. 4. The difference between cases and controls for adjusted H(e), i.e., determined from the difference of the antilogs, was 2.0 \( \mu \text{mol/L} \) \( (P = 0.0001) \). When we repeated these analyses, restricting the evaluation to subjects of ages 35–70 years so that cases and controls compared would have the same age range (163 cases, 160 controls), we saw no effect on the H(e) difference between cases and controls or its statistical significance.

Fig. 3. Plasma homocyst(e)line as a function of serum creatinine in (top) unrelated case probands \((r = 0.35, P < 0.00001)\) and (bottom) control subjects \((r = 0.36, P < 0.00001)\).

○, women; △, man.

Fig. 4. Antilog of logarithmically adjusted homocyst(e)line in cases and controls after multivariable adjustment for serum creatinine, uric acid, and measured plasma LDL cholesterol by analysis of covariance. Results are shown as least squares mean ± SE.

Familial H(e) and Plasma Vitamin Concentrations

To assess the role that nutrient intake may have played, we determined the plasma concentrations of vitamins \( B_6 \), \( B_{12} \), and folate in all cases and their affected siblings. There were significant inverse correlations between the concentrations of each vitamin and of H(e) in plasma. In addition, the vitamin concentrations were positively correlated with each other (Table 5). Accordingly, we performed step-wise regression among all cases and their affected siblings, using log-transformed H(e) as the dependent variable and the above-named vitamins and the previously considered potential covariates as independent variables. Again, creatinine was the first independent variable to enter \((F = 42.7, P < 0.0001)\). Plasma folate concentration was next \((F = 20.6, P < 0.0001)\), followed by vitamin \( B_{12} \) \((F = 11.6, P = 0.0008)\), uric acid \((F = 10.9, P = 0.001)\), gender \((F = 9.6, P = 0.002)\), and total protein \((F = 8.1, P = 0.005)\). Vitamin \( B_6 \) concentrations did not add significantly to the model once folate and vitamin \( B_{12} \) were included.

To further evaluate H(e) concentrations in cases and controls by utilizing all available cases, we used a general linear model with intraclass correlation. This model accounts for similarities between siblings (non-independent observations) as well as contributions from the above-named covariates. The regression coefficient for case status without adjusting for vitamin concentrations was 0.161 \( (\text{SE 0.030, } P < 0.00001) \). Taking the antilog of the regression coefficient for case status provides an estimate of the adjusted ratio of H(e) in cases to H(e) in controls (the slope of the regression line). If the control mean is taken as 10 \( \mu \text{mol/L} \), the expected mean

<table>
<thead>
<tr>
<th>Pearson ( r )</th>
<th>VIT. ( B_6 )</th>
<th>VIT. ( B_{12} )</th>
<th>Folate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \ln[H(e)] )</td>
<td>-0.18*</td>
<td>-0.34</td>
<td>-0.25</td>
</tr>
<tr>
<td>VIT. ( B_6 )</td>
<td>0.39</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>VIT. ( B_{12} )</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* \( P = 0.0028 \); all other correlations, \( P < 0.0001 \).
case value would be 11.75 μmol/L, almost identical to the above estimate obtained by using a general linear model without intraclass correlation.

Among individual unrelated cases, 28% had H(e) concentrations greater than the 90th percentile, and 17% had values greater than the 95th percentile. This provides an estimate of the prevalence of sporadic high concentrations of H(e) among early familial CAD cases. The presence of two or more case siblings having increased H(e) in a given family may serve to diagnose familial hyperhomocyst(e)inemia leading to early familial CAD. Initially, we used as cutoffs the sex-specific 90th percentiles derived from the control group. When plasma samples were available for two or more affected siblings in a family (85 families), we found 8 (9.4%) families with two or more cases having H(e) greater than the 90th percentile (1% expected). In 3 (3.6%) of these 85 families two or more siblings had H(e) greater than the control 95th percentile (0.25% expected). After adjustment for vitamin content (folate, vitamin B_{12}, and vitamin B_{9}), concordant H(e) concentrations exceeding the control 90th percentile were found in 13 (15%) of the families, and 3 (3.6%) families still showed concordant concentrations greater than the 95th percentile. Vitamin content below the reference range was seen in only three cases: two with low vitamin B_{9} values (8.5 and 19 nmol/L) and another with a low folate concentration (1.4 nmol/L). Eliminating the 3 families with vitamin-deficient members yielded 10 families (12%) still with concordant, vitamin-adjusted H(e) concentrations above the 90th percentile of the control group.

The intraclass correlation of H(e) among sibling cases (based only on families with two or more affected cases) was calculated as the ANOVA estimator of sibling correlations for log-transformed H(e) values adjusted for serum creatinine, uric acid, and measured LDL cholesterol (24). The estimated intraclass correlation coefficient was significant both before (r = 0.30, P = 0.001) and after adjusting for plasma folate and vitamin B_{12} (r = 0.26, P = 0.005).

The probands with early familial CAD appeared to display a bimodal distribution of H(e) values. We formally tested this by commingling analyses, using the maximum likelihood method. H(e) was log-transformed and adjusted for the same variables (both excluding and including plasma vitamin concentrations) that had been found to be significantly associated with H(e) in stepwise regression. Results are depicted in Fig. 5. The best fit to the data unadjusted for plasma vitamin concentrations was two distributions having equal SDs, with the means separated by 2.16 SD (x^2 = 6.85, P = 0.03). The higher mode contained 14% of cases. After adjusting for plasma vitamin concentrations, the distribution was even more significantly bimodal (x^2 = 9.2, P = 0.01), with the modes separated by 2.5 SD; however, only 8% remained in the upper mode. These results were obtained by considering only unrelated cases.

When all coronary cases were included without adjustment for plasma vitamin concentrations, the separation of modes was 2.7 SD (x^2 = 18.6, P = 0.00009). The higher mode in this commingling analysis included 7.5% of cases. After adjustment for plasma vitamin concentrations, the separation of modes was 2.9 SD—even more significant (x^2 = 25.0, P = 0.000004)—with the higher mode including 7.8% of cases. However, these x^2 values derived from calculations including affected siblings are inflated, due lack of independence of subjects.

**Discussion**

This is the first study to examine H(e) concentrations in a large number of families ascertained by the presence of multiple siblings with early CAD. It is also the first study to report clear evidence for bimodality in the H(e) distribution from such cases. This bimodality was present both before and after controlling for intake of vitamins B_{9}, B_{12}, and folate. Finding 9–12% of 85 families with two or more affected early coronary cases having increased concentrations of H(e) corresponded remarkably well with the results of commingling analysis, which found ~8% of coronary cases in the higher H(e) mode after adjustment for plasma vitamin concentrations. These results suggest that a major gene influencing H(e) concentrations may be present in ~10% of early familial CAD cases. The absence of the higher H(e) mode in controls further supports the relevance of high H(e) in the pathogenesis of early CAD.
The mechanism(s) for the association of hyperhomocyst(e)inemia with atherosclerosis are still unclear and under intense investigation. Reported pathophysiological effects of homocysteine have generally fallen into three categories: endothelial cell toxicity, increased platelet adhesiveness, and modification of clotting factors. Recently, evidence for effects on lipid metabolism has also been presented. Here we briefly summarize some of these findings.

Chronic infusion of homocysteine into baboons has resulted in extensive endothelial desquamation and a reduction in platelet survival (25). These findings were thought to be a result of direct cytotoxicity of homocysteine and its thiolactone, largely through the actions of copper-catalyzed hydrogen peroxide generated by autoxidation of the SH group (26, 27). Endothelial cytotoxicity of homocysteine may be modulated by the presence of endothelial-derived relaxing factor or nitric oxide; nitric oxide and homocysteine react under physiological conditions to form S-nitrosohomocysteine. Both this product and S-nitrosocysteine possess potent antiplatelet and vasodilatory activity. Stamler et al. (28) demonstrated that homocysteine did not cause platelet aggregation directly but could, after prolonged incubation, inhibit the release of nitric oxide from endothelial cells. Thus, homocysteine may initially disarm the protective capacity of endothelial cells to secrete endothelial-derived relaxing factor and thereafter cause direct injury with subsequent desquamation. These findings may help explain prior contradictory studies in which platelets from patients with homocystinuria frequently manifested increased adhesiveness, whereas addition of homocysteine or its derivatives to isolated platelets had inconsistent effects (28, 29).

Patients with homozgyous homocystinuria have long been noted to have frequent and early venous as well as arterial thromboses. Homocysteine may alter the balance between procoagulant and anticoagulant by selectively inhibiting the processing and secretion of thrombomodulin (30), reducing the activation of protein C (31), and inducing a protease activator of coagulation factor V (32). Processing of von Willebrand factor (33) and possibly factor VII (29) is similarly impaired. Other studies have reported reductions in antithrombin III activities in patients with homocystinuria (29). Decreased binding to its receptor and impaired proteolytic activity of tissue plasminogen activator resulted from H(e) incubations (29, 34). Finally, homocysteine at concentrations as low as 8 μmol/L significantly increased the affinity of lipoprotein(a) for fibrin. Such a complex may inhibit plasmin and favor thrombosis (35). Thus, multiple mechanisms may contribute to the thrombotic diathesis associated with increased H(e).

The reaction of thiol compounds to form hydrogen peroxide involves generation of free radical intermediates. Increased concentrations of H(e) have therefore been hypothesized to lead to increased production of oxidized lipoproteins, which may promote foam cell formation by way of the scavenger receptor (36, 37). This concept has recently been challenged, however, because no increases in cholesterol hydroperoxide were found in HDL of patients with homozygous cystathionine β-synthase deficiency (38).

There were some limitations in our analyses. The age and sex distributions were considerably different between cases and controls. The older average ages of the cases probably contributed importantly to the much higher prevalence of hypertension and diabetes in this group than in the controls. However, after adjustment for serum creatinine, H(e) concentrations were not related to age or gender. Furthermore, when examined within case- and gender-specific groups, age did not correlate significantly with logarithmically transformed H(e) concentrations (data not shown). Finally, none of the results were materially affected by constraining the age range of the cases and the controls to be entirely overlapping.

The paucity of women among the early familial coronary cases was somewhat surprising, given our use of an older age cut-point to allow for the well-known difference in coronary incidence rates between men and women. Possibly, women with early familial coronary disease did not survive as frequently as their male counterparts and thus were not available for screening in this study. However, despite the excess of male cases, sex differences could not explain the H(e) discrepancy between cases and controls. Serum creatinine concentration was clearly the strongest covariate of H(e) and explained sex differences within the case and control groups (see Fig. 1). Case–control differences in H(e) were still highly significant after adjustment for relevant covariates. Furthermore, sex- and age-specific differences between cases and controls were highly significant (Fig. 2). In addition, a marked excess of high H(e) values was seen among cases (relative odds, 4.9), exceeding the sex-specific 95th percentile derived from controls (P <.001).

Other studies have noted a fairly strong negative correlation between vitamin intake or concentrations in plasma (especially vitamins B_{12}, B_{13}, and folate) and H(e) concentrations among free-living persons (8–10, 39). Indeed, very high H(e) concentrations (up to 467 μmol/L in one coronary patient, and many in the 20–40 μmol/L range) have been reported in patients with vitamin B_{12} and (or) folate deficiency, especially when multiple vitamin deficiencies were present (10, 40–42). Vitamin supplements containing these B vitamins effectively lower H(e) concentrations in many cases (10, 42). Because plasma vitamin concentrations were not measured in the controls, we cannot exclude that lower average intake of B vitamins among coronary cases could have contributed with the higher concentrations of H(e) in cases compared with controls. However, the plasma concentrations of vitamins in our cases tended to be higher than in the Framingham Study population (data not shown) (39). Furthermore, the mean plasma values for these vitamins were in the upper reference range, with only a few cases having values below the lower limits of normal.

The presence of bimodality, even after correcting for
plasma vitamin concentrations, strongly suggests that one or more major genes affecting H(e) is contributing to risk of early familial CAD. The lack of H(e) values >20 µmol/L in our controls suggests that such genes are not common. Two candidates are the genes affecting cystathionine β-synthase deficiency (3) and thermolabile tetrahydrofolate reductase (11). Family studies with genetic markers for candidate genes should prove most revealing. Such studies should include a careful examination of relevant vitamin nutrure by utilizing appropriate dietary assessment and (or) blood measurements.

Treatment of lipid abnormalities was much more frequently encountered among early familial CAD cases than controls. In fact, none of the controls was taking medication for hyperlipidemia. Although H(e) concentrations were higher in persons taking lipid-lowering medications than in those not taking these medications when the entire group of cases and controls were considered together, this difference vanished when the same comparison was restricted to cases only. This observation raises the question of overadjustment in multivariate analyses. By combining cases and controls, spurious associations can be introduced between H(e) and other coronary risk factors simply because such risk factors, as well as H(e), may be higher in cases, as one might expect. Statistical adjustment of H(e) for risk factors associated with early coronary disease, but not necessarily associated with H(e), would tend to lower the effect of H(e) concentrations in cases and raise the effect in controls. To avoid this problem, we first examined interactions with case status. The covariates included in the final general linear model did not show interactions with case status. Nevertheless, adjustments for covariates such as serum creatinine and uric acid raise another issue. If serum H(e) is a causal factor leading to early CAD, persons with higher concentrations of H(e) due to diminished renal clearance may be at higher risk. Indeed, the increased risk for CAD among patients with renal disease may, in fact, be mediated by H(e). The strong correlation between serum uric acid and H(e) may help elucidate the long-observed association of serum uric acid with CAD risk. Furthermore, higher mean concentrations of H(e) in men may help explain the well-known gender difference in CAD incidence. After adjusting for sex differences, serum uric acid or even serum creatinine may thereby obscure physiologically relevant differences in H(e) between cases and controls. Nevertheless, such adjustment is a conservative way to compare H(e) concentrations and did not reduce the statistical significance of the case-control difference.

Measured plasma LDL cholesterol concentrations were positively correlated with H(e) within case and control groups considered individually. Such a correlation has not been reported in previous studies. Interestingly, we found a strongly positive correlation between serum total protein and measured LDL cholesterol. Possibly the positive correlation between H(e) and measured LDL cholesterol was a reflection of increasing amounts of homocysteine bound to serum proteins in general and particularly to LDL apolipoprotein B. A chance association between measured LDL cholesterol and H(e) cannot be ruled out, however.

This and most other studies of H(e) in relation to coronary disease have been retrospective. The one prospective study available, which used a nested, case-control design, found positive results similar to the present study (8). In that study there appeared to be an overrepresentation of markedly high H(e) concentrations among male physicians who subsequently developed coronary disease. Thus, the high H(e) concentrations were apparent before the diagnosis of CAD and were not merely a consequence of the disease.

In conclusion, our study provides an estimate of the percentage of early familial coronary disease that may be attributed to high H(e) concentrations. Among unrelated early familial CAD cases, 28% had a H(e) content exceeding the 90th percentile of controls and 18% exceeded the 95th percentile. Among the 85 families screened with two or more early cases, 9–12% had two or more members with H(e) concentrations above the 90th percentile (vs 1% expected) and 3.6% of the families had two or more cases with H(e) exceeding the 95th percentile (vs 0.25% expected). Additionally, there was significant intraclass correlation of H(e) among sibships before and after adjustment for plasma vitamin concentrations. Our results are consistent with those of Genest et al. (12), who reported that 20% of 71 probands with premature CAD had H(e) concentrations greater than the 90th percentile of a control population. When they screened first-degree relatives of the probands, 14% of the families were found to have familial hyperhomocyst(e)inemia. These results parallel the 14% of unrelated probands we found in the higher H(e) mode before adjustment for vitamin concentrations. After this adjustment, 8% of our unrelated probands remained in the higher H(e) mode. Further family studies of H(e) are needed to determine the contributions of specific genetic defects and how these might be modified by vitamin intake.

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References