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Agreement among Four Homocysteine Assays and Results in Patients with Coronary Atherosclerosis and Controls

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Background: **Hyperhomocysteinemia has been associated with coronary atherosclerosis in many, but not all, prospective and retrospective studies. Some on these inconsistencies may be attributed to methodological variabilities.**

Methods: **In the present study, three newly commercially available assays and one in-house HPLC assay for total homocysteine (tHcy) were utilized in 99 subjects with angiographically documented atherosclerosis and in 91 community controls matched by age, gender, and smoking history. The in-house assay, a modified Fortin and Genest HPLC method, was compared with the Bio-Rad HPLC assay, the Abbott IMx® fluorescence polarization immunoassay, and a Bio-Rad enzyme-linked immunoassay (EIA) microtiter method.**

Results: **Correlation coefficient values between the in-house HPLC assay and the Bio-Rad HPLC, the Abbott IMx, and the Bio-Rad EIA assays were 0.95, 0.96 and 0.90, respectively. Although tHcy concentrations were higher in cases compared with controls by all four methods, the difference reached statistical significance only with the in-house HPLC procedure (median, 13.5 ± 6.7 μmol/L in cases vs 10.9 ± 4.8 μmol/L in controls; $P < 0.01$, adjusting for covariates), where it was an independent predictor of case or control status, along with hypertension, total cholesterol, and triglycerides. The tHcy distributions in cases and controls demonstrated significant overlap. The number of atherosclerotic major coronary vessels was associated with significantly higher tHcy ($P < 0.01$ for trend) in all four methods.**

Conclusions: **The three commercial assays for tHcy differed in analytical and clinical performance. Analytically, the Abbott IMx method showed the best comparability with the in-house assay, but clinically, the three commercial methods were similar and did not distinguish cases from controls.**

Homocysteine (Hcy)[5] is a thiol-containing amino acid produced by cellular demethylation of methionine. Homozygous homocystinuria, a rare genetic disorder usually caused by cystathionine β-synthase deficiency, leads to severe increases of serum Hcy to concentrations >100 μmol/L and is associated with venous thrombosis and premature atherosclerosis *(1)*. In 1969, McCully *(2)* postulated that hyperhomocysteinemia causes the profound vascular changes seen in this disorder. Subsequent cross-sectional epidemiological studies have demonstrated that even mild increases in total Hcy (tHcy; 15–25 μmol/L) may pose a significant, independent risk factor for coronary heart disease (CHD), peripheral vascular disease, and acute coronary and cerebrovascular events. However, prospective studies have been far less consistent in their conclusions, with many failing to support Hcy as a significant risk factor for coronary atherosclerosis *(3–6)*.

Some of these inconsistencies may be attributed to differences in methodologies. Hcy exists in several major forms (free Hcy, homocystine, mixed disulfides involving Hcy, homocysteine thiolactone, and protein-bound Hcy). A variety of methods have been utilized in the literature to measure the fasting or post methionine-loading con-

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[5] Nonstandard abbreviations: Hcy, homocysteine; tHcy, total Hcy; CHD, coronary heart disease; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; SAH, *S*-adenosyl-L-homocysteine; and EIA, enzyme-linked immunoassay.

centrations of some or all of these potentially atherogenic variants. Most recent studies have focused on the measurement of fasting tHcy, the sum of these free and protein-bound moieties of Hcy. In the present study, we evaluate the relationship between tHcy and coronary artery disease by utilizing three newly commercially available assays and our in-house HPLC assay in patients with angiographically documented coronary atherosclerosis and in appropriate controls.

Materials and Methods

From January through March 1997, 99 subjects (82 men and 17 women) were enrolled after undergoing cardiac catheterization for symptoms or stress tests compatible with myocardial ischemia and found to have a stenotic lesion of >50% in at least one major coronary vessel (left anterior descending, left circumflex, or right coronary arteries). These subjects were categorized based on the number of vessels involved. From January through March 1997, 91 controls (73 men and 18 women) were recruited from the same neighborhoods in Damascus as the case subjects and matched on age, gender, and smoking history. Two patients in the case group had myocardial infarctions more than 6 months before enrollment.

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. Controls had no prior history of CHD or symptoms compatible with angina pectoris and had normal resting electrocardiograms. Hypertension was defined as resting systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥95 mmHg. Diabetes mellitus was established by prior diagnosis, a fasting blood glucose of >1200 mg/L, or a glycosylated hemoglobin concentration >6%.

Blood samples were collected after a 12-h fast for the determination of total cholesterol, HDL-cholesterol (HDL-C), triglycerides, and tHcy. In cases, samples were obtained on the day before the angiography. Blood specimens containing EDTA were placed on ice within 10 min of collection, and blood specimens containing no anticoagulant 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. Serum samples were used for the measurement of lipids and lipoproteins at the University of Damascus Hospitals, and plasma EDTA samples were used for the determination of tHcy at Children's Hospital in Boston. Analysts were blinded to the identity of the samples, which were coded and run anonymously with alternating cases and controls. Cholesterol and triglycerides were measured on a Hitachi 911 automated analyzer (Roche Diagnostics) according to the manufacturer's recommendations. Triglyceride measurements were corrected for endogenous glycerol. HDL-C was measured after phosphotungstic acid-$MgCl_2$ precipitation (Roche Diagnostics). LDL-cholesterol (LDL-C) was estimated using the Friedewald equation.

The in-house HPLC assay is a modification of the method of Fortin and Genest *(7)*. Briefly, the Hcy, mixed-disulfide, and protein-bound Hcy in 120 μL of sample were reduced to free Hcy with tri-*n*-butylphosphine. After protein precipitation with perchloric acid, the preparation underwent derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4 sulfonate. tHcy was measured photometrically after separation with a reversed-phase column. The in-house method demonstrated run-to-run interassay imprecision (CVs) of 5.8%, 7.5%, and 3.9% for 18 samples at each of tHcy concentrations of 9, 34, and 59 μmol/L, respectively.

The three commercially available assays were performed according to manufacturers' recommendations. We compared our assay with the Bio-Rad HPLC Hcy assay (Bio-Rad Laboratories). In this assay, 50 μL of sample is subjected to reduction and derivatization with a water-soluble trialkylphosphine and a fluorescent thio-specific dye (ABD-F), respectively. After precipitation of protein with trichloroacetic acid, the supernatant is injected on a reversed-phase column heated to 45 °C. tHcy is detected fluorometrically (λ_{ex}, 385 nm; λ_{em}, 515 nm). The run-to-run CVs (n = 18) for the Bio-Rad HPLC were 7.7% and 3.8% at tHcy concentrations of 8 and 30 μmol/L, respectively.

The Abbott IMx® homocysteine assay (Abbott Laboratories) is a fully automated, fluorescence polarization immunoassay. This assay involves the initial reduction of Hcy, mixed-disulfide, and protein-bound Hcy to free Hcy with dithiothreitol, followed by conversion to *S*-adenosyl-L-homocysteine (SAH) by bovine SAH hydrolase and excess adenosine. After mouse monoclonal SAH antibody is added to the prepared sample, *S*-adenosyl-L-cysteine fluorescein tracer is added, which competes with SAH for antibody binding sites. Finally, tHcy is quantified by the intensity of the polarized fluorescent light measured by the IMx optical assembly. Run-to-run CVs (n = 22) were 2.9%, 0.8%, and 1.7% at tHcy concentrations of 7, 12.5, and 25 μmol/L, respectively.

The Bio-Rad (Axis®) enzyme-linked immunoassay (EIA) is a microtiter assay that involves four steps: (*a*) reduction of Hcy, mixed disulfides, and protein-bound forms of Hcy to free Hcy by dithiothreitol; (*b*) conversion of free Hcy and adenosine to SAH by bovine SAH hydrolase; (*c*) competitive binding of sample SAH and immobilized SAH with monoclonal mouse anti-SAH, and spectrophotometric measurement of peroxidase activity after the addition of anti-mouse antibody labeled with horseradish peroxidase. Run-to-run CVs (n = 15) for the Bio-Rad EIA procedure were 11% and 8.1% at mean tHcy concentrations of 6.43 and 25.6 μmol/L, respectively.

The study protocol was approved by the Committee on the Protection of Human Subjects of the University of Damascus and informed consents were obtained from all participants.

STATISTICAL METHODS

The results for gaussian distributed data are reported as means ± SD. Because tHcy and triglyceride distributions were skewed rightward, their values are expressed as medians ± SD and were natural log-transformed to normalize the data for subsequent analyses. The remaining variables are expressed as means ± SD. tHcy bias is calculated as the test method minus the in-house method.

Using previously published Hcy studies *(4–6)*, we estimated that there was 90% power ($\alpha = 0.05$, two-sided) to find a 1.6 μmol/L difference in Hcy between cases and controls. Case-control differences were evaluated with the unpaired Student *t*-test and a general linear model to adjust for covariates. Pearson correlation coefficients and linear regression with least-squares method were used to evaluate correlations between patient characteristics, the tHcy assays, and the other analytes. To define independent predictors of case and control status, we used stepwise, forward logistic regression with a model that included age, gender, diabetes, smoking, hypertension, lipids, and tHcy.

All *P* are two-tailed with $P < 0.05$ regarded as statistically significant. Analyses were performed with SPSS 9.0 (SPSS Inc.).

Results

Baseline patient characteristics and lipid measurements are presented in Table 1. Age, gender, and current smoking status were not significantly different between controls and cases. However, subjects who reported no past use of tobacco were relatively higher in the control vs case group (60% vs 41%, respectively; $P = 0.007$). In addition, the prevalence of diabetes and hypertension was higher in the case group. Total cholesterol, LDL-C, and the total cholesterol: HDL-C ratio were significantly higher in subjects with coronary disease.

Table 1. Baseline characteristics.[a]

	Controls (n = 91)	Cases (n = 99)	*P*
Age, years	47.0 ± 11.1	52.0 ± 8.8	NS[b]
Gender			NS
Male	73	82	
Female	18	17	
Hypertension	1	16	<0.001
Diabetes	0	17	<0.001
Current smoker	36	39	NS
History of MI	0	2	NS
Cholesterol, mg/L	2090 ± 429	2220 ± 531	<0.001
LDL-C, mg/L	1440 ± 381	1500 ± 451	0.041
HDL-C, mg/L	320 ± 91	330 ± 71	NS
Triglycerides, mg/L	1300 ± 965	1960 ± 1641	<0.001
Cholesterol/HDL-C	6.3 ± 2.4	7.1 ± 1.6	0.04
Angiography			
One-VD		31	
Two-VD		40	
Three-VD		28	

[a] Lipid values given in mean ± SD.

[b] NS, not significant; MI, myocardial infarction; VD, coronary vessel(s) diseased.

Correlations between our in-house method and the commercially available assays were generally good. Compared with the in-house procedure (x), the following linear regression equations and correlations were observed: Abbott IMx, $y = 0.96x + 1.19$ ($r = 0.96$; $P < 0.0001$); Bio-Rad HPLC, $y = 0.81x + 1.38$ ($r = 0.95$; $P < 0.0001$); and Bio-Rad EIA, $y = 0.95x + 1.66$ ($r = 0.90$; $P < 0.0001$). Weak correlations were noted between the HDL-C and the Abbott IMx tHcy ($r = -0.14$; $P = 0.06$), Bio-Rad HPLC tHcy ($r = -0.16$; $P = 0.03$), and Bio-Rad EIA tHcy ($r = -0.16$; $P = 0.03$), but not with the in-house method ($r = -0.11$; $P = 0.14$).

Mean tHcy bias was 0.59 ± 1.74 μmol/L for the Abbott IMx, −1.21 ± 2.03 μmol/L for the Bio-Rad HPLC, and 1.01 ± 2.76 μmol/L for the Bio-Rad EIA. LDL-C was weakly correlated with Abbott IMx bias ($r = -0.21$; $P = 0.005$) and Bio-Rad EIA bias ($r = 0.19$; $P = 0.01$). The mean of the in-house method and comparison method was positively correlated with Bio-Rad HPLC bias ($r = -0.436$; $P < 0.001$) but not with the Bio-Rad EIA ($r = 0.125$; $P = 0.85$) or Abbott IMx biases (Fig. 1). The Bio-Rad HPLC method tended to overestimate tHcy at low concentrations and underestimate tHcy at higher concentrations compared with the in-house assay.

We compared the tHcy measured with the four assays in the study groups. tHcy in case subjects was significantly higher than in control subjects only when measured with the in-house HPLC assay ($P = 0.01$) after controlling for age, gender, smoking, diabetes, hypertension, and lipids (Table 2). When cases were classified into quartiles derived from tHcy of controls, cases demonstrated an increased likelihood of being in the top two quartiles compared with the lower two quartiles in all assays (Fig. 2). In nondiabetic subjects, tHcy (in-house HPLC), hypertension, cholesterol, and triglycerides were independent predictors of case or control status.

Overall, male subjects had significantly higher tHcy concentrations than did women with the in-house HPLC (12.9 μmol/L for men vs 10.8 μmol/L for women; $P < 0.05$), Bio-Rad HPLC (11.6 vs 9.5 μmol/L; $P < 0.05$), and Abbott IMx (13.4 vs 12.1 μmol/L; $P < 0.05$) in univariate analysis. In the control group, men had significantly higher tHcy than women for all assays: in-house HPLC, 11.1 μmol/L for male controls vs 10.3 μmol/L for female controls ($P = 0.045$); Bio-Rad HPLC, 11.3 vs 9.3 μmol/L ($P = 0.02$); Abbott IMx, 12.5 vs 9.9 μmol/L ($P = 0.02$); Bio-Rad EIA, 13.7 vs 11.6 μmol/L ($P = 0.03$). However, these differences were no longer statistically significant when adjusted for influence of age, smoking status, hypertension, diabetes, and lipids. In the case subjects, there were no significant gender differences in tHcy.

Compared with male controls, male cases had significantly higher total cholesterol (2350 vs 2080 mg/L, respectively; $P = 0.001$), triglycerides (2140 vs 1550 mg/L,

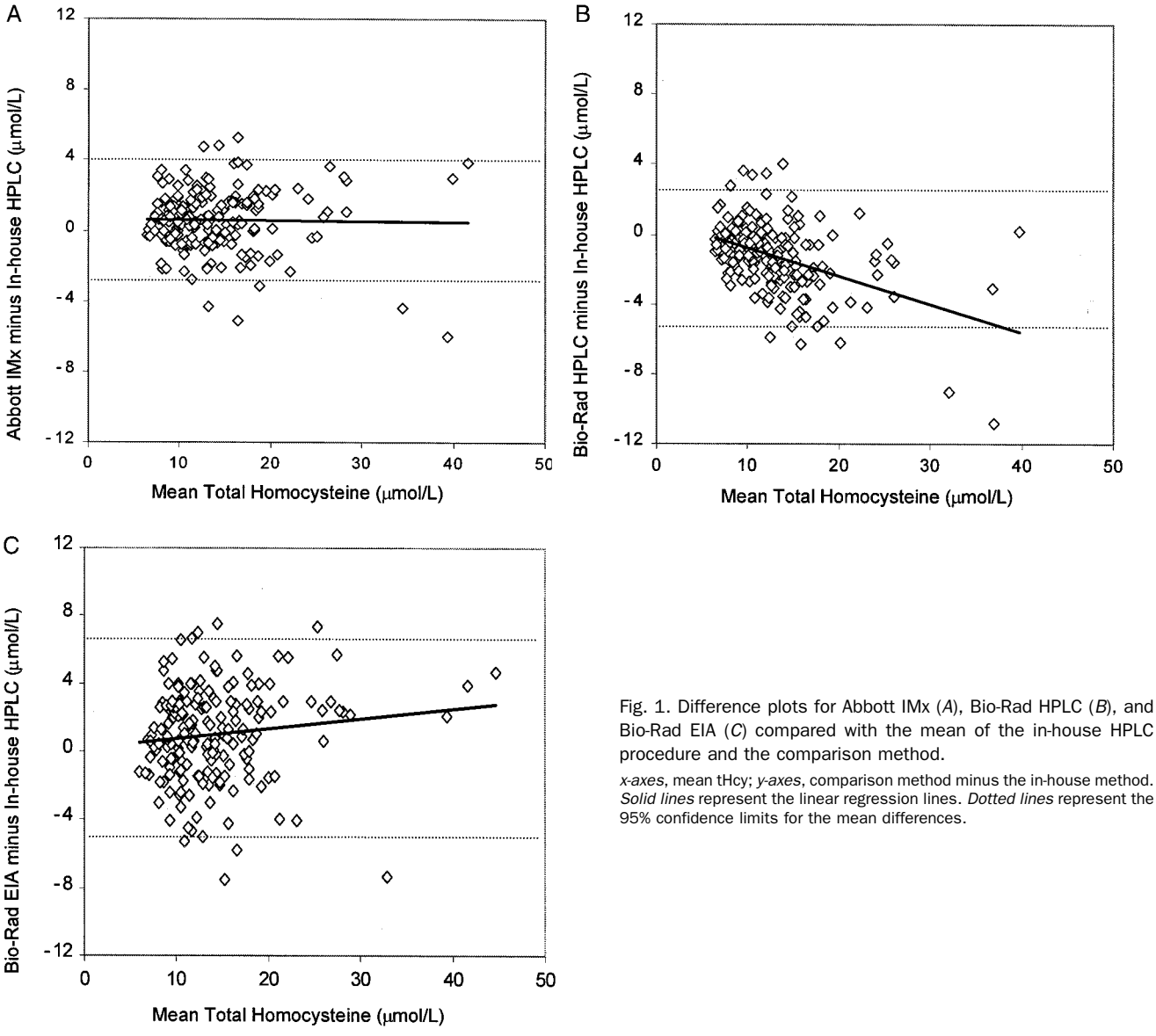

Fig. 1. Difference plots for Abbott IMx (*A*), Bio-Rad HPLC (*B*), and Bio-Rad EIA (*C*) compared with the mean of the in-house HPLC procedure and the comparison method.

x-axes, mean tHcy; *y-axes*, comparison method minus the in-house method. *Solid lines* represent the linear regression lines. *Dotted lines* represent the 95% confidence limits for the mean differences.

respectively; $P < 0.001$), and in-house HPLC tHcy (13.8 vs 11.1 μmol/L, respectively; $P = 0.02$). Although not statistically significant, tHcy was also higher in female cases compared with controls: in-house, 10.8 μmol/L vs 10.3 μmol/L ($P = 0.11$); Bio-Rad HPLC, 10.6 vs 9.3 μmol/L ($P = 0.14$); Abbott IMx, 12.4 vs 9.9 μmol/L ($P = 0.15$); Bio-Rad EIA, 12.9 vs 11.6 μmol/L ($P = 0.25$). Triglycerides were higher in female cases vs controls (1590 vs 1000 mg/L, respectively; $P = 0.001$).

Active smoking appeared to be associated with higher tHcy with the Bio-Rad EIA (14.2 μmol/L for smokers vs 12.2 μmol/L for nonsmokers; $P < 0.05$) but not in the other assays. Hypertensive subjects also had higher triglycerides (2140 mg/L for hypertensives vs 1570 mg/L for normotensives; $P = 0.028$). These differences were not statistically significant after controlling for the effects of potential covariates. In subjects with atherosclerosis, diabetic status was not associated with significantly different tHcy or lipids.

We next examined the tHcy concentrations in subjects with different numbers of major coronary vessels affected by atherosclerosis (Table 2). An increased number of diseased coronary arteries was associated with increased tHcy in all assays ($P = 0.003$ for Bio-Rad EIA and $P < 0.001$ for others for positive linear trend). Subjects with two-vessel CHD had higher tHcy than those with one-vessel involvement (in-house, $P = 0.007$; Abbott IMx, $P = 0.014$; Bio-Rad HPLC, $P = 0.004$; Bio-Rad EIA, $P = 0.051$). tHcy in subjects with three-vessel disease was significantly higher subjects with than one-vessel disease (in-house, $P = 0.0004$; Abbott IMx, $P = 0.0002$; Bio-Rad HPLC, $P = 0.0001$; Bio-Rad EIA, $P = 0.007$), but not significantly higher than subjects with two-vessel CHD. Subjects with one-vessel CHD did not have significantly increased tHcy

Table 2. Comparison of four tHcy methods in subjects with CHD and appropriate controls.[a]

	In-house HPLC	Bio-Rad HPLC	Abbott IMx	Bio-Rad EIA
Controls	10.9 ± 4.8	10.8 ± 4.3	12.1 ± 5.2	12.6 ± 5.3
Cases	13.5 ± 6.7[b]	11.6 ± 5.7	13.2 ± 6.6	13.2 ± 7.2
CHD severity				
One-VD[e]	10.8 ± 3.8	9.6 ± 3.2	12.0 ± 4.0	11.9 ± 4.3
Two-VD	13.9 ± 4.2[b]	12.1 ± 3.5	13.2 ± 4.1	13.0 ± 4.7
Three-VD	15.0 ± 9.9[c]	12.5 ± 8.5[b]	15.7 ± 9.7[b]	15.8 ± 10.6[d]

[a] Values given as median ± SD (μmol/L).
[b–d] Significance compared with controls: [b] $P < 0.01$; [c] $P < 0.001$; [d] $P < 0.05$ after adjustment for patient characteristics and lipids.
[e] VD, coronary vessel(s) diseased.

compared with controls. However, three-vessel CHD was associated with significantly higher tHcy compared with controls with all assays. tHcy was higher in two-vessel CHD subjects than in controls only with the in-house HPLC method.

Discussion

Prior work has demonstrated that severe hyperhomocysteinemia is a strong risk factor for the development of premature atherosclerosis. However, the data have been less compelling for smaller increases in tHcy, possibly because of differences in methodologies. In the present study, despite the trend toward higher tHcy in cases in all assays, the performance of the four assays varied enough to give slightly different results in specimens from the same subjects. Quartiles, defined by the distribution of tHcy values in our controls, differed between assays, especially between the immunoassays and the HPLC methods (Fig. 2). All comparison methods demonstrated a positive intercept and reduced slope when compared with the in-house HPLC. The Bio-Rad EIA demonstrated the greatest scatter and lowest correlation with the in-house method.

Defining normal and abnormal ranges for tHcy maybe problematic when (*a*) physiological concentrations of cases and controls show a great deal of overlap, (*b*) no gold standard or reference method has been established, and (*c*) some variability exists between assays. Genetic variations also influence the cardiovascular risk associated with hyperhomocysteinemia. Approximately 40% and 12% of the white male population are heterozygous and homozygous, respectively, for the *MTHFR* mutation (677C→T), which leads to mild-to-moderately increased tHcy, particularly when plasma folate concentrations are low *(8, 9)*. Moreover, the results of several studies, including a recent metaanalysis *(10)*, indicate that this mutation does not independently increase cardiovascular risk. The prevalence of the *MTHFR* mutation in our study population or its relative distribution between controls and cases is not known. Mean and median tHcy concentrations were slightly higher than those reported previously in adult American males *(11, 12)*. With the in-house assay, 75% and 60% of the case and control subjects, respectively, had tHcy considered to be increased by current standards (tHcy ≥10 μmol/L). Therefore, tHcy reference ranges derived from one population may not be valid in other populations. Given the significant overlap in fasting tHcy between controls and subjects with documented CHD, the varying prevalence of such polymorphisms in study populations may influence the observed relationships between tHcy and cardiovascular disease.

Although tHcy was statistically increased only in the in-house HPLC method, all assays demonstrated a positive trend for tHcy as coronary involvement increased. In subjects with three-vessel coronary artery disease, tHcy was significantly higher than in controls with all three assays. This is consistent with prior studies that also demonstrated higher tHcy in multivessel CHD compared with single-vessel disease *(13)* and in subjects with a higher coronary score of overall severity *(14)*. In addition, a graded relationship between hyperhomocysteinemia and extracoronary atherosclerosis has also been demonstrated. For example, significant correlations have been noted between the Hcy concentration and the degree of carotid intimal-medial wall-thickening *(15)*, the severity of lower-limb peripheral vascular disease *(16)*, and the number of vascular sites affected by atherosclerosis [i.e., coronary, cerebral, or peripheral arteries *(14)*]. Therefore, higher tHcy is associated with greater systemic atherosclerosis.

Folate and vitamin B_6 consumption can decrease Hcy concentrations and are associated with decreased risk of atherosclerosis *(6, 17, 18)*, with substantial risk reduction observed at a folate intake of ~400 μg/day in women *(18)*. In response to data supporting the relationship between neural tube birth defects and folate deficiency and demonstrating the failure of >30% of adults over age 20 to meet the prior US Department of Agriculture recommended dietary allowance of folate *(19, 20)*, the US Department of Agriculture began mandating fortification of cereal grains in 1998 and recently increased the recommended dietary allowance to 600 μg/day for pregnant women and 400 μg/day for the general adult population *(21)*. Fortification may lead to average increases of folate intake by ~100 μg/day; however, whether this will lead to significant reductions in Hcy in subjects at highest risk for cardiovascular disease has yet to be determined. In addition, because of the difficulty in discriminating which

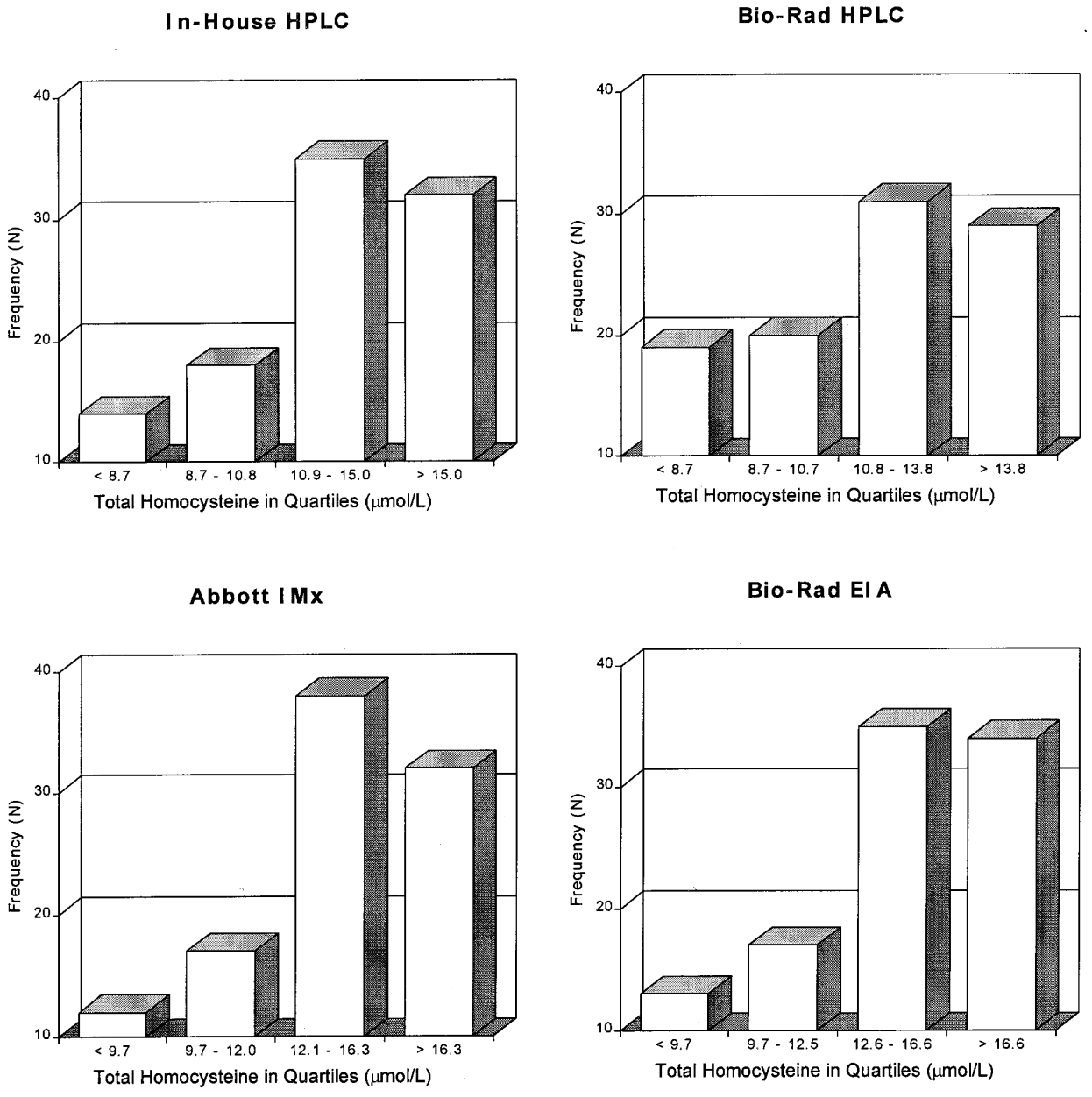

Fig. 2. Distribution of tHcy in case subjects.

Quartiles were defined by tHcy values (μmol/L) in control subjects as measured by the corresponding comparison methods.

subjects are at risk from hyperhomocysteinemia and defining what constitutes abnormal tHcy concentrations, selective targeting of treatment with folate and vitamin B_6 may prove challenging. If prospective data suggest cardiovascular benefit from folate, B_6, and/or B_{12} supplementation, probably more aggressive fortification of the US diet maybe indicated for the primary prevention of CHD.

Limitations in the present study merit consideration. With the comparison assays, differences between cases and controls might have achieved statistical significance with a larger sample size. The presence of occult coronary disease in our controls could not be excluded and may have attenuated the differences demonstrated between the groups. Therefore, more stringent evaluation of controls likely would have enhanced the encountered differences. In addition, we had an unexpected absence of diabetics in our control group. However, no statistical heterogeneity was found between nondiabetic and diabetic cases, and analyses when diabetics were excluded were consistent with when diabetics were included. Other studies have demonstrated no significant differences in tHcy between diabetics compared with nondiabetics when controlling for other potential covariates *(11)*. Fi-

nally, we are unable to comment on gender-specific differences, although a larger sample size might produce a statistically significant difference between female cases and controls. Previous studies have demonstrated substantially higher tHcy concentrations in men *(22, 23)*, however. As a result, separate reference ranges based on sex have been proposed *(24)*.

In conclusion, although increasing severity of coronary involvement was associated with significantly higher tHcy for all assays, three of the four methods failed to detect statistically significant differences between subjects with coronary disease and controls. The present study demonstrates that the clinical applicability of tHcy testing may be hampered by assay variability, the lack of an accepted reference method, and the influence of patient characteristics on Hcy concentrations.

Reagents for this study were generously provided by Bio-Rad Laboratories (Hercules, CA) and Abbott Laboratories (Abbott Park, IL).

Supplemental Information

Data from the HCY study are available as a supplement from the Clinical Chemistry Web Site. The file can be accessed by a link from the on-line Table of Contents (http://www.clinchem.org/content/vol46/issue2/).

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