Clinical Chemistry 54:5
801–813 (2008)

Trends in Circulating Concentrations of Total Homocysteine among US Adolescents and Adults: Findings from the 1991–1994 and 1999–2004 National Health and Nutrition Examination Surveys

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BACKGROUND: The National Health and Nutrition Examination Survey (NHANES) has monitored total homocysteine (tHcy) concentrations in a nationally-representative sample of the US population since 1991. Until recently, however, data could not be compared across survey periods because of changes in analytical methods and specimen matrices. Such an analysis of these data could supplement current knowledge regarding whether the US folic acid fortification program has modified national plasma tHcy concentrations.

METHODS: We examined tHcy data in the prefortification NHANES III survey (phase II, 1991–1994) and in 3 postfortification survey periods (1999–2000, 2001–2002, and 2003–2004). We applied method adjustment equations to the survey data based on method comparison studies of separate samples. Persons with chronic kidney disease were excluded from the analyses.

RESULTS: Mean plasma tHcy concentrations decreased by 8%, 9%, and 10% for adolescent, adult, and older men and by 6%, 3%, and 13% for women, respectively, from before to after fortification. Concentrations remained unchanged between the first and third postfortification survey periods. Prevalence estimates of increased plasma tHcy concentrations (>13 μmol/L) for older men and women decreased from prefortification (32% and 20%, respectively) to postfortification (14% and 5%, respectively) but remained unchanged thereafter (16% and 14%, respectively [males] and 5% and 9%, respectively [females]).

CONCLUSIONS: After adjusting for method changes, we quantified a prefortification to postfortification decrease in circulating tHcy concentrations of about 10% in a national sample of the US population. This change is similar to effects seen in intervention trials with folic acid and in smaller observational studies.

Genetic variations in enzymes involved in homocysteine metabolism; inadequate intakes of folate, riboflavin, vitamins B6 or B12; and impaired renal function lead to increased total homocysteine (tHcy)[5] concentrations in blood *(1)*. Concentrations are also increased by behaviors such as smoking, lack of exercise, excessive alcohol intake, and high coffee consumption *(2)*. Alhough tHcy concentrations have been positively associated with cardiovascular disease in early observational studies *(3)*, subsequent prospective population studies *(4)* and folate intervention trials *(5)* have suggested a weaker or even tenuous relationship. Results from pooled analysis of additional clinical trials are expected to overcome the current lack of statistical power to detect small effects *(6)*. Thus far, however, there appears to be evidence of the efficacy of folic acid supple-

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The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official views or positions of the US federal government including the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry, the National Institutes of Health, the Food and Drug Administration, or the Department of Health and Human Services.

Received November 9, 2007; accepted January 15, 2008.
Previously published online at DOI: 10.1373/clinchem.2007.100214

[5] Nonstandard abbreviations: tHcy, total homocysteine; NTD, neural tube defects; NHANES, National Health and Nutrition Examination Survey; NHW, non-Hispanic white; NHB, non-Hispanic black; MA, Mexican-American; USDA, US Department of Agriculture; CKD, chronic kidney disease.

mentation in the prevention of stroke *(7)*. Increased concentrations of tHcy have also been associated with the development of dementia, Alzheimer disease, and cognitive dysfunction; decline in physical function; and osteoporosis and fractures in elderly individuals *(1)*.

Since January 1998, the US Food and Drug Administration has required fortification of enriched cereal-grain products with folic acid to reduce the risk of pregnancy complicated by neural tube defects (NTD) *(8)*. Recent observational studies have suggested associations between folate fortification in the US population and reduced NTD rates *(9)*, decreased prevalence of inadequate serum and erythrocyte folate concentrations *(10–13)*, and decreases in the incidence of stroke *(14)*. Decreases in tHcy concentrations were reported in the Framingham *(12)* and Chilean populations *(15)* after introduction of folate fortification in the US and in Chile.

The National Health and Nutrition Examination Survey (NHANES) began to monitor tHcy concentrations in the US population in 1991. Several papers have described distributions of tHcy concentrations *(16, 17)* and their determinants *(18–20)* obtained by measurements performed on surplus serum from the second phase of NHANES III (1991–1994). In 1999, continuous monitoring of plasma tHcy concentrations was implemented. At present, data are available for 3 postfortification survey periods: 1999–2000, 2001–2002, and 2003–2004. Several reports have presented information about tHcy concentrations for the first 2 survey periods *(10, 11, 21, 22)*. However, the use of different laboratories, methods, and specimen matrices for the measurement of tHcy has precluded direct comparison of tHcy results from prefortification to postfortification *(23)*, as well as among the 3 postfortification survey periods. We describe the method adjustments necessary to allow comparison of tHcy data across time, and we provide an account of changes in tHcy concentrations in the US population over the last 13 years.

Study Participants and Methods

SURVEY DESIGN AND STUDY PARTCIPANTS

NHANES constitutes a series of periodic nationally representative cross-sectional probability surveys of the noninstitutionalized civilian US population. Conducted by the National Center for Health Statistics at the Centers for Disease Control and Prevention, NHANES obtains a stratified, multistage, probability sample designed to represent the US population based on age, sex, and race-ethnicity *(24)*. During each survey period, certain subpopulations are oversampled to allow for more precise estimates. Race-ethnicity categories [non-Hispanic white (NHW), non-Hispanic black (NHB), and Mexican-American (MA)] are based on self-reported data *(25)*. All respondents gave their informed consent, and the NHANES protocol for each survey period was reviewed and approved by the National Center for Health Statistics Institutional Review Board.

LABORATORY METHODS

Methods for determining tHcy concentrations changed across the 4 survey periods (see Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue5). Because optimally prepared EDTA plasma was not available during NHANES III, tHcy concentrations were analyzed by HPLC in surplus serum from phase II of NHANES III (1991–1994) at the US Department of Agriculture (USDA) Human Nutrition Research Center on Aging *(26)*. Whole blood was allowed to clot for 30–60 min before centrifugation and removal of serum. From 1999–2004, EDTA whole blood was processed within 30 min of collection to avoid an artificial increase in tHcy from continuous production in, and release from erythrocytes. Plasma tHcy was analyzed at the CDC Nutrition Laboratory using a commercial fluorescence polarization immunoassay reagent set (Abbott Laboratories) on the Abbott IMx® analyzer during 1999–2001 *(27)*, then on the Abbott AxSYM® analyzer (same reagent set, newer fully-automated model) during 2002–2004 *(28)*. Interrun CVs for plasma tHcy at 6.70–26.0 μmol/L were 3%–5% for both Abbott platforms during 1999–2004.

METHODS ADJUSTMENTS

We adjusted all tHcy data forward to the Abbott AxSYM® because that method was used for the longest time period in NHANES and is the most widely used method in clinical laboratories. Owing to error in both analytical methods, we performed method adjustments by Deming regression analysis using Analyze-it, a statistical plug-in for Microsoft Excel (Analyze-it Software), after log-transforming the data to account for nongaussian distributions.

Because a different specimen matrix was used for NHANES III and no direct method comparison data were available between the USDA HPLC and the Abbott AxSYM method, we adjusted tHcy data from NHANES III in 3 stages: USDA HPLC serum to USDA HPLC plasma; USDA HPLC plasma to CDC HPLC plasma; and CDC HPLC plasma to AxSYM® plasma. The first 2 adjustments were based on data from an earlier report *(23)* in which USDA and CDC laboratories performed a method comparison of their 2 HPLC assays *(26, 29)* to determine the magnitude of differ-

ences in tHcy concentrations between plasma and serum. Blood specimens from 30 donors were separated into EDTA plasma (optimal processing) and serum (delayed processing) *(23)*. For the serum-to-plasma data adjustment, we used the USDA data for serum after allowing the blood to clot for 60 min (n = 30). For the USDA HPLC plasma–to–CDC HPLC plasma data adjustment, we used the entire sample set (n = 150). Finally, the CDC HPLC–to–AxSYM conversion was based on a set of plasma samples that were measured as part of NHANES with these 2 methods and the IMx method (n = 361). Therefore, the data from this sample set were also used to compare the 2 Abbott platforms.

STATISTICAL METHODS

Statistical analyses on method adjusted tHcy data were performed using SAS (version 9, SAS Institute) and SUDAAN (version 9, RTI) software using sample weights to account for differences in nonresponse or noncoverage and to adjust for planned oversampling of some groups *(24)*. The 95% CIs for all survey periods were estimated with SUDAAN software by using Taylor series linearization, a method that incorporates the sample weights and accounts for the sample design. We used 3 age strata in our data analyses: 12–19 years (adolescents), 20–59 years (adults), and ≥60 years (older persons).

Because impaired renal function has an appreciable influence on plasma tHcy concentrations *(30)*, data were excluded from analyses for all participants with chronic kidney disease (CKD) of any stage (568, 532, 613, and 620 persons in 1991–1994, 1999–2000, 2001–2002, and 2003–2004, respectively) and persons for whom insufficient information was available to determine whether they had CKD (127, 73, 77, and 84 persons, respectively). We followed the recommendations of the National Kidney Disease Education Program to estimate glomerular filtration rate as the best overall index of kidney function *(31, 32)* and used the National Kidney Foundation classification system to determine stages of CKD *(33)* (see Supplemental Text 1 in the online Data Supplement).

We generated frequency distribution curves for the entire population and for older persons for each survey period. Medians or geometric means ($\log_{10}$-transformed data) were evaluated owing to the skewness of the distribution curves. Prevalence estimates for individuals with increased tHcy concentrations (>13 μmol/L) *(12, 15)* and individuals with desirable tHcy concentrations (≤9 μmol/L) *(34)* were determined for each survey period.

Using an ANOVA model that included age (3 age groups), sex (male or female), racial-ethnic group (NHW, NHB, MA, or other), and survey period (1991–1994, 1999–2000, 2001–2002, or 2003–2004), we found significant ($P < 0.05$) age × survey period and sex × survey period interactions. To assess whether circulating tHcy concentrations (geometric means) or prevalence estimates changed between consecutive survey periods (i.e., 1991–1994 compared with 1999–2000, 1999–2000 compared with 2001–2002, and 2001–2002 compared with 2003–2004), and to assess whether prevalence estimates of increased or desirable tHcy values were significantly different between the 3 age groups (i.e., 12–19 years compared with 20–50 years, 20–59 years compared with ≥60 years, and ≥60 years compared with 12–19 years), we performed pairwise comparisons using 2-tailed, 2-group *t* tests on age- and sex-specific subgroups. We considered the *P* value of each comparison significant if it was ≤0.017 (Bonferroni multiple comparison adjustment, 0.05 divided by 3, the total number of comparisons). We determined selected population percentile values (5th, 50th, and 95th) for tHcy for the prefortification period (1991–1994) and for the postfortification period (1999–2004). Strata with <220 individuals were considered to produce imprecise estimates of the 5th and 95th percentiles *(35)*.

Results

METHOD ADJUSTMENTS

USDA HPLC plasma results (n = 30) were on average 9% lower than their serum results, with a constant relative difference across all concentrations; all predicted plasma results were within 15% of the measured results (Table 1 and Supplemental Fig. 1A in the online Data Supplement for Deming regression plot). CDC HPLC plasma results (n = 150) were on average 6% higher than USDA HPLC plasma results. However, the relative difference varied with tHcy concentration (8% and 12% at the median and at the 13 μmol/L cutoff value, respectively). Because 97% of all predicted CDC HPLC results were within 20% of the measured results, the method adjustment equation appeared to correct for this concentration-dependent difference (Table 1 and Supplemental Fig. 1B in the online Data Supplement). AxSYM results (n = 361) were on average 1% higher than CDC HPLC results; 99.7% of all predicted AxSYM results were within 20% of the measured results (Table 1 and Supplemental Fig. 1C in the online Data Supplement). Finally, AxSYM results (n = 361) were on average 6% higher than IMx results, with a constant relative difference across all concentrations; 99% of all predicted AxSYM results were within 20% of the measured results (Table 1 and Supplemental Fig. 1D in the online Data Supplement).

Table 1. Method adjustment equations to allow comparison of tHcy data from various survey periods of the NHANES.

Survey period	Conversion	Adjustment equation[a]
1991–1994	USDA HPLC serum to USDA HPLC plasma	Log_{10} USDA HPLC plasma = 1.042 * log_{10} USDA HPLC serum − 0.079; r = 0.98; 95% CI for slope (0.957–1.126); 95% CI for intercept (−0.156 to −0.002)
	USDA HPLC plasma to CDC HPLC	Log_{10} CDC HPLC = 1.165 * log_{10} USDA HPLC plasma − 0.120; r = 0.97; 95% CI for slope (1.110–1.220); 95% CI for intercept (−0.170 to −0.070)
	CDC HPLC to AxSYM	Log_{10} AxSYM = 1.014 * log_{10} CDC HPLC − 0.006; r = 0.99; 95% CI for slope (0.998–1.029); 95% CI for intercept (−0.020 to 0.008)
1999–2000 & 2001	IMx to AxSYM	Log_{10} AxSYM = 0.983 * log_{10} IMx + 0.042; r = 0.99; 95% CI for slope (0.969–0.997); 95% CI for intercept (0.030–0.054)
2002 & 2003–2004	No conversion needed since AxSYM was used	NA

[a] Adjustment equations are based on Deming regression analysis of log_{10}-transformed data.

DISTRIBUTIONS OF PLASMA tHcy CONCENTRATIONS, 1991–2004

During 1991–1994, 7% of the entire population and 22% of older persons were classified as having CKD (all stages). During the 3 postfortification survey periods, 8% (1999–2000), 9% (2001–2002), and 9% (2003–2004) of the entire population and 27%, 29%, and 31%, respectively, of older persons were classified as having CKD. Exclusion of persons with CKD had a noticeable impact on the frequency distribution curves (Fig. 1) and on the prevalence estimates of increased plasma tHcy concentrations, especially for older persons (approximately 10 percentage points lower prevalence estimates for increased tHcy concentrations). Frequency distributions after excluding persons with CKD from the entire population and from older persons for each of the 4 survey periods illustrate how the distribution in circulating tHcy concentrations changed over time (Fig. 2). The width of the distribution curve narrowed from before to after fortification, and the long upper tail disappeared almost entirely. The distribution curves for 1999–2000 and 2001–2002 were almost entirely overlapping.

TRENDS IN PLASMA tHcy CONCENTRATIONS, 1991–2004

Because of the age × survey period and the sex × survey period interactions, we present median concentrations of plasma tHcy and time trend analysis for age- and sex-specific subgroups in the 4 survey periods (Table 2). Except for adolescent women ($P = 0.0725$), concentrations of plasma tHcy decreased significantly in every sex-age subgroup after the introduction of folate fortification in 1998 (0.2–1.2 μmol/L based on medians, corresponding 3%–13% decreases). These pre- to postfortification decreases were largest for older persons: 1.0 μmol/L (10%) for older men and 1.2 μmol/L (13%) for older women. When stratified further by race-ethnicity, concentrations of plasma tHcy decreased significantly in most subgroups except for adolescent NHW and MA women ($P = 0.1573$ and $P = 0.0626$, respectively), older NHB men ($P = 0.0284$), and older NHB and MA women ($P = 0.0782$ and $P = 0.0763$, respectively). We found similar decreases based on geometric means as reported here for medians. There were no significant changes in tHcy concentrations across the 3 postfortification survey periods.

TRENDS IN PREVALENCE ESTIMATES OF INCREASED OR DESIRABLE PLASMA tHcy CONCENTRATIONS, 1991–2004

Among the 3 age groups, the prevalence of increased plasma tHcy concentrations (>13 μmol/L) was always higher in older persons compared to the other 2 age groups, both prefortification (approximately 20%–30% in older persons vs approximately 10%–20% in adults, and <10% in adolescents) and postfortification (approximately 5%–15% in older persons vs approximately 3%–7% in adults, and <3% in adolescents) (Table 3). Prevalence estimates of increased plasma tHcy values decreased significantly in all subgroups from before to after fortification, except for decreases of borderline significance in adolescent women ($P = 0.0431$) and older NHB women ($P = 0.0178$). Prevalence estimates did not change significantly across the 3 postfortification survey periods.

Among the 3 age groups, the prevalence of desirable plasma tHcy concentrations (≤9 μmol/L) was always lower in older persons compared to the other 2 age groups, both prefortification (approximately 25%–50% in older persons vs approximately 50%–75% in

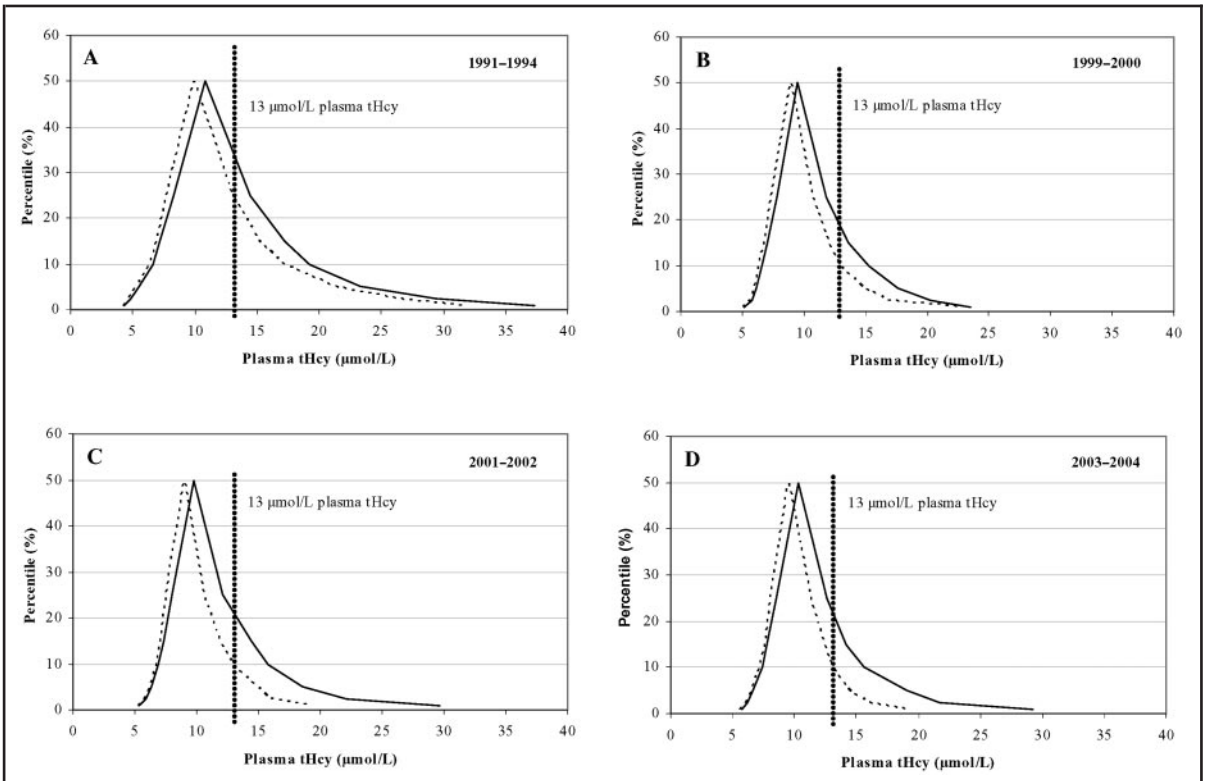

Fig. 1. Frequency distribution of plasma tHcy among older persons in the US including (solid line) and excluding (dotted line) those with chronic kidney disease (CKD) according to the NHANES spanning 1991–1994 (A), 1999–2000 (B), 2001–2002 (C), and 2003–2004 (D).

The vertical dotted line in each panel indicates the cutoff for increased tHcy values.

adults, and >75% in adolescents) and postfortification (approximately 25%–70% in older persons vs approximately 60%–90% in adults, and >90% in adolescents) (Table 3). Prevalence estimates of desirable plasma tHcy values increased significantly from before to after fortification in about 50% of the sex- and age-specific subgroups but did not change for adolescent women ($P = 0.0354$), older men ($P = 0.3947$), older NHW men ($P = 0.353$), older NHB men and women ($P = 0.4275$ and $P = 0.8926$, respectively), and older MA women ($P = 0.3345$). They also did not change significantly across the 3 postfortification survey periods, except for an increase in adult women between 1999–2000 and 2001–2002 and decreases in adult and older women and older NHW women between 2001–2002 and 2003–2004.

SELECTED POPULATION PERCENTILE VALUES FOR PLASMA tHcy CONCENTRATIONS FOR PRE- AND POSTFORTIFICATION

The 5th, 50th, and 95th percentiles for the prefortification survey (1991–1994) and the postfortification time period (1999–2004) by sex, race-ethnicity, and age group are shown in Table 4. The postfortification downward shift in the 95th percentile in all subgroups is readily apparent.

Discussion

Circulating tHcy concentrations have been measured as part of the NHANES survey for 3 years before and 6 years after the introduction of folate fortification. Until now, however, tHcy data could not be compared across survey periods because of changes in analytical methods and specimen matrices. We derived method adjustment equations to allow for time trend analysis. This process had several strengths: (*a*) sample sizes for method comparisons were of sufficient size, except the EDTA plasma-to-serum comparison; (*b*) all 3 method comparisons spanned the reference interval of tHcy concentrations; (*c*) each comparison was conducted over a period of several days to capture the interrun variability; (*d*) the small adjustment between the Abbott IMx and AxSYM platforms was consistent with a

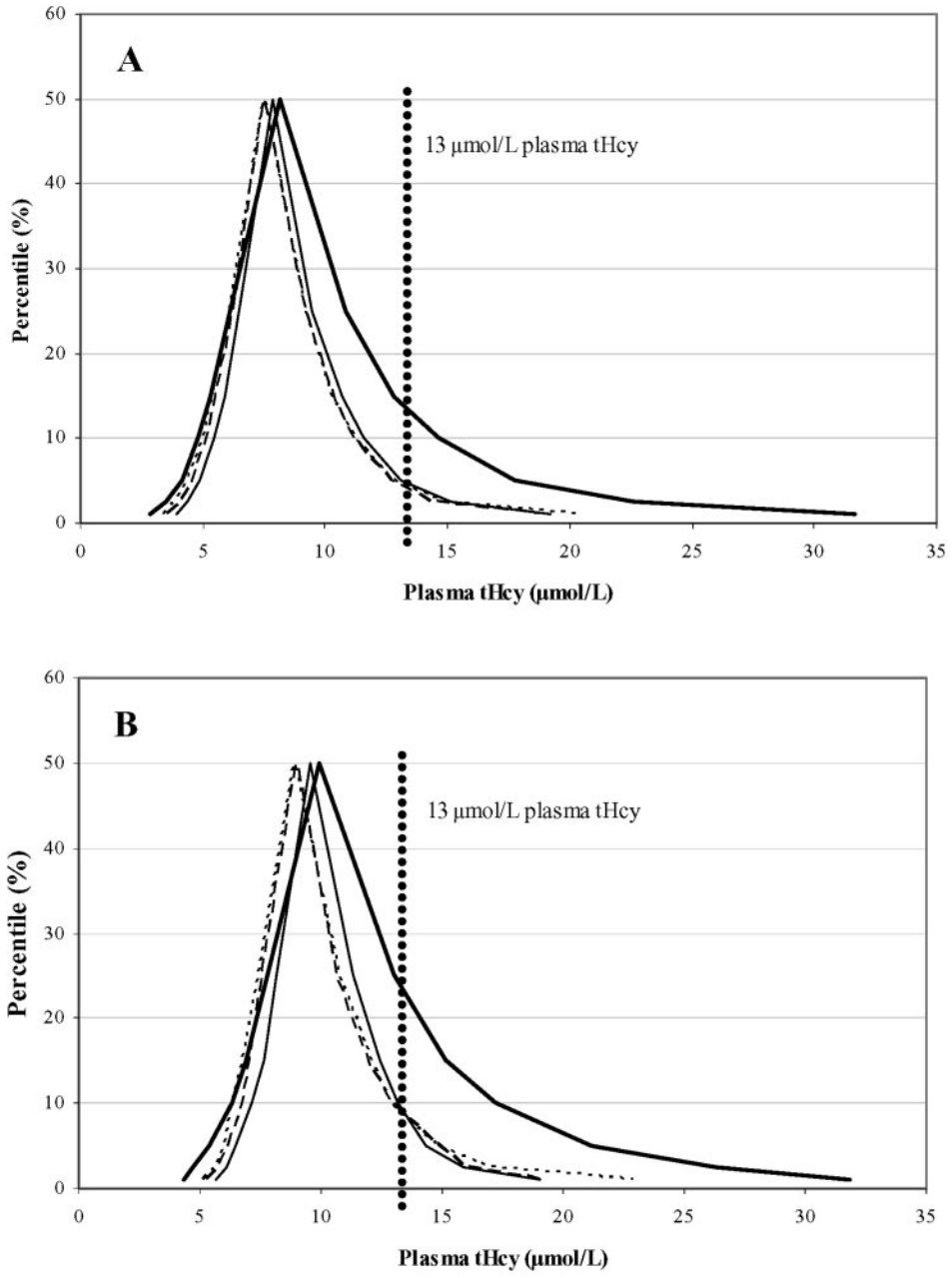

Fig. 2. Frequency distribution of plasma tHcy among the entire population (A) and older persons (B) in the US according to the NHANES spanning 1991–1994 (bold solid line), 1999–2000 (dotted line), 2001–2002 (dashed line), and 2003–2004 (solid line).

Persons with CKD were excluded. The vertical dotted line in each panel indicates the cutoff for increased tHcy values.

previous report *(5, 28)*; (*e*) all measurements were adjusted to the AxSYM method, which has shown accurate agreement with international standard reference material *(36)*; and (*f*) the processing time of whole blood was standardized separately in NHANES 1991–1994 and NHANES 1999–2004, minimizing imprecision in tHcy values due to inconsistent sample handling.

One limitation in our method adjustments includes a potential bias in the resulting adjustment that

Table 2. Trends in method-adjusted plasma tHcy concentrations by sex, race-ethnicity, and age group during the NHANES, 1991–2004.[a,b]

Race-ethnicity	Sex	Age group, years	Median, μmol/L (95% CI), n				*P*[c]		
			1991–1994[d]	1999–2000[e]	2001–2002[e]	2003–2004	1991–1994 vs 1999–2000[f]	1999–2000 vs 2001–2002[g]	2001–2002 vs 2003–2004[h]
All	Both	All	8.11 (7.90–8.36)	7.49 (7.38–7.59)	7.53 (7.37–7.71)	7.88 (7.67–8.07)	—	—	—
			7781	5719	6294	5878			
All	M	12–19	6.70 (6.32–7.12)	6.20 (6.05–6.46)	6.36 (6.18–6.57)	6.58 (6.36–6.79)	<0.001	NS	NS
			631	1054	1069	1027			
All	M	20–59	9.14 (8.76–9.59)	8.31 (8.14–8.59)	8.48 (8.26–8.65)	8.63 (8.45–8.88)	<0.001	NS	NS
			2030	1146	1438	1316			
All	M	≥60	10.7 (10.0–11.4)	9.67 (9.34–10.0)	9.64 (9.22–10.0)	10.1 (9.59–10.6)	<0.001	NS	NS
			787	564	536	581			
All	F	12–19	5.84 (5.41–6.51)	5.48 (5.20–5.77)	5.55 (5.42–5.78)	5.84 (5.71–5.95)	NS	NS	NS
			737	1022	1091	978			
All	F	20–59	7.02 (6.74–7.31)	6.80 (6.65–6.96)	6.74 (6.56–6.92)	7.07 (6.88–7.24)	<0.001	NS	NS
			2780	1420	1619	1427			
All	F	≥60	9.21 (8.85–9.98)	8.03 (7.57–8.52)	8.40 (7.97–8.78)	9.07 (8.71–9.61)	<0.001	NS	NS
			816	513	541	549			
NHW	M	12–19	6.50 (6.10–7.47)	6.16 (5.94–6.43)	6.22 (6.03–6.58)	6.67 (6.48–6.88)	0.0118	NS	NS
			117	213	326	262			
NHW	M	20–59	9.22 (8.83–9.91)	8.45 (8.22–8.76)	8.50 (8.29–8.66)	8.70 (8.54–9.02)	<0.001	NS	NS
			564	502	707	651			
NHW	M	≥60	10.6 (9.84–11.4)	9.65 (9.27–10.0)	9.62 (9.18–10.0)	10.1 (9.49–10.6)	0.0014	NS	NS
			393	280	301	330			
NHW	F	12–19	5.75 (5.27–6.63)	5.47 (5.15–5.87)	5.64 (5.41–5.95)	5.95 (5.84–6.33)	NS	NS	NS
			143	205	329	266			
NHW	F	20–59	7.16 (6.84–7.59)	6.84 (6.66–7.11)	6.91 (6.68–7.08)	7.17 (6.97–7.38)	<0.001	NS	NS
			865	572	789	717			
NHW	F	≥60	9.21 (8.70–10.2)	7.96 (7.49–8.44)	8.42 (7.88–8.80)	9.18 (8.76–9.70)	<0.001	NS	NS
			436	242	304	298			
NHB	M	12–19	7.34 (7.07–7.99)	6.27 (5.99–6.48)	6.38 (6.25–6.63)	6.41 (6.15–6.74)	<0.001	NS	NS
			262	302	339	398			
NHB	M	20–59	9.25 (8.84–9.54)	8.23 (7.75–8.67)	8.62 (8.32–8.83)	8.78 (8.45–9.12)	0.0029	NS	NS
			649	225	273	295			
NHB	M	≥60	11.2 (9.98–13.5)	10.1 (8.91–10.7)	9.99 (9.68–10.7)	10.6 (9.85–11.4)	NS	NS	NS
			163	76	99	85			
NHB	F	12–19	6.10 (5.90–6.57)	5.56 (5.24–5.79)	5.58 (5.28–5.82)	5.79 (5.60–6.03)	<0.001	NS	NS
			320	287	326	354			
NHB	F	20–59	7.14 (6.92–7.62)	6.85 (6.45–7.44)	6.86 (6.56–7.02)	7.17 (6.89–7.53)	<0.001	NS	NS
			983	282	313	321			
NHB	F	≥60	9.53 (8.53–10.6)	9.43 (8.84–9.90)	9.01 (8.54–9.41)	9.39 (8.61–10.5)	NS	NS	NS
			151	85	95	88			
MA	M	12–19	6.96 (6.41–8.03)	6.07 (5.78–6.37)	6.36 (6.19–6.52)	6.19 (5.94–6.46)	0.0109	NS	NS
			218	473	321	317			

Continued on page 808

Table 2. Trends in method-adjusted plasma tHcy concentrations by sex, race-ethnicity, and age group during the NHANES, 1991–2004.[a,b] (*Continued from page 807*)

Race-ethnicity	Sex	Age group, years	Median, μmol/L (95% CI), n				*P*[c]		
			1991–1994[d]	1999–2000[e]	2001–2002[e]	2003–2004	1991–1994 vs 1999–2000[f]	1999–2000 vs 2001–2002[g]	2001–2002 vs 2003–2004[h]
MA	M	20–59	8.87 (8.51–9.41)	7.64 (7.25–8.10)	7.77 (7.32–8.19)	7.71 (7.48–8.07)	<0.001	NS	NS
			725	310	350	266			
MA	M	≥60	10.9 (10.7–12.1)	9.53 (8.93–10.5)	9.63 (8.57–10.4)	9.66 (9.19–10.2)	0.0036	NS	NS
			205	169	108	142			
MA	F	12–19	5.69 (5.39–6.16)	5.16 (4.85–5.57)	5.14 (4.93–5.36)	5.29 (5.14–5.46)	NS	NS	NS
			226	447	360	303			
MA	F	20–59	6.30 (6.10–6.75)	6.13 (5.96–6.42)	5.87 (5.64–6.21)	6.32 (6.10–6.59)	0.003	NS	NS
			777	419	378	285			
MA	F	≥60	8.79 (8.32–9.66)	8.31 (7.72–9.15)	8.23 (7.61–9.65)	8.29 (7.96–8.99)	NS	NS	NS
			189	150	111	134			

[a] All indicates racial-ethnic groups are not shown separately; NS, not significant; NHW, non-Hispanic white; NHB, non-Hispanic black; MA, Mexican American.
[b] Persons with CKD and persons with incomplete information to determine whether they had CKD were excluded.
[c] Time trend analysis on groups comprising the entire age range and both sexes could not be performed because of significant age × survey period and sex × survey period interactions.
[d] For 1991–1994, USDA data for serum tHcy by HPLC has been adjusted to plasma, then to CDC HPLC, and then to Abbott AxSYM.
[e] For 1999–2001, CDC data for plasma tHcy by Abbott IMx have been adjusted to Abbott AxSYM.
[f] Pairwise comparison (*t*-test) of geometric means from 1991–1994 and 1999–2000 ($P < 0.017$ significant).
[g] Pairwise comparison (*t*-test) of geometric means from 1999–2000 and 2001–2002 ($P < 0.017$ significant).
[h] Pairwise comparison (*t*-test) of geometric means from 2001–2002 and 2003–2004 ($P < 0.017$ significant).

accommodates both differences in matrix and processing time based on the comparison of USDA HPLC serum to USDA HPLC plasma. However, our findings that serum samples had approximately 10% higher tHcy concentrations than optimally prepared plasma samples correspond well with previous findings *(37)*, indicating that this source of bias was properly adjusted. Another limitation is the uncertainty generated in the 3-stage adjustment on the prefortification data to make it comparable to postfortification data. The calculated propagated error for the 3-stage adjustment, as computed from the r^2, is 9%, small enough to allow the detection of a decrease in tHcy concentrations between pre- and postfortification. Because the 3 method regression conversions increase the imprecision of the final estimated tHcy values for 1991–1994, such imprecision would reduce the statistical power to detect differences (toward the null) between the survey periods.

Excluding persons with CKD was an important step in limiting our data analysis to the healthy US population. Otherwise, we would have overestimated prevalence rates of increased tHcy in older persons by approximately 10 percentage points, and we would have confounded our time trend analysis because the prevalence of CKD has increased in the older US population during the past 13 years (22% in 1991–1994 and 31% in 2003–2004), possibly as a result of the increasing prevalence of obesity and related type II diabetes. Although other conditions, genetic variations, unfavorable lifestyles, and the consumption of dietary supplements are known to affect tHcy values, we considered those factors to be part of the wide spectrum of lifestyle choices to which the healthy US population is exposed.

Jacques et al. reported a moderate decrease of 7% in tHcy concentrations in the Framingham population (from 10.1–9.4 μmol/L) for the time period 1995–1998, a finding that reflected the beginning of the voluntary phase of folate fortification in the US *(12)*. After the introduction of folate fortification in Chile, a 12% decrease in tHcy concentrations (from 13.0–11.4 μmol/L) was observed in an elderly Chilean population *(15)*. The pre- (9–11 μmol/L) and postfortification (8–10 μmol/L) tHcy concentrations in older persons in our analysis were similar to those found in the Framingham population but lower than those in Chile. The decrease from pre- to postfortification tHcy concentrations in our analysis (10% in older men and 13% in older women) was similar to

Table 3. Trends in method-adjusted prevalence of increased plasma tHcy and desirable plasma tHcy concentrations by sex, race-ethnicity, and age group during the NHANES, 1991–2004.[a,b]

Race-ethnicity	Sex	Age group, years	Prevalence, % (95% CI)				*P*		
			1991–1994[c]	1999–2000[d]	2001–2002[d]	2003–2004	1991–1994 vs 1999–2000[e]	1999–2000 vs 2001–2002[f]	2001–2002 vs 2003–2004[g]
Plasma tHcy >13 μmol/L									
All	M	12–19	5.79 (3.81–8.71)[h]	2.22 (1.02–4.76)	1.52 (0.78–2.96)[h]	1.60 (0.72–3.49)[h]	0.0158	NS	NS
All	M	20–59	19.0 (16.6–21.6)[j]	4.98 (3.28–7.50)[j]	5.28 (3.89–7.14)[j]	6.79 (4.98–9.19)[j]	<0.001	NS	NS
All	M	≥60	31.8 (25.1–39.2)[k]	14.2 (10.5–19.0)[k]	15.8 (12.4–19.9)[k]	13.6 (9.78–18.6)[k]	<0.001	NS	NS
All	F	12–19	3.04 (1.36–6.68)[h]	0.49 (0.12–2.00)[h]	0.33 (0.09–1.23)[h]	0.39 (0.09–1.73)[h]	NS	NS	NS
All	F	20–59	9.68 (8.35–11.2)[j]	2.69 (1.71–4.21)[j]	2.38 (1.62–3.50)	3.35 (2.32–4.82)[j]	<0.001	NS	NS
All	F	≥60	19.8 (15.2–25.4)[k]	5.44 (4.01–7.36)[k]	4.66 (2.53–8.44)[k]	8.67 (5.80–12.8)[k]	<0.001	NS	NS
NHW	M	≥60	30.2 (23.3–38.2)	13.5 (9.61–18.6)	15.4 (11.3–20.5)	12.3 (8.09–18.4)	<0.001	NS	NS
NHW	F	≥60	18.8 (13.7–25.2)	5.51 (3.76–8.01)	4.51 (2.14–9.24)	8.61 (5.17–14.0)	<0.001	NS	NS
NHB	M	≥60	43.1 (32.5–54.4)	16.7 (9.79–27.0)	23.5 (15.9–33.3)	24.6 (16.4–35.2)	<0.001	NS	NS
NHB	F	≥60	26.4 (19.7–34.3)	10.1 (2.91–29.4)	5.40 (1.91–14.3)	15.9 (9.35–25.7)	NS	NS	NS
MA	M	≥60	35.8 (26.2–46.6)	11.4 (6.89–18.3)	10.1 (5.29–18.5)	13.1 (8.42–19.8)	<0.001	NS	NS
MA	F	≥60	17.2 (12.2–23.6)	9.00 (6.14–13.0)	8.29 (4.49–14.8)	5.30 (3.05–9.08)	0.0144	NS	NS
Plasma tHcy ≤9 μmol/L									
All	M	12–19	77.7 (71.9–82.5)[h]	90.1 (87.2–92.4)[h]	89.4 (85.3–92.5)[h]	89.5 (85.8–92.3)[h]	<0.001	NS	NS
All	M	20–59	48.4 (43.3–53.6)[j]	62.0 (57.7–66.1)[j]	61.2 (57.1–65.1)[j]	58.6 (52.8–64.1)[j]	<0.001	NS	NS
All	M	≥60	32.3 (25.7–39.7)[k]	36.2 (30.0–42.8)[k]	37.3 (30.3–45.0)[k]	35.4 (29.3–42.0)[k]	NS	NS	NS
All	F	12–19	88.5 (82.1–92.8)[h]	95.0 (90.7–97.3)[h]	95.8 (93.7–97.2)[h]	94.6 (91.9–96.5)[h]	NS	NS	NS
All	F	20–59	73.6 (71.1–75.9)[j]	83.4 (81.1–85.5)[j]	87.0 (85.3–88.5)[j]	82.0 (78.3–85.3)[j]	<0.001	0.0083	0.0097
All	F	≥60	45.8 (40.1–51.7)[k]	64.8 (57.5–71.5)[k]	61.0 (54.4–67.3)[k]	48.8 (41.4–56.2)[k]	<0.001	NS	0.0124
NHW	M	≥60	32.0 (24.5–40.5)	36.8 (30.0–44.1)	38.0 (30.5–46.2)	36.3 (29.5–43.7)	NS	NS	NS
NHW	F	≥60	45.6 (38.5–52.9)	67.9 (60.2–74.7)	61.7 (53.7–69.2)	47.3 (39.1–55.7)	<0.001	NS	0.0114
NHB	M	≥60	30.8 (24.4–38.0)	37.3 (23.2–54.0)	28.6 (21.7–36.7)	26.0 (15.6–40.1)	NS	NS	NS
HB	F	≥60	43.3 (33.1–54.1)	44.2 (36.5–52.1)	48.8 (40.8–56.9)	43.5 (30.0–58.1)	NS	NS	NS
MA	M	≥60	24.7 (20.1–29.9)	41.4 (32.1–51.5)	39.0 (25.3–54.8)	37.8 (29.1–47.3)	0.0024	NS	NS
MA	F	≥60	52.7 (45.9–59.4)	57.8 (49.0–66.2)	58.7 (36.2–78.0)	61.3 (50.1–71.4)	NS	NS	NS

[a] All indicates racial-ethnic groups are not shown separately; NS, not significant; NHW, non-Hispanic white; NHB, non-Hispanic black; MA, Mexican American.
[b] Persons with CKD and persons with incomplete information to determine whether they had CKD were excluded.
[c] For 1991–1994, USDA data for serum tHcy by HPLC has been adjusted to plasma, then to CDC HPLC, and then to Abbott AxSYM.
[d] For 1999–2001, CDC data for plasma tHcy by Abbott IMx has been adjusted to Abbott AxSYM.
[e] Pairwise comparison (*t*-test) of prevalence estimates from 1991–1994 and 1999–2000 ($P < 0.017$ significant).
[f] Pairwise comparison (*t*-test) of prevalence estimates from 1999–2000 and 2001–2002 ($P < 0.017$ significant).
[g] Pairwise comparison (*t*-test) of prevalence estimates from 2001–2002 and 2003–2004 ($P < 0.017$ significant).
[h] Significantly different from 20–59 y old age group, $P < 0.017$.
[j] Significantly different from ≥60 y old age group, $P < 0.017$.
[k] Significantly different from 12–19 y old age group, $P < 0.017$.

the decrease found in Chile but larger than that found in the Framingham population, possibly because tHcy concentrations may have decreased further after 1998.

Jacques et al. *(12)* reported changes in the prevalence of increased tHcy concentrations (>13 μmol/L) from 19% (prefortification) to 10% (postfortification). The changes in prevalence for older men (32% to 14%) and older women (20% to 5%) in our analysis appear larger. However, the average prevalence estimates for men and women prefortification (25%) and postforti-

Table 4. Method-adjusted selected population percentile values for plasma tHcy concentrations by sex, race-ethnicity, and age group during the NHANES, 1991–1994 and 1999–2004.[a,b]

Race-Ethnicity	Sex	Age group, years	Survey[c]	n	tHcy concentration, μmol/L (95% CI) at selected percentile		
					5th	50th	95th
All	Both	All	1991–1994	7781	4.14 (3.78–4.30)	8.11 (7.90–8.36)	17.7 (17.2–30.1)
			1999–2004	17891	4.65 (4.36–4.73)	7.66 (7.56–7.76)	12.9 (12.7–20.9)
All	M	All	1991–1994	3448	4.93 (4.44–5.14)	8.95 (8.72–9.38)	19.8 (18.7–35.8)
			1999–2004	8731	5.33 (5.01–5.41)	8.39 (8.28–8.50)	13.6 (13.3–21.7)
All	F	All	1991–1994	4333	3.57 (3.07–3.83)	7.17 (7.02–7.37)	15.8 (15.3–23.0)
			1999–2004	9160	4.30 (4.00–4.38)	6.95 (6.85–7.06)	11.9 (11.6–16.9)
All	M	12–19	1991–1994	631	3.69 (3.03–4.08)	6.70 (6.32–7.12)	14.1 (12.1–27.8)
			1999–2004	3150	4.14 (3.76–4.29)	6.37 (6.26–6.50)	10.2 (9.86–15.0)
All	M	20–59	1991–1994	2030	5.32 (4.99–5.53)	9.14 (8.76–9.59)	20.2 (18.5–32.1)
			1999–2004	3900	5.87 (5.45–5.99)	8.51 (8.39–8.62)	13.3 (13.0–21.6)
All	M	≥60	1991–1994	787	5.87 (5.07–6.71)	10.7 (10.1–11.4)	21.5 (20.1–34.5)
			1999–2004	1681	6.67 (6.02–6.94)	9.78 (9.59–10.1)	15.4 (15.0–22.4)
All	F	12–19	1991–1994	737	2.78 (2.56–3.31)	5.84 (5.41–6.51)	10.8 (9.43–15.4)
			1999–2004	3091	3.82 (3.43–3.91)	5.68 (5.57–5.79)	8.92 (8.60–10.6)
All	F	20–59	1991–1994	2780	3.68 (2.92–4.02)	7.02 (6.74–7.31)	15.3 (14.9–26.0)
			1999–2004	4466	4.36 (4.05–4.45)	6.89 (6.80–6.99)	11.6 (11.3–16.2)
All	F	≥60	1991–1994	816	4.94 (4.18–5.51)	9.21 (8.85–9.98)	20.3 (18.1–25.4)
			1999–2004	1603	6.00 (5.29–6.12)	8.57 (8.34–8.82)	13.4 (13.0–16.9)
NHW	M	12–19	1991–1994	117[d]	3.57 (2.60–4.25)	6.50 (6.10–7.47)	12.8 (11.4–.)
			1999–2004	801	4.10 (3.60–4.32)	6.37 (6.24–6.52)	9.84 (9.49–13.8)
NHW	M	20–59	1991–1994	564	5.31 (4.95–5.66)	9.22 (8.83–9.91)	20.2 (18.0–30.6)
			1999–2004	1860	5.99 (5.37–6.14)	8.59 (8.45–8.72)	13.3 (12.9–19.9)
NHW	M	≥60	1991–1994	393	5.82 (4.90–6.65)	10.6 (9.84–11.4)	21.4 (20.0–33.9)
			1999–2004	911	6.74 (5.99–7.09)	9.75 (9.54–10.1)	15.4 (14.9–18.9)
NHW	F	12–19	1991–1994	143[d]	2.69 (2.54–3.30)	5.75 (5.27–6.63)	10.8 (8.68–15.5)
			1999–2004	800	3.89 (3.48–4.04)	5.78 (5.62–5.94)	9.02 (8.69–9.88)
NHW	F	20–59	1991–1994	865	3.82 (2.90–4.18)	7.16 (6.84–7.59)	15.3 (15.0–21.3)
			1999–2004	2078	4.53 (4.02–4.66)	7.02 (6.89–7.14)	11.9 (11.4–15.1)
NHW	F	≥60	1991–1994	436	4.87 (4.01–5.60)	9.21 (8.70–10.2)	19.9 (17.3–24.0)
			1999–2004	844	6.00 (5.16–6.17)	8.53 (8.31–8.82)	13.4 (12.8–17.0)
NHB	M	12–19	1991–1994	262	3.94 (3.02–4.31)	7.34 (7.07–7.99)	17.3 (12.6–23.8)
			1999–2004	1039	4.03 (3.73–4.15)	6.35 (6.23–6.50)	10.6 (10.2–14.9)
NHB	M	20–59	1991–1994	649	5.58 (4.67–6.06)	9.25 (8.84–9.54)	20.2 (18.0–27.9)
			1999–2004	793	5.80 (5.43–6.04)	8.56 (8.40–8.75)	14.5 (13.8–20.9)
NHB	M	≥60	1991–1994	163[d]	6.36 (5.39–7.23)	11.2 (10.0–13.5)	24.0 (20.0–30.9)
			1999–2004	260	6.76 (6.08–7.22)	10.2 (9.82–10.7)	15.9 (15.4–19.7)
NHB	F	12–19	1991–1994	320	3.45 (2.89–3.99)	6.10 (5.90–6.57)	13.5 (11.7–15.9)
			1999–2004	967	3.72 (3.27–3.88)	5.65 (5.55–5.78)	8.76 (8.35–9.60)
NHB	F	20–59	1991–1994	983	3.89 (3.54–4.17)	7.14 (6.92–7.62)	15.5 (14.0–21.8)
			1999–2004	916	4.34 (3.82–4.51)	7.01 (6.85–7.19)	11.6 (11.1–17.0)
NHB	F	≥60	1991–1994	151[d]	5.14 (4.52–5.77)	9.53 (8.53–10.6)	21.1 (17.0–31.2)
			1999–2004	268	6.12 (5.59–6.53)	9.32 (8.92–9.64)	14.9 (13.6–16.3)

Continued on page 811

Table 4. Method-adjusted selected population percentile values for plasma tHcy concentrations by sex, race-ethnicity, and age group during the NHANES, 1991–1994 and 1999–2004.[a,b] (*Continued from page 810*)

Race-Ethnicity	Sex	Age group, years	Survey[c]	n	tHcy concentration, μmol/L (95% CI) at selected percentile		
					5th	50th	95th
MA	M	12–19	1991–1994	218[d]	3.63 (2.63–4.21)	6.96 (6.41–8.03)	15.0 (12.3–20.8)
			1999–2004	1111	4.16 (3.83–4.29)	6.22 (6.06–6.36)	9.85 (9.45–13.5)
MA	M	20–59	1991–1994	725	5.06 (4.35–5.47)	8.87 (8.51–9.41)	16.2 (15.3–21.7)
			1999–2004	926	5.37 (4.87–5.54)	7.72 (7.53–7.92)	12.1 (11.8–14.2)
MA	M	≥60	1991–1994	205[d]	6.40 (4.91–6.99)	10.9 (10.7–12.1)	20.9 (17.1–.)
			1999–2004	419	6.45 (5.52–6.84)	9.66 (9.21–10.1)	15.1 (14.5–20.1)
MA	F	12–19	1991–1994	226	3.22 (2.76–3.53)	5.69 (5.39–6.16)	9.40 (8.65–10.8)
			1999–2004	1110	3.61 (3.23–3.74)	5.21 (5.11–5.37)	7.80 (7.57–9.10)
MA	F	20–59	1991–1994	777	3.47 (3.17–3.68)	6.30 (6.10–6.75)	12.9 (11.7–18.8)
			1999–2004	1082	3.87 (3.55–4.02)	6.13 (6.00–6.30)	9.53 (9.19–12.3)
MA	F	≥60	1991–1994	189[d]	5.15 (4.89–6.06)	8.79 (8.32–9.66)	17.0 (15.2–19.0)
			1999–2004	395	5.43 (4.73–5.74)	8.29 (7.97–8.88)	13.9 (13.1–15.2)

[a] All indicates racial-ethnic groups are not shown separately; NHW, non-Hispanic white; NHB, non-Hispanic black; MA, Mexican American.
[b] Persons with CKD and persons with incomplete information to determine whether they had CKD were excluded.
[c] For 1991–1994, USDA data for serum tHcy by HPLC has been adjusted to plasma, then to CDC HPLC, and finally to Abbott AxSYM. For 1999–2001, CDC data for plasma tHcy by Abbott IMx has been adjusted to Abbott AxSYM.
[d] Minimum cell size to provide robust 5th and 95th percentile estimates ($n = 220$) is not fulfilled.

fication (10%) are similar to prevalence estimates in the Framingham population (data not shown).

A decrease in tHcy concentrations as a result of fortification was expected, particularly because serum and erythrocyte folate concentrations increased significantly during this period *(10–13, 38)*, and high tHcy concentrations can be responsive to improvements in folate status. Our results are in good agreement with a metaanalysis of 25 randomized controlled trials *(39)*. The Homocysteine Lowering Trialists' Collaboration assessed the effect of different doses of folic acid on reduction of plasma tHcy concentrations in approximately 2500 inividuals and found that daily doses of 200 μg are associated with 60% of the maximal reduction achieved with doses of ≥800 μg. At a prefortification tHcy concentration of 10 μmol/L, as found in our current analysis, and serum folate concentration of 12 nmol/L, as shown in our last analysis *(38)*, the predicted reduction in plasma tHcy concentration was 12.6% at a dose of 200 μg of folic acid/day. We found reductions in tHcy concentrations of approximately 10%.

It remains to be seen whether lowering tHcy concentrations in the US population will have any effects on primary and/or secondary prevention of cardiovascular disease. Yang et al. reported recently on what might be a first effect of fortification on stroke mortality: the ongoing decline in stroke mortality observed in the US between 1990 and 1997 accelerated in 1998–2002 in nearly all population strata *(14)*. Similar effects were seen in Canada, whereas the decline in stroke mortality in England and Wales did not change significantly between 1990 and 2002 *(14)*. Interestingly, a recent metaanalysis of randomized trials assessing the efficacy of folic acid supplementation in the prevention of stroke found a significantly reduced risk of stroke of 18% *(7)*. A greater beneficial effect was seen in trials with treatment duration of more than 36 months; a decrease in the concentration of tHcy of more than 20% was found in trials done in regions without grain fortification and among individuals without a history of stroke.

Although estimates of median tHcy concentrations appeared to increase during the 3 postfortification survey periods, the changes were too small to reach statistical significance. Slight increases in tHcy concentrations during postfortification could be expected owing to moderate decreases in serum and erythrocyte folate concentrations during the same time period *(38)*. It is unlikely that there were other significant changes in lifestyle behaviors (e.g., diet, exercise, supplement use) over such a short period of time. However, secular changes in lifestyle behaviors associated with tHcy concentrations were not studied specifically

in our analysis. Furthermore, our results can be generalized to the part of the US population that is free of CKD, but not the overall US population.

We have presented a detailed analysis of circulating tHcy concentrations in the US population. Although the complex method adjustment performed to allow for time trend analysis generated some uncertainty, this uncertainty was small enough to allow the quantification of a modest decrease in plasma tHcy concentrations from before to after fortification. The decrease of approximately 10% corresponds well with effects seen in intervention trials with folic acid and in smaller observational studies. We saw no significant changes in tHcy concentrations during the 6 postfortification years. Stabilization of tHcy concentrations may largely depend on the stabilization of blood folate concentrations as well as on other possible causes of increased concentrations, including but not limited to vitamin B_{12} status. Therefore, monitoring of all related biomarkers should continue, and further investigations of other possible causes of high tHcy concentrations should be undertaken.

Grant/Funding Support: None declared.
Financial Disclosures: None declared.
Acknowledgments: We thank Sonja Strider (CDC's National Center for Environmental Health) for performing the plasma homocysteine assays. CMP and CLJ developed the study concept and design and supervised the collection of data. JKS, CMP, and CLJ performed and guided the statistical data analysis. CMP, JDO, CLJ, MFP, EAY, and JIR were involved in the data analysis and interpretation. CMP drafted the manuscript. CMP, JDO, CLJ, MFP, EAY, and JIR provided critical revision of the manuscript for important intellectual content. All authors contributed to the final manuscript.

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