Original Contribution

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# Distribution of and Factors Associated With Serum Homocysteine Levels in Children

## Child and Adolescent Trial for Cardiovascular Health

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## Abstract

**Context** Although evidence suggests that homocysteine is a risk factor for cardiovascular disease in adults, little information exists on homocysteine levels in children.

**Objectives** To describe the distribution of serum homocysteine concentrations among children and to examine the association between homocysteine levels and several characteristics, including serum levels of folic acid and vitamins $B_{12}$ and $B_6$.

**Design** Cross-sectional analysis.

**Setting** School-based cohort from California, Louisiana, Minnesota, and Texas.

**Participants** A total of 3524 US schoolchildren, aged 13 and 14 years, from the Child and Adolescent Trial for Cardiovascular Health (completed in 1994). Measurement was conducted in 1997.

**Main Outcome Measure** Nonfasting serum total homocysteine concentration.

**Results** The distribution of homocysteine values ranged from 0.1 to 25.7 μmol/L (median, 4.9 μmol/L). Geometric mean homocysteine concentration was significantly higher in boys (5.22 μmol/L) than girls (4.84 μmol/L); blacks (5.51 μmol/L) than whites (4.96 μmol/L) or Hispanics (4.93 μmol/L); nonusers of multivitamins (5.09 μmol/L) than users (4.82 μmol/L); and smokers (5.19 μmol/L) than nonsmokers (5.00 μmol/L). Serum homocysteine was significantly inversely correlated with serum levels of folic acid ($r=-0.26$, $P=.001$), vitamin $B_{12}$ ($r=-0.21$, $P=.001$)

pressure. After multivariate adjustment, homocysteine remained independently associated with sex, race, serum folic acid and vitamin $B_{12}$ levels, and systolic blood pressure.

**Conclusions** The distribution of homocysteine levels in children is substantially lower than that observed for adults; however, a small percentage of children are still potentially at elevated risk for future cardiovascular disease. Serum folic acid may be an important determinant of homocysteine levels in children.

The opportunity for primary prevention of cardiovascular disease (CVD) begins during childhood.[1] Specifically, early lesions of atherosclerosis are present in the arteries of children and adolescents[2]; some children display moderate to high levels of physiologic risk factors[3]; and childhood levels of physiologic risk factors may predict adult levels.[3-5] Recent studies suggest that plasma homocysteine concentration may be an independent and modifiable risk factor for CVD in adults.[6] A meta-analysis found for every 5-µmol/L increase in homocysteine, the odds ratio for risk of coronary artery disease was 1.6 (95% confidence interval [CI], 1.4-1.7) for men and 1.8 (95% CI, 1.4-2.3) for women.[7] Although conclusive evidence for a causal association with CVD in adults is lacking,[7-17] the potential for prevention provides a strong rationale for understanding the determinants of homocysteine in children.

Homocysteine is a sulfhydryl amino acid formed during the conversion of methionine to cysteine.[18,19] Its main metabolic pathways require folic acid, vitamin $B_{12}$, and vitamin $B_6$. Serum homocysteine is inversely correlated with dietary intake or serum levels of vitamins $B_6$ and $B_{12}$ and folic acid.[20-26] Moreover, homocysteine levels respond rapidly to nutrient supplementation with reductions as great as 40% due to folic acid supplementation alone.[7,27,28] In adults, increasing age, being male, and cigarette smoking are associated with higher homocysteine levels.[25,26]

Few studies have examined homocysteine levels and their predictors in children. The largest study, among 756 Norwegian children aged 8 to 12 years, identified serum folic acid and family history of premature CVD as important correlates of homocysteine levels.[29] A study of South African children (n=433) aged 7 to 15 years found significantly higher homocysteine concentrations in black children from the Venda tribe in South Africa (5.8 µmol/L) compared with white children (5.1 µmol/L).[30] Vilaseca et al[31] reported that plasma homocysteine levels increased with age in cross-sectional samples of infants aged 2 months to adults aged 18 years residing in Spain. The few studies of homocysteine levels in children have either been conducted with relatively small or homogeneous samples or lacked information on a comprehensive set of predictors. To date, no data have been reported on large, multiethnic samples of healthy children living in the United States.

In the present study, we describe the distribution of serum homocysteine levels among 3524 US schoolchildren aged 13 to 14 years. Furthermore, we examine the association between homocysteine levels and a variety of demographic, physiologic, and behavioral variables.

# Methods

## Study Participants

The subjects for this analysis were part of a larger study, the Child and Adolescent Trial for Cardiovascular Health (CATCH). The original CATCH cohort (n=5106 third-graders) was recruited at baseline (1991-1992) from 96 public

promotion program in 56 intervention schools and 40 control schools from grades 3 to 5 (1991-1994). The design and results of the trial are described in detail elsewhere.[33]

Physiologic, behavioral, and psychosocial measurements were conducted on the original CATCH cohort students postintervention during grades 6 to 8 (1994-1997). At the eighth-grade (1997) risk factor screening survey, 3714 cohort students (73%) had written parental consent to participate in all CATCH measurements. The 3524 cohort students (69%) with serum available for homocysteine analysis were included in the present study **Table 1**. Complete laboratory data, including serum levels of homocysteine, folic acid, vitamin $B_{12}$, and vitamin $B_6$ were available for 3321 children (65%).

**Table 1. Characteristics of Participants**

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## Data Collection

All methods, training techniques, and quality control programs of CATCH are described in detail elsewhere.[32,34] Data were collected through classroom-administered questionnaires and risk factor examinations. The risk factor screening profile included blood pressure (BP), height, weight, and nonfasting serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, apolipoprotein B, total homocysteine, vitamin $B_6$, vitamin $B_{12}$, and folic acid. Parents also completed a mailed questionnaire on family history of CVD and multivitamin use, with telephone follow-up of nonrespondents. Of the 3524 children with homocysteine results, 3209 children (91%) had a questionnaire completed by a parent.

**Blood Pressure.** Five recordings (taken 1 minute apart) of seated systolic and diastolic BP and heart rate were obtained after a 5-minute rest period using the Dinamap automatic BP device (Model 8100XT, Critikon Inc, Tampa, Fla). Cuff size selection was based on arm length and circumference measurements. The average of the last 3 readings was used for analysis.

**Height and Weight.** Height was measured using a portable stadiometer and weight was measured using the SECA Integra 815 portable scale (SECA, Rumilly, France). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

**Smoking Behavior.** Current smokers were defined as those children who reported smoking on 1 day or more in the past 30 days.

**Family History of CVD.** Children were classified as having a family history of CVD if the parent reported either myocardial infarction or stroke in any biological relative and as having premature CVD if an event occurred before age 60 years.

**Intake of Multivitamins.** Parents reported whether their child usually took a multivitamin as well as the brand

Frequency of multivitamin intake was categorized as: less than 1, 1 to 2, 3 to 4, 5 to 6, 7, or more than 7 tablets per week. Children were classified as multivitamin users if they took 1 or more multivitamins in a week. The majority of users took multivitamin preparations containing 0.4 mg of folic acid, 2 mg of vitamin $B_6$, and 6 μg of vitamin $B_{12}$ (87%, 75%, and 73%, respectively).

## Blood Sample Collection and Biochemical Analyses

Nonfasting blood samples were obtained via venipuncture with the child seated. Whole blood was collected into serum separator Vacutainer tubes (Becton Dickinson, Franklin, NJ) and allowed to clot in a covered container for 20 minutes at room temperature. Clotted blood samples were then placed on ice and centrifuged approximately 2 to 4 hours after blood collection. Based on a pilot study of our blood handling procedure, we found a 5% increase in average serum homocysteine concentration after blood was allowed to clot at room temperature for 20 minutes when compared with immediate sample chilling. Serum was stored in Nalgene cryule vials (Nalgene Co, Rochester, NY) and shipped by overnight carrier on refrigerant packs to the central laboratory for immediate analysis of total and HDL cholesterol and apolipoprotein B. Serum was frozen at −70°C and covered from light until analyzed for homocysteine, folic acid, vitamin $B_6$, and vitamin $B_{12}$, which was approximately 5 to 10 months after blood collection.

All serum samples were analyzed at Miriam Hospital, Providence, RI. Total cholesterol was determined by the method of Allain et al[35] on a Beckman CX4 autoanalyzer (Beckman Instruments Inc, Fullerton, Calif). High-density lipoprotein was determined following precipitation with heparin sodium and manganese chloride. Apolipoprotein B was assayed by nephelometry (Behring Diagnostics Inc, Westwood, Mass) using antisera raised by injecting goats with low-density lipoprotein. Total homocysteine was determined by the fluorimetric method of Vester and Rasmussen[36] with the exception that 20% methanol was used in buffer B in the high-performance liquid chromatography procedure. Vitamin $B_{12}$ and folic acid were measured by a solid phase, no-boil radioimmunoassay using a commercial kit (Diagnostic Products Corporation, Los Angeles, Calif).[37,38] Vitamin $B_6$ was analyzed by a radioassay kit (ALPCO, Windham, NH), which measures the conversion of tritiated tyrosine to tyramine by the vitamin $B_6$–dependent enzyme tyrosine decarboxylase.[39]

Laboratory reliability was assessed by taking blind duplicate blood samples from a 10% random sample of subjects. Intraclass correlation coefficients for serum lipids were 0.99 for total cholesterol, 0.98 for HDL cholesterol, and 0.99 for apolipoprotein B. Intraclass correlation coefficients were 0.91 for serum homocysteine, 0.97 for vitamin $B_6$, 0.96 for vitamin $B_{12}$, and 0.98 for folic acid.

## Statistical Methods

We tabulated the percentile distribution of serum homocysteine levels for all children by sex and ethnic subgroups. Mean homocysteine concentration was calculated for the overall cohort and compared using the 2-sample *t* test among the following subgroups: boys vs girls; whites vs blacks vs Hispanics; multivitamin users vs nonusers; family history of CVD vs no family history; and smokers vs nonsmokers. Mean homocysteine levels were also compared for quintiles of the distribution of serum vitamin $B_{12}$, vitamin $B_6$, and folic acid levels. We calculated Spearman correlation coefficients to assess the association of serum homocysteine levels with serum vitamin levels (folic acid, $B_{12}$, and $B_6$) and physiologic cardiovascular risk factors (serum lipids, BMI, and BP).

where indicated. We calculated the 95% CIs for the geometric means by taking the antilogarithms of the transformed 95% CIs.

The assumption of a linear dose-response relationship between each continuous variable and homocysteine was examined using an analysis of variance–based test for linear trend.[40] We fit a segmented regression model to the relationship between serum homocysteine and serum folic acid using nonlinear least squares regression to estimate the change point, defined as the point where the relationship between homocysteine and folic acid changes slope.

We conducted a mixed-model analysis of covariance to evaluate the simultaneous influence of the various predictors on serum homocysteine levels. Serum homocysteine concentration was modeled as the dependent variable. Sex, ethnicity, family history of CVD, multivitamin use, and smoking were represented as indicator variables in the models; age, BMI, BP, and serum lipids were analyzed as continuous variables. Serum vitamin levels were categorized according to quintiles of their respective distributions and represented as indicator variables. The analysis was adjusted for the fixed effects of CATCH field site and original CATCH intervention condition and the random effect of CATCH school within field site and intervention condition. Subjects with missing covariate data were excluded from analyses. There were no differences in the results after excluding 2 residual outliers. We used SAS statistical software for all analyses.[41]

# Results

## Distribution of Serum Homocysteine

Table 1 presents the characteristics of study participants. The percentile distribution of serum homocysteine levels for all children by sex and ethnic subgroups is shown in Table 2. The distribution of values was positively skewed and ranged from 0.1 to 25.7 μmol/L. Less than 1% (n=12) of the children had levels above 14.0 μmol/L. The mean homocysteine concentration for all subjects was 5.29 μmol/L (median, 4.9 μmol/L). The distribution of serum homocysteine was shifted toward higher values for boys compared with girls and blacks compared with whites and Hispanics.

### Table 2. Percentile Distribution of Total Serum Homocysteine Levels Overall and by Sex and Race*

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Figure 1 displays the percentile distribution of homocysteine values among multivitamin users compared with nonusers. Among nonusers, the distribution was positively skewed with a long tail toward high values; in contrast, among multivitamin users, the distribution was more symmetrical. Only 3.3% of children who took a multivitamin exceeded the 95th percentile of the overall homocysteine distribution (8.5 μmol/L) vs 5.7% among nonusers (*P*=.01; 2-tailed Fisher exact test).

**Figure 1.** Distribution of Serum Homocysteine

Sample concentrations were taken from 695 students who took at least 1 multivitamin weekly and 2503 students who did not take a multivitamin weekly.

## Serum Vitamin Status

Serum vitamin levels ranged from 8.6 to 324 nmol/L for folic acid, 21 to 1747 pmol/L for vitamin $B_{12}$, and 2.2 to 960 nmol/L for vitamin $B_6$. None of the children's levels were below the minimum of the laboratory reference range for folic acid, but a small percentage were below the minimum for vitamins $B_{12}$ and $B_6$ (1.7% and 3.2%, respectively). There were significant positive correlations between folic acid and vitamin $B_{12}$ ($r$=0.20; $P$=.001), folic acid and vitamin $B_6$ ($r$=0.48; $P$=.001), and vitamins $B_{12}$ and $B_6$ ($r$=0.16; $P$=.001). Boys had significantly higher levels of folic acid than girls (40.8 vs 35.1 nmol/L; $P$<.001), whereas girls had higher levels of vitamin $B_{12}$ (379.8 vs 352.4 pmol/L; $P$<.001). Black children had significantly lower levels of folic acid and vitamin $B_6$ than either white or Hispanic children (folic acid: 29.4 vs 40.1 and 36.5 nmol/L, respectively, and vitamin $B_6$: 26.1 vs 38.7 and 37.4 nmol/L, respectively; $P$<.001) but higher levels of vitamin $B_{12}$ (402 vs 359.2 and 359.5 pmol/L, respectively; $P$<.001). Multivitamin users had significantly higher levels of all vitamins than nonusers, and smokers had significantly lower levels than nonsmokers (data not shown).

## Factors Associated With Serum Homocysteine Levels

In unadjusted analyses, we observed significantly higher mean homocysteine levels for boys than girls (5.22 vs 4.84 μmol/L; $P$<.001) and for black compared with white or Hispanic children (5.51 vs 4.96 and 4.93 μmol/L, respectively; $P$=.001). Mean homocysteine levels were approximately 6% lower among multivitamin users compared with nonusers (4.82 vs 5.09 μmol/L; $P$=.001). There was a significant inverse linear dose-response relationship between serum homocysteine and frequency of multivitamin use ($P$<.001) (data not shown). Children who smoked had somewhat higher mean homocysteine levels than nonsmokers (5.19 vs 5.00 μmol/L; $P$=.03). There were no significant differences in homocysteine levels between subgroups of children with or without a family history of CVD (5.04 vs 4.99 μmol/L; $P$=.40) or premature CVD (5.01 vs 5.03 μmol/L; $P$=.80). In an analysis of covariance that also adjusted for serum levels of vitamins (**Table 3**), the difference in mean homocysteine levels between multivitamin users and nonusers and smokers and nonsmokers was attenuated and nonsignificant; however, sex and ethnic subgroup differences remained.

### Table 3. Sociodemographic and Behavioral Factors Associated With Serum Homocysteine Levels*

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We examined the correlation between serum homocysteine and several physiologic CVD risk factors. Weak significant positive correlations were observed for homocysteine with age ($r$=0.06; $P$<.001), systolic BP ($r$=0.08; $P$=.001), and BMI ($r$=0.09; $P$=.001). There was no correlation with diastolic BP or with serum lipids, including total cholesterol, HDL cholesterol, and apolipoprotein B. **Table 4** shows the results from an analysis of covariance,

relationship with systolic BP remained significant. The results did not differ when apolipoprotein B was included in the models instead of total cholesterol (data not shown).

**Table 4. Association of Total Homocysteine With Physiologic Cardiovascular Risk Factors***

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Serum homocysteine was inversely correlated with serum levels of all 3 vitamins. The correlations with homocysteine were somewhat stronger for folic acid ($r$=−0.36; $P$=.001) than vitamins $B_{12}$ ($r$=−0.21; $P$=.001) and $B_6$ ($r$=−0.18; $P$=.001). The inverse relationship of homocysteine with folic acid appeared segmented with an initial steep slope and plateau at higher concentrations of folic acid (**Figure 2**). Based on the results of the segmented regression, a change point was estimated at folic acid levels of 30.6 nmol/L (95% CI, 28.9-32.2 nmol/L), at which point the linear relationship with a slope of −0.16 µmol/L of homocysteine per 1 nmol/L of folic acid changed to an apparent plateau. **Table 5** presents the association of homocysteine levels with serum levels of vitamins according to quintiles of their distributions. Mean homocysteine was 30% lower in the highest compared with the lowest quintile of folic acid (4.48 vs 6.15 µmol/L; $P$<.001). Similarly, the homocysteine level was approximately 15% lower in the highest compared with the lowest quintiles of both vitamin $B_{12}$ and vitamin $B_6$ ($P$<.001). Results from the mixed-model analysis of covariance revealed the association of homocysteine levels with folic acid remained significant and unchanged after controlling for levels of the other 2 vitamins simultaneously. The association of homocysteine with vitamin $B_{12}$ was attenuated but remained significant, whereas the association with vitamin $B_6$ disappeared.

**Figure 2.** Segmented Regression of Serum Homocysteine Concentration on Serum Folic Acid Concentration

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**Table 5. Association of Serum Folic Acid, Vitamin B12, and Vitamin B6 With Serum Homocysteine Levels***

Table 5. Association of Serum Folic Acid, Vitamin B
12
, and Vitamin B
6
With Serum Homocysteine Levels*

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## Comment

children are potentially at elevated risk for future CVD, and there are important sex and ethnic subgroups at higher risk. Our results strongly support the importance of folic acid as a determinant of homocysteine levels in children.

The distribution of homocysteine values in our sample was substantially lower than the distribution of values observed in adults; median homocysteine concentration (4.9 μmol/L) was approximately half of adult levels. Tonstad et al[29] found comparable median levels (5.1 μmol/L) in a sample of 8- to 12-year-old Norwegian children. The lower homocysteine values in children are consistent with an age-dependent increase in plasma homocysteine concentration.[21,22,25,26,31] Data from 195 Spanish children revealed a clear increase in plasma homocysteine levels with age; median homocysteine was 5.8 μmol/L for infants aged 2 months to children aged 10 years, 6.6 μmol/L for adolescents aged 11 to 15 years, and 8.1 μmol/L for adolescents aged 16 to 18 years.[31] Nygard et al[25] observed a nearly 2-μmol/L increase in average homocysteine concentration between ages 40 to 42 years and 65 to 67 years in adults.

Although the children's overall distribution of homocysteine concentration was lower than that of adults, many children are still at levels that may confer elevated risk for CVD. In general, the relationship between homocysteine level and risk for CVD is graded and continuous across the entire distribution of homocysteine values with no evidence of a threshold level.[7,13,14,21] For example, Nygard et al[14] demonstrated a dose-response relationship for risk of death among patients with coronary heart disease within the range of 5 to 20 μmol/L, with a substantial increase in risk even at levels below 15 μmol/L. When compared with patients with levels below 9.0 μmol/L, the adjusted odds ratio for risk of death was 1.9 for those with homocysteine levels from 9 to 14.9 μmol/L and 2.8 for those with levels from 15 to 19.9 μmol/L. In our sample, approximately 5% of children had homocysteine levels at or above 9.0 μmol/L. Furthermore, our data indicate there may be demographic subgroups at higher risk; boys had higher homocysteine levels than girls and black children had higher homocysteine levels then white and Hispanic children.

The inverse association between serum homocysteine concentration and serum levels of folic acid and vitamin $B_{12}$ is consistent with several previous studies.[20,22,24,42,43] The dose-response relationship appears to plateau at higher levels of serum folic acid. We demonstrated a plateau between decreasing homocysteine with higher levels of serum folic acid at approximately 30.6 nmol/L of folic acid. Pancharuniti et al[43] similarly observed a plateau for decreasing homocysteine concentration at plasma folic acid levels of 12.5 nmol/L. This relationship with serum folic acid levels may correspond to the plateau in homocysteine concentration observed in response to increasing amounts of folic acid supplementation. In a review of 9 intervention studies, reduction in plasma homocysteine reached a plateau at folic acid doses of about 400 μg/d, beyond which homocysteine levels remained stable even with increasing doses of folic acid.[7] Malinow et al[44] found similar homocysteine-lowering effects with cereals containing 499 μg or 665 μg of folic acid per 30 g of cereal.

Our results suggest that multivitamin intake or supplementation with folic acid and possibly vitamin $B_{12}$ may reduce homocysteine levels, especially for children with extremely high levels. Among our multivitamin users, the distribution was less skewed and only 23 children (3.3%) exceeded the 95th percentile for homocysteine (8.5 μmol/L). Shimakawa et al[23] also observed lower levels of homocysteine among supplement users; plasma homocysteine was 1.5 μmol/L lower among supplement users compared with nonusers. In addition, several short-term intervention trials with combinations of folic acid and vitamins $B_{12}$ or $B_6$ have demonstrated significant reductions in plasma homocysteine levels, particularly after treatment with folic acid alone.[7,27,28] In view of these

products be fortified with 140 μg of folic acid per 100 g of cereal or grain product.[45] Although this originally targeted women of childbearing age to prevent neural tube defects, it will affect all consumers of enriched grain products.[46,47] The effect of folic acid fortification of the food supply on children's homocysteine levels warrants investigation.

In the present study, we found no association between serum vitamin $B_6$ and homocysteine levels independently of folic acid and vitamin $B_{12}$. This finding is consistent with our knowledge about the metabolism of homocysteine. Nonfasting homocysteine levels, as measured in our study, are regulated mainly by the remethylation pathway,[6] which depends on adequate amounts of folic acid and vitamin $B_{12}$. Conversely, the transsulfuration pathway, which requires vitamin $B_6$, is thought to be more important in regulating postprandial increases in homocysteine levels.[7]

Unlike Tonstad et al,[29] we did not find a relationship between homocysteine and family history of CVD. Underlying genetic defects in the enzymes regulating homocysteine metabolism cause elevated levels of homocysteine[48-50] that may be inheritable and account for the extreme values observed in our data. Tonstad et al[29] found that children whose father, grandfather, or uncle died of myocardial infarction or sudden cardiac death before age 55 years had higher levels of homocysteine (mean homocysteine level, 5.92 μmol/L) compared with children without a family history (mean homocysteine level, 5.25 μmol/L). A lack of association in our data may be due to our less specific measure of family history for which we included history of stroke or myocardial infarction in all relatives.

In our data, most of the association of smoking with homocysteine was attributable to lower serum vitamin levels. Smokers tend to have lower intake of fruits and vegetables and antioxidant vitamins.[51,52] We found that children who smoked had significantly lower levels of all serum vitamins. Nygard et al[25] found in 7591 men and 8586 women, aged 40 to 67 years without a history of CVD, that current smokers had significantly higher plasma homocysteine levels, and the relationship increased almost linearly with daily number of cigarettes smoked. We could not adequately assess a dose-response relationship due to the small number of children who smoked and the lack of sufficient variation in the amount smoked per day.

Most studies have found no association between homocysteine levels and clinical cardiovascular risk factors, including serum lipids, BP, and BMI.[21,25,53,54] In multivariate analyses, we observed a weak positive relationship with systolic BP and no relationship with serum lipids or BMI. A mechanism for increasing homocysteine levels with BP has not been described; however, BP has been shown to be associated with diet. Specifically, a diet rich in fruits and vegetables and low-fat dairy products substantially lowered systolic and diastolic BP in both hypertensive and normotensive adults.[55] The association of homocysteine levels with BP in our data may be due to confounding by diet.

A limitation of our study is the cross-sectional design. The simultaneous assessment of various exposures and homocysteine levels limits our ability to determine a true cause-effect relationship. Serum levels of folic acid are also not as reliable an index of tissue body stores as red blood cell folate[37,55-57]; therefore, measurement error in serum folic acid as a surrogate for tissue body stores may have attenuated our estimate. Furthermore, unmeasured factors, such as diet, which simultaneously determine levels of vitamins and homocysteine, could explain the observed associations. Finally, the CATCH cohort is not a random sample of US children. Accordingly, the distribution of homocysteine levels reported in our sample may not be generalizable or reflect norms; however, the distribution of other risk factors, such as cholesterol and BMI, are comparable with results from other large

Future research should determine the relationship between dietary intake of folic acid and homocysteine levels and the longitudinal tracking of children's homocysteine levels into adulthood. These data are important to the planning and evaluation of future prevention initiatives that target dietary interventions in youth.

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