Influence of methylenetetrahydrofolate reductase genotype, age, vitamin B-12, and folate status on plasma homocysteine in children[1–3]

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ABSTRACT

Background: Several studies have examined the association of the methylenetetrahydrofolate reductase (*MTHFR*) genotype with plasma homocysteine in adults, but few studies have been performed in children.

Objective: We measured the concentrations of plasma total homocysteine, folate, and vitamin B-12 in a group of healthy fasting children and related these to *MTHFR* genotype.

Design: After the subjects fasted, blood samples were collected into EDTA-containing tubes. Plasma, red blood cells, and the buffy coat were immediately stored at −80°C for biochemical and molecular analyses. Plasma total homocysteine was determined by HPLC. Folate and vitamin B-12 were measured by a double-labeled radioimmunoassay, and the genotypic analysis was performed by polymerase chain reaction amplification of genomic DNA extracted from blood leukocytes.

Results: Plasma homocysteine concentrations correlated negatively with folate and vitamin B-12, but positively with age ($P < 0.0001$). Whereas folate and vitamin B-12 accounted for 27% and 19% of the variation in homocysteine, respectively, age accounted for 48% of the variation. When the cohort was divided into older (>10 y) and younger (≤10 y) individuals, folate was significantly lower in the older individuals who were homozygous for the mutation (*T/T*) than in those who were homozygous for the wild-type allele (*C/C*). Homocysteine was higher in the *T/T* group than in both the *C/C* and *C/T* subgroups aged >10 y.

Conclusion: Our data show that in a healthy pediatric population, *MTHFR* genotype played a significant role in determining homocysteine concentrations in older (>10 y), nutritionally stressed children. *Am J Clin Nutr* 2000;72:1469–73.

KEY WORDS Homocysteine, methylenetetrahydrofolate reductase, *MTHFR* genotype, folate, vitamin B-12, children

INTRODUCTION

Homocysteine, a sulfur amino acid, is metabolized to cysteine by transsulfuration or to methionine by remethylation. Impairment of these pathways, either of genetic or nutritional origin, can lead to hyperhomocysteinemia. Epidemiologic studies showing that a marginal increase in plasma homocysteine is associated with a higher risk of coronary artery or cerebrovascular disease have revived interest in the metabolism of this amino acid (1–4). Selhub et al (5) showed clearly, in a cross-sectional study involving 1041 elderly subjects, a graded increase in the prevalence of carotid-artery stenosis with increasing plasma homocysteine concentrations. In the same study, the authors showed that inadequate intake of folic acid was the main determinant of this condition. Furthermore, a study by Tonstad et al (6) showed that a modest elevation in plasma homocysteine in children correlated with premature cardiovascular death in their male relatives and, to some extent, could account for the contribution of family history to the risk of cardiovascular disease.

The genetic background of individuals is another factor to consider when evaluating dietary folic acid requirements. In this respect, the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is of prime interest because it converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate and major methyl donor for homocysteine remethylation to methionine. Frosst et al (7) identified a missense mutation that converted an alanine codon to a valine codon [a C-to-T substitution at nucleotide 677 (677C→T)] and showed that this mutation encoded the thermolabile form of MTHFR that had previously been suggested to be a risk factor for coronary artery disease (CAD) (8). In French Canadians, this mutation has a heterozygous and homozygous prevalence of 51% and 12%, respectively. Similar frequencies are observed in other North Americans of other ethnic backgrounds (9–11). More importantly, individuals who are homozygous mutant have significantly higher plasma homocysteine concentrations. Although several studies reported that the thermolabile variant of the enzyme is a risk factor for CAD (8, 12, 13), this subject remains controversial. A meta-analysis by Kluijtmans et al (14), involving 735 CAD patients, concluded that the *MTHFR* genotype

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TABLE 1
Plasma folate, vitamin B-12, and total homocysteine in subjects of both sexes[1]

	Folate	Vitamin B-12	Homocysteine
	nmol/L	*pmol/L*	*μmol/L*
Median	14.4	308	5.8
Range	9.4–39.7	112–870	2.6–24.3
5th–95th Percentile	10.2–27.9	162–570	3.1–14.0

[1] Because the distribution was not Gaussian and the data were skewed, the values are expressed as medians, ranges, and percentiles. To convert folate to μg/L, multiply by 0.44; to convert vitamin B-12 to ng/L, multiply by 1.35; and to convert homocysteine to μg/L, multiply by 20.1.

was a modest but significant risk factor for CAD. On the other hand, Brattström et al (15) concluded, from their meta-analysis of 23 case-control studies involving 5869 CAD patients and 6644 control subjects, that although the thermolabile genotype was a major cause of mild hyperhomocysteinemia, it did not increase cardiovascular risk. The discrepancies between these studies were discussed previously (16).

Several studies confirmed the effect of the *MTHFR* genotype on plasma homocysteine in adults (17, 18), but few studies examined this association in children. In this study, we measured the concentrations of plasma total homocysteine, folate, and vitamin B-12 in a group of apparently healthy children and related these to *MTHFR* genotype.

SUBJECTS AND METHODS

Subjects

The protocol was approved by the Ste-Justine Hospital Review Board on Investigation of Human Subjects. All children were recruited from the Ste-Justine Hospital Day Surgery Center at the time of minor elective surgery and were aged 24 mo to 18.75 y. The criteria for inclusion were as follows: French Canadian ancestry, no personal or parental history of metabolic disorders, no intake of drugs that affect lipid or protein metabolism (oral contraceptives or anticonvulsants), and no acute illness ≤3 before entry into the study.

Methods

After all 127 subjects (58 boys and 69 girls) fasted overnight, blood samples were collected into EDTA-containing tubes (Vacutainer; Becton Dickinson and Co, Orangeburg, NJ). Plasma was isolated by centrifugation (3000 × *g* at 4 °C for 20 min) and multiple aliquots were immediately stored at –80 °C in conical Eppendorf tubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) until analyzed. Red blood cells and the buffy coat were also kept frozen at −80 °C for further molecular analyses. Plasma total homocysteine concentrations were determined by HPLC and electrochemical detection (19). Genotypic analysis was performed by using polymerase chain reaction amplification (20) of genomic DNA extracted from blood leukocytes. The primers and polymerase chain reaction conditions were described previously (8). Polymerase chain reaction products were digested with *Hinf* I restriction enzyme to identify the 677C→T substitution of the *MTHFR* gene (7). Plasma total folate and vitamin B-12 were measured by a double-labeled radioimmunoassay (Ciba-Corning, Toronto, Canada) of which interassay variations were respectively 6.8% at 5.6 nmol/L and 4.0% at 553 pmol/L.

TABLE 2
Correlation between plasma total homocysteine, folate, vitamin B-12, and age[1]

	r	*P*
Homocysteine and folate	−0.515	<0.0001
Homocysteine and vitamin B-12	−0.440	<0.0001
Homocysteine and age	0.688	<0.0001

[1] The Spearman correlation test was used to establish the relations between plasma homocysteine (dependent variable) and folate, vitamin B-12, and age (independent variables). $n = 127$.

TABLE 3
Distribution of *MTHFR* genotype by sex[1]

Genotype	Boys	Girls	Total
		n (%)	
C/C	26 (50.0)	26 (50.0)	52 (100.0)
C/T	20 (37.7)	33 (62.3)	53 (100.0)
T/T	12 (54.5)	10 (45.5)	22 (100.0)

[1] There were no significant differences by chi-square test. *C/C*, homozygous for the wild-type allele; *C/T*, heterozygous; *T/T*, homozygous for the mutant allele; MTHFR, methylenetetrahydrofolate reductase.

Statistical analyses

Wilcoxon's rank-sum test was used to evaluate the effect of sex on plasma folate, vitamin B-12, and homocysteine concentrations. The Kruskal-Wallis test was used to study the effect of the *MTHFR* genotype on the 3 variables. The chi-square test was used to assess whether the proportion of girls and boys differed in the 3 genotype subgroups. Spearman's correlation test was used to correlate homocysteine concentrations with folate, vitamin B-12, and age. Interaction between age (≤10 or >10 y) and enzyme genotype on plasma homocysteine and folate was assessed by two-factor analysis of variance after a neperian log transformation of the data. Multiple comparisons were performed with Tukey's test for each age group when there was a significant interaction between age and *MTHFR* genotype (21). P values < 0.05 indicated statistical significance.

TABLE 4
Distribution of plasma total folate, vitamin B-12, and homocysteine concentrations by *MTHFR* genotype[1]

Genotype	Folate	Vitamin B-12	Homocysteine
	nmol/L	*pmol/L*	*μmol/L*
C/C ($n = 52$)			
Median	14.8	304	6.44
5th–95th Percentile	11.2–26.5	188–620	3.07–13.96
C/T ($n = 53$)			
Median	14.1	305	5.37
5th–95th Percentile	9.8–28.3	137–534	3.09–11.02
T/T ($n = 22$)			
Median	13.6	328	5.46
5th–95th Percentile	10.2–28.2	190–643	3.22–17.41

[1] There were no significant differences between genotype groups by the Kruskal-Wallis test. MTHFR, methylenetetrahydrofolate reductase.

TABLE 5
Effect of the interaction between age and *MTHFR* genotype on plasma folate and homocysteine concentrations[1]

Genotype	Folate		Homocysteine	
	≤10 y	>10 y	≤10 y	>10 y
	nmol/L		*μmol/L*	
C/C				
Median	17.1	14.7	4.7	8.1
Range	11.2–30.2	9.7–26.5	2.7–8.4	3.9–14.9
n	21	31	21	31
C/T				
Median	18.1	12.9	4.6	7.2
Range	10.3–35.9	9.4–17.2	2.6–7.4	4.1–21.1
n	28	25	28	25
T/T				
Median	17.4	10.9	4.9	10.6
Range	11.4–39.7	9.4–12.9	2.8–8.9	4.7–24.3
n	14	8	14	8

[1] Ten years of age represents the 50th percentile of the group studied. On the basis of ANOVA, after log transformation of the data, and multiple comparisons by Tukey's test, there was a significant interaction between age (≤10 or >10 y) and *MTHFR* genotype ($P < 0.03$) and *MTHFR* genotype had a significant effect on folate concentrations ($P < 0.05$). The effect of the interaction between age and *MTHFR* genotype on homocysteine concentrations was not significant. MTHFR, methylenetetrahydrofolate reductase.

RESULTS

Descriptive statistics showed that folate, vitamin B-12, and homocysteine concentrations for the 127 subjects were distributed asymmetrically with skewness factors of 1.783, 1.242, and 2.089, respectively. Hence, medians, ranges, and percentiles were used to report values for the 3 analytes (**Table 1**). There was no significant difference in plasma folate, vitamin B-12, or homocysteine concentrations between boys and girls. As shown in **Table 2**, the dependent variable plasma homocysteine concentration correlated negatively with folate and vitamin B-12 but positively with age. Whereas folate and vitamin B-12 accounted for 27% and 19% (R^2) of the variation, respectively, age accounted for 48% of the variation. As can be derived from the data in **Table 3**, 40.9% of the subjects were homozygous for the *MTHFR* wild-type allele (*C/C*), 41.7% were heterozygous (*C/T*), and 17.3% were homozygous for the mutant allele (*T/T*). The genotype frequency analysis showed that the distribution of boys and girls among the 3 groups was not significantly different and there was no bias generated by sex. When folate, vitamin B-12, and homocysteine were analyzed as a function of *MTHFR* genotype, with the 2 sexes combined, there were no significant differences between groups (**Table 4**).

To introduce age as a dependent variable, the group was divided into subjects aged ≤ and >10 y (50th percentile). As shown in **Table 5**, there was an interaction between age and *MTHFR* genotype and the *MTHFR* genotype had a significant effect on folate concentrations. However, no such interaction between age and *MTHFR* genotype was observed for homocysteine. The age, *MTHFR* genotype, and folate and vitamin B-12 distributions of 6 subjects of both sexes with homocysteine concentrations above the 95th percentile are shown in **Table 6**. Four individuals had plasma folate concentrations below the 5th percentile and 2 had concentrations below the 25th percentile. Three individuals were homozygous for the mutant allele and one was heterozygous. Although the number of these hyperhomocysteinemic subjects was small, there was a higher frequency of the *MTHFR* mutant allele in this group, suggesting that it was the cause of the elevated homocysteine concentrations.

DISCUSSION

As for many biological variables in humans, homocysteine concentrations reflect the balance between environmental and polygenic factors. It was shown previously that elevated plasma total homocysteine concentrations are an independent risk factor for premature cardiovascular disease (2–5, 22). Most of the epidemiologic studies showing the influence of age, sex, and vitamin B-12 and folate statuses were conducted in adult and elderly populations (1, 4, 23, 24). In the present study we obtained data in healthy children. All subjects had fasted overnight before blood samples were taken because the blood concentrations of these variables are influenced by food intake (25). Because the blood samples were obtained at the time of elective surgery, nutrition questionnaires were not administered. However, the median folate and vitamin B-12 concentrations were similar to those reported by Wright et al (26) for an American population. This observation supports the hypothesis that the intake of these nutrients was adequate in the study group. The frequency of homozygotes for the thermolabile variant was higher than reported in our earlier studies (7) and those of others (27–29). This difference was not definitively explained but may have stemmed from the ethnic background of our subjects, who were exclusively French Canadian and came from regions of Quebec with known founder effects. In an earlier study, Tonstad et al (6) found total homocysteine concentrations (10th–90th percentiles) of 3.5–6.5 μmol/L and serum folate concentrations of 1.1–50.7 nmol/L in a control group of children. The upper limit of folate

TABLE 6
Biological data for subjects with plasma homocysteine concentrations above the 95th percentile

Homocysteine (μmol/L)	Age	*MTHFR* genotype	Folate	Vitamin B-12
	y		*nmol/L*	*pmol/L*
14.2 (>95th)	16	*C/C*	9.4 (<5th)	298 (<50th)
14.9 (>95th)	18	*C/C*	11.4 (<25th)	512 (<90th)
16.1 (>95th)	15	*T/T*	8.7 (<1st)	230 (<25th)
17.4 (>95th)	14	*T/T*	9.9 (<5th)	305 (<50th)
21.1 (>99th)	18	*C/T*	12.1 (<25th)	112 (<1st)
24.4 (>99th)	16	*T/T*	9.7 (<5th)	190 (<25th)

[1] MTHFR, methylenetetrahydrofolate reductase. 5th–95th percentiles: homocysteine, 3.1–14.0 μmol/L; folate, 10.2–27.9 nmol/L; vitamin B-12, 162–570 pmol/L.

was much higher than that observed in our group. The children studied by Tonstad et al (6) were between 8 and 12 y of age, whereas the age of our population extended to 18 y. Because there is a direct relation between age and homocysteine, it stands to reason that our range of values would be greater. When we considered only the individuals aged ≤12 y in our study, the 10th–90th percentiles for homocysteine were 3.2–7.8 μmol/L, values more similar to those reported by Tonstad et al. Another study, conducted by Schneede et al (30) in a small group of infants, reported homocysteine concentrations of 5.3–11.0 μmol/L. However, folate concentrations were not available.

If, as defined by Brouwer et al (31), a plasma folate concentration of 10 nmol/L is accepted as the threshold for subclinical deficiency, only 6 individuals in the present study, independently of *MTHFR* genotype (3 girls and 3 boys), would have qualified as being deficient. The homocysteine concentrations of these individuals ranged from 6.6 to 16.1 μmol/L; 3 concentrations were above the 90th percentile and 2 were above the 95th percentile.

Our cohort was aged 3–18 y and had a relatively uniform distribution. Age showed a strong association with plasma homocysteine. Similar results were reported recently by Balasa et al (32), who showed by multiple regression analysis that homocysteine was positively associated with age. In their study, *MTHFR* genotype accounted for only 2.9% of the variance in homocysteine concentrations for the entire group, but these authors did not fractionate their data according to age and did not report values for folate and vitamin B-12. In our population, we observed an interaction between age and *MTHFR* genotype as well as a significant effect of genotype on folate concentrations. However, a similar interaction for homocysteine was not significant. As shown previously for adults, folate and probably vitamin B-12 are strong determinants of homocysteine concentrations (9). The fact that we did not observe an interaction between age and *MTHFR* genotype for homocysteine concentrations was explained by the small size of the homozygous mutant subgroup and the wide variation in homocysteine concentrations in the older age group. However, the importance of nutrition in genetically stressed individuals should not be discounted. Nonetheless, considering the numerous studies that showed an association of homocysteine with cardiovascular disease in adults, additional investigations in children are clearly warranted to identify the factors that influence homocysteine concentrations and thus allow possible early intervention.

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