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Polymorphisms in the Methylenetetrahydrofolate Reductase and Methionine Synthase Reductase Genes and Homocysteine Levels in Brazilian Children

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Hyperhomocysteinemia is a risk factor for thrombosis, and methylenetetrahydrofolate reductase (*MTHFR*) and methionine synthase reductase (*MTRR*) polymorphisms, folate, and B_{12} levels could contribute to plasma homocysteine (Hcy) variation. Although well established in adults, few studies have been performed in childhood. In this study, we investigated association of polymorphisms C677T and A1298C in the *MTHFR* gene and A66G in the *MTRR* gene with Hcy levels in children. These polymorphisms, as well as Hcy, folate, and vitamin B_{12} levels were investigated in 220 normal children with ages ranging from 1 to 8 years. Plasma Hcy, folate, and vitamin B_{12} levels were normal in all children. None of the polymorphisms could be considered an independent risk factor for hyperhomocysteinemia during childhood. The median Hcy levels in 37 children (17%) doubly heterozygous for C677T and A1298C mutations in the *MTHFR* gene were not different from the other genotypes. However, the association of the different genotypes with Hcy, folate, and vitamin B_{12} levels demonstrated significant *P*-values. The folate levels demonstrated a statistically significant decrease ($P = 0.0477$) from the C677T mutation in the *MTHFR* gene (TT genotype) when compared to the other groups. Folate was the only independent risk factor for hyperhomocysteinemia. Thus, monitoring the concentrations of folate would be more helpful for evaluating hyperhomocysteinemia and for preventing cardiovascular disease.

KEY WORDS: homocysteine; methylenetetrahydrofolate reductase; methionine synthase reductase; children

INTRODUCTION

The metabolism of homocysteine (Hcy) and folate and their relation to birth defects, thrombembolic phenomena, and other health problems have been the subject of many publications in the last two decades. Recent studies have established that an elevated level of plasma total Hcy is a risk factor for premature cardiovascular disease and venous thrombosis [Boushey et al., 1995; Boers, 1997; D'Angelo and Selhub, 1997; Bautista et al., 2002].

Increase in the level of Hcy can result from genetic- or nutrient-related disorders in the two pathways of Hcy metabolism, namely remethylation and transsulfuration [Selhub and Miller, 1992]. Some studies have shown that Hcy concentrations are inversely correlated with the level of vitamin B_{12} and folate [Verhoef et al., 1996; Folsom et al., 1998].

Nuss et al. [1995] described a missense mutation (alanine to valine) in base pair (bp) 677 of the gene for methylenetetrahydrofolate reductase (*MTHFR*), the enzyme that provides the folate derivative for the conversion of Hcy to methionine, that could contribute to an elevation in plasma Hcy. This mutation results in mild hyperhomocysteinemia, primarily when folate levels are low. Additional genetic variants in the *MTHFR* and methionine synthase reductase (*MTRR*) genes have been identified. These variants include a glutamate to alanine mutation (bp 1298) in *MTHFR*, and an isoleucine to methionine mutation (bp 66) in *MTRR*.

Lifestyle factors seem to have a greater influence upon Hcy concentrations than do common *MTHFR* polymorphisms [Infante-Rivard et al., 2002]. The study of genetic factors in infancy, a phase in which few acquired factors are present, may enable the role of congenital factors in the development of thrombosis to be understood. A previous study in a French-Canadian population, stratified 127 children into two groups ($<$10 year and $>$10 year) and measured folate, vitamin B_{12}, homocysteine levels, and the mutation C677T in the *MTHFR* gene [Delvin et al., 2000].

In this study, in addition to C677T in the *MTHFR* gene, we investigated whether the A1298C polymorphism in the *MTHFR* gene and the A66G polymorphism in the *MTRR* gene were associated with alterations in the concentrations of Hcy, folate, and B_{12} in a large group of healthy Brazilian children under 8 year.

MATERIALS AND METHODS

The study was approved by the institutional Ethics Committee at UNICAMP, and informed consent was obtained from all mothers. The study group consisted of normal children attended at the Pediatric Preventive Clinic at the university hospital, UNICAMP. The children were invited to participate in the study during a routine visit to the out patient clinic. The

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exclusion criteria were the presence of any disease, the use of medication, and vitamin supplementation.

After all 220 children fasted overnight, blood samples were collected into EDTA-containing tubes (Vacutainer; Becton Dickinson and Co., Orangeburg, NJ). This indication was taken because the blood concentrations of B_{12}, folate, and homocysteine are influenced by food intake [Tucker et al., 1996].

Genomic DNA was extracted from blood leukocytes by the phenol/chloroform method as described [Blin and Stafford, 1976]. The mutations, C677T and A1298C, in the *MTHFR* gene and the mutation, A66G, in the *MTRR* gene were determined by the polymerase chain reaction (PCR) [Saiki et al., 1988] using specific primers, as previously described [Arruda et al., 1997; Weisberg et al., 1998; Brown et al., 2000]. The amplified fragments of the C677T, A1298C, and A66G mutations were digested with the restriction enzymes HinfI, MboII, and NdeI, respectively.

The quantification of homocysteine in human plasma was performed by HPLC with fluorescence detection [Ducros et al., 2002]. A total of 63 plasma samples were analyzed concurrently by DIMP-T&R-MIMS (direct insertion membrane probe using trap and release membrane introduction mass spectrometry) after derivatization with ethyl chloroformate [Haddad et al., 2001; Vellasco et al., 2002], used as a reference, presenting relative deviations of lower than 8% and HPLC with fluorescence detection. Our laboratory data for reference value for Hcy is 4–12 μmol/L.

Folate and vitamin B_{12} levels were determined by the ECLIA analysis. Our laboratory reference values for these two substances were 3.2–9 ng/ml and 202–900 pg/ml, respectively.

Statistical Analysis

Descriptive analysis was used in frequency tables for categorical (absolute) variables, and positional and dispersion measures for continuous variables. The Mann–Whitney test was used to compare continuous variables between two groups and the Kruskal–Wallis test for three groups. Identification of the factors which affect the Hcy levels was done using linear regression analysis for the continuous Hcy variable, and multivariate logistic regression analysis for the classified Hcy variable. The variables included vitamin B_{12}, folate, and the C677T and A1298C polymorphisms in the *MTHFR* gene and A66G in the *MTRR* gene. A significance level of 5% was adopted. Hardy–Weinberg equilibrium was determined for all of the genotypes using the Chi square test.

RESULTS

The study group consisted of 220 children (130 F, 90 M) with an age range of 1–8 years (median age, 4.8 years). None of the children studied were using any kind of medication and had no clinical symptoms of disease.

The genotype polymorphisms are summarized in Table I. Except for the polymorphism A66G in the *MTRR* gene, the distribution of the C677T MTHFR and A1298C MTHFR alleles was in Hardy–Weinberg equilibrium.

Since the detection of individuals homozygous for A66G can be misinterpreted using enzyme digestion, an SSCP assay was done for each DNA sample analyzed. There was no disagreement among all of the samples tested. The prevalence of homozygosity for C677T, A1298C, and A66G was 9.5, 5.5, and 15.0%, respectively (Table I). No children homozygous for both C677T and A1298C mutations in the *MTHFR* gene were observed, only one case that was homozygous for C677T and heterozygous for A1298C in the *MTHFR* gene was identified. We observed 37 children (17%) doubly heterozygous for C677T and A1298C mutations in the *MTHFR* gene. There was no statistical difference in the prevalence of A1298C and C677T mutations of the *MTHFR* gene and A66G of the *MTRR* gene, according to age and infant gender.

TABLE I. Prevalence for the Polymorphisms C677T and A1298C in the *MTHFR* Gene and A66G in the *MTRR* Gene

Polymorphisms	Prevalence [n (%)]	Hardy–Weinberg equilibrium
C677T *MTHFR*	TT = 21 (9.5) CT = 97 (44) CC = 102 (46.5)	$P = 0.766$
A1298C *MTHFR*	CC = 12 (5.5) AC = 83 (37.5) AA = 125 (57)	$P = 0.711$
A66G *MTRR*	GG = 33 (15) AG = 158 (72) AA = 29 (13)	$P < 0.001$

The summary of genotypes identified in this study is shown in Table II. The most common genotypes determined in this population were CTAAAG (20.91%), CCACAG (15.45%), CCAAAG (13.18%), and CTACAG (11.82%).

Table III summarizes the Hcy, vitamin B_{12}, and folate levels according to genotypes of the polymorphisms. The mean Hcy, folate, and vitamin B_{12} levels were according to the reference values in all the groups analyzed. With the exception of the C677T mutation in the *MTHFR* gene, there was no statistical difference in these variables between all genotypes identified. Folate concentrations demonstrated a significant ($P = 0.047$) progressive increase from genotype TT to CC.

We then investigated whether the combination of the three genotypes of C677T polymorphism with A1298C or A66G was associated with Hcy levels, considering folate and vitamin B_{12} levels (Table IV). The results did not show any significant difference between the parameters analyzed.

Hcy, B_{12}, and folate levels were compared between the different genotypes. When the number of children with a determined genotype was low, two groups of genotypes with a difference in only one polymorphism were grouped. Hcy levels were significantly increased in the TTAAAG and TTAAAA genotypes compared to genotype CCACAG ($P = 0.019$). When folate and vitamin B_{12} levels were compared between these two groups, folate was seen to be significantly decreased in the group with greater Hcy levels. The Hcy levels were statistically increased in genotypes TTAAAG and TTAAAA ($P = 0.019$), or TTAAAG ($P = 0.03$) than in CCACGG genotype, but there was no significant difference in the folate or vitamin B_{12} levels between these genotypes. Folate levels were significantly decreased in subjects with genotype CCAAAA when compared with CCAAAG ($P = 0.03$), CCACAG ($P = 0.003$), and CTAAAG ($P = 0.02$). Vitamin B_{12} levels were significantly lower in CCAAAG when compared with CCACAG ($P = 0.04$) and CTACAG ($P = 0.04$), in CCACAA compared with CCACAG ($P = 0.01$), CCACGG ($P = 0.03$), CTACAG ($P = 0.01$), and TTAAAA with TTAAAG ($P = 0.025$); and in CCCCGG when compared with CCACGG ($P < 0.01$).

By classifying Hcy levels as variable in <10 or >10 (value of the 75th percentile) and using multivariate logistic regression to identify the factors which influence Hcy levels, we obtained the following results: the lower the levels of folate, the higher the risk of having a Hcy concentration >10 μmol/L. Thus, this was the only independent risk factor for hyperhomocysteinemia.

TABLE II. Homocysteine, Vitamin B_{12}, and Folate Levels for the Genotype of the *MTHFR* Polymorphisms, C677T and A1298C, and for the *MTRR* Polymorphism, A66G, Respectively

Polymorphism C677T/A1298C/A66G	n (%)	Homocysteine (μmol/L)	Vitamin B_{12} (pg/ml)	Folate (ng/ml)
CCAAAA	8 (3.64)	8.21 ± 4.30	863 ± 296	3.73 ± 1.84
CCAAAG	29 (13.18)	8.72 ± 5.72	778 ± 240	5.56 ± 2.37
CCAAGG	8 (3.64)	8.64 ± 2.17	873 ± 219	6.38 ± 3.22
CCACAA	4 (1.82)	9.07 ± 2.14	633 ± 156	5.83 ± 1.76
CCACAG	34 (15.45)	8.26 ± 2.56	930 ± 351	6.64 ± 3.36
CCACGG	7 (3.18)	6.11 ± 2.62	1123 ± 474	7.83 ± 5.40
CCCCAG	9 (4.09)	9.87 ± 4.68	803 ± 300	4.80 ± 2.36
CCCCGG	3 (1.36)	8.9 ± 6.82	650 ± 494	6.23 ± 1.47
CTAAAA	4 (1.82)	8.9 ± 3.34	779 ± 217	5.17 ± 2.24
CTAAAG	46 (20.91)	8.23 ± 2.95	831 ± 306	5.60 ± 2.60
CTAAGG	10 (4.55)	8.98 ± 2.07	889 ± 355	5.04 ± 2.14
CTACAA	7 (3.18)	7.35 ± 2.18	723 ± 243	5.45 ± 2.77
CTACAG	26 (11.82)	8.07 ± 1.87	939 ± 335	5.49 ± 3.36
CTACGG	4 (1.82)	9.75 ± 3.99	797 ± 304	5.09 ± 1.99
TTAAAA	6 (2.73)	10.2 ± 4.46	1031 ± 408	4.65 ± 3.04
TTAAAG	13 (5.91)	9.29 ± 3.18	901 ± 418	4.73 ± 2.78
TTAAGG	1 (0.45)			
TTACAG	1 (0.45)			

The values are the mean ± SD.

DISCUSSION

Since the identification of hyperhomocysteinemia as a risk factor for thrombosis, understanding the determinants of plasma Hcy levels has been important for the prevention and treatment of this condition. Various studies in adults have demonstrated an association between polymorphisms in the *MTHFR* and *MTRR* genes and plasma Hcy levels. Of special clinical interest is the observation that the association between genotype and mild hyperhomocysteinemia is seen primarily when folate levels are low. Lifestyle factors, such as smoking, sedentarism, arterial hypertension, drug intake, caffeine consumption, and others also have great influence upon Hcy levels.

Although this condition is well established in adults, few studies have been performed in children, and these have only analyzed the *MTHFR* polymorphism C677T. Mainou et al. [2002] and Dalmau et al. [2002] suggested that Hcy concentrations were significantly higher in children with the TT genotype, and multiple linear regression analysis revealed an inverse correlation with folate levels. Balasa et al. [1999] demonstrated no effect of C677T genotype on Hcy levels in younger children, although folate levels were not measured in this group. The study by Delvin et al. [2000] showed the influence of *MTHFR* genotype, age, vitamin B_{12}, and folate status on plasma Hcy in a healthy pediatric population (age 24 month to 18.75 year). Their data showed that *MTHFR* genotype played a significant role in determining Hcy concentrations in older (>10 year), nutritionally stressed children.

In this study we measured Hcy, folate, and B_{12} levels in a representative group of 220 children and related these to polymorphisms of both the *MTHFR* and *MTRR* genes, as well as folate and vitamin B_{12} levels.

Previously we reported that the prevalence of individuals homozygous for the C677T polymorphism in the *MTHFR* gene was 10% [Arruda et al., 1998]. The finding of 9.5% homozygous children in this study is in agreement with the expected level, suggesting that this is representative of the Brazilian population.

As expected, the high miscegenation of our population explains the 5.5% prevalence of individuals homozygous for the A1298C polymorphism in the *MTHFR* gene, a lower value than that observed among Ashkenazi Jewish individuals (8.1%) and Caucasians (8.8%), and a higher value than among African-Americans (2.1%) [Rady et al., 2002].

The same could be observed when we analyzed the prevalence of homozygosity for A66G polymorphism in the *MTRR* gene: 15% compared to 19.5% among Ashkenazi

TABLE III. Homocysteine, Vitamin B_{12}, and Folate Levels for the *MTHFR* Polymorphisms, C677T and A1298C, and for *MTRR* Polymorphism, A66G

A1298C *MTHFR*	AA (n = 125)	AC (n = 83)	CC (n = 12)	*P*
Homocysteine (μmol/L)	8.63 ± 3.82	8.11 ± 2.5	9.6 ± 4.98	0.82
Folate (ng/ml)	5.31 ± 2.53	6.14 ± 3.4	5.15 ± 2.20	
B_{12} (pg/ml)	843 ± 302	911 ± 347	765 ± 339	
A66G *MTRR*	AA (n = 29)	AG (n = 158)	GG (n = 33)	
Homocysteine (μmol/L)	8.6 ± 3.43	8.51 ± 3.53	8.27 ± 3.16	0.9896
Folate (ng/ml)	4.83 ± 2.37	5.68 ± 2.89	6.08 ± 3.25	
B_{12} (pg/ml)	821 ± 297	865 ± 320	899 ± 365	
C677T *MTHFR*	CC (n = 102)	CT (n = 97)	TT (n = 21)	
Homocysteine (μmol/L)	8.45 ± 4.07	8.29 ± 2.61	9.61 ± 3.43	0.3164
Folate (ng/ml)	5.96 ± 3.08	5.47 ± 2.71	4.67 ± 2.64	0.047
B_{12} (pg/ml)	859 ± 322	855 ± 311	938 ± 387	

The values are the mean ± SD.

TABLE IV. Homocysteine, Vitamin B_{12}, and Folate Levels for the *MTHFR* Polymorphisms, C677T and A1298C, and for the *MTRR* Polymorphism, A66G

C677T *MTHFR*			%	Homocysteine (μmol/L)	Folate (ng/ml)	Vitamin B_{12} (pg/ml)
TT (n = 21)	A1298C *MTHFR*	AC (n = 1)	0.45			
		AA (n = 20)	9	9.42 ± 3.44	4.68 ± 2.71	935 ± 398
	A66G *MTRR*	AA (n = 6)	2.72	10.2 ± 4.46	4.65 ± 3.04	1031 ± 408
		AG (n = 14)	6.36	9.57 ± 3.19	4.71 ± 2.67	908 ± 401
		GG (n = 1)	0.45			
CT (n = 97)	A1298C *MTHFR*	AC (n = 37)	16.81	8.14 ± 2.25	5.44 ± 3.08	883 ± 322
		AA (n = 60)	27.27	8.38 ± 2.82	5.48 ± 2.48	837 ± 306
	A66G *MTRR*	AA (n = 11)	5	7.86 ± 2.52	5.35 ± 2.47	743 ± 224
		AG (n = 72)	32.72	8.17 ± 2.60	5.56 ± 2.87	870 ± 318
		GG (n = 14)	6.36	9.24 ± 2.68	5.05 ± 2.02	863 ± 333
CC (n = 102)	A1298C *MTHFR*	AC (n = 45)	20.45	7.98 ± 2.63	6.75 ± 3.59	934 ± 372
		AA (n = 45)	20.45	8.6 ± 4.95	5.36 ± 2.52	809 ± 243
		CC (n = 12)	5.45	9.6 ± 4.98	5.15 ± 2.20	765 ± 339
	A66G *MTRR*	AA (n = 12)	5.45	8.5 ± 3.63	4.43 ± 2.02	786 ± 268
		AG (n = 72)	32.72	8.64 ± 4.32	5.95 ± 2.94	864 ± 309
		GG (n = 18)	8.18	7.7 ± 3.43	7.04 ± 3.88	889 ± 411

The values are the mean ± SD.

Jewish, 29.6% among Caucasians, 7.3% among Hispanics, and 10.3% among African-Americans [Rady et al., 2002].

None of the children demonstrated hyperhomocysteinemia, and all of the parameters analyzed were within the normal ranges.

The C677T polymorphism in *MTHFR* seems not to be a risk factor for increased plasma Hcy levels during childhood, since when we analyzed the C677T mutation alone there was no correlation with Hcy level and the concentration of Hcy was in the normal range in all infants with the TT genotype. These results could be explained by the fact that the concentration of folate was within the reference values in all these children, although the group with the TT genotype presented lower folate levels when compared to the other groups ($P = 0.047$).

The second missense mutation in the *MTHFR* gene, A1298C, rarely occurs in combination with the C677T polymorphism, suggesting that the two mutations occur independently. The 1298 mutation did not alter plasma Hcy levels, although adults heterozygous for both mutations may be at risk of mild hyperhomocysteinemia [van der Put et al., 1998], particularly when associated with low folate levels. In this study, we identified one child (0.48%) homozygous for the C677T polymorphism and heterozygous for the A1298C polymorphism, and 37 individuals (17%) heterozygous for both mutations. In contrast to adults, the doubly heterozygous children did not demonstrate increased Hcy levels when compared with the other children, and folate and vitamin B_{12} levels were not decreased in this group. These results suggest that genetic factors *per se* are not associated with plasma Hcy levels.

The A66G mutation in the *MTRR* gene, which is related to an increased risk of spine bifida when homozygous, has not been associated with hyperhomocysteinemia. However, recent reports in adults revealed that homozygosity for the G allele could contribute to a moderate increase in Hcy levels, independent of the serum levels of folate, vitamin B_6 and B_{12} [Gaughan et al., 2001]. Wilson et al. [1999] described that individuals doubly homozygous for A66G in the *MTRR* gene and C677T in the *MTHFR* gene had an increased risk of neural tube defects, especially when the blood vitamin B_{12} level was low. In our study, the only child homozygous for both the C677T mutation in the *MTHFR* gene and the A66G mutation in the *MTRR* gene did not demonstrate hyperhomocysteinemia. Fifteen percent of the children were homozygous for the A66G mutation. For these, Hcy, folate, and B_{12} levels were within the normal range, with no statistical difference in Hcy concentration among the three genotypes ($P = 0.98$). Therefore, the isolated analysis of A66G polymorphism did not show any genetic effect on plasma Hcy levels.

In multivariate analysis, none of the polymorphisms proved to be an independent risk factor for hyperhomocysteinemia. Therefore, we performed an analysis to identify additive or synergistic effects of these polymorphisms on plasma Hcy levels. The TTAAAG and TTAAAA genotypes demonstrated a significant increase in plasma Hcy levels when compared with CCACAG genotype, independent of vitamin B_{12} and folate levels. On the other hand, some genotypes demonstrated a significant decrease in vitamin B_{12} or folate levels, with no alteration in the Hcy level. These results indicated that although no single polymorphism could be identified as an independent risk factor for hyperhomocysteinemia, in association they could have an effect.

In the future, a screening test of several genetic markers could provide a better evaluation of the risk of venous and arterial disease, and data of the type obtained here could be included in such as screening test.

The contribution of our data to the understanding of the genetic factors related to hyperhomocysteinemia is of great importance, since we analyzed a representative population of infants under 8 years. This study evaluated the effect of three polymorphisms on Hcy, folate, and vitamin B_{12} levels in the same population.

When this study was planned, the aim was to evaluate the importance of genetic factors *per se* in hyperhomocysteinemia. However, contrary to expectations, our results demonstrated that acquired factors were more important, and that monitoring the serum concentrations of folate would be more helpful for evaluating hyperhomocysteinemia and for preventing cardiovascular or venous disease.

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