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Role of folic acid depletion on homocysteine serum level in children and adolescents with epilepsy and different MTHFR C677T genotypes

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ABSTRACT

Homocysteine (Hcy) is a sulfur-containing amino acid involved in methionine metabolism. An elevated total plasma Hcy concentration (tHcy) is a risk factor for vascular disease. The present study aimed to assess the role of antiepileptic drugs (AEDs) and C677T methylenetetrahydrofolate (MTHFR) polymorphisms on tHcy in pediatric patients with epilepsy treated for at least 6 months with various treatment regimens protocols including the newer AEDs.

The study group was recruited from children and adolescents with epilepsy followed up in the Child Neuropsychiatry Clinic of the Second University of Naples, between January 2007 and March 2008. Inclusion criteria were: (1) patients with epilepsy, treated with one or more anticonvulsant drugs for at least 6 months; (2) age between 2 and 16 years. Plasma tHcy concentrations were considered elevated when they exceeded 10.4 μmol/L, and folate concentrations <3 ng/mL were considered deficient. Serum vitamin B12 levels were considered normal between 230 and 1200 pg/mL. The study group was composed of 78 patients (35 males, 43 females), aged between 3 and 15 years (mean 8.9 years). Thirty-five patients were taking AED monotherapy, 43 polytherapy. Sixty-three healthy sex- and age-matched children and adolescents served as controls. The mean tHcy value in the patient group was higher than the mean value in the control group (12.11 ± 7.68 μmol/L vs 7.4 ± 4.01 μmol/L; $p < 0.01$).

DNA analysis for the MTHFR C677T polymorphism showed the CT genotype in 46%, CC in 35% and TT in 17.8% of cases. Decreased folic acid serum levels significantly correlated with increased tHcy levels ($p < 0.003$). Female sex was a less significant risk factor for increased tHcy levels ($p = 0.039$).

Our study confirms the association between hyperhomocysteinemia and epilepsy. The elevation of tHcy is essentially related to low folate levels. Correction of poor folate status, through supplementation, remains the most effective approach to normalize tHcy levels in patients on AED mono- or polytherapy.

1. Introduction

In the last decade, a relationship between increased total homocysteine plasma (tHcy) levels and antiepileptic treatments, has been recognized.[1–9]

The folate level may be one of the determinants of serum tHcy, in particular in older children treated with more than one antiepileptic drugs (AEDs).[1,4,6,10]

The duration of therapy has also been recognized as a potential risk factor.[6] The are conflicting[11] results about the role of methylenetetrahydrofolate reductase (MTHFR) polymorphisms (particularly the C677T and A1298C) as determinants of high tHcy levels in this patient group. MTHFR is a key enzyme in the production of 5-methyltetrahydrofolate, which is required as the methyl donor for Hcy remethylation to methionine. The C677T mutation of the MTHFR gene decreases the activity of this enzyme. High homocysteine blood levels for age have been found to have potential NMDA-mediated proconvulsant effects and are acknowledged as a vascular risk factor linked to toxic effects on the arterial endothelium.[12] Previous studies have demonstrated the effect of anticonvulsant monotherapy on tHyc levels in adult patients.[6–12]

It was the aim of the present study to assess the effects of monotherapy versus polytherapy and the role of MTHFR

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polymorphism on tHcy levels in a pediatric population affected by epilepsy treated with older and new generation AEDs.

2. Methods

The patients reported in this study were identified prospectively at the Department of Child Neuropsychiatry of the Second University of Naples and were enrolled between January 2007 and March 2008.

Inclusion criteria were: (1) patients with epilepsy, treated with one or more than one AEDs for at least 6 months; (2) age from 2 years old. Exclusion criteria included: neurometabolic or systemic diseases, chronic therapy with other than antiepileptic drugs and current or previous treatment with folic acid and/or other vitamins.

Sixty-three healthy controls who had no clinical and/or laboratory evidence of metabolic and/or endocrine diseases were studied. Controls were recruited from outpatients of the Department of Child Neuropsychiatry of the Second University of Naples: these subjects had been referred for non-epileptic problems e.g. episodic headache, minor head trauma, and dizziness.

2.1. Vitamin preparations were not prescribed to any subjects

Informed consent was obtained by parents and controls and, when possible, by patients. The study was approved by the Ethical Committee of the Faculty of Medicine, Second University of Naples, Italy.

All patients and controls underwent the following examinations: haematochemistry and liver/kidney function evaluation, blood and urine amino acids and urine organic acids assessment, dosage of serum levels of folic acid, vitamin B12, tHcy and anticonvulsant drugs. DNA was extracted from peripheral blood by using a previously published method[14] of genotypization (MTHFR) and was available from all subjects who agreed to participate.

Venous blood samples were collected in the morning, after an overnight fast. Blood samples were immediately processed in order to prevent artefactual variations of tHcy, due to the products of in vitro erythrocyte metabolism.[13] Plasma and serum aliquots were quickly separated and frozen at −80 °C for batch analysis. tHcy levels were determined via fluorescence polarization immunoassay (AXYM ABBOT Laboratories).[14]

Serum vitamin B12, and serum folate concentrations were determined using commercial kits (ACS Ciba-Corning, and Immulite, DPC).

In each patient, isolation of DNA was performed by using Wizard Genomic DNA Purification kit according to the manufacturer. The MTHFR C677T mutation was detected via polymerase chain reaction (PCR) amplification of genomic DNA, followed by restriction fragment length polymorphism (RFLP) analysis.[15] Genotypization was accomplished examining the occurrence of a HinfI recognition site. Briefly, PCR 198 bp product was obtained via amplification using a mastercycler gradient thermal cycler (eppendorf). The primer pair used was: sense 5′-CGAAGCAGGGAGCTTTGAGG-3′, reverse 5′-AGGACGGTGCGGTGAGAGTG-3′. PCR product was digested with HinfI endonuclease, yielding a mayor 175 bp fragment in the presence of the C677T mutation. Bands were resolved on 3% agarose gel electrophoresis. All determinations were repeated twice in two separate runs.

Plasma tHcy concentrations were considered elevated when they exceeded 10.4 μmol/L, according with Huemer et al.[16] Folate concentrations <3 ng/mL were considered deficient, as reported by Ono et al.[17] Serum vitamin B12 levels were considered in normal range between 230 and 1200 pg/mL.

2.2. Statistical analysis

Student's *t*-test for unpaired data was used to compare tHcy concentrations of patients and controls.

Fisher's exact test was performed for relation of univariate analysis and multiple logistic regression analysis was used to analyze factors of influence on high and low tHcy concentrations, odds ratio and confidence interval were calculated for each variable. SPSS version[17] was used for statistical analysis (SPSS, Chicago).

$p < 0.05$ values were considered significant.

3. Results

Seventy-eight (35 male, 43 female) children and adolescents, ranging in age from 3.1 to 15.0 (mean + SD, 8.9 + 6.4) years were included in the study. The patients suffered from the following types of epilepsy: generalized epilepsy, 40 (cryptogenic, 23); partial epilepsy, 18 (cryptogenic, 10); refractory epileptic encephalopathies, 20 (Dravet syndrome, 3; Lennox-Gastaut syndrome, 4; lissencephaly, 2; brain atrophy, 11).

Thirty-five patients were treated with monotherapy (Valproic Acid (VPA), 20; Carbamazepine (CBZ), 10; Levetiracetam (LEV), 5), and 43 patients with polytherapy. Mean duration of drug treatment was of 4.8 years (range 1–10 years). Forty-six patients (58.9%) were seizure-free.

Patients' mean tHcy value was significantly higher than that of controls (11.31 ± 6.68 μmol/L vs 7.4 ± 4.01 μmol/L; $p < 0.01$).

Overall, 32 patients (41.0%) showed tHcy ≥10.4 μmol/L (mean 15.11 ± 4.68; median 13.97). Out of 32 patients with hyperhomocysteinemia, 10 were treated with monotherapy (VPA, 6; CBZ, 3; LEV, 1) and 22 with polytherapy (12 on 2 AEDs and 10 on three drugs) (Table 1). Three out of 10 patients (30%) on monotherapy and 8 out of 22 (36.4%) on polytherapy were taking enzyme-inducing anticonvulsant drugs. The most frequent drug combinations were: VPA + Lamotrigine (LTG) (6), CBZ + Topiramate (TPM) (4), VPA + LTG + Clobazam (2) and VPA + LTG + TPM (2). Table 2 shows the most frequently used AEDs.

Our patients showed the following distribution of MTHFR polymorphism: CT (46%), CC (35%), and TT (17.8%). Folic acid concentration was <3 ng/mL in 33 patients (range 1.3–2.9, mean 1.9); 19 out of 33 patients (59.4%) showed increased tHcy levels. Vitamin B12 was increased in 17 patients (21.8%) (range 1270–1980 pg/mL, mean 1621) and 7 of these had abnormal tHcy levels (Table 1). Multiple logistic regression data analysis showed a significant negative correlation between folic acid serum levels and tHcy levels ($p < 0.003$); to a lesser extent, female sex proved to be a significant risk factor for high tHcy levels ($p = 0.039$) (Table 3).

4. Discussion

In the present study, pediatric patients with epilepsy treated with old and/or newer AEDs showed tHcy plasma levels higher than control subjects. More specifically about 40% of children and adolescents had hyperhomocysteinemia. These data are in agreement with previous reports,[9,18,19] showing tHcy increase in 10–30% patients taking AEDs.

It is noteworthy that most previous reports described patients treated with VPA, CBZ or Phenobarbital monotherapy in children[1,3–5,20] or adults.[6,21] In our series, there was no significant relationship between C677T polymorphism and plasma tHcy levels. In this respect, data emerging from literature are still controversial. Indeed, while some authors found that the MTHFR polymorphisms C677T, A1298C and G1793A play no role in determining abnormal levels of plasma tHcy in patients treated with CBZ or VPA monotherapy[11,16,20] or combination therapy,[11] other authors[8,19,23,24] reported that patients with epilepsy and the

Table 1
Comparison between patients with tHcy blood level < or > 10.4 μmol/L.

Pts. with homocysteine ≥10.4 μmol/L (tot. 32)*		Pts. with homocysteine ≤10.4 μmol/L (tot. 46)°	*p*
Homocysteine blood value (mean ± SD) (median)	15.11 ± 4.68 (13.97)	6.71 ± 2.44 (7.01)	
Duration of therapy (mean-SD) (median)	5 ± 2.59 (5)	4.67 ± 2.89 (4.0)	NS
Monotherapy (*n.* pts)	10*	25°	
Politherapy (*n.* pts)	22*	21°	
MTHFR polymorphisms			
C677T	16	20	
T677T	9	5	
C677C	7	21	
Age (mean ± SD) (median)	9.71 ± 4.08 (10.5)	8.56 ± 4.30 (7.5)	NS
Sex (M/F)	12/20	23/23	NS
B12 vitamin (>800 ng/mL)	7	10	0.218
Folic acid (<3 ng/mL)	19	14	0.001

* $p = 0.006$ (at Fisher's exact test).
° $p = 0.381$ (at Fisher's exact test).

Table 2
Most frequently used anticonvulsant drugs in our series (tot. 78 pts).

Anticonvulsant drug	No. of patients	%
Valproic acid	43	55.1
Carbamazepine	24	30.7
Lamotrigine	16	20.5
Topiramate	14	17.9
Levetiracetam	7	8.9
Zonisamide	7	8.9
Clobazam	7	8.9
Phenobarbital	5	6.4
Clonazepam	4	5.1
Ethosuccimide	1	1.3

C677T allele were at higher risk of hyperhomocysteinemia when treated with CBZ or PHT. In addition, C677T/A1298C polymorphisms of MTHFR were significantly more frequent in epilepsy patients treated with enzyme-inducing drugs and lower plasma level of folic acid. Therefore, Caccamo et al.,[19] state that the TT677/AA1298 diplotype is mostly closely linked to developing hyperhomocysteinemia, while CC677/AA1298 may play a protective role, being present more frequently in controls.

Furthermore, Sniezawska et al.[24] reported polymorphisms of MTHFD1GG (G1958A) related to increased tHcy levels in epileptic patients.

In keeping with other studies, treatment duration had not clear effect on tHcy values in our data.[11,20] In contrast, Sener et al.[6] found a significant relationship between therapy duration and folic acid levels, thus excluding any link with tHcy levels. Furthermore, no relationship was found between plasma levels of AEDSs and serum tHcy.[1,5]

Polytherapy (≥2 AEDs) has been reported as being associated with higher levels of tHcy by Huemer et al.[16] and Tümer et al.[3] while in another study,[11] multidrug therapy was significantly correlated with higher tHcy serum levels, especially in patients who were TT homozygous for the C677T mutation and less in the CT heterozygous or CC wild type patients. Conversely, in our sample, there was no significant correlation between mono/polytherapy and tHcy levels.

Table 3
Multiple logistic regression (odds ratio and confidence interval were calculated for each variable).

Model term	Odds ratio	95% CI		*p*-Value
		Lower	Upper	
% Const	0.55	0.15	1.94	0.350
Age	1.38	0.35	5.40	0.645
Folate	4.89	1.70	14.05	0.003
B12 vitamin	0.87	0.21	3.57	0.852
MTHFR genotype	1.36	0.69	2.70	0.376
Therapy	1.18	0.36	3.87	0.788
VPA	0.65	0.22	1.90	0.428
Therapy duration	1.05	0.29	3.79	0.945
Sex	0.26	0.07	0.93	0.039

In the literature, CBZ and VPA have been associated with higher tHcy levels and lower serum folate levels than those found in controls.[1,4,5,20] These differences were more pronounced in patients treated with CBZ.[5] In addition, the enzyme inducing AEDs (CBZ, PHT, PB) were associated with higher levels of tHcy compared with VPA.[2,22]

In almost all studies, old generation AEDs have predominantly been used monotherapy; only few patients were taking more recently introduced AEDs, like TPM, LTG and oxcarbazepine.[16]

In relation to folate levels, several studies have found a significant inverse association between low levels of folate and higher tHcy levels in patients with epilepsy, taking either mono- or polytherapy.[1,2,4–6,8,11,16,20]

Folate supplementation induced tHcy normalization in all patients.[8,21] A key role for circulating folate levels is also confirmed in our study; in fact, folic acid was the only factor significantly related to serum tHcy levels by multiple logistic regression analysis. Accordingly, the relationship between the C677T mutation and increased tHcy was strongly influenced by folate status. In fact, the large majority of homozygous individuals with low plasma folate had increased tHcy, while those with high plasma folate levels showed normal tHcy levels.[25]

Our study has at least three limitations: first of all, the number of patients on different drugs is too small to come to firm conclusions about the effects of individual drugs; moreover, our group of patients is inhomogeneous because almost all patients were taking new AEDs in combination with old AEDs; finally, this is a cross-sectional (rather than prospective longitudinal) study. Therefore, no final conclusion can then be drawn about low folate levels as a possible risk factors for high tHcy levels. Further investigations are needed to verify our data.

In conclusion, our experience confirms that hyperhomocysteinemia is common in children receiving antiepileptic drug treatment for epilepsy. This increase is mainly related to low folate levels, which are the main determinant for tHcy normalization in patients on antiepileptic therapy.

Considering the vascular risk associated with high tHcy the assessment of blood levels of tHcy and folate is warranted in the routine evaluation of treated epileptic patients and folate substitution should be considered.

Conflict of interest

None of the authors has any conflict of interest to disclose

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