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ORIGINAL ARTICLE

Anti-epileptic drug treatment in children: hyperhomocysteinaemia, B-vitamins and the 677C→T mutation of the methylenetetrahydrofolate reductase gene

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The aim of the study was to observe the influence of carbamazepine and valproic acid on plasma total homocysteine and B-vitamin status and the gene–drug interaction with the 677C→T mutation of the methylenetetrahydrofolate reductase (MTHFR) gene. Plasma total homocysteine concentrations were determined in 136 epileptic children taking anti-epileptic drugs as monotherapy. Nutritional (folate, B_{12} and B_6 vitamins) and genetic (MTHFR 677 C→T) determinants of plasma homocysteine were studied in a random sample of 59 of the 136 epileptic children. Total homocysteine concentrations were significantly increased ($p < 0.05$) and folate and vitamin B_6 levels were significantly decreased ($p < 0.01$) in the children taking anti-epileptic drugs compared with our reference ranges. In the carbamazepine-treated group, significantly positive correlation was found between duration of treatment and homocysteine concentration ($p < 0.01$). Homocysteine concentrations showed a significantly negative correlation with vitamin levels (folate: $p = 0.002$, and vitamin B_{12}: $p = 0.017$) only in the carbamazepine treated group. In children treated with carbamazepine up to 3 years, total homocysteine concentration correlated negatively only with folate ($p = 0.003$), while in patients treated for more than 3 years, total homocysteine correlated negatively only with vitamin B_{12} values ($p = 0.007$). The lowering action of carbamazepine treatment on folate levels seems to be associated with hyperhomocysteinaemia, which seems to be related to the homozygous condition for the MTHFR 677C→T mutation. Valproic acid treatment, although also associated with hyperhomocysteinaemia, only shows a lowering effect on vitamin B_6 levels, which seems to be independent of the MTHFR genotype.

Keywords: Hyperhomocysteinaemia. Folate. Vitamin B_{12}. Vitamin B_6. MTHFR 677C→T mutation. Anti-epileptic drugs.

Introduction

Mild hyperhomocysteinaemia is an independent risk factor for atherosclerosis and thrombosis in adults,[1–2] and has been associated with the risk of stroke even in paediatric patients.[3–4] Plasma homocysteine concentration is determined by genetic and acquired factors.[5] Homozygosity of the 677C→T mutation of the 5,10 methylenetetrahydrofolate reductase (MTHFR) gene is the most common inherited cause of mild hyperhomocysteinaemia.[6] This polymorphism causes a thermolabile variant with a 50% reduction of MTHFR activity,[7] with the consequence of an impairment of 5-methyltetrahydrofolate synthesis, which is indispensable for homocysteine remethylation. Serum folate, vitamin B_{12} and vitamin B_6,

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involved in homocysteine metabolism, are the most important nutritional determinants of its plasma concentration.[8]

Long-term anti-epileptic drug treatment is associated with hyperhomocysteinaemia probably caused by B-vitamin deficiency[9–10] which seems more evident in epileptic adult patients with the 677C→T mutation of the MTHFR gene.[11] The influence of treatment with anti-epileptic drugs on B-vitamin status has largely been described,[12–16] although the exact mechanism remains controversial.[17] It has been proposed that anti-epileptic drugs induce folate depletion by the induction of liver enzymes, the impairment of folate absorption, a competitive interaction between folate coenzymes and drugs, an increased demand for folate as a cofactor in the hydroxylation of the anticonvulsant, and an altered activity of some enzymes involved in one-carbon transfer, such as MTHFR.[18–19] There are few data in the literature on the mechanism of interaction between anti-epileptic drugs and vitamins B_{12} and B_6.[10,20]

In a previous study we found an association between hyperhomocysteinaemia and anticonvulsant therapy in children.[3] However, we could not analyse the genetic or nutritional determinants of hyperhomocysteinaemia at that time. The aim of this present study was to further investigate the relationship between anti-epileptic drugs and hyperhomocysteinaemia in children. We study the influence of carbamazepine and valproic acid on plasma total homocysteine and B-vitamin (folate and vitamin B_{12} and B_6) status and the gene–drug interaction with the 677C→T mutation at the MTHFR gene.

Materials and methods

Patients

We analysed plasma total homocysteine concentrations in 136 epileptic children (age range: 1–18 years; median: 10 years) undergoing anticonvulsant treatment, who were regularly evaluated as outpatients at the Neuropediatrics Department of the Hospital Sant Joan de Déu, Barcelona. The patients suffered from partial epilepsy, partial epilepsy with secondary generalized seizures and generalized epilepsy. Anti-epileptic treatment, valproic acid ($n = 74$ patients) or carbamazepine ($n = 62$ patients) was introduced as monotherapy from 10 months to 16 years (median: 3 years) before the study, and serum anti-epileptic drug levels were regularly monitored, along with hepatic and haematologic function. Doses were adjusted to achieve seizure control and they ranged from 20 to 30 mg/kg/day for carbamazepine and from 20 to 40 mg/kg/day for valproic acid. Exclusion criteria were a history of stroke episode, diabetes mellitus, liver or renal failure, malnutrition, anaemia, inborn errors of intermediary metabolism and vitamin supplementation.

Nutritional (folate, B_{12} and B_6 vitamins) and genetic (MTHFR 677 C→T) determinants of plasma homocysteine were studied in a random sample of 59 of the 136 epileptic children.

Blood for this study was obtained in the course of anti-epileptic drug monitoring. The Hospital Ethics Committee approved the study and blood samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in 1996.

Methods

Biochemical analysis

Homocysteine

Fasting plasma total homocysteine (the sum of all homocysteine forms which generate this amino acid by reduction) was determined by high performance liquid chromatography (HPLC) with fluorescence detection of the 7-fluorobenzo-2oxa-1,3-diazole-4-sulphonate (SBDF) derivatives.[21] Reference values were established in a paediatric population by measurement of plasma total homocysteine in apparently healthy children ($n = 185$) who underwent pre-surgical analysis for minor surgery. Total homocysteine was independent of sex and increased significantly with age in the general paediatric population ($r = 0.53$; $p < 0.001$).[21] After statistical analysis applied to all age groups (Kruskal–Wallis) we established three age groups (1–10 years, 11–15 years and 16–18 years) whose total homocysteine concentrations were the most significantly different from one another (Mann–Whitney; $p < 0.001$).[21] Therefore, the epileptic patients were distributed in the same age-groups to compare their total homocysteine concentrations with our reference values with similar ages. Hyperhomocysteinaemia was defined as total homocysteine values above the 95th percentile (P_{95}) of the distribution within the paediatric population (1–10 years: 8.0 μmol/litre; 11–15 years: 9.2 μmol/litre; 16-18 years: 10.8 μmol/litre).

Vitamins

Serum total folates and cobalamin concentrations were determined after a preliminary heat

denaturation step by a competitive protein binding chemiluminiscent assay (IMMULITE, Diagnostic Products Corporation). Vitamin B_6 (pyridoxal-5-phosphate) was analysed by HPLC (Perkin Elmer Integral 4000) with fluorescence detection (LC 240, Chromsystem Kit). Patients' serum vitamin concentrations ($n = 59$) were compared with our reference values ($n = 56$; age range: 1–18 years; median: 10 years), which are independent of age and sex for the paediatric age. We considered low vitamin values to be those below the 5th percentile (P_5) of the distribution within the paediatric reference values (P_5 folate: 10 nmol/litre; P_5 vitamin B_{12}: 222 pmol/litre; P_5 vitamin B_6: 23 nmol/litre).

Anti-epileptic drugs

Carbamazepine and valproic acid concentrations were determined by fluorescence polarization immunoassay (FPIA) automated in the Cobas Integra analyser (Roche Diagnostic Systems). Samples for anti-epileptic drug monitoring were collected on pre-doses, and all patients were in the steady-state with regard to carbamazepine and valproic acid. Duration of treatment was considered as a variable and patients were grouped according to it (treatment for $\leqslant 3$ years and for > 3 years).

Mutation detection

Genomic DNA was prepared from peripheral blood leukocytes using a standard phenol extraction and ethanol precipitation method.[22] Primers for polymerase chain reaction (PCR) amplification were those described by Frost *et al*.[6] Template DNA (100 ng) was amplified by using 23 μl of PCR Supermix (Life Technologies) and 10 pmol of each primer in a final volume of 25 μl. The PCR conditions were as follows: 30 cycles of denaturation at 94°C for 40 seconds, a single annealing/extension step at 56°C for 30 seconds, and a final elongation step of 5 minutes at 72°C. The PCR products were digested with HinfI (New England Biolabs) and analysed by non-denaturing polyacrylamide gel electrophoresis and ethidium bromide staining. Positive homozygous and heterozygous individuals were used to control the digestion process. For genetic analysis, 28 healthy Spanish children (age range: 1–18 years) were chosen as a reference population.

Statistical methods

The Hardy–Weinberg equilibrium test was applied to allelic frequencies. Statistical analyses were performed using the statistical package SPSS (version 6.1.2). The χ^2 test was used to analyse allele and genotype differences between control and epileptic children and the association of genotype to hyperhomocysteinaemia and low vitamin levels. The relationship between total homocysteine or anti-epileptic drugs and B-vitamin concentrations was analysed by the Spearman correlation coeficient, and this coefficient was also used to calculate the correlation between the duration of treatment and total homocysteine and vitamin levels. Non-parametric Mann–Whitney U-test was applied to compare total homocysteine and vitamin values of patients with those of reference values, with a 95% confidence interval.

Results

Total homocysteine and anti-epileptic drugs

Total homocysteine values in the children undergoing anti-epileptic drug treatment (carbamazepine or valproic acid) were significantly increased compared with our reference population (Table 1). Total homocysteine concentrations were above the P_{95} of the reference values in 55 of the 136 patients (40.4%). The total percentage of children with hyperhomocysteinaemia treated with carbamazepine (41.9%) or valproic acid (39.2%) was not significantly different. No significant correlation was found between total homocysteine values and carbamazepine or valproic acid levels (carbamazepine: 36.7 μmol/litre (SD: 8.8); valproic acid: 532.5 μmol/litre (SD: 173.6)).

Vitamins and anti-epileptic drugs

As regards vitamin levels in 59 of the 136 epileptic children (Table 2), serum folate and B_6 concentrations were significantly lower in the anti-epileptic drug treated patients compared with our reference values. The percentage of children with vitamin levels in the low range ($< P_5$) is higher for folate than for B_6 and very low for vitamin B_{12}. When we considered either the carbamazepine or the valproic acid-treated children, folate and vitamin B_6 concentrations were significantly decreased in the epileptic children compared with the reference ranges in both groups, while vitamin B_{12} levels were significantly increased only in the children treated with valproic acid. However, the percentage of children with folate levels in the low range ($< P_5$ of our reference values) is especially high

Table 1 Plasma total homocysteine in 136 paediatric patients taking anti-epileptic drugs, compared with our reference values

	Total homocysteine median (range) μmol/litre		
Age (years)	1–10	11–15	16–18
Anti-epileptic drugs	7.6[a] (3.9–19.1) $N = 75$	8.9[b] (4.6–18.0) $N = 33$	9.4[e] (5.1-39.8) $N = 28$
HHC	40.5%	48.5%	30.0%
CBZ	7.7[b] (4.4–19.1) $N = 31$	7.9[d] (4.6–18.0) N = 17	9.4[NS] (5.1–39.8) N = 14
HHC	48.4%	41.2%	28.6%
Valproic acid	7.4[a] (3.9–13.5) N = 44	9.3[c] (5.0–15.2) $N = 16$	9.9[NS] (6.5–37.3) N = 14
HHC	34.1%	56.2%	36.0%
Reference values	5.8 (3.7–8.0) $N = 96$	6.6 (5.1–9.3) $N = 58$	8.1 (5.7–10.8) $N = 31$

N: number of individuals; HHC: hyperhomocysteinaemia (tHcy > P_{95} of the reference values); CBZ: carbamazepine.
NS: Non-significant differences; [a]$p < 0.00001$; [b]$p < 0.0001$; [c]$p < 0.001$; [d]$p < 0.01$; [e]$p < 0.05$.

(62%) in the carbamazepine-treated children, while the higher percentage (30.8%) of children with low vitamin B_6 levels is within the valproic acid-treated group.

Relationship between total homocysteine, vitamins and carbamazepine

A significantly positive correlation was found between total homocysteine concentrations and the duration of treatment with anti-epileptic drugs ($p = 0.013$), particularly with carbamazepine treatment ($p = 0.015$), but not with valproic acid (Table 3). Moreover, a negative correlation was observed between duration of carbamazepine treatment and vitamin B_{12} values ($p = 0.013$). Total homocysteine concentrations showed a significantly negative correlation with vitamin levels (folate: $p = 0.002$, and vitamin B_{12}: $p = 0.017$) only in the carbamazepine treated group.

Regarding the duration of carbamazepine treatment, in children treated up to 3 years total homocysteine concentration correlated negatively only with folate ($p = 0.003$) while, in patients treated for more than 3 years, total homocysteine values correlated negatively only with vitamin B_{12} ($p = 0.007$).

Relationship between total homocysteine, vitamins and MTHFR genotypes

The allele and genotype frequencies for the thermolabile 677C→T mutation at the MTHFR gene in the 59 children undergoing anti-epileptic drug treatment and 28 healthy children of the same age and geographic origin are summarized in Table 4. No differences were detected between the two populations with regards to the allelic frequencies, both being in Hardy–Weinberg equilibrium. The prevalence of homozygous and heterozygous 677C→T is not significantly different in the anti-epileptic drug population compared with the healthy control group.

The prevalence of hyperhomocysteinaemia and low B-vitamin levels (< P_5 of our reference values) related to the MTHFR genotypes is shown in Fig.1. In epileptic children treated with anti-epileptic drugs, hyperhomocysteinaemia was more frequent in the MTHFR T/T genotype than in the C/T or C/C genotypes, both in children treated with

Table 2 Folate, vitamin B_{12} and vitamin B_6 concentrations in paediatric patients taking anti-epileptic drugs compared with our reference values. Relation of anti-epileptic drugs with low vitamin levels

	Folate nmol/litre	Vitamin B_{12} pmol/litre	Vitamin B_6 nmol/litre
Anti-epileptic drugs $N = 59$	11.9[a] (4.9–30.6)	518[NS] (137–885)	33.1[a] (4.1–88.9)
Vit % < P_5	37	11.3	28
Carbamazepine $N = 32$	9.3[a] (4.9–26.2)	391[NS] (137–885)	31.8[b] (4.1–88.9)
Vit % < P_5	62	21.8	25
Valproic acid $N = 27$	14.1[c] (6.2–30.6)	630[b] (256–885)	32.5[b] (6.9–88.4)
Vit % < P_5	10	0	30.8
Reference values $N = 56$	17.4 (8.6–37.8)	445 (136–886)	50.8 (17.0–89.7)

N: number of individuals; Vit %: vitamin values < P_5 of the reference values.
NS: Non-significant differences: [a]$p < 0.00001$; [b]$p < 0.0001$; [c]$p < 0.05$.

Table 3 Relationship between total homocysteine, vitamins and duration of anti-epileptic drug treatment[a]

Variables	*N*	Correlation coefficient[b]	Significance
AED duration vs tHcy	136	r = 0.2133	0.013
THcy vs folate	59	r = −0.3761	0.007
THcy vs vitamin B12	59	r = −0.4144	0.003
Folate vs vitamin B12	59	r = 0.3926	0.004
Carbamazepine treatment			
CBZ duration vs tHcy	59	r = 0.3166	0.015
CBZ duration vs B_{12}	32	r = −0.4358	0.017
tHcy vs folate	32	r = −0.5182	0.002
tHcy vs vitamin B_{12}	32	r = −0.4193	0.017
Carbamazepine treatment for ⩽3 years			
tHcy vs folate	14	r = −0.7247	0.003
Carbamazepine treatment for >3 years			
tHcy vs vitamin B_{12}	18	r = −0.6129	0.007

[a]Only significantly related variables were included in the table.
[b]Spearman correlation coefficients.
AED: anti-epileptic drugs; CBZ: carbamazepine; tHcy: total homocysteine.

Table 4 Prevalence of the 677C→T mutation of the MTHFR gene in 59 children treated with anti-epileptic drugs and a control paediatric population

MTHFR	Epileptic children	Healthy/control children
677C→T	*N* (%)	*N* (%)
C/C	19 (32.2)	7 (25.0)
T/C	30 (50.8)	17 (60.7)
T/T	10 (16.9)	4 (14.3)
Allele frequency		
C	0.58	0.55
T	0.42	0.45

carbamazepine (100% vs 60% and 41.7%) and valproic acid (100% vs 53.3% and 0%), although the differences were non-significant. As regards the drug–vitamin interaction, in carbamazepine-treated patients folate levels decreased according to the MTHFR genotype (median (range) T/T: 6.6 (5.9–16.5); C/T: 9.3 (5.1–13.8); C/C: 11.2 (4.9–26.2) μmol/litre), although the differences among these are not significant. The influence of carbamazepine on vitamins B_{12} and B_6 and of valproic acid on the three vitamins does not appear to be related to the MTHFR genotype (Fig.1).

Discussion

The present study confirms that mild hyperhomocysteinaemia is a common condition in epileptic children taking anticonvulsants (carbamazepine or valproic acid) as monotherapy.[3] Significant differences were found in total homocysteine values of anticonvulsant treated children in the three age groups compared with our reference values. High plasma total homocysteine concentrations (15–39.8 μmol/litre) were observed in epileptic children from 8 to 18 years old (Table 1), but especially in the eldest groups, taking either valproic acid or carbamazepine. This observation suggested that long-term anti-epileptic drug treatment might progressively increase total homocysteine concentrations,[9] which was confirmed by the positive correlation found between the duration of treatment with carbamazepine and total homocysteine concentrations. This observation is supported by some authors[9] but not by others,[11] and would have to be further proved by means of prospective studies.

Since the effect of the two anti-epileptic drugs on homocysteine concentration might be different, we stratified the patients according to the treatment type. However, plasma total homocysteine concentrations were similarly increased in children treated with valproic acid or carbamazepine (Table 1). In addition, the percentage of children with hyperhomocysteinaemia was similar for both anti-epileptic drugs (Table 1), except for the subgroup of children with the MTHFR C/C genotype (Fig.1). Our results differed from those of other authors that found significantly increased total homocysteine values, particularly in adult patients treated with carbamazepine.[10] In the eldest

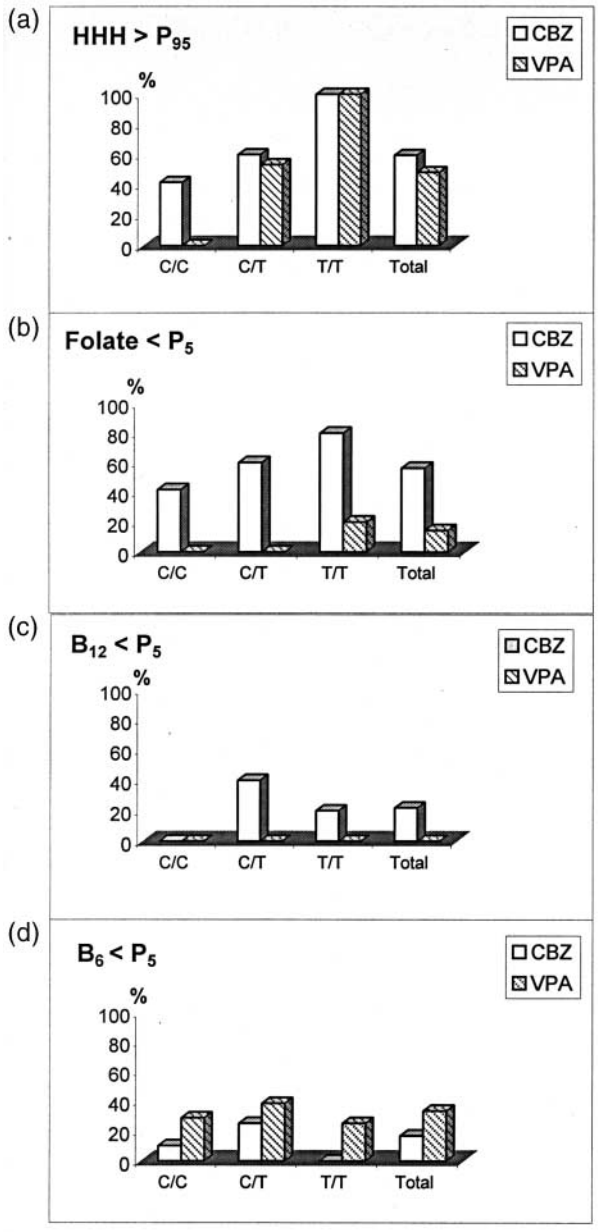

Fig. 1. Hyperhomocysteinaemia (plasma tHcy > P_{95} of our reference values) and low B-vitamin levels (< P_5 of our reference values) related to MTHFR 677C→T genotype in epileptic children undergoing treatment with carbamazepine (CBZ) and valproic acid (VPA). a) Frequency of children with hyperhomocysteinaemia. b) Frequency of children with low folate values. c) Frequency of children with low vitamin B_{12} levels. d) Frequency of children with low vitamin B_6 levels.

group of children (taking valproic acid or carbamazepine) the differences in total homocysteine concentrations between patients and reference values were not significant, probably because of the small number of children studied (Table 1).

The influence of anti-epileptic drugs on B-vitamins was evaluated by stratifying patients treated with valproic acid or carbamazepine to avoid the confusing effect of the two drugs (Table 2). We considered as low vitamin levels the values below the P_5 of our reference values, because B-vitamin deficiency is uncommon in children on normal diets, at least in the Mediterranean countries. However, vitamin levels in the low range might be observed in children on special diets,[23] suffering from anorexia[24] or having some genetic abnormalities such as the 677C→T mutation of the MTHFR gene, which interferes with folate metabolism.[25] We observed a clear lowering effect of carbamazepine on folate levels: a high percentage (62%) of the children taking carbamazepine showed folate levels in the low range, even below our reference values (< 8.6 nmol/litre), although none had a marked folate deficiency. The lowering effect of carbamazepine on vitamin B_6 concentrations has also been demonstrated by other authors,[10] although the mechanism of the interaction between carbamazepine and B_6 is still unclear, and it does not appear to be related to MTHFR genotype (Fig.1). In our experience, carbamazepine also has a lowering effect on vitamin B_{12} concentrations (21.8% of children below the P_5), although the decrease is not significant. Moreover, we found a significantly negative correlation between duration of carbamazepine treatment and vitamin B_{12} levels, and between total homocysteine and vitamin B_{12} concentrations in the long-term treated patients. Conversely, in children treated with carbamazepine up to 3 years, we only observed a correlation between total homocysteine and folate levels. The hyperhomocysteinaemia associated with carbamazepine treatment seems to be related to defective folate in the short-term and to defective vitamin B_{12} in the long-term treatment. However, prospective studies including more patients treated for a long time were needed to better understand this observation.

Regarding valproic acid, it does not seem to decrease folate levels, but it is associated with low vitamin B_6 values (30.8% of valproic acid treated children were in the low-normal range, six of them with marked vitamin B_6 deficiency). We found significantly increased vitamin B_{12} levels in valproic acid treated children compared with our reference values, a finding which has also been observed in adult patients by other authors.[20] There was no correlation between vitamin B_{12} and valproic acid levels, or between vitamin B_{12} and total homocysteine concentrations in valproic acid treated patients, so that high vitamin B_{12} levels could not be attributed to a disturbance in B_{12} intracellular metabolism causing hyperhomocysteinaemia.[26]

As regards the mutation analysis, in our study all the epileptic children carrying the T/T genotype showed hyperhomocysteinaemia (either with carbamazepine or valproic acid treatment), and total homocysteine concentrations of some of them were very high (9–39.8). In contrast, the percentage of children with hyperhomocysteinaemia and T/C or C/C genotypes was clearly lower (Fig. 1). Either carbamazepine or valproic acid seems to have an effect on the prevalence of hyperhomocysteinaemia related to the 677C→T genotype, which in the case of carbamazepine treatment appears to be related to low folate levels, as observed also by other authors in adult patients.[11] However, valproic acid treatment seems to have an effect only on vitamin B_6 levels, which is independent of the MTHFR genotype. According to our results, the influence of valproic acid treatment on plasma total homocysteine concentrations would appear to be independent of vitamin B_6.

The gene–drug interaction observed by us and other authors[11] has important implications, especially for epileptic children with the T/T genotype, since anti-epileptic drugs often involve long-term treatment that could increase vitamin requirements and thus cause hyperhomocysteinaemia. This effect should be carefully considered in the general population, particularly in geographical areas with a high prevalence of the 677C→T mutation.[25,27–29] Although only some children taking anti-epileptic drugs show hyperhomocysteinaemia, it is associated with premature vascular risk with a gradual and continuous relationship, and without a threshold value.[30] Although hyperhomocysteinaemia is independent of other conventional risk factors, it potentiates their atherogenic effect.[5] Therefore, other determinants of hyperhomocysteinaemia not present in childhood, such as tobacco or coffee,[31] might add their effect to the mild folate deficiency caused by anti-epileptic drugs, increasing total homocysteine levels and, consequently, the vascular risk.

Moreover, the effect of anti-epileptic drugs on folate and total homocysteine concentrations has important implications for pregnant women carrying the MTHFR T/T genotype.[32] Folate has a protective effect against congenital anomalies,[33] and its defective metabolism in anti-epileptic drug treatment might have adverse consequences for the fetus. Hyperhomocysteinaemia has been associated with the risk of spina bifida,[34–35] preeclampsia,[36–37] recurrent miscarriages,[31] intrauterine growth retardation[38] and Down syndrome.[39]

An epileptogenic effect of hyperhomocysteinaemia has been reported[10,40] and this might be supported by the high prevalence of the homozygous thermolabile mutation at the MTHFR gene found by some authors.[11] We found only a slightly higher prevalence of the T/T genotype in epileptic patients (16.9%) which is not significantly different from that of the healthy children (14.3%). It is worth noting that the prevalence of the T/T genotype is quite high in our paediatric population compared with other geographical areas.[41] Nevertheless, the relatively low total homocysteine concentrations observed in epileptic children undergoing anticonvulsant treatment, compared with the high total homocysteine concentrations detected in homocystinuric patients,[40] might imply an alteration of the seizure threshold with only long-term consequences.[10]

In conclusion, mild hyperhomocysteinaemia is a common condition in epileptic children taking anticonvulsants (carbamazepine or valproic acid) as monotherapy. The lowering action of carbamazepine treatment on folate levels is associated with hyperhomocysteinaemia, which is related to the homozygous condition for the MTHFR 677C→T mutation. Valproic acid treatment, although also associated with hyperhomocysteinaemia, only shows a lowering effect on vitamin B6 levels, which is independent of the MTHFR genotype. The possibility of easily correcting hyperhomocysteinaemia by folate supplementation[42] would justify tHcy determination in anti-epileptic treated patients, even in childhood.

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