Hyperhomocysteinemia in epileptic patients on new antiepileptic drugs

*Vincenzo Belcastro, †Pasquale Striano, ‡Gaetano Gorgone, *Cinzia Costa, §Clotilde Ciampa, ¶Daniela Caccamo, **Laura R. Pisani, #Giancarla Oteri, **Maria G. Marciani, ††Umberto Aguglia, §§Salvatore Striano, ¶¶Riccardo Ientile, *Paolo Calabresi, and #Francesco Pisani

*Neurology Clinic, University of Perugia, Perugia and Fondazione Santa Lucia – I.R.C.C.S., Roma, Italy; †Muscular and Neurodegenerative Diseases Unit, G. Gaslini Institute, University of Genova, Genova, Italy; ‡Associazione Fatebenefratelli per la ricerca AFaR, DPT of Neurosciences, Ospedale Fatebenefratelli, Isola Tiberina, Roma, Italy; §Epilepsy Center, “Federico II” University, Napoli, Italy; ¶Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy; #Department of Neurosciences, Neurology Clinic, University of Messina, Messina, Italy; **Neurology Clinic, University Tor Vergata, Roma, Italy; and ††Epilepsy Center, Reggio Calabria and “Magna Grecia” University of Catanzaro, Catanzaro, Italy

SUMMARY

Purpose: Older enzyme-inducing antiepileptic drugs (AEDs) may induce supraphysiologic plasma concentrations of total (t) homocysteine (Hcy). The aim of the present study was to investigate the effect of new AEDs on plasma tHcy levels.

Methods: Patients 18–50 years of age, on AEDs monotherapy, with no other known cause of hyper-tHcy were enrolled. Plasma tHcy, folate, vitamin B₁₂, and AEDs levels were determined by standard high-performance liquid chromatography (HPLC) methods. Methylene tetrahydrofolate-reductase (MTHFR) polymorphisms were checked using Puregene genomic DNA purification system (Gentra, Celfio, Italy). A group of healthy volunteers matched for age and sex was taken as control.

Results: Two hundred fifty-nine patients (151 on newer and 108 on older AEDs) and 231 controls were enrolled. Plasma tHcy levels were significantly higher [mean values, standard error (SE) 16.8, 0.4 vs. 9.1, 0.2 μM; physiologic range 5–13 μM] and folate lower (6.3, 0.1 vs. 9.3, 0.1 nM; normal > 6.8 nM) in patients compared to controls. Patients treated with oxcarbazepine, topiramate, carbamazepine, and phenobarbital exhibited mean plasma tHcy levels above the physiologic range [mean values (SE) 16 (0.8), 19.1 (0.8), 20.5 (1.0), and 18.5 (1.5) μM, respectively]. Conversely, normal tHcy concentrations were observed in the lamotrigine and levetiracetam groups [both 11.1 (0.5) μM].

Discussion: Oxcarbazepine and topiramate might cause hyper-tHcy, most likely because of the capacity of these agents to induce the hepatic enzymes. Because literature data suggest that hyper-tHcy may contribute to the development of cerebrovascular diseases and brain atrophy, a supplement of folate can be considered in these patients to normalize plasma tHcy.

KEY WORDS: Hyperhomocysteinemia, Topiramate, Oxcarbazepine, Hepatic enzyme induction, Folate deficiency.
with folate and other B vitamins did not provide any appreciable benefit in preventing cardiovascular events (Bønaa et al., 2006; Lonn et al., 2006). In the neurologic field, an increasing body of data has indicated that supraphysiologic Hcy levels are a risk factor for cerebrovascular diseases and brain atrophy (Homocysteine studies collaboration, 2002; Seshadri et al., 2002; Hassan et al., 2004; Ravaglia et al., 2005; Spence, 2007). Patients with epilepsy exhibit elevated plasma tHcy levels more frequently than the general population does (10–40% vs. approximately 5%) (Caccamo et al., 2004; Huemer et al., 2005; Belcastro et al., 2007; Mintzer et al., 2009; Tan et al., 2009). This is mainly due to (1) the reduced activity of the key enzyme 5,10-methylenetetrahydrofolate-reductase (MTHFR, which regulates 5-methyltetrahydrofolate, required for the remethylation of Hcy to methionine), caused by polymorphisms in the MTHFR gene (Chango et al., 2000; Caccamo et al., 2004; Belcastro et al., 2007), and (2) a deficiency of folate, an important cofactor in the metabolism of Hcy, induced by older antiepileptic drugs (AEDs), such as carbamazepine, phenytoin, phenobarbital, and primidone (Patsalos & Perucca, 2003; Reynolds, 2006). These drugs stimulate many cytochrome P450 (CYP) and glucuronyl transferase (GT) enzymes of the liver, and hence the metabolism of folate (Patsalos & Perucca, 2003). Compared with the older AEDs, most of the recently developed AEDs are less likely to influence the activity of the hepatic enzymes (Patsalos & Perucca, 2003), and hence the risk of developing hyper-tHcy with these drugs should be negligible or even absent (Reynolds, 2006). This topic has not yet been investigated formally, and the few observations in the literature are insufficient to draw definite conclusions. The present study has been conducted to look at this matter more deeply and, specifically, to investigate whether patients treated with newer AEDs as monotherapy exhibit supraphysiologic levels of plasma Hcy concentrations.

**Subjects and Methods**

The study was conducted from April 2006 to May 2008 at the outpatient Epilepsy Centres of Perugia, Messina, Napoli, Rome, and Catanzaro University sites, Italy. The final protocol was approved by the local ethics committees of each center and informed consent was signed by the participants. Blood samples were analyzed in the lab of the Department of Biochemical, Physiological and Nutritional Sciences, University of Messina.

**Patients**

Patients were recruited with the following inclusion criteria: (1) 18–50 years of age, this age range was chosen to minimize the effect of age on tHcy levels (Belcastro et al., 2007); (2) established diagnosis of epilepsy; (3) approximately good compliance to therapy as evaluated on the basis of history and monitoring of plasma AED levels; (4) stable (i.e., unchanged for at least 6 months) monotherapy with AEDs; (5) no regular consumption of vitamins or any other drugs, other than AEDs, known to affect tHcy levels in the last 6 months (i.e., levodopa, fibrates, niacin, statins, metformin, methotrexate, sulfasalazine, and so on); (6) no clinical evidence for any condition known to affect tHcy levels (i.e., renal insufficiency, cardiovascular disease, cancer, diabetes, malabsorption, hypertension, inborn errors of Hcy, cobalamin or folate metabolism, use of tobacco products, and chronic alcohol consumption); (7) Mediterranean diet only with no vegetarian or particular diet requirements/changes during the last 6 months.

**Measurement of tHcy, folate, and vitamin B12 levels**

Blood samples were collected after an overnight fast, cooled on ice immediately, and centrifuged at 4°C (800 g per 10 min). Plasma was separated within one hour and stored at −20°C until analysis. Plasma total levels of Hcy, folate, and cobalamin were assessed by use of commercially available kit for high performance liquid chromatography (HPLC) measurements (Bio-Rad, Milan, Italy), and Simul-TRAC-SNBI125 RIA (DRG Diagnostics, Marburg/Lahn, Germany). Normal reference intervals in fasting conditions were 5–13 μM (tHcy), 6.8–76.6 nM (folate), and 118–716 pm (vitamin B12). The intradaily and interdaily coefficients of variation for these determinations were <10%. Details of procedures are given in previous papers (Caccamo et al., 2004; Belcastro et al., 2007).

**AED assay**

AED plasma levels were determined, each sample in duplicate, by EMIT (Syva Corporation, Palo Alto, CA, U.S.A.) (older AEDs) or by standard HPLC methods (lamotrigine, topiramate, mono-hydroxy-derivative (MHD), i.e., the active oxcarbazepine monohydroxy derivative, and levetiracetam) (Bahrami et al., 2005; Contin et al., 2005; Jueneke et al., 2006). The intra- and interdaily coefficients of variation for these determinations were <10%.

**MTHFR polymorphism analysis**

C677T and A1298C MTHFR polymorphism analysis was carried out on DNA extracted from white cells of freshly drawn peripheral blood samples using Puregene genomic DNA purification system (Gentra, Celbio, Italy). Double gradient-denaturing gradient gel (DG-DGG) was used as described (Caccamo et al., 2004; Belcastro et al., 2007).

**Statistical analysis**

Comparison of discrete variables (sex, MTHFR C677T genotype distribution) was made using the Pearson chi-square test. Continuous data (age, plasma tHcy, folate, vitamin B12) were investigated using the Mann-Whitney
U test. Kruskal-Wallis analysis of variance (ANOVA) was used when appropriate. Kruskal-Wallis ANOVA was also implemented to explore the variation of plasma tHcy across patients receiving AEDs. The effect of the product term “patient/control category × MTHFR genotypes” on plasma tHcy was explored by a generalized linear model (GLZ) (Green & Silverman, 1994). The likelihood ratio test (LRT) was used to determine the significance of the product term after adjusting for the effects of sex, age, folate, and vitamin B_{12}. Subsequently, the influence of AED administration on plasma tHcy was investigated in patients by implementing a multiple regression design including also sex, age, folate, vitamin B_{12}, and the presence of TT677 MTHFR genotypes as independent variables. Before applying GLZ and multiple regression, a natural logarithm transformation of tHcy values was made to account for the rightward skew of the data. Compliance to normal distribution of the residuals in these analyses was verified by a chi-square fitting test. Differences were considered significant according to a threshold of p < 0.05. All statistics were implemented using STATISTICA v.6 (Statsoft, Tulsa, OK, U.S.A.).

Results

Two hundred fifty-nine consecutive epileptic patients stabilized on monotherapy with older (n = 108) or new AEDs (n = 151) and 231 healthy drug-free controls matched for age and sex were studied. Demographic details are given in Table 1.

Elevated plasma tHcy levels were found in 87 patients (33.6%) and in 19 controls (8.2%) (χ² = 53.0, d.f. = 1, p < 0.0001); 46 of these patients were stabilized on monotherapy with new AEDs and the remaining 41 patients with older AEDs (Table 2).

There were no statistically significant differences in sex (χ² = 5.8, d.f. = 5, p = 0.3), the proportions of MTHFR C677T genotypes (χ² = 0.07, d.f. = 2, p = 0.9), age (Z = −1.1, p = 0.2), and in vitamin B_{12} (Z = −1.6, p = 0.1). Age (χ² = 5.8, d.f. = 5, p = 0.3) and vitamin B_{12} (χ² = 7.3, d.f. = 5, p = 0.2) were not significantly different, even after stratifying patients and controls in relation to the MTHFR genotypes.

Patients exhibited higher plasma tHcy (Z = 12.6, p < 0.0001) and lower folate (Z = −13.0, p < 0.0001) than controls, and these differences remained significant even if patients and controls were grouped according to the MTHFR genotypes (χ² = 170.9, d.f. = 5, p < 0.0001 and χ² = 126.6, d.f. = 5, p < 0.0001, respectively) (Table 1).

tHcy was associated with folate (parameter estimate = −0.1, Wald statistic = 81.5, d.f. = 1, p < 0.0001), age (parameter estimate = 0.004, Wald statistic = 11.3, d.f. = 1, p < 0.001), and the product term case/control category on tHcy (d.f. = 1, p < 0.0001).

Table 1. Demographic, genetic, and biochemical data of patients and controls

<table>
<thead>
<tr>
<th>MTHFR genotype</th>
<th>Cases</th>
<th>Controls</th>
<th>M/F (n)</th>
<th>Age (years)</th>
<th>tHcy (µM)</th>
<th>Folate (µM)</th>
<th>Vitamin B_{12} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC677</td>
<td>68</td>
<td>60</td>
<td>38/30</td>
<td>39.1 (1.4)</td>
<td>124.0 (6.9)</td>
<td>7.1 (0.4)</td>
<td>9.7 (0.2)</td>
</tr>
<tr>
<td>CT677</td>
<td>119</td>
<td>110</td>
<td>73/46</td>
<td>42.0 (1.0)</td>
<td>138.0 (4.2)</td>
<td>8.8 (0.3)</td>
<td>9.0 (0.2)</td>
</tr>
<tr>
<td>TT677</td>
<td>72</td>
<td>61</td>
<td>44/28</td>
<td>41.2 (1.1)</td>
<td>213.0 (7.9)</td>
<td>11.3 (0.4)</td>
<td>5.1 (0.2)</td>
</tr>
<tr>
<td>Overall</td>
<td>259</td>
<td>231</td>
<td>155/104</td>
<td>41.8 (0.6)</td>
<td>168.0 (4.9)</td>
<td>9.1 (0.2)</td>
<td>6.3 (0.1)</td>
</tr>
</tbody>
</table>

Values are given as mean and standard error (SE). M, male; F, female.

Table 2. Drug dose and plasma levels, plasma tHcy levels, hyper-tHcy patients, and C677T MTHFR genotype frequencies in drug group patients

<table>
<thead>
<tr>
<th>AEDs</th>
<th>Patients (n)</th>
<th>AED dose (mg/day)</th>
<th>Plasma AED (µg/ml)</th>
<th>tHcy (µM)</th>
<th>Hyper-tHcy patients (n, %)</th>
<th>CC677MTHFR (n)</th>
<th>CT677MTHFR (n)</th>
<th>TT677MTHFR (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXC</td>
<td>45</td>
<td>1,486 (70.8)</td>
<td>28.5 (2.2)</td>
<td>16.0 (0.8)</td>
<td>15; 33.3</td>
<td>15</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>TPM</td>
<td>41</td>
<td>283.7 (16.6)</td>
<td>6.8 (0.6)</td>
<td>19.1 (0.8)</td>
<td>27; 65.8</td>
<td>8</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>LEV</td>
<td>32</td>
<td>1,781.3 (80.1)</td>
<td>26.8 (2.9)</td>
<td>11.1 (0.5)</td>
<td>2; 6.2</td>
<td>9</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>LTG</td>
<td>33</td>
<td>307.6 (13.6)</td>
<td>9.2 (1.0)</td>
<td>11.1 (0.5)</td>
<td>2; 6.1</td>
<td>9</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>CBZ</td>
<td>40</td>
<td>829.9 (25.5)</td>
<td>9.9 (1.3)</td>
<td>20.5 (1.0)</td>
<td>25; 62.5</td>
<td>9</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>PB</td>
<td>31</td>
<td>136.4 (9.8)</td>
<td>27.8 (2.4)</td>
<td>18.5 (1.5)</td>
<td>13; 41.9</td>
<td>9</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>VPA</td>
<td>37</td>
<td>946.4 (28.3)</td>
<td>79.2 (9.4)</td>
<td>10.4 (0.5)</td>
<td>3; 8.1</td>
<td>10</td>
<td>17</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are given as means (SE). OXC, oxcarbazepine; TPM, topiramate; LEV, levetiracetam; LTG, lamotrigine; CBZ, carbamazepine; PB, Phenobarbital; VPA, valproate; MTHFR, methylenetetrahydrofolate-reductase.

At least <0.05 as compared to LEV, LTG, and VPA group (see the section “Results” and Table 3 for further details).
A significant difference in plasma tHcy values was also seen across patients stratified on the basis of pharmacotherapy ($\chi^2 = 63.4$, d.f. = 6, $p < 0.0001$) (Table 2). Increase in tHcy was associated with carbamazepine (CBZ), phenobarbital (PB), topiramate (TPM), and oxcarbazepine (OXC) therapy, even after controlling for sex, age, vitamin B$_{12}$, folate, and presence of the TT677 MTHFR genotype (Table 3). The residuals of both GLZ ($p = 0.5$) and multiple regression designs ($p = 0.4$) had a normal distribution, as evaluated by a chi-square-fitting test.

**DISCUSSION**

The present data confirm previously published data showing that epileptic patients on chronic AED therapy are more prone than the general population to develop hyper-tHcy and low folate levels (Verrotti et al., 2000; Caccamo et al., 2004; Belcastro et al., 2007; Tan et al., 2009). The novel finding deriving from the present investigation is that TPM and OXC among of the recent AEDs can cause hyper-tHcy, whereas lamotrigine (LTG) and levetiracetam (LEV) are devoid of this effect. TPM is eliminated predominantly through renal excretion (approximately 80% in monotherapy), and is a mild inducer of drug metabolizing enzymes of the liver (Patsalos & Perucca, 2003; Bialer et al., 2004). One of the few well-demonstrated interactions in this sense is that with ethinylestradiol, the oral clearance of which is significantly increased in presence of TPM when given at doses of >200 mg/day; below this dose, the induction effect of TPM is negligible (Patsalos & Perucca, 2003; Bialer et al., 2004). The majority of our patients were taking doses above 200 mg/day, and this is most likely the reason for which plasma tHcy levels in the TPM group were significantly higher than those of the control group (Table 2).

OXC, which is structurally similar to CBZ, is a prodrug that metabolizes to the active mono hydroxy-derivative 10-hydroxy carbazepine. In recent times, this drug has to some extent replaced CBZ in clinical practice. As compared to the latter, OXC exhibits a reduced capacity of inducing the hepatic metabolism, and hence of interacting with other drugs (Patsalos & Perucca, 2003). In particular, a daily OXC dose of 1,200 mg has been observed to have an induction potential of less than 50% as compared to that of CBZ at a dose of 800 mg (Andreasen et al., 2007). Our patients were taking a mean daily OXC dose of approximately 1,500 mg, and in this group about one-third exhibited supraphysiologic levels of tHcy (Table 2).

As expected, plasma tHcy levels were higher in carriers of the TT677 MTHFR mutation (Tables 1 and 3). Homozygous 677TT genotype individuals, in fact, exhibit the lowest (i.e., a reduction of >50%) MTHFR enzymatic activity as compared to other genotypes (Chango et al., 2000).

Plasma tHcy levels in patients stabilized on LTG and LEV therapy were significantly lower than those observed in the other drug groups and not significantly different from those observed in the controls and in the valproate (VPA) group (Tables 2 and 3). Both drugs are, in fact, devoid of inducing effects (Patsalos & Perucca, 2003). VPA, a known inhibitor of liver metabolism, has been seen to cause elevated tHcy levels in pediatric populations through a mechanism not yet clarified (Verrotti et al., 2000; Attilakos et al., 2006). In a recent study on adult patients, however, LTG and VPA, in monotherapy, did not induce increased tHcy levels (Gidal et al., 2005).

Concerning the hypothesized pathogenic role of hyper-tHcy in vascular diseases and brain atrophy, this still remains a medical dilemma. Early epidemiologic studies have suggested that patients with epilepsy have a greater prevalence of cardiovascular and cerebrovascular disease than seen in the general population (Annegers et al., 1984; Nilsson et al., 1997). This risk has been confirmed by more recent investigations (Cleary et al., 2004; Gaitatzis et al., 2004; Elliott et al., 2007; Hamed et al., 2007). Among the various variables analyzed, increased total homocysteine has been indicated as an independent risk factor for the above-mentioned diseases (Hassan et al., 2004; Elliott et al., 2007; Hamed et al., 2007;
Gorgone et al., 2009). This is a very attractive hypothesis, since a condition of hyper-tHcy would be easily corrected by intake of folate and other B vitamins (Huemer et al., 2005; Reynolds, 2006; Belcastro et al., 2007). Despite of the entirety of the aforementioned observations, however, a clear association between elevated plasma tHcy levels and vascular disease/brain atrophy has not been definitely demonstrated. A recent meta-analysis on data from recent large randomized controlled trials has concluded that lowering plasma tHcy concentrations with folic acid and other B vitamins does not produce any clinical benefit in the prevention of stroke and coronary heart disease (Bazzano et al., 2006). However, some criticism has been raised concerning the design and the statistical power of these studies, the main concern being the insufficient size of the populations studied to draw firm conclusions (Clarke et al., 2006). The hypothesis that hyper-tHcy could be a mere indicator of vascular disease rather than a causative agent has been also advanced (Herrmann et al., 2007; Moat, 2008).

A recent study (Tan et al., 2009) showed that the duration of AED therapy is significantly associated with the acceleration of atherosclerosis in patients with epilepsy, as revealed by measurement of intima media thickness of the common carotid artery and other parameters. These results are in line with those of other studies (Elliott et al., 2007; Hamed et al., 2007). In the study of Tan et al. (2009), hyper-tHcy, although not significantly associated with atherosclerosis, has been suggested to play a contribution role through indirect complex mechanisms. These may include overproduction of reactive oxygen species, impairment of cellular oxidative defense mechanisms, and increased release of inflammatory mediators (Wang et al., 2005; Tyagi et al., 2006).

An additional aspect to be considered, and strictly associated to hyper-tHcy, is the AED-induced folate deficiency, a condition that itself may have different clinical implications (Reynolds, 2006; McNulty & Scott, 2008). In the general population, for example, maternal folate deficiency has been linked with the development of neural tube defects and periconceptional folate supplementation with a reduction of risk (Czeizel, 2004; Wolff et al., 2009). Although this has not been firmly established in women with epilepsy (Morrow et al., 2009), preconceptional and gestational folate supplementation at a daily dose of 0.4 mg has been recommended also in this population (Harden et al., 2009). Concerning folate dose to be prescribed in various clinical conditions, however, no unanimous consensus exists among experts, and a wide range, from <0.5 to 5 mg daily, can be found in the literature (McNulty & Scott, 2008; Sauer et al., 2009). In our previous study (Belcastro et al., 2007), for example, we used a daily folate dose of 5 mg, which was sufficient to normalize, in a population of hyper-tHcy epileptic patients, both plasma tHcy (ranging from 16.6–38 μM) and folate levels (ranging from 3.8–7.5 nM). Given the general agreement that the bioavailability of natural food folate is incomplete, folate fortification has been adopted in the United States, and this strategy should be extended in other countries to minimize the problem. More recently, concern has been raised about possible adverse effects of excessive folate supplementation, which might be harmful in individuals at higher risk, for example, for cancer and cardiovascular disease (Sauer et al., 2009). Further research is needed to clarify this important aspect.

In this context, our data might have practical implications. In fact, we have found that, apart from patients taking older AEDs, a percentage of 30–60% of patients stabilized on chronic OXC or TPM therapy exhibit supraphysiologic levels of tHcy (Table 2) and low folate values (Tables 1 and 3). In the case of well-controlled patients, a supplement of folate could be prescribed to normalize plasma tHcy levels (Huemer et al., 2005; Reynolds, 2006; Belcastro et al., 2007). Conversely, if patients are not satisfactorily controlled by OXC and or TPM, a switch to another drug, like LTG or LEV among the new AEDs, or VPA among the older ones, could be taken into consideration, as indicated by the present data and those of other recent studies (Gidal et al., 2005; Mintzer et al., 2009).

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We confirm that we have read the Journal’s position on issue involved in ethical publication and affirm that this report is consistent with those guidelines.

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