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Chapter IX

Methionine, Homocysteine and Cysteine, and Antiepileptic Drugs in Epilepsy

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Abstract

The last decade has seen many studies on hyperhomocysteinemia (HHcy) in epileptic patients treated with antiepileptic drugs (AEDs). It has been shown that HHcy occurs in 10-40% of the epileptic population and that AEDs pharmacotherapy has a fundamental effect on the levels of homocysteine (Hcy). The literature indicates that carbamazepine (CBZ) and valproic acid (VPA) therapy in epileptics leads to an increase of Hcy levels. It has also been indicated that the levels of cysteine (Cys) in epileptic patients treated with AEDs change, which may result in disturbed transformation of Cys to glutathione (GSH), an important factor in the maintenance of redox homeostasis in the body.

The study included 63 epileptic patients and 38-61 controls. The levels of Hcy, Met, Cys and GSH were determined by high-performance liquid chromatography (HPLC) with electrochemical detection.

The present study demonstrates that AEDs treatment in epileptics leads to an increase in Hcy ($p<0.001$) and GSH ($p<0.01$), a decrease in Cys ($p<0.001$), and relatively unchanged methionine (Met) concentrations, with disturbed levels of Met:Hcy ($p<0.01$) and Cys:Hcy ($p<0.001$) ratios. Carbamazepine monotherapy ($p<0.05$) and AEDs polytherapy ($p<0.001$) and long-term therapy ($p<0.001$) compared to controls appear to be related to disturbances in both Met:Hcy, and Cys:Hcy ratios. Additionally, it was found that after VPA or CBZ monotherapy and long-term therapy of the two AEDs together lead to a reduction of the level in Cys (VPA, $p<0.05$; long-term therapy, $p<0.01$

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compared to controls) and increased the level of GSH (CBZ, $p<0.001$; long-term therapy, $p<0.01$ compared to controls). In patients with epilepsy before treatment with AEDs, there were slightly changed Hcy and Cys levels but significantly reduced levels of Met ($p<0.05$) and statistically insignificantly increased levels of GSH.

It seems that AEDs pharmacotherapy, rather than epilepsy, leads to increases in Hcy levels, especially in patients treated in polytherapy and long-term. In patients with epilepsy before AEDs therapy, the remethylation process of Hcy appears to be disrupted and after AEDs treatment, especially after VPA therapy, there seems to be a disruption in the transsulfuration process of this thiol. Our study indicated that oxidative stress (measured as levels of GSH) was increased in patients with epilepsy both before and after treatment with AEDs (in particular CBZ and long-term therapy), but it appears to occur via different mechanisms. It also seems that, in epileptic patients treated with AEDs, only therapy with B-vitamins and folates can effectively help regulate the levels of the vascular disease factor Hcy and the metabolism of biothiols (Hcy, Met, Cys, GSH).

Introduction

The last decade has seen many studies on hyperhomocysteinemia (HHcy) in epileptic patients treated with antiepileptic drugs (AEDs). It has been shown that HHcy occurs in 10-40% of the epileptic population [1,2], and that AEDs pharmacotherapy has a fundamental effect on the levels of homocysteine (Hcy) in both children [3-5] and adults [6-10].

The literature indicates that carbamazepine (CBZ) therapy in epileptics leads to an increase of Hcy levels [4,5,8,11]. Treatment with valproic acid (VPA), however, has variable effects; in some cases, the Hcy level is decreased [12], in others it is increased [3-5,8], and in yet others VPA has no effect on Hcy levels [7,13,14]. Lamotrigine (LTG) treatment of epileptic patients has not been shown to lead to an increase in Hcy [12,15].

Furthermore, it has been demonstrated that a specific predisposition to generate circulating Hcy in patients with gene polymorphisms in both the cystathionine beta-synthase (*CBS*) and methylenetetrahydrofolate reductase (*MTHFR*) C677T [2,7] and A1298C [16,17] genes is an important factor in the levels of HHcy in epileptics treated with AEDs. The study by Mudd et al.. [18] shows that 20% of the heterozygote CBS patients have epileptic seizures when circulating Hcy increases from 50 to 200 μM. It has also been shown that, in women with generalized idiopathic epilepsy, the frequency of the *MTHFR*, TT (C677T) homozygote is increased, with this polymorphism also possibly increasing the risk of becoming an epileptic [19]. The literature indicates that Hcy formation in epileptics treated with AEDs may also involve 5,10-methylenetetrahydrofolate reductase (MTR). Studies on animal models have shown that phenytoin administration can both decrease [20] and increase [21] MTR activity.

It is also known that the three-functional enzyme methylenetetrahydrofolate dehydrogenase/ methynyltetrahydrofolate cyclohydrolase/ formyltetrahydrofolate synthetaze (MTHFD1) is involved in the regulation of circulating Hcy. It has been shown that *MTHFD1* homozygotes experience an increase in Hcy levels in heart disease and dysgenesis of the neural tube dependent on folate (FA) levels [22], while *MTHFD1* GA (G1958A) heterozygotes with Alzheimer's or Parkinson's disease have variable cysteine (Cys) to Hcy ratios (Cys:Hcy) [23].

Moreover, it has been demonstrated that both heterozygote and homozygote individuals with the *MTHFR* (C677T) polymorphism have a significantly increased level of Hcy and decreased levels of FA, especially in polytherapy with AEDs [7,13,24]. However, the study by Sniezawska et al.. [10] shows greater increases in Hcy concentration during AEDs treatment of epileptics with the *MTHFR* CT (C677T) and *MTHFD1* GG (G1958A) genotypes.

Additionally, it has been shown that the MTHFT, MTR, and MTHFD1 enzymes take part in converting Hcy to methionine (Met), and changes in the activity of these enzymes can have an effect on the effectiveness of the process of remethylation from Hcy to Met, as well as the levels of these compounds [21]. Ono et al.. [25] demonstrated that the level of Met is decreased in epileptics treated with AEDs. However, the study by Sniezawska et al.. [10] shows greater decreases in Met concentration during AEDs treatment of epileptics with the *MTHFR* CC and CT (C677T), *MTHFD1* GG and GA (G1958A), and *MTR* AA and AG (A2756G) genotypes.

In the body, Hcy is a point of intersection of two main metabolic pathways: transsulfuration and remethylation. Under physiological conditions, approximately 50% of Hcy is catabolized by transsulfuration and undergoes transformation to Cys and then to glutathione (GSH). The remaining 50% of Hcy undergoes remethylation to Met. Methionine is supplied with food and its transformation to Hcy involves several steps. At the first step, Met is transformed to SAM (S-adenosylmethionine) and then to SAH (S-adenosylhomocysteine), and hydrolyzed to Hcy (Figure 1). Agnati et al.. [26] showed that Hcy may pass the blood/brain barrier and that the level of plasma Hcy corresponds to the Hcy concentration in the brain. Elevated levels of Hcy in the central nervous system (CNS) may damage vascular endothelium, deteriorate the functionality of the blood/brain barrier, lead to disturbed production of nitrogen oxide, and to neurotoxic effects both in the senescent brain and in neurological diseases.

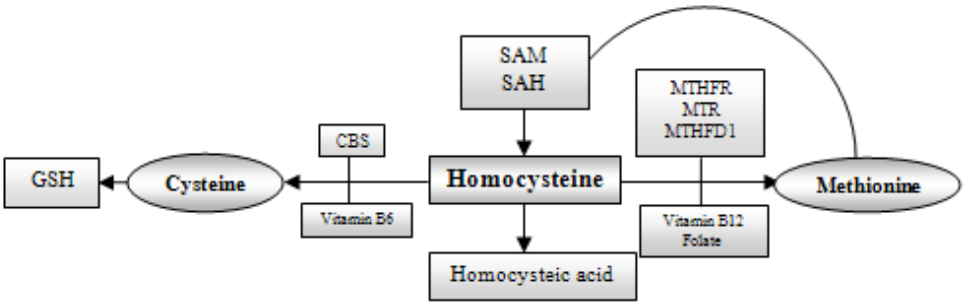


Figure 1. Synthesis and metabolic pathways of homocysteine, CBS- cystationine β-synthase, GSH-glutathione, MTHFR- 5,10-methylenetetrahydrofolate reductase, MTR- methionine synthase, MTHFD1- methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase, SAH- S-adenosylhomocysteine, SAM-S-adenosylmethionine.

Homocysteine or it oxidative product, homocysteic acid (Figure 1), are reported as producers of convulsions (Figure 2). In animal models, administration of Hcy leads to generalized seizures that can be prevented by N-methyl-D-aspartate (NMDA) or non-NMDA receptor antagonists [27,28]. The study by Folbergrova et al.. [29] demonstrates

prevention of seizures induced by homocysteic acid in rats using a group II metabotropic glutamate receptor (mGluR) agonist. Simultaneously, in the rat cortex, hippocampus, and brain stem, a cyclic product of Hcy, thiolactone, significantly inhibits Na+/K+-ATPase activity, which may contribute at least in part to the understanding of the excitotoxic and convulsive properties of Hcy [30]. In humans, only in homocystinuric patients but not in patients with HHcy, seizures have been observed due to a CBS deficit [31]. The role of HHcy in the development of oxidative stress and DNA damage to the genes encoding proteins involved in epileptogenesis must also be considered.

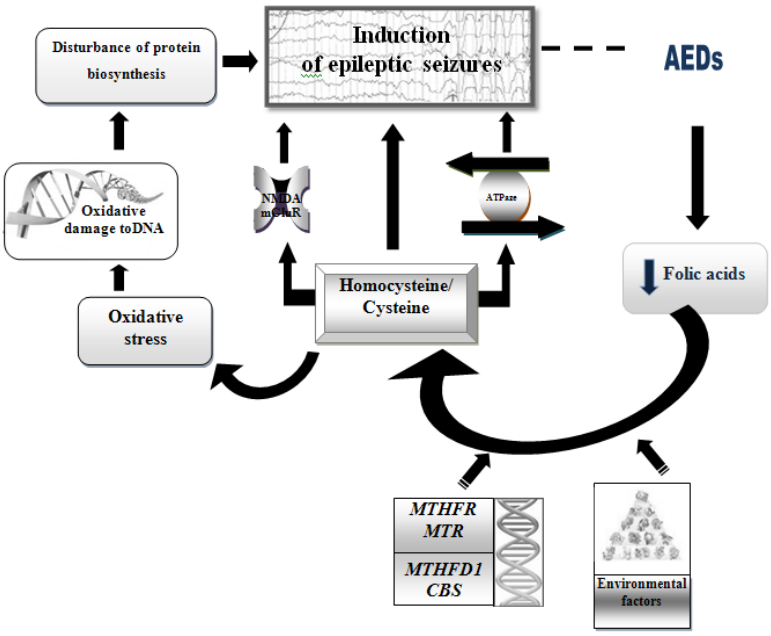


Figure 2. Homocysteine, cysteine and epileptic seizures, AEDs- antiepileptic drugs, CBS- cystationine β-synthase, MTHFR- 5,10-methylenetetrahydrofolate reductase, MTR- methionine synthase, MTHFD1- methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase, NMDA- N-methyl-D-aspartate receptor, mGluR- group II metabotropic glutamate receptor.

Biothiols and Antiepileptic Drugs in Epileptic Patients

There is much published literature demonstrating an increase in the plasma concentration of Hcy in epileptic patients treated with AEDs. These articles indicate that pharmacologic treatment in epileptic patients can lead to an increase in Hcy concentration that may be due to various factors, including diet, the type of pharmacologic agents used in treatment, the length of treatment, and genetic factors.

It seems that one of the significant factors affecting the generation of Hcy in epileptic patients treated with AEDs is the patient's diet, especially the presence of essential cofactors

for the metabolism of Hcy (vitamins B6 and B12, FA). It also appears that the demonstrated lack of Hcy concentration increase after AEDs seen in studied American populations [12,32, 33], in contrast to European (Greece, [5]; Germany, [6]; Italy, [3]; Norway, [7]; Poland, [10,34]), Japanese [25], Korean [13], Turkish [4,15], and Egyptian [8] populations, is most likely due to the diet consumed by the majority of Americans, a diet rich in the cofactors necessary for remethylation of Hcy to Met (FA) [35].

It is also known that insufficiency of vitamin B12 and low FA levels may lead to a decreased efficiency in the remethylation process of Hcy, which results in a decrease of Met concentration. The studies by Ono et al.. [25] and Sniezawska et al.. [10] show decreased Met concentrations in patients with epilepsy after AEDs treatment. Additionally, the study by Badiou et al.. [11] in epileptic patients with HHcy shows increased Cys levels after CBZ treatment as compared to oxcarbazepine (OXCBZ) therapy. However, the study by Apeland et al.. [9] in patients with epilepsy treated with AEDs demonstrates an increased level of total Cys with perturbation of the redox status of biothiols. Similar changes have been observed in the aminothiol redox status in patients with cardiovascular disease, where an alteration in plasma redox was evidenced by the decrease in thiol/disulfide ratios of Cys, Hcy, and cysteinylglycine. In all analyzed groups in this study, Cys was directly correlated with Hcy but not with GSH or cysteinylglycine, which in turn were correlated each other. It seems that in patients with heart diseases the levels of plasma Cys were more linked to Hcy than to the metabolism of GSH [36].

There is little information in the literature on the correlation between Hcy, Cys and GSH in patients with epilepsy treated with AEDs.

Homocysteine and Methionine Levels and Antiepileptic Drugs

The purpose of this study was to analyse the levels of the sulphur-containing amino acids Hcy and Met in epileptic patients treated with VPA, CBZ, new generation (NG) AEDs and patients treated by polytherapy, as well as patients before AEDs treatment and control group.

Patients

The studies were conducted on 63 patients with idiopathic, cryptogenic epilepsy, including 28 women and 35 men, aged 18-65 years (mean age: 35.3±13.8 years). Among the epileptic patients, 55 of them, 24 women and 31 men aged 18-65 years (mean age: 35.8±13.4 years), were treated with variable AEDs. Eight patients, 4 women and 4 men aged 18-65 years (mean age: 31.7±17.2 years), were preparing to begin AEDs treatment.

Among the patients treated with AEDs, 67% of them were on monotherapy [VPA, 59%; CBZ, 27%; NG – mainly LTG and OXCBZ – 14%], and 33% were receiving polytherapy. In the polytherapy group, 61% were taking VPA and NG AEDs, 22% were taking VPA and CBZ, and 17% were taking CBZ and NG AEDs. In the overall group of patients receiving AEDs, 31% had been receiving AEDs for fewer than 5 years, while 69% had been receiving AEDs for over 5 years.

In the group of patients treated with AEDs, the mean levels of VPA and CBZ were within the reference values and averaged 57.1 μg/ml and 6.8 μg/ml, respectively.

The control group included 61 individuals, 41 women and 20 men, aged 22-67 years (mean age: 44.3±14.2 years).

The classification of epilepsy and/or epileptic syndrome suffered by each patient was verified according to the criteria and terminology recommended by the Commission on Classification and Terminology of The International League Against Epilepsy [37].

None of the epileptic patients and control subjects (epileptic patients and controls – Caucasian), were diagnosed with impaired liver or kidney function. None of the patients and controls had been receiving B group vitamin or FA supplementation, and testing was conducted a few hours before the patients took their AEDs dose.

None of the control subjects had verifiable symptoms of dementia or any other neurological disorders or smoking and drinking habits, and receiving B group vitamin or FA supplementation.

A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

Analysis of Hcy and Met Concentrations

Preparation of Samples

The analyzed plasma thiol compounds (Hcy, Fluka Germany; Met, Sigma, USA) were diluted with water at a 2:1 ratio and reduced using 1% TCEP [Tris-(2-carboxyethyl)-phosphin-hydrochloride; Applichem, Germany] at a 1:9 ratio. Subsequently, the sample was deproteinized using 1M $HClO_4$ (at a 2:1 ratio) and applied to the HPLC/EC system.

Determination of Hcy and Met Concentration

The samples were fed to the HPLC system (P580A; Dionex, Germany) coupled to an electrochemical detector (CoulArray 5600; ESA, USA). The analysis was performed in Termo Hypersil BDS C18 column (250mm x 4.6mm x 5μm) [Germany] in isocratic conditions, using the mobile phase of 0.15 M phosphate buffer, pH 2.9, supplemented with 12.5-17% acetonitrile for estimation of Hcy and Met [38].

The system was controlled and the data were collected and processed using Chromeleon software (Dionex, Germany).

Results

The levels of Hcy and Met were studied in the plasma of epileptic patients treated by AEDs in monotherapy (VPA, CBZ, NG AEDs), epileptics treated by polytherapy (VPA+NG AEDs, CBZ+NG AEDs, VPA+CBZ), epileptics before AEDs treatment, and the control group.

The study revealed that the epileptics being treated with various AEDs have a significant increase in their plasma concentration of Hcy (one-way ANOVA test, $p<0.05$ compared to controls) [Table 1]. It was also shown that an increase in Hcy concentration above the minimum required to diagnose mild HHcy (above 16 μM) occurred in 27% of the patients,

while an increase above the normal range (above 15 μM) occurred in 36% of epileptics treated with AEDs. Furthermore, the concentration of Met was significantly decreased only in epileptics who had not yet commenced treatment with AEDs (one-way ANOVA test, $p<0.05$ compared to controls). In both patients treated with AEDs and awaiting AEDs treatment, a decreased Met:Hcy ratio was observed (one-way ANOVA test, for AEDs-treated patients, $p<0.01$ compared to controls).

Table 1. Concentrations of Hcy and Met in epileptic patients before AEDs treatment AEDs(-), after beginning AEDs treatment AEDs(+), and controls

Analyzed compound	Control group	Epileptic patients		p
		AEDs(-)	AEDs(+)	
Hcy [μM]	11.9 ± 4.0	12.9 ± 3.2	14.5 ± 6.7*	0.0383
Met [μM]	22.6 ± 6.0	15.7 ± 9.2*	19.7 ± 7.9	0.0180
Met:Hcy	2.1 ± 0.9	1.4 ± 0.6	1.5 ± 0.8**	0.0011

Results are presented as mean ± SD.
One-way ANOVA for independent variables was used.
Statistically significant differences at *p<0.05, **p<0.01 compared to controls.

Statistically significant differences in Spearman test:

Control group:

- Spearman R coefficient +0.6286, $p<0.0001$ between Met and Met:Hcy.

AED(-) patients:

- Spearman R coefficient +0.8108, $p=0.0269$ between Met and Met:Hcy.

AED(+) patients:

- Spearman R coefficient -0.3479, $p=0.0099$ between Hcy and Met:Hcy.
- Spearman R coefficient +0.6215, $p<0.0001$ between Met and Met:Hcy.

Taking into account the type of pharmacotherapy used in epileptic patients, it was observed (Table 2) that the concentration of Hcy was significantly increased only in the patients undergoing polytherapy (one-way ANOVA test, $p<0.01$ compared to controls) and there was an essentially unchanged Hcy concentration only in the patients taking NG AEDs. The concentration of Met was decreased the most in patients taking CBZ and essentially unchanged in those treated with VPA. Moreover, the patients treated with VPA did not demonstrate a decrease in the Met:Hcy ratio (one-way ANOVA test, for patients treated with CBZ, $p<0.05$ compared to controls; for patients treated by polytherapy, $p<0.001$ compared to controls).

Considering the length of time AEDs treatment was used, long-term (more than 5 years) treatment of epileptics with various AEDs (Table 3, Figure 3) lead to a significant increase of Hcy concentration (Mann-Whitney test, $p<0.001$ compared to patients treated for less than 5 years with AEDs). Furthermore, it was shown that long-term treatment with AEDs insignificantly decreased the levels of Met while significantly decreasing the Met:Hcy ratio (Mann-Whitney test, $p<0.001$ compared to short-term treatment with AEDs).

Table 2. Concentrations of Hcy and Met in epileptic patients treated with VPA, CBZ, NG AEDs, and polytherapy, AEDs(+) before starting AEDs(-) treatment, and controls

Analyzed compound	Control group	AEDs(-)	Epileptic patients				p
			VPA	CBZ	NG AEDs	Polytherapy	
Hcy [μM]	11.9 ± 4.0	12.9 ± 3.2	13.0 ± 5.0	14.2 ± 4.8	11.6 ± 4.1	17.2 ± 9.0**	0.0126
Met [μM]	22.6 ± 6.0	15.7 ± 9.2	22.9 ± 9.0	16.1 ± 5.4	17.6 ± 7.4	18.5 ± 6.5	0.0069
Met:Hcy	2.1 ± 0.9	1.4 ± 0.6	2.0 ± 0.9	1.2 ± 0.5*	1.6 ± 0.9	1.1 ± 0.3***	0.0001

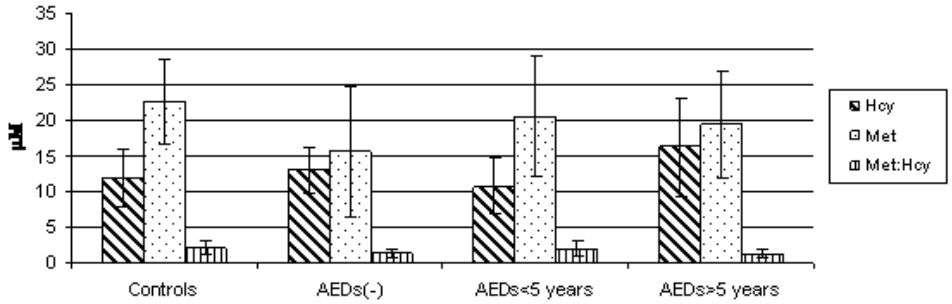
Results are presented as mean ± SD.
One-way ANOVA for independent variables was used.
Statistically significant differences at *$p<0.05$ between patients treated with CBZ and controls, and at **$p<0.01$, ***$p<0.001$ between patients treated with polytherapy and controls.

Table 3. Concentrations of Hcy and Met in epileptic patients, treated with various AEDs, depending on length of treatment

Analyzed compound	Epileptic patients treated with AEDs		P
	Less than 5 years	More than 5 years	
Hcy [μM]	10.7 ± 3.9	16.2 ± 7.0***	0.0001
Met [μM]	20.5 ± 8.5	19.4 ± 7.6	0.2603
Met:Hcy	2.0 ± 1.0	1.3 ± 0.6***	0.0010

Results are presented as mean ± SD.
Non-parametric Mann-Whitney test for independent variables was used.
Statistically significant differences at ***p<0.001 compared to patients treated with AEDs for less than 5 years.

It seems that generation of Hcy in epileptic patients is affected mainly not by the disease itself, but the pharmacologic treatment for the disease. This is demonstrated by studies conducted on a Greek population, in epileptic children aged 4.5 to 14 years treated by AEDs for 20 weeks [5], by studies conducted on epileptic adult patients treated with AEDs in mono- and polytherapy for over 30 days [6], and by a study conducted in patients awaiting treatment with AEDs. The last mentioned study was conducted by Verrotti et al.. [3] in children, and is similar to our present study that was conducted on patients aged 18 to 65 years.



Results are presented as mean ± SD, one-way ANOVA test was used, for Hcy and Met:Hcy, p<0.001 between AEDs>5 years and controls, p<0.01 between AEDs<5 and AEDs>5 years.

Figure 3. Concentrations of Hcy and Met in epileptic patients with AEDs treatment less and more than 5 years, before AEDs(-) treatment, and controls.

The generation of Hcy in epileptic patients, as demonstrated by the literature, is related both to AEDs (such as phenytoin, phenobarbital, primidone, and CBZ) inducing microsomal liver enzymes and increasing cytochrome P450 enzyme activity, which leads to a decrease in FA serum concentration, as well as AEDs (such as VPA) that induce liver enzymes to a smaller degree and have a lesser effect on the concentration of FA in blood serum [39]. An increase in Hcy and accompanying decrease in FA concentration in epileptic patients after receiving CBZ was demonstrated in the studies by Verrotti et al.. [3], Karabiber et al.. [4] and Attilakos et al.. [5]. In epileptics taking VPA, an increase in Hcy concentration was demonstrated both accompanying a decrease in FA levels [3,4] and without FA level changes [5].

It is believed that a decrease in FA concentration during AEDs treatment is due to impaired intestinal absorption of FA, an increase in FA usage required for AEDs

hydroxylation, the activation of liver enzymes leading to a final decrease in FA concentration, direct interactions between FA and AEDs caused by structural similarities between coenzymes for FA and AEDs, or a direct effect of AEDs on Hcy metabolism and kidney function [40].

However, there are also reports in the literature demonstrating a lack of AEDs effect both on inducing liver enzymes and on live enzyme activity changing the levels of circulating Hcy. Both in Sener et al.. [15] and in the present study, no significant increase in Hcy concentration was detected in epileptic patients treated with CBZ, while the articles by Apeland et al.. [9], Unal et al.. [14], and in the present study, there was no significant increase in Hcy after VPA treatment. The lack of significant increase in Hcy concentration in epileptic patients treated with CBZ and VPA may be due to these AEDs having only a small influence on the concentration of cofactors (the literature has shown definite effects of AEDs on the levels of FA and vitamin B12) essential to the remethylation of Hcy [4,5], and additionally in the case of VPA, the articles by Apeland et al.. [7], Gidal et al.. [12], and Attilakos et al.. [5] have shown that VPA increases the concentration of vitamin B12, which most probably prevents the accumulation of Hcy in the blood of epileptic patients.

It is also believed that NG AEDs such as LTG [12,15] and OXCBZ [11] do not change the concentrations of vitamin B12 and FA [12] in epileptic patients, nor do they increase the concentration of Hcy. The current study confirms these observations through the analysis of Hcy levels in epileptic patients receiving NG AEDs in monotherapy. Furthermore, many studies demonstrate that combining NG AEDs therapy, especially with VPA, can lead to increased formation of Hcy [6,8,17,25].

Another predisposing factor for increased formation of serum Hcy seems to be long-term (more than 5 years, [13]) therapy of epilepsy using AEDs. Increased levels of Hcy in patients receiving long-term treatment of CBZ, phenytoin, phenobarbital, and VPA and/or in polytherapy have also been demonstrated in the literature [8,25,41,42, present study]. The study by Hamed et al.. [8] demonstrates that long-term CBZ and VPA therapy take part in blood vessel damage via disturbed metabolism of Hcy and FA.

It is also known that insufficiency of the cofactors needed for the remethylation of Hcy (vitamin B12, FA) can lead to a decreased efficiency of this process, which results in a decrease of Met concentration [10]. A decrease in Met concentration in epileptic patients treated by mono- and polytherapy was demonstrated by Ono et al.. [25] as well as the present study, especially when using medication that decreases the levels of FA (CBZ). This study has also shown that a decrease in the Met:Hcy ratio accompanied the decrease of Met concentration. According to one of the newer theories [43,44], a decrease in the Met:Hcy ratio may be related to the transformation of Hcy to thiolactone in endothelium and mediate Hcy-induced atherosclerotic changes. Folates, which may increase Met and decrease Hcy levels, in vascular endothelial cells also inhibit conversion of Hcy to thiolactone by changing the relative levels accounting for the Hcy: Met ratio [44].

Homocysteine, Cysteine and Glutathione Levels and Antiepileptic Drugs

The purpose of this study was to analyse the levels of the sulphur-containing amino acids Cys and GSH in epileptic patients treated with VPA, CBZ, NG AEDs and patients treated by polytherapy, as well as patients before AEDs treatment and control group.

Patients

Epileptic patients: see point- Homocysteine and methionine levels and antiepileptic drugs.

The control group included 38 individuals, 28 women and 10 men, aged 22-67 years (mean age: 41.8±14.5 years).

Analysis of Hcy Concentrations

Analysis of Hcy levels: see point- Homocysteine and methionine levels and antiepileptic drugs.

Analysis of Cys and GSH Concentrations

Preparation of Samples

The analyzed plasma thiol compounds (Cys and GSH, Sigma, USA) were diluted with water at a 2:1 ratio and reduced using 1% TCEP [Tris-(2-carboxyethyl)-phosphin-hydrochloride; Applichem, Germany] at a 1:9 ratio. Subsequently, the sample was deproteinized using 1M $HClO_4$ (at a 2:1 ratio) and applied to the HPLC/EC system.

Determination of Cys and GSH Concentration

The samples were fed to the HPLC system (P580A; Dionex, Germany) coupled to an electrochemical detector (CoulArray 5600; ESA, USA). The analysis was performed in Termo Hypersil BDS C18 column (250mm x 4.6mm x 5µm) [Germany] in isocratic conditions, using the mobile phase of 0.15 M phosphate buffer, pH 2.9, supplemented with 12.5-17% acetonitrile for estimation of GSH and 0.15 M phosphate buffer, pH 2.8 with 10 % acetonitrile for estimation of Cys [33].

The system was controlled and the data were collected and processed using Chromeleon software (Dionex, Germany).

Results

The levels of Cys, GSH and Hcy were studied in the plasma of epileptic patients treated by AEDs in monotherapy (VPA, CBZ, NG AEDs), epileptics treated by polytherapy

(VPA+NG AEDs, CBZ+NG AEDs, VPA+CBZ), epileptics before AEDs treatment, and the control group.

The study revealed that epileptics being treated with various AEDs have a significant increase in their plasma concentration of Hcy (one-way ANOVA test, $p<0.001$ compared to controls) and GSH (one-way ANOVA test, $p<0.01$ compared to controls), and decreased Cys level (one-way ANOVA test, $p<0.001$ compared to controls) [Table 4]. Furthermore, the concentration of GSH was significantly increased only in epileptics who had been treated with AEDs. In both patients treated with AEDs and awaiting AEDs treatment, a decreased Cys:Hcy ratio was observed (one-way ANOVA test, for AEDs-treated patients, $p<0.001$ compared to controls).

Table 4. Concentrations of Hcy, Cys and GSH in epileptic patients before AEDs treatment AEDs(-), after beginning AEDs treatment AEDs(+), and controls

Analyzed compound	Control group	Epileptic patients		P
		AEDs(-)	AEDs(+)	
Hcy [µM]	10.0 ± 3.5	12.9 ± 3.2	14.5 ± 6.7***	0.0003
Cys [µM]	266.9 ± 56.3	264.8 ± 71.9	221.2 ± 56.6***	0.0009
Cys:Hcy	29.0 ± 13.3	23.2 ± 9.4	17.5 ± 7.6***	0.0011
GSH [µM]	888.3 ± 151.9	1071.7 ± 199.0	1042.3 ± 251.4**	0.0029

Results are presented as mean ± SD.
One-way ANOVA for independent variables was used.
Statistically significant differences at **p<0.01, ***p<0.001 compared to controls.

Statistically significant differences in Spearman test:

Control group:

- Spearman R coefficient +0.6665, $p<0.0001$ between Cys and Cys:Hcy.

AED(+) patients:

- Spearman R coefficient -0.4295, $p=0.0012$ between Hcy and Cys:Hcy.
- Spearman R coefficient +0.6035, $p<0.0001$ between Cys and Cys:Hcy.

Taking into account the type of pharmacotherapy used in epileptic patients, it was shown (Table 5) that the concentration of Hcy was significantly increased only in the patients undergoing polytherapy (one-way ANOVA test, $p<0.001$ compared to controls). The concentration of Cys was decreased the most in patients taking NG AEDs and a significant in those treated with VPA (one-way ANOVA test, $p<0.05$ compared to controls). Moreover, on the patients treated only with NG AEDs did not demonstrated a significant decrease in the Cys:Hcy ratio (one-way ANOVA test, for patients treated with VPA, $p<0.01$; for patients treated with CBZ, $p<0.05$; for patients treated by polytherapy, $p<0.001$ compared to controls). Furthermore, the concentration of GSH was significantly increased only in epileptics who had treated with CBZ (one-way ANOVA test, $p<0.001$ compared to controls).

Considering the length of time AEDs treatment was used, long-term (more than 5 years) treatment of epileptics with various AEDs (Table 6, Figure 4) lead to a significant increase of Hcy concentration (Mann-Whitney test, $p<0.001$ compared to patients treated for less than 5 years with AEDs). Furthermore, it was shown that long-term treatment with AEDs did not

Table 5. Concentrations of Hcy, Cys and GSH in epileptic patients treated with VPA, CBZ, NG AEDs, and polytherapy, AEDs(+) before starting AEDs(-) treatment, and controls

Analyzed compound	Control group	AEDs(-)	Epileptic patients				p
			VPA	CBZ	NG AEDs	Polytherapy	
Hcy [µM]	10.0 ± 3.5	12.9 ± 3.2	13.0 ± 5.0	14.2 ± 4.8	11.6 ± 4.1	17.2 ± 9.0***	0.0002
Cys [µM]	266.9 ± 56.3	264.8 ± 71.9	220.7 ± 55.8*	210.1 ± 51.7	208.0 ± 49.9	232.2 ± 64.4	0.0099
Cys:Hcy	29.0 ± 13.3	23.2 ± 9.4	18.8 ± 7.3**	17.1 ± 8.8*	20.1 ± 8.4	15.4 ± 7.0***	0.0001
GSH [µM]	888.3 ± 151.9	1071.7 ±199.0	1014.1 ± 261.3	1232.3 ± 247.8***	930.0 ± 244.1	1000.2 ± 202.0	0.0005

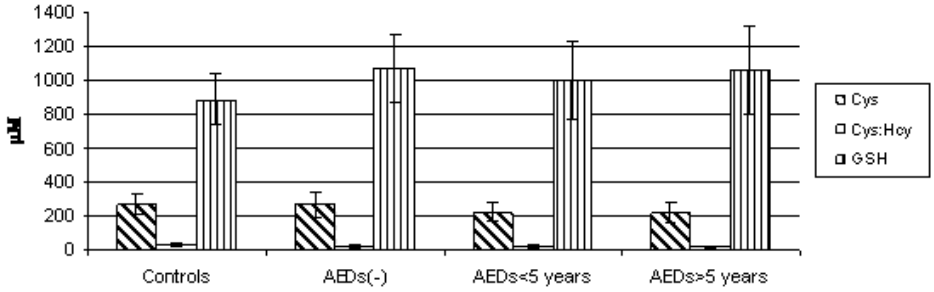
Results are presented as mean ± SD.
One-way ANOVA for independent variables was used.
Statistically significant differences at *p<0.05 between patients treated with VPA, CBZ and controls, and at **p<0.01 between patients treated with VPA and controls, ***p<0.001 between patients treated with CBZ, polytherapy and controls.

change the levels of Cys but did significantly decrease the Cys:Hcy ratio (Mann-Whitney test, $p<0.01$ compared short-term treatment with AEDs), and insignificantly increased GSH concentrations.

Table 6. Concentrations of Hcy, Cys and GSH in epileptic patients, treated with various AEDs, depending on length of treatment

Analyzed compound	Epileptic patients treated with AEDs		P
	Less than 5 years	More than 5 years	
Hcy [µM]	10.7 ± 3.9	16.2 ± 7.0***	0.0001
Cys [µM]	221.0 ± 55.7	221.2 ± 57.9	0.4334
Cys:Hcy	22.3 ± 7.9	15.4 ± 6.4**	0.0030
GSH [µM]	1000.4 ± 235.4	1061.6 ± 259.2	0.3936

Results are presented as mean ± SD.
Non-parametric Mann-Whitney test for independent variables was used.
Statistically significant differences at **p<0.01, ***p<0.001 compared to patients treated with AEDs for less than 5 years.



Results are presented as mean ± SD, one-way ANOVA test was used, for Cys, GSH, $p<0.01$ and Cys:Hcy $p<0.001$ between AEDs>5 years and controls; for Cys, $p<0.05$ between AEDs<5 years and controls.

Figure 4. Concentrations of Cys and GSH in epileptic patients with AEDs treatment less and more than 5 years, before AEDs(-) treatment, and controls.

Under physiological conditions, half of Hcy undergoes transsulfuration and provides Cys. The study by Bugaj et al.. [45] and the present study show that AEDs pharmacotherapy, rather than epilepsy, affects the transformation of Hcy to Cys and Hcy to Met. It also seems that AEDs therapy (especially CBZ and VPA therapies), which leads to decreased levels of Cys and slightly unchanged Met concentrations, is most likely associated with increased metabolism of Cys [45, present study]. However, the study by Badiou et al.. [11] demonstrated an increase in the level of Cys in epileptic patients with HHcy after more than 1 month of therapy with CBZ, which is probably associated with impaired remethylation of Hcy to Met. It appears that the length of AEDs treatment in epileptic patients may affect the level of biothiols. The study by Bochynska et al.. [34] shows a significantly increased level of Hcy in epileptic patients after 1 year of treatment with VPA and unchanged Hcy concentrations in patients with chronic epilepsy treated with VPA for at least 2 years.

The present study also showed a decreased Cys:Hcy ratio, especially in patients with epilepsy treated with AEDs in polytherapy and after long-term therapy. Changed levels of

Cys and Hcy may be associated with impaired saturation in protein and enzymes by the reaction of thiol-disulfide exchange and further modification of protein properties [46]. Additionally, the increase in Hcy and decrease in Cys levels after AEDs treatment of patients with epilepsy may indicate a disturbance of the transsulfuration process of Hcy to Cys in these patients, and diminished transformation of Cys to GSH. Cultured human hepatocytes have demonstrated a transformation of 50% of Cys into GSH [47].

Vitamin B6 is involved in the transsulfuration process of Hcy to Cys, whereas vitamins B2, B12 and FA act as cofactors for its remethylation. A decrease in enzyme or cofactor efficiency, e.g. vitamin B6, may lead to an increase in Cys levels. The study by Apeland et al.. [9] in epileptic patients before B-vitamin supplementation showed an increased total Cys level (total Cys: 260.30±38.62), which was similar to the level in patients with epilepsy in the present study before commencing AEDs therapy. Furthermore, following treatment with B-vitamins, the Cys levels of Apeland et al..'s [9] patients decreased (total Cys: 225.23±41.14) to the level seen in epileptic patients with AEDs treatment in the present study. It seems that B-vitamin supplementation normalized Cys levels and improved the redox status of biothiol.

Homocysteine is redox-active, and its toxic effects have been shown to attribute to direct or indirect perturbation of redox homeostasis [48]. Cysteine is less redox-active than Hcy, and it is converted more slowly and not so completely to its oxidized form and has no demonstrable effect on the glutathione peroxidase activity [48,49]. Upchurch et al.. [49] indicated that cells treated with Hcy show a significant reduction in glutathione peroxidase and this pathway may lead to selective impairment of endothelial cells by this thiol. It is known that, in epileptic patients treated with AEDs, Hcy may lead to endothelium dysfunction [7], evaluation of serum lipids and carotid artery intima media thickness [14,50,51], acceleration of atherosclerosis [8,42], to early stroke or thrombotic events [14], and increase the risk for cardiovascular and cerebrovascular diseases [52].

Perturbations of Hcy and Cys redox-status have been reported after treating pregnant mice intraperitoneally with VPA [53]. In the plasma of these mice, Met and serine levels decreased, while Hcy and Cys concentrations increased. Additionally, a decrease of reduced GSH with no total GSH change has been demonstrated in experimental animals receiving AEDs. The reduced GSH removes hydrogen peroxide in a reaction catalyzed by glutathione peroxidase and the depletion of GSH, a common event in damage-induced apoptosis, is necessary and sufficient to induce cytochrome c release, the key event in apoptotic cell death via this pathway [54]. The results of studies evaluating the effects of VPA on oxidant status in epileptic patients are varied. The study by Yis et al.. [55] in epileptic patients treated with VPA shows an imbalance between oxidant-antioxidant status with unchanged levels of glutathione peroxidase activity. A generation of oxidative stress by VPA as a potentially hepatotoxic factor was demonstrated in the study by Chang & Abbott [56]. On the other hand, Sobaniec et al.. [57] found lower levels of glutathione peroxidase in patients receiving VPA, and Verrotii at al. [58] found that VPA therapy does not appear to cause oxidative stress in epileptic children.

It seems that epileptic patients with active epilepsy can show an increase in cell contents of reactive oxygen species and a low antioxidant status. In patients with progressive myoclonus epilepsy of the Unverricht-Lundborg type, oxidative stress has been shown to be due to increased intracellular GSH concentration [59]. The present study demonstrated increased levels of GSH both in patients with epilepsy before AEDs treatment with unchanged Cys levels and after treatment with AEDs with decreased Cys level (in particular

CBZ and long-term therapy), which may confirm that oxidative stress participates in the pathogenesis of epilepsy but appears in different mechanisms involving therapy. Generation of oxidative stress in patients with epilepsy before AEDs treatment is also confirmed by the study of Yuksel et al.. [60], which showed increased lipid peroxidation in newly diagnosed patients.

Many studies have demonstrated the success of B-vitamin and FA supplementation in decreasing Hcy levels in epileptic patients treated with AEDs [1,2,34]. However, the study by Sydow et al.. [61], conducted on HHcy patients with peripheral vascular disease, demonstrated that B-vitamin supplementation helps regulate only Hcy levels and doesn't have a sufficient effect in decreasing different risk factors of vascular disease e.g. asymmetric dimethylarginine (ADMA) levels [10]. Moreover, the studies by Sydow et al.. [61] and Koga et al.. [62] indicate that, to regulate both Hcy and ADMA, patients require combined therapy with B-vitamins, FA, and arginine (Arg). The effectiveness of this combined therapy on epileptic patients treated with AEDs appears to also be indicated by the result analysis obtained for Met and Cys after treatment with this therapy in the present study.

Conclusion

The present study demonstrates that AEDs pharmacotherapy rather than epilepsy leads to increases in Hcy levels in epileptic patients, especially in those treated in polytherapy and long-term. In patients with epilepsy before AEDs therapy, the remethylation process of Hcy (decreased level of Met) appears to be disrupted, and after AEDs treatment, especially after VPA therapy, so is the transsulfuration process of this Hcy (resulting in a decreased level of Cys). CBZ monotherapy and AEDs polytherapy and long-term therapy appear to be related to disturbances in both Met:Hcy and Cys:Hcy ratios. Our study indicated that oxidative stress (measured as GSH levels) increased both in patients with epilepsy before AEDs treatment and after treatment with AEDs, in particular CBZ and long-term therapy, but this oxidative stress appears in different mechanisms.

It also seems that, in epileptic patients treated with AEDs, only therapy with B-vitamins and FA can effectively help regulate the levels of the vascular disease factor Hcy and the metabolism of biothiols (Hcy, Met, Cys, GSH).

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