Epilepsy is a chronic disorder, characterized by recurrent, unprovoked epileptic seizures with a prevalence of 5 to 10 per 1000 persons and an incidence of 50 to 120 per 100,000 persons per year.¹ Epilepsy affects 70 million people worldwide.² Its prevalence is about 1% in North the Indian population.³ Studies to estimate prevalence and patterns of epilepsy in India found that urban men and women had a higher prevalence of epilepsy compared with rural areas.³ The exact pathophysiology of epilepsy remains unknown, but hyperexcitability and altered synaptic transmission of brain neurons are known to be cardinal features in epileptogenesis.⁴ Accumulating evidence suggest that the sulfur-containing amino acid homocysteine plays a role in various developmental disorders.⁵ Two main factors affect homocysteine concentration in humans: diet (mainly intake of folate and vitamin B₁₂) and polymorphisms in genes that encode enzymes or transport proteins involved in folate and vitamin B₁₂-dependent homocysteine metabolism. This is a complex series of metabolic pathways crucial for DNA synthesis repair and a wide range of methylation reactions. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in one-carbon
metabolism. This enzyme catalyzes the conversion of 5,10-methylentetrahydrofolate into 5-methyltetrahydrofolate, the predominating circulating form of folate. 5-Methyltetrahydrofolate participates in the vitamin B12-dependent remethylation of homocysteine to methionine, which in turn is converted to S-adenosylmethionine that serves as a methyl group donor in the methylation of DNA, proteins, neurotransmitters, and phospholipids. MTHFR gene polymorphisms are commonly associated with hyperhomocysteinemia. The MTHFR C677T (rs 1801133) is a common mutation of the MTHFR gene with a C to T transition, located at nucleotide 677. This mutation results in a change of amino acid from alanine to valine, leading to the reduced activity of the enzyme. A 10% to 40% proportion of epileptic patients develop hyper-total-homocysteinemia as a consequence of the intake of enzyme-inducing antiepileptic drugs (AEDs), which deplete organisms of vitamin B serving as cofactors/substrates in homocysteine metabolism, and the presence of C677T polymorphisms implies the reduced activity of 5,10-methyltetrahydrofolate reductase (MTHFR), which converts homocysteine to methionine. Epileptic patients exhibit elevated plasma total homocysteine (tHcy) levels more frequently compared to the general population. This is mainly due to the reduced activity of the key enzyme MTHFR (which regulates 5-methyltetrahydrofolate required for the remethylation of homocysteine to methionine) caused by polymorphisms in the MTHFR gene. As homozygosity of the thermolabile MTHFR mutant allele occurs commonly in 5% to 15% of the various populations, it was postulated that the existence of homozygotes for the mutant allele of MTHFR predisposes epileptic patients to elevated tHcy and further augments the severity of hyperhomocysteinemia in epileptic patients receiving AEDs. The aim of this study was to evaluate the levels of serum folate, vitamin B12, and homocysteine in epileptic patients receiving AED (phenytoin, carbamazepine, and sodium valproate) monotherapy in toxicity and non-toxicity groups, and to evaluate the probable contribution of C677T variant of MTHFR gene polymorphism in hyperhomocysteinemia.

PATIENTS AND METHODS

Study subjects
Epileptic patients were recruited from outpatient and wards of neurology department, AIIMS and Safdarjung Hospital, New Delhi. The diagnoses of epilepsy were based on the classification systems for seizure types provided by the international league against epilepsy. The study protocol was approved by the ethics committee of the institute, and all studies were performed with full and informed consent of the patients.

Group 1: A total number of 100 epileptic patients receiving phenytoin (n=50), carbamazepine (n=25), and valproate (n=25) monotherapy showing adverse drug reactions and toxicity were recruited.

Group 2: A total number of 100 epileptic patients receiving phenytoin (n=50), carbamazepine (n=25), and valproate (n=25) monotherapy without showing adverse drug reactions and toxicity were recruited.

Group 3: A total number of 100 age- and sex-matched normal healthy controls from the general population of North Indian origin were recruited.

Inclusion criteria
Patients of either gender above 5 years of age taking one of the first line AEDs including phenytoin, carbamazepine, and valproate monotherapy were included in the study. Patients with normal therapeutic doses who developed toxicity and adverse drug reactions in the form of nystagmus, ataxia, dysarthria, fatigue, cognitive impairments, tremor, diplopia, gingival hyperplasia, and hirutism were included. Only patients of North Indian origin (Jammu and Kashmir, Uttarakhand, Haryana, Himachal Pradesh, Bihar, and Punjab) were included in the study. An equal number (=100) of age- (±5 years) and sex-matched normal healthy controls were collected from the North Indian origin.

Exclusion criteria
Epilepsy associated with gross neurological deficits (mental retardation, motor/speech) and imaging abnormalities including tumors, tuberculosis, multiple neurocysticercosis, vascular malformations, and atrophic lesions were excluded. Other anticonvulsant drugs, cytochrome P450 inducers like haloperidol, diazepam, rifampicin and CYP inhibitors like sulfonamides, ranitidine, and antipsychotics were excluded from the study. Patients with severe liver/kidney dysfunctions or diabetes mellitus and patients taking phenytoin, carbamazepine, and sodium valproate for less than 1 month were also excluded from the study. Patients taking any form of medication or had surgery or suffered from trauma in the past 30 days were also excluded from the study. Patients of origin other than North India were also excluded from the study.

Sample collection
Venous blood (10 mL) was collected in 2% EDTA tubes (5 mL) and plain vial (5 mL) from each subjects.
participated in the study; serum was isolated within 2 hours by centrifugation (2000g) at 4°C for 20 minutes and stored at −20°C for assaying levels of homocysteine, folic acid, vitamin B$_{12}$, and therapeutic drugs. Peripheral blood leukocytes were also stored at −20°C for extracting DNA and performing molecular tests.

**Therapeutic drug monitoring assay**

For determining serum levels of phenytoin, carbamazepine, and sodium valproate, the blood was collected between 8 AM and 10 AM before the morning dose, and the drug levels were measured by enhanced chemiluminescence enzyme immunoassay method (ImmuliteR, 1000 systems, DPC, USA) following the manufacturer’s protocol. The principle of the assay is based on the competitive displacement of the drug in a sample and the drug labeled with enzyme glucose 6-phosphate dehydrogenase for binding with antibody specific to AEDs. Finally, the absorbance was measured at 340 nm spectrophotometrically (reference values: the normal therapeutic range was 10-20 µg/mL for serum phenytoin, 4-12 µg/mL for carbamazepine, and 50-100 µg/mL level for sodium valproate). The drug level above these therapeutic ranges was considered as toxic level.

**Determination of homocysteine, folate, and vitamin B$_{12}$ levels**

Blood samples (5 mL) were collected from each subject following an overnight fasting period between 8 and 10 AM before the morning dose of anti-epileptic drug. Serum homocysteine, vitamin B$_{12}$, and folic acid concentrations were measured using chemiluminescence enzyme immunoassay method (reference values: 5-15 µmol/L for homocysteine, 3-17 ng/mL for folic acid, and 174-878 pg/mL for vitamin B$_{12}$).

**MTHFR (C677T) genotyping**

DNA was isolated by the conventional phenol–chloroform method. DNA purity, integrity, and damage were assessed using the nanodrop method. Genomic DNA (40 ng) was amplified by the polymerase chain reaction (PCR) in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, Connecticut, USA) using the MTHFR primers as specified earlier (5′-TGA AGG AGA AGT TG1 CTG CGG GA-3′ for the sense oligonucleotide primer and 5′-AGG ACG GTG CGG TGA GAG TG-3′ for the antisense primer). Both primers were synthesized by Geneworks in the following singleplex reaction: 1.75 mM of MgCl$_2$, 1× standard PCR buffer, 0.2 mM of dNTPs, 0.2 µM each of forward and reverse primers, 0.16 g/L of bovine serum albumin, 1 unit of Taq polymerase (Perkin-Elmer, Cetus, USA), 50 ng of genomics DNA, made to a final volume of 25 µL with sterile distilled water. The cycle parameters were as follows: 1 cycle for an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation for 1 minutes at 94°C, primer annealing for 1 minute at 58°C, primer extension for 2 minutes at 72°C, and a final extension for 10 min at 72°C. This amplification reaction resulted in the 198-base pair (bp) fragment. For the restriction digestion, 7 units of Hinfl, 2 µL of NE buffer II (New England Bio labs Inc., USA), and 2.3 µL of sterile water were added to the extension mixture to a final volume of 20 µL, and the samples were digested overnight at 37°C. Hinfl did not digest the fragment derived from the C allele, whereas Hinfl digested the fragment of the same length from the T allele into 175- and 23-bp fragments. These fragments were then electrophoresed using a 12% polyacrylamide gel, visualized under UV light. MTHFR genotypes were detected on the basis of restriction fragment length and unique genotype-dependent band combination (Figures 1 and 2).

**Statistical analysis**

The sample size was calculated by using the software Stat Direct (http://www.statsdirect.com). The study group consisted of 200 epileptic patients (16-60) years and 24 (6) (range 21-42) years, respectively. Demographic and clinical characteristics of epileptic patients enrolled in this study are shown in (Table 1). Of the total 200 epileptic patients, 50 had phenytoin toxicity, 50 had phenytoin non-toxicity, 25 had carbamazepine toxicity, 25 had carbamazepine non-toxicity, 25 had sodium valproate toxicity, and 25 had sodium valproate non-toxicity. The seizure types were generalized tonic–clonic seizures (67%), simple partial seizures with secondary generalization (SPS...
sec. gen., 19.5%), complex partial seizures with secondary generalization (CPS sec. gen., 8.5%), and SPS (3%). The serum phenytoin levels in the toxicity group ranged from 17.3 to 39 µg/mL and those in non-toxicity group ranged from 10.2 to 20.5 µg/mL (the normal therapeutic range was 10–20 mg/mL). The serum carbamazepine drug levels in the toxicity group ranged from 11.4 to 17 µg/mL and those in the non-toxicity group ranged from 5.3 to 12.3 mg/mL (the normal therapeutic range was 4-12 mg/mL). The serum sodium valproate drug levels in the toxicity group ranged from 97 to 131 µg/mL and those in the non-toxicity group ranged from 50.2 to 101 mg/mL (the normal therapeutic range was 50–100 mg/mL). The distribution of the MTHFR (C677T) genotypes for non-toxicity and toxicity groups and control was found to be in Hardy–Weinberg equilibrium (P > .01). The allelic and genotypic frequency for MTHFR (C677T) showed a significant association between toxicity and non-toxicity groups (Table 2): allelic chi square = 9.62, P = .002, odds ratio (OR): 2.84 (1.43-5.68); genotypic chi square = 16.01, P = .0006, OR: 0.31 (0.16-0.57).

However, the allelic and genotypic frequency between the non-toxicity group and controls did not show a significant difference (P > .01). The findings of this study showed a significant increase in mean homocysteine levels in epileptic patients receiving anti-epileptic drug monotherapy (phenytoin carbamazepine, sodium valproate) in toxicity and non-toxicity groups (P < .05) and reduction in mean folic acid and vitamin B12 in toxicity and non-toxicity groups (P < .05) (Table 3).

Homocysteine, folate, and vitamin B12 concentrations according to MTHFR gene polymorphism CC, CT, and TT in epileptic cases and controls are shown in (Table 4).

**DISCUSSION**

There are many published reports demonstrating an increase in the plasma concentration of homocysteine in epileptic patients treated with AEDs. Pharmacologic treatment in epileptic patients can lead to an increase in homocysteine that may be due to various factors, including pharmacotherapy and genetic factors. Furthermore, it appears that the generation of homocysteine in epileptic patients is affected not mainly by the disease itself, but by the pharmacologic treatment for the disease. This is demonstrated by studies conducted on epileptic adult patients treated with AEDs in mono- and polytherapy.18 The elevated plasma level of homocysteine is induced either by genetic or environmental factors. Because MTHFR 677 C>T mutation is known to be associated with reduced enzyme activity and AEDs decrease MTHFR enzyme activity, this study examined whether the presence of MTHFR 677 C>T mutation and AEDs in epileptic patients causes elevated tHcy. The 677C→T (change of an alanine to a valine) is the most common MTHFR polymorphism that, in the homozygous state, implies a decrease of 50% to 60% in the enzymatic activity and low folate levels.17,13 The 677C→T frequency varies among ethnic groups with a T allele frequency oscillating from 1% in African Americans to 30% in Japanese and Europeans (the highest prevalence).13 In the Indian population, the variant T allele in the toxicity group was found to be
Table 2. MTHFR (C677T) allelic and genotype frequencies in epileptic cases and controls.

| Gene polymorphism | Epilepsy with toxicity cases N=100 | Epilepsy with non-toxicity cases (NT) N=100 | Controls N=100 | Allelic chi-square, P value, odds ratio and 95% CI | Genotypic frequency | Genotypic chi-square value, P value, odds ratio and 95% CI |
|-------------------|------------------------------------|---------------------------------------------|---------------|-----------------------------------------------|---------------------|---------------------------------------------------------|
| MTHFR (C677T)     | C=0.60 T=0.40                      | C=0.81 T=0.19                               | C=0.90 T=0.10 | Tox vs NT* 9.62, 0.002, 2.84 (1.43-5.68)        | CC=44               | Tox vs NT* 16.01, 0.0006, 0.31 (0.16-0.57)               |
|                   |                                   |                                             |               | NT vs Con 2.58, 0.180, 2.11 (0.87-5.21)         | CT+TT=56            | NT vs controls 2.82, 0.092, 1.77 (0.86-3.66)           |
|                   |                                   |                                             |               |                                               | CC=72               |                                                         |
|                   |                                   |                                             |               |                                               | CT+TT=28            |                                                         |
|                   |                                   |                                             |               |                                               | CC=82               |                                                         |
|                   |                                   |                                             |               |                                               |                     |                                                         |

*P<.001

Table 3. Mean homocysteine, folic acid, and vitamin B12 levels in toxicity, non–toxicity, and controls.

| Parameters                      | Epilepsy with toxicity (N=100) | Epilepsy with non-toxicity (N=100) | Normal controls N=100 | P value one-way ANOVA |
|---------------------------------|-------------------------------|-----------------------------------|-----------------------|-----------------------|
|                                 | PHT n=50                       | CBZ n=25                          | Val n=25              |                       |
| Homocysteine levels µmol/L mean (SD) | 25.2 (3.6)                    | 28.6 (4.2)                        | 23.9±2.8              |                       |
| Folic acid levels ng/mL mean (SD) | 2.2 (1.2)                     | 2.1 (1.0)                         | 2.3 (1.3)             |                       |
| Vitamin B12 levels pg/mL mean (SD) | 142 (21.6)                    | 151 (19.5)                        | 144 (260)             |                       |

PHT: Phenytoin; CBZ: carbamazepine; Val: valproate; ANOVA: analysis of variance; SD: standard deviation.

(Ref: Values 5-15 µmol/L for homocysteine, 3-17 ng/ml for folic acid, and 174-478 pg/ml for vit B12)

0.40, whereas that in the non-toxicity group was found to be 0.19 and in controls 0.10. A higher increase in mean tHcy levels was observed in epileptic patients receiving AEDs monotherapy (phenytoin, carbamazepine, and sodium valproate) in the toxicity group as compared with non-toxicity and controls groups. The correlations between the levels of serum folate, vitamin B12, and tHcy were shown in numerous studies and was reported that in patients receiving AED, the plasma tHcy level increased due to low levels of folate and vitamin B12.19 The findings of this study are similar to the findings of previous studies as far as serum folate, vitamin B12, and tHcy levels are concerned.

Homocysteine, a thiol-containing amino acid formed by the demethylation of methionine, is an intermediate product in one-carbon metabolism (OCM). Folic acid and vitamin B12 are cofactors of OCM.20 Deficiency of folic acid and vitamin B12 may lead to elevated plasma homocysteine concentrations. However, prolonged VPA therapy is often associated with a wide range of chronic adverse effects including metabolic and endocrine disturbances, atherosclerotic vascular diseases, and major congenital malformations.21 As an important metabolic product in OCM, homocysteine is a risk factor for atherosclerotic vascular diseases (e.g., stroke, myocardial infarction) and major congenital malformations (e.g., neural tube defects-NTDs).22 Hyperhomocysteinemia is frequently caused not only...
by folic acid deficiency but also by genetic polymorphisms coding for enzymes involved in the OCM. Comparing the plasma homocysteine, folate level, and MTHFR C677T mutation in epileptic patients with those in normal controls, Yoo and Hong found that a common MTHFR C677T mutation was a determinant of hyperhomocysteinemia in epileptic patients receiving AEDs, which suggests that a gene–drug interaction induced hyperhomocysteinemia.23 One research indicated that the prevalence of the 677 T allele varied from 9.34% to 40.53% in different ethnic groups, the lowest being demonstrated for South Asians and the highest for East Asians.24

It has been suggested that phenytoin and carbamazepine as enzyme inducers can directly modulate the activity of different liver enzymes. Liver enzyme induction may cause depletion of the cofactors involved—follic acid, pyridoxal 5’-phosphate, and vitamin B12—leading to the alterations observed in the homocysteine status. It is reported that patients on Phenyltoin often have low serum folic acid levels. Some studies also revealed that carbamazepine use was associated with low folic acid status. However, in the current study, patients with MTHFR CT and TT genotypes receiving AED monotherapy showed significantly lower folate and vitamin B12 levels both in toxicity and non-toxicity groups compared with controls. The present data confirm the 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.25,26 As expected in this study, plasma tHcy levels were higher in carriers of the TT677 MTHFR mutation in the toxicity group as compared to the non-toxicity group and controls. Increased homocysteine levels associated with anti-epileptic drugs are reported to be a risk factor for systemic vascular events including stroke, and chronic vascular toxicity is an important concern. Increased homocysteine may contribute to the development of anti-epileptic drug–related side effects such as impaired cognitive function and fetal malformations including neural tube defect. It is also reported that increased homocysteine may also be involved in poor seizure control in epileptic patients based on the fact that its systemic application is known to cause an animal model of epilepsy. Significant negative correlations were reported between plasma homocysteine and plasma folate or vitamin B12 concentrations in patients who received carbamazepine. The findings of this study showed a significant increase in homocysteine levels in epileptic patients receiving anti-epileptic drug monotherapy in toxicity and non-toxicity groups (P ≤ .005), and significant reductions were found in the level of co-factors folic acid and vitamin B12 against increased homocysteine levels. In terms of the anti epileptic drug used, the level of homocysteine concentration was more in patients with carbamazepine monotherapy (28.6 ± 4.2) in the toxicity group. There was no relationship between seizure frequency and homocysteine levels in epilepsy patients. The observation in this study also indicates that in AED-treated patients, some other mechanism unrelated to co-factors may take role in developing hyperhomocysteinemia. The limitation of this study was that the number of patients on different drugs was too small to yield firm conclusions about the effects of individual drugs. Further investigations are needed to verify the data by increasing the number of patients on phenytoin, carbamazepine, and sodium valproate toxicity and non-toxicity samples to confirm conclusions about the effects of individual drugs.

In conclusion, the results of this study confirm that

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### Table 4. Homocysteine, folate, and vitamin B12 concentrations according to MTHFR gene polymorphisms in cases and controls.

| Genotypes | MTHFR (C677T) |
|-----------|---------------|
|           | cc            | ct            | tt            |
|           | Epilepsy with toxicity cases | Epilepsy with non-toxicity cases | Controls | Epilepsy with toxicity cases | Epilepsy with non-toxicity cases | Controls |
| homocysteine µmol/L | 18.2 (4.6) | 16.2 (2.6) | 10.2 (1.6) | 2.6 (1.8) | 2.8 (0.5) | 5.6 (1.4) | 144 (19.4) | 164 (13.6) | 212 (23.6) |
| folate ng/mL | 23.1 (5.1) | 19.2 (4.6) | 11.2 (4.8) | 2.1 (0.5) | 2.6 (1.8) | 5.8 (1.9) | 136 (22.6) | 158 (18.6) | 206 (22.6) |
| vitamin B12 pg/ml | 26.8 (5.8) | 21.4 (5.6) | 12.8 (5.2) | 2.0 (0.9) | 2.2 (1.9) | 6.3 (1.4) | 134 (14.8) | 148 (21.6) | 198 (22.2) |

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AED pharmacotherapy in epileptic patients showing toxicity and non-toxicity leads to increase in tHcy levels. Individuals with the variant MTHFR (C677T) genotypes seem to have greater predisposition to tHcy concentration increase during AED treatment. Measurement and early management of hyperhomocysteinemia are recommended in epileptic patients, particularly the mutants for MTHFR gene, aiming to prevent its devastating sequel. Further studies including a large number of patients to determine other gene mutations and assess vitamin B12 and folate levels are needed to establish the relationship between epilepsy, homocysteine, and MTHFR gene mutation in the North Indian population.

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