

A COMPARATIVE STUDY ON RISK OF OSTEOPOROSIS ASSOCIATED WITH THE USE OF VALPROATE VERSUS LEVETIRACETAM IN EPILEPTIC PATIENTS AND ITS RELATIONSHIP WITH METHYLENE TETRA-HYDRO FOLATE REDUCTASE GENOTYPES

MOHAMMED FAREEDULLAH^{1*}, REHAB RAFI¹, NEHA NAAZ¹, MOHAMMED BARKATH ALI SHAMS¹, SYEDA SHAISTA BABAR¹, MOHAMMED ISHAQ², MOHAMMED MOHIUDDIN JALAAL³, SRIKANTH JAWALKAR⁴

¹Department of Pharmacy Practice, Deccan School of Pharmacy, Hyderabad, Telangana State, India. ²Department of Research, Salar-e-Millat Research Lab, Princess Esra Hospital (Owaisi Group of Hospitals), Hyderabad, Telangana State, India. ³Department of Orthopaedics, Princess Esra Hospital (Owaisi Group of Hospitals), Hyderabad, Telangana State, India. ⁴Department of Neurology, Princess Esra Hospital (Owaisi group of hospitals), Hyderabad, Telangana State, India. E-mail: fareedullah29@gmail.com

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ABSTRACT

Objective: The study objects at assessing and comparing the intensity of the effect of valproate (VPA) and levetiracetam (LV) on the bone mass in young adult epileptic patients while distinguishing their methylene tetra-hydro folate reductase (MTHFR) genotypes and correlating MTHFR polymorphism and antiepileptic drugs (AEDs) usage with the risk of development of osteoporosis.

Methods: The study design was a comparative, prospective, and observational study. It was conducted at Princess Esra Hospital (PEH), Hyderabad and genotype testing was carried out at Salar-e-Millat Research lab (PEH). The consent was obtained from total 70 subjects, divided into three groups: Group 1: 18 patients receiving sodium VPA monotherapy
Group 2: 17 patients receiving LV monotherapy
Group 3: 35 healthy control subjects from general population.
Patients of either gender within age group of 15–40 years, experiencing generalized tonic-clonic seizures or focal seizures, receiving the AED for duration of time ≥ 2 years were included in the study.

Results: Our study showed significant correlation between the AEDs treatment and MTHFR polymorphism in predisposing osteoporosis.

Conclusion: The variants of MTHFR gene (C677T) are prone to develop increased levels of homocysteine as a result of decreased activity of the enzyme in their bodies which are further increased in patients receiving AEDs. Monitoring of homocysteine levels in epileptic patients especially in the mutants of MTHFR gene along with their periodic testing of bone mineral density levels is recommended. Treatment for low folate and calcium levels is recommended in these patients to correct their deficiencies.

Keywords: Hyperhomocysteinemia, Epilepsy, Levetiracetam, Sodium valproate, Osteopenia, Methylene tetrahydrofolate reductase.

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INTRODUCTION

Epilepsy is one of the most prevalent neurological disorders which are unpredictable in character due to incomplete understanding of the pathophysiology of seizures [1]. This mysterious misfiring of the neurons is a complex neurological condition which burdens more than 70 million persons worldwide. Epileptic patients in India are expected to be nearly 12 million contributing to approximately one-sixth of the global burden [2]. Recent studies attest the role of homocysteine in multiple developmental disorders including epileptogenesis [3]. Homocysteine being a sulfur containing amino acid plays a crucial role in the cell protein formation pathway and other complex metabolic pathways [4]. The concentration of this amino acid is affected mainly by diet (rich in folates and Vitamin B₁₂) and changes in type and concentration of enzymes involved with the metabolism of homocysteine. The Enzyme 5, 10 - Methylentetrahydrofolate reductase (MTHFR) acts as prime catalyst that removes the circulating homocysteine through a methionine cycle. S-adenosylmethionine, end product of methionine cycle, serves as a methyl group donor in the methylation of DNA, proteins, neurotransmitters, and phospholipids [5]. The MTHFR gene exhibits polymorphism due to the simple change of gene at exon-4 resulting into valine instead of alanine at position codon 677 (C677T) resulting in the known T-allele variant (valine type), a heat-labile form with lesser enzymatic activity

in contrast to the wild type, that is, C - allele variant (alanine type) which regulates a normal methionine cycle moderating the circulating homocysteine [6,7]. MTHFR polymorphism emanates increased levels of circulating homocysteine (hyperhomocysteinemia) that propounds to be a risk factor for fractures (independent of age and bone mineral density [BMD]), osteoporosis, and cardiovascular disorders [6,8]. Hyperhomocysteinemia effects bone by altering the bone calcification due to inhibition in the crosslinking of collagen [9,10].

Recent studies prove a deteriorating effect of anti-epileptic drugs (AEDs) on bone health. Epileptic patients treated with sodium valproate (VPA) monotherapy presented elevated plasma homocysteine levels VPA [11] attributed to the inhibition of folate absorption in the intestine by VPA. Such enzyme inducing AEDs depletes organism of Vitamin B and alters Vitamin B¹² dependent homocysteine metabolism causing hyper-total-homocysteinemia in about 10–40% of epileptic patients [12–14]. The presence of a C677T polymorphism entails hyperhomocysteinemia and the added effect of enzyme inducing AEDs evokes hyper-total-homocysteinemia [15]. On the other hand, it is found that non-enzyme inducing AED, levetiracetam (LV) decreases the bone strength and bone formation without any significant effects on BMD [16]. However, relatively a smaller number of studies have been carried out regarding the effect of LV on BMD and further studies are encouraged to conclude about its effect on bone health.

Osteoporosis is a condition with compromised bone density due to the imbalance of bone remodeling process condition predisposing an individual to fragile bones susceptible to fractures frequently [17]. Early onset of generalized osteoporosis is pertinent with hyperhomocysteinemia and presence of homocystinuria [18,19] which is assumed to be due to interference with crosslinking of collagen in bones [20-22]. Primary osteoporosis is an age-related process whereas secondary osteoporosis occurs when other factors such as clinical disorders, genetic disorders, impaired Vitamin D supply, and drugs are involved. Vitamin D stimulates intestinal calcium absorption and promotes mineralization of the skeleton [16]. A decrease in sunlight exposure or an abate in Vitamin D intake through dietary route, drugs such as Gonadotropin releasing hormone, Warfarin, Vitamin A, Cyclosporine, gastric acid lowering drugs, AEDs, Anti-psychotics, loop diuretics, and thyroid replacing therapy interrupt hepatic and/or renal metabolism curtailing Vitamin D activity [23]. Apart from these any imbalance in either local factors (cytokines, growth factors, and prostaglandins) or systemic factors (parathyroid hormone, calcitonin, calcitriol, glucocorticoids, etc.) or both causes osteoporosis. When the genes that are involved in absorption and metabolism of Vitamin D and other pathways helping in the formation of bones are affected, it leads to osteoporosis. Osteoporosis can be detected by measuring the BMD using either dual energy X-ray absorptiometry test or ultrasonography test or by quantitative computed tomography (quantitative CT). The T score levels of these tests interpret the BMD of the bone tested that could be either normal (+2 to -1 levels) or osteopenic (-1 to -2.4 levels) or osteoporotic (<-2.5 levels).

This study aims to assess and compare the intensity of the effect of valproate and LV on the bone mass and to interpret the risk of osteoporosis in these patients along with identifying the MTHFR genotypes in these patients and correlating MTHFR polymorphism with the risk of development of osteoporosis.

METHODOLOGY AND SUBJECTS

Epileptic patients were selected for the study from the Outpatient Department of Neurology, Princess Esra Hospital (PEH) (Owaisi group of Hospitals). The patients diagnosed with epilepsy based on the ILAE classification receiving monotherapy of either sodium VPA or LV for over 2 years were selected. Their BMD was evaluated by ultrasonography BMD Testing in peripheral site, that is, radius. All their blood samples were studied for the MTHFR activity at the Salar-e-Millat Research Lab at PEH (Owaisi group of Hospitals). 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.

Study population

The study population included the following three groups:

- Group 1: A total number of 18 epileptic patients receiving VPA monotherapy were recruited
- Group 2: A total number of 17 epileptic patients receiving LV were recruited
- Group 3: A total number of 35 age and sex matched normal healthy controls from the general population of South Indian origin were recruited.

Around 35 subjects from the out-patient, department of neurology, were selected based on the following criteria:

Inclusion criteria

The patients diagnosed with generalized tonic-clonic seizures and focal seizures (based on the ILAE classification) that were on monotherapy of either sodium VPA or LV for more than 2 years were included in the study. The selected age group of patients ranged from 15-40 years of both the genders of South Indian origin. A total number of 35 age- and sex-matched normal healthy individuals of South Indian origin were also included in the study.

Exclusion criteria

Subjects unwilling to participate in the study, pregnant women, neonates were excluded from the study. Patients with severe neurological deficits,

cardiovascular diseases, and known history of osteoporosis were also excluded from the study. Epileptic patients receiving combination therapy (VPA or LV with other AEDs) or monotherapy of other AEDs were also excluded from the study. Controls who received surgery or other drugs in the past 30 days before the study were also excluded from the study.

Sample collection

BMD test by ultrasonography was carried out at orthopedic department (PEH).

Non-fasting venous blood (2 ml) was collected in 2% EDTA coated container from each subject, mixed well and stored at 4°C. DNA was isolated by a rapid non-enzymatic method by salting out cellular proteins with saturated solution and precipitation by dehydration. Blood samples were brought to room temperature before beginning with the procedure and repeated freezing and thawing of the samples was avoided. 200-300 µL of sample blood was added to a 1.5 ml of Eppendorf using a micropipette and 1 ml of TKM1 (lysis) solution and 100 µL Triton-X was added and mixed by inversion 50-60 times for the primary wash of sample resulting in red blood cell (RBC) lysis. These samples were incubated at room temperature until they become clear with no visible lumps then centrifuged at 3000 rpm for 2 min. The supernatant was discarded carefully, without disturbing the pellet of beds by angling the pipette such that the tip is pointed away from the pellet.

The pellet was re-suspended by adding 1 mL TKM1 solution and 100 µL Triton-X, mixed by inversion 50-60 times and centrifuged at 3000 rpm for 2 min. RBC lysis step was repeated until white blood cell (WBC) pellets were obtained. To the WBC pellets 600 µL of TKM2 (lysis) solution and 10 µL of SDS were added and mixed by inversion. The samples were again incubated at 55°C for 30 min and the tubes were brought to room temperature. To salt out cell proteins, 90 µL of 6M NaCl was added to the Eppendorf tubes and mixed well, and then centrifuged at 800 rpm for 5 min. The supernatant was then collected in a fresh tube to which 1 ml of ethanol was added and mixed by gentle inversion to give white threads like smear of DNA which was then centrifuged at 800 for 5 min. A white DNA pellet sedimented at the bottom of the tube and the supernatant was discarded slowly.

Eluting DNA

To the pellet 1 ml of 70% ethanol was added and mixed well to wash the tube properly and centrifuged at 800 rpm for 5 min. Then, the supernatant was discarded, and the tubes were air dried.

Dissolving DNA

To dissolve the DNA, 50-100 µL of TE buffer was added to the DNA pellet [24]. The purified DNA was stored at -20°C for downstream analysis. DNA quantification purity and integrity were assessed by fluorescent dye tagging using ethidium bromide.

Amplification

DNA amplification by polymerase chain reaction (PCR) was carried out in a Bio-Rad machine - Bio-rad T100 Thermocycler using the MTHFR primers, that is, 5.e. i AGG AGA AGG TGT CTG CCG GA-3' for the sense oligonucleotide primer and 5'-AGG ACG GTG CCG TGA GAG TG-3' for the antisense primer. A PCR master mix by Thermofisher company, USA was used for this study which is a mixture of Taq DNA Polymerase (0.05 U/µL), 0.4 mM of each dNTP, reaction buffer, 4 mM MgCl₂, and 4 mL × 1.25 mL Nuclease water required for PCR except primers. The cycle parameters were as follows: One cycle for an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 58°C, primer extension for 2 min at 72°C, and a final extension for 10 min at 72°C. This amplification reaction resulted in the 198-base pair (bp) fragment. These amplified DNA fragments obtained were then electrophoresed using 3% agarose gel and visualized under UV light (Bio-Rad).

RFLP: (MTHFR (C677T) genotyping

For the restricted digestion of the amplified DNAs, 1–2 μ L of HinfI (7–10 Units), 2 μ L of R buffer (ThermoFisher company, USA), and 18 μ L of nuclease free water were added to 10 μ L of extension mixture to make the volume 30–32 μ L. Samples digested at 37°C for 16 h show that HinfI digested the fragment of the same length from the T allele into 175-bp and 23-bp fragment while the fragment derived from the C allele remained undigested. The fragments obtained were then electrophoresed using 3% agarose gel and visualized under light (Bio Rad). Detection of MTHFR genotypes was done based on restriction fragment length and unique genotype dependent band combination.

statistical analysis

Statistical analysis was performed using SPSS software (Version 20.0). Un-paired t-test was used to find out the significant difference between two groups which was illustrated as Mean and standard deviation (SD). Odds ratio analysis was used to find the risk variables under the study with a 95% confidence interval limit from 2x2 contingency table. $p < 0.05$ was considered significant. Unpaired t-test was used to find out the significant difference between two groups (VPA and LV) with respect to BMD score as well as to analyze which drug group's BMD score influenced significantly. Deviations from the Hardy-Weinberg equilibrium were calculated to find out genetic variations and Allelic frequencies. Statistical analysis was performed based on Chi-square distribution.

RESULTS

The study group consisted of 35 epileptic patients and 35 controls with the mean age of 23.88 (range 15–40) years in patient group and 26.17 (range 18–40) years in control group.

Of the 35 Epileptic patients who were on a long-term monotherapy of valproic acid and LV, 18 (51.43%) were on VPA and 17 (48.57%) on LV. The seizure types were generalized tonic-clonic seizures (77.14%) and focal seizures (22.86%). Family history of epilepsy, exposure to light and milk intake, was also studied. The association between the MTHFR gene polymorphism and epidemiological and clinical variables is summarized in Table 1.

The effect of duration of usage of VPA and LV on BMD was also calculated. The results are shown in Table 2.

Details of genotype frequencies as well as allele frequencies in the patients and controls are shown in Table 3. It was observed that the frequency of CC genotype (associated with normal folate reductase activity) was found to be 97.14% in the controls. However, it was observed to be reduced to 45.71% in the patient's group. In contrast to this, the frequency of CT genotype was 2.85% in the controls compared to 51.43% in the patient's group. These results indicate that there appears to be significantly increased no. of CT genotype in the patients group compared to controls group. It is inferred that individuals with the CT genotype have a high risk of developing not only epilepsy but also osteopenia, a process that ultimately leads to osteoporosis.

Odds ratio analysis by comparing the genotype frequency in the test and control groups revealed a high risk of 38.25% (CI - 24.68–312.21) ($p=0.007$). These results were obtained when the genotypes were compared in a codominant model. The frequency of TT homozygotes appears to be relatively very low in healthy population. The frequency for TT genotype of developing osteoporosis was also found to be higher than CC. In the dominant model, when CT and TT genotypes were compared with CC, the odds ratio was found to be even higher, that is, 40.37 (CI - 4.95–328.67) ($p=0.005$). However, recessive model did not portray any such risk. So far as allelic frequencies are concerned, the frequency of C allele was 98.57% in controls compared to 71.43% in patients. However, there was a significant increase in the frequency of T allele in the patient group which was 28.57% when compared to 1.43% in control group. Since T allele is associated with reduced folate reductase activity, individuals with CT and TT genotypes have high risk of developing epilepsy and osteoporosis (OR=27.00 [3.58–212.49] [$p=0.0001$]). Both genotypic and allelic frequency results were significant as $p < 0.05$ favoring the opinion.

DISCUSSION

The increased levels of homocysteine in epileptic patients have been reported by several studies. The leading causes to this can be the pharmacological treatment of these patients as AEDs have been found to affect the folate metabolism. Another cause proposed for hyperhomocysteinemia in these patients is the presence of MTHFR polymorphism in their genetic makeup. MTHFR enzyme catalyzes the reaction that converts homocysteine to methionine thereby reducing the homocysteine levels and a polymorphism in the gene coding for this enzyme leads to decreased clearance

Table 1: Association between the MTHFR gene polymorphism and epidemiological and clinical variables

Variables	Genotype (%)				p value
	n (%)	CC (n [%])	CT (n [%])	TT (n [%])	
Gender	35				0.98
Male	18 (51.43)	9 (50)	9 (50)	0	
Female	17 (48.57)	7 (41.18)	9 (52.94)	1 (5.88)	
OR (95%CI)		1.00 (Ref)	1.28 (0.33–4.72)		
Type of epilepsy	35				0.79
Focal	8 (22.86)	4 (50)	4 (50)	0	
Generalized tonic-clonic seizures	27 (77.14)	12 (44.44)	14 (51.86)	1 (3.70)	
OR (95%CI)		1.00 (Ref)	1.16 (0.23–5.60)	1.08 (0.03–31.63)	
Anti-epileptic drugs drug	35				0.78
Sodium valproate	18 (51.43)	7 (38.89)	11 (61.11)	0	
Levetiracetam	17 (48.57)	9 (52.94)	7 (41.18)	1 (5.88)	
OR (95%CI)		1.00 (Ref)	0.49 (0.12–1.94)	2.36 (0.08–66.88)	
Family history	35				0.47
Yes	8 (22.86)	2 (25)	6 (75)	0	
No	27 (77.14)	14 (51.86)	12 (44.44)	1 (3.70)	
OR (95%CI)		1.00 (Ref)	0.28 (0.04–1.68)	0.51 (0.01–16.63)	
Light exposed	35				0.74
Yes	10 (28.57)	6 (60)	4 (40)	0	
No	25 (71.43)	10 (40)	14 (56)	1 (4)	
Milk intake		1.00 (Ref)	2.10 (0.46–9.44)	1.85 (0.06–52.76)	0.15
Yes	35				
No	17 (48.57)	7 (41.18)	10 (58.82)	0	

of homocysteine. Hyperhomocysteinemia have been found to be associated with declination of the bone strength along with its other effects on the vascular system. The gene-drug interaction induced hyperhomocysteinemia has been found in patients receiving AEDs and having mutation of MTHFR C677T. In our study, we have focused in analyzing the combined effect of AED treatment and MTHFR gene (C677T) polymorphism in epileptic patients that could pre-dispose them to osteoporosis thereby increasing the risk of fractures in this population.

In our study, we have included two AEDs, an older AED, sodium VPA and a newer AED, LV to assess their effect on BMD. Thirty-five patients were recruited in this study of which 17 were receiving LV and 18 were on VPA monotherapy. All the patients receiving LV were found to develop osteopenia, whereas one out of 12 patients receiving VPA developed osteopenia along with one patient who developed osteoporosis and others were normal.

From the literature survey, it has been found that the individuals with either homozygosity (TT) or heterozygosity (CT) of T allele of MTHFR gene polymorphism are associated with decreased enzyme activity, whereas the wild form (CC) is not found to interrupt in its mechanism. The decreased enzyme activity leads to increased homocysteine levels along with decreased folate levels. In our study, out of 35 patients, 18 patients were heterozygous for the C677T mutation (CT), one patient was homozygous for C677T mutation

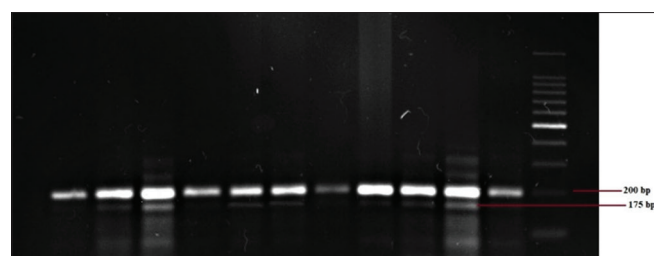


Figure 1: C677T mutation analysis

Table 2: Effect of VPA and LV on AED usage duration and BMD score

Variable	VPA	LV	t value	p value
AED usage duration (mean)	4.11±4.4.26	2.50±0.80	1.51	0.13
BMD score (mean)	-1.42±0.91	-1.83±0.29	1.78	0.08

LV: Levetiracetam, AEDs: Anti-epileptic drugs, VPA: Valproate, BMD: Bone mineral density

Table 3: Genotype frequency of the MTHFR C>T gene polymorphism in disease and controls

MTHFR C>T	No: of individuals (%)		OR (95% CI)	p
	Controls (n=21)	Disease (n=21)		
Genotype	n (%)	n (%)		
CC ^a	34 (97.14)	16 (45.71)	1.00 (Ref)	
CT	1 (2.85)	18 (51.43)	38.25 (4.68-312.21)	0.007
TT	0 (0)	1 (2.85)	6.27 (0.24-162.42)	
CC ^b	34 (97.14)	16 (45.71)	1.00 (Ref)	
CT+TT	1 (2.85)	19 (54.29)	40.37 (4.95-328.67)	0.005
CC+CT ^c	35 (100.00)	34 (97.14)	1.00 (Ref)	
TT	0 (0)	1 (2.85)	3.08 (0.12-78.41)	0.50
CC+TT ^d	34 (97.14)	17 (48.57)	1.00 (Ref)	
CT	1 (2.85)	18 (51.43)	36.00 (4.42-292.86)	0.008
Allele				
C	69 (98.57)	50 (71.43)	1.00 (Ref)	
T	1 (1.43)	20 (28.57)	27.00 (3.58-212.49)	0.0001

^aCodominant model of inheritance (wild-type homozygous genotype serves as the reference). ^bDominant inheritance model (combined heterozygous and homozygous for the minor allele versus wild-type homozygous). ^cRecessive inheritance model (minor allele homozygous versus combined heterozygous and homozygous for the wild-type allele). ^dOver dominant model of inheritance (heterozygous vs. combined homozygous wild type genotype and homozygous minor allele)

(TT) while 16 patients were non-mutants (CC). The risk associated with CT when compared with CC was found to be greater (O.R =38.25) indicating the involvement of MTHFR polymorphism in contributing to hyperhomocysteinemia.

The bone strength of all these patients was identified using BMD test that resulted in diagnosis of 26 patients with osteopenia and one with osteoporosis. Out of these 26 patients, 14 were found to have the CT genotype and one was with TT genotype. Among the 16 patients that were bearing CC genotype, there were 11 patients that were diagnosed with osteopenia and one patient was diagnosed with osteoporosis showing that the drugs alone have a potent effect on bone strength. However, the duration of AEDs received by these patients was greater than the individuals bearing mutant genotypes that resulted in developing osteopenia with a shorter interval of AED treatment. A longer duration of treatment with AEDs also induced osteopenia in the wild CC genotype individuals. This reveals a close relationship of MTHFR gene polymorphism and AEDs treatment in inducing osteopenia and increasing the risk of fractures.

The present study is the first study to depict the synergistic effect of AEDs and MTHFR polymorphism on bone health in epileptic patients below 40 years. It has been found that the peak bone mass is attained at the age of 30 years. There is steady decline of around 0.3-0.5% bone loss per year after the age of 40 years [25]. Bone turnover needs to be balanced during the critical period of childhood and adolescents as alterations in bone mineralization and bone growth during this period can lead to lower peak bone mass in early adulthood [26]. AEDs are found to even hasten the age-dependent bone loss. Among the 35 patients selected for the study, 31 patients were below the age of 30 years from which 23 were diagnosed with osteopenia. Regular monitoring of BMD, especially of young patients receiving AEDs along with MTHFR gene polymorphism, is recommended to avoid any further complications related to bone health.

The results of our study showed significant correlation between the AEDs treatment and MTHFR polymorphism in predisposing osteoporosis. The limitations of our study were small no. of patients receiving individual drug that limits us to give firm conclusions about their effects. From our study, it could be said that the variants of MTHFR gene (C677T) are prone to develop increased levels of homocysteine as a result of decreased activity of the enzyme in their bodies which is further increased in patients receiving AEDs. The levels of homocysteine must be monitored in epileptic patients especially in the mutants of MTHFR gene along with their periodic testing of BMD levels. Treatment for low folate and calcium levels is recommended in these patients to correct their deficiencies and avoid further problems in the future.

CONCLUSION

The collected data and results conclude that the effect of VPA versus LV on the BMD of an epileptic patient was found to be higher in case of LV with lower margins. The patients receiving LV have all shown osteopenia despite the MTHFR polymorphism whereas epileptic patients on VPA showed diversity in their effect with a fraction of VPA subjects showing osteopenia and osteoporosis. The deteriorating effect of LV was found to be higher than VPA despite the MTHFR polymorphism. Patients with CC genotype had normal BMD compared to those with CT and TT genotype indicating the protective nature of CC genotype on the BMD. CT and TT MTHFR genotype predispose individuals to a higher risk of precipitating osteoporosis. The results of this study suggest a BMD scan in patients receiving AEDs for a longer duration (like few months to years) to measure the BMD levels. In case of a lower BMD, serum calcium and folate levels should be identified to diagnose deficiencies and alleviate the condition.

AUTHORS CONTRIBUTIONS**CONFLICT OF INTEREST**

The authors declared that they have no conflicts of interest regarding the research, authorship, and publication of this article.

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