

# Vitamin B<sub>6</sub> and homocysteine levels in carbamazepine treated epilepsy of Khyber Pakhtunkhwa.

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## Abstract

**Objectives:** The study focused on the plasma levels of vitamin B<sub>6</sub> and homocysteine in different genotypes of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes in carbamazepine resistant epilepsy in the population of Khyber Pakhtunkhwa.

**Methodology:** Patients who were possible candidates for carbamazepine therapy were followed for six months for their seizure control. Plasma levels of vitamin B<sub>6</sub> and homocysteine were determined using immunoassay based techniques at baseline and after six months. MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes were genotyped using restriction fragment length polymorphisms. Seizure control during therapy was recorded on a standardized proforma.

**Results:** Low vitamin B<sub>6</sub> levels and hyperhomocysteinemia were found in 61.7% of resistant patients (n=34). Resistant patients had the following frequencies of variant genotypes (677CT=38.1% and 677TT=24.4%; 1298AC=42.2% and 1298CC=26.1%; 588CT= 47.6% and 315TT= 33.3%) of MTHFR (C677T and A1298C) and GABRG2 (C588T and C315T) genes. A significant decline in vitamin B<sub>6</sub> (P<0.0001) and hyperhomocysteinemia were found in variant genotypes of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes.

**Conclusion:** Following six months of carbamazepine of therapy in heterozygous variant genotypes of MTHFR (677CT and 1298AC) and GABRG2 (588CT and 315CT) genes, we observed a significant fall in vitamin B<sub>6</sub> levels and hyperhomocysteinemia.

**Keywords:** Carbamazepine, epileptics, homocysteine, seizure control, RFLP, vitamin B<sub>6</sub>.

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## Introduction

Epilepsy is a neurological disorder manifested by repeated, unprovoked seizures that affects one percent of the population globally<sup>1</sup>. Different factors are responsible for resistant epilepsy, and these need to be explored<sup>2</sup>. Genetic factors and nutrition are considered one of the main causes of resistant epilepsy<sup>3,4</sup>. Nutrients that reduce seizure frequencies include vitamin B<sub>6</sub>, magnesium, vitamin E, manganese, taurine, dimethylglycine and omega-3 fatty acids<sup>5</sup>. Moreover, vitamin B<sub>6</sub> plays an important role in the development and differentiation of central nervous

system (CNS)<sup>6</sup>. Deficiency of vitamin B<sub>6</sub> level leads to decrease in synthesis of GABA, which is an inhibitory neurotransmitter in CNS<sup>7,8</sup>. Low GABA is also associated with CNS disorders such as epilepsy, anxiety and depression<sup>9</sup>. On the other hand, methylenetetrahydrofolate reductase (MTHFR) plays an important role in folate and homocysteine metabolism<sup>10</sup>. Reports say that MTHFR (C677T and A1298C) gene polymorphism is associated with elevated levels of homocysteine. Moreover, deficiency of vitamin B<sub>6</sub>, B<sub>12</sub>, and folic acid is also responsible for hyperhomocysteinemia<sup>7,8</sup>. There is a reports about the association of C677T and A1298C of MTHFR gene polymorphisms with elevated levels of homocysteine due to vitamin B<sub>6</sub>, vitamin B<sub>12</sub> and folic acid deficiency in patients with myocardial infarction in Pakistani population<sup>11</sup>. However, as vitamin B<sub>6</sub> helps in synthesis of GABA, an inhibitory neurotransmitter and whereas there are no reports for levels of vitamin B<sub>6</sub> in epileptics treated with carbamazepine, hence, the current study was designed to know about the levels of vitamin B<sub>6</sub> and homo-

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cysteine in epileptic patients treated with carbamazepine. The study also focused to know the variant of MTHFR and GABRG2 genes frequently experiencing vitamin B<sub>6</sub> deficiency with ultimate poor seizure control.

## **Materials and methods**

### **Epileptic patients**

The study was approved by the Ethical Board of Khyber Medical University, Peshawar via approval no: DIR/KMU-EB/AC/000047. Seventy nine patients suffering from different types of epilepsy who were to be treated with carbamazepine were selected at out-patient Department of Neurology of Government Lady Reading Hospital, Peshawar, Pakistan. Medical history, careful physical examination and complete investigations were performed by respective ward/OPD neurologists at time of enrollment into the study. All patients signed written consent after an explanation of the steps and aims of the study in the context of their local language (s).

### **Study design and protocol**

It was a hospital based longitudinal study. Patients who received carbamazepine as monotherapy were enrolled in the study. Baseline interviews were carried out for selection, evaluation of seizures on the initiation of the therapy. Patients were asked to visit the epilepsy out-patient Department of Neurology on monthly basis for seizure control. Patients with epilepsy were then evaluated in the sixth month of the therapy for possible shift in their plasma levels of vitamin B<sub>6</sub> as well as their clinical screening for better epilepsy management.

### **Collection of blood and sampling techniques**

Patients were purposively enrolled in the study. Blood samples were collected at baseline and in the 6<sup>th</sup> month of the therapy. The blood was taken in gel tubes following administration of the drug in the morning. The plasma was separated and subjected to biochemical analysis for levels of vitamin B<sub>6</sub> and homocysteine. For genotyping blood samples were taken in EDTA tubes which were subjected for DNA extraction and genotyping.

### **Genotyping of MTHFR and GABRG2 Genes**

Genomic DNA was extracted using kit method (NucleoSpin® Blood, Germany). Briefly describing, the extraction of DNA was carried out through series of

reactions which included deproteination of blood (proteinase K), lysis of blood cells (lysis buffer), purification of DNA (washing buffer), and finally elution of DNA (through elution buffer) according to standard protocols provided by respective manufacturers. The extracted DNA was stored on -20 °C for further analysis. MTHFR (C677T and A1298C) and GABRG2 (C588T and C315T) genes were amplified using gradient thermo cycler. After amplification, the PCR products were run on 2% agarose gel and assessed their size with 50Pb ladder. MTHFR (C677T) gene was amplified by using primers 5-TTTGAGGCTGACCTGAAGCACTTGAAGGAG-3 and 5-GAGTGGTAGCCCTGGATGGGAAAGATCCG-3. The amplified product was digested using restriction enzyme Hinf I. Similarly, MTHFR (A1298C) gene was amplified by using primers 5-CTTTGGGGA GCTGAA GGACTACTAC-3 and 5-CACTTTGTG ACCATTCCGGTTTG-3. The resulted product was digested using restriction enzyme MboII. GABRG2 (C588T) gene was amplified by using primers 5- AATCACCTTTTAT-TCTAATGGTC-3 and 5-CAGTGAAGGCAACT-TACTAAGA-3. The amplified product was digested using restriction enzyme ApoI. GABRG2 (C315T) gene was amplified using primers 5-CAAATGTGGTGAATTAGTAACTGG-3 and 5- TCACATTTTCTCTCAAACATGC-3. The PCR product was digested using restriction enzyme BamHI. The digested products of all amplified exons were run on 5% agarose gel and respective fragment sizes were assessed with 50Pb ladder.

### **Analysis of plasma levels of vitamin B<sub>6</sub>**

Vitamin B<sub>6</sub> levels were determined using immunoassay based Kit (Alpha Diagnostic International USA). Serial dilutions of standard solutions and samples were added into 96 wells using ELISA vitamin B<sub>6</sub> plate. The plate was incubated for 1 hour at 37 °C. Biotinylated detection antibody working solution (1X) was added into each well and incubated again for 45 minutes at 37 °C. The plate was washed 3 times after incubation. HRP conjugate antibodies (1X) were added into each well. Mixed gently and incubated for 30 minutes at 37 °C. The plate was washed 5 times. Finally, we added TMB substrate into each well. Mixed gently and incubated the plate for 15 minutes again at 37 °C. Reaction stop solution was added to all wells. Absorbances of wells were read at 450 nm using micro plate reader.

### Analysis of plasma levels of homocysteine

Homocysteine levels were also determined using enzyme linked Immunosorbant assay (ELISA) (Kit Abbott Laboratories Ltd; Pakistan) technique. Standard serial dilutions, primary, secondary antibody dilutions and buffer were prepared according to the manufacturers' procedures. Serial dilutions of standard and samples were added into a 96 well plate. The plate was incubated for 2 hours at 37 °C. Then added Biotin-antibody (1X) was added into each well of the plate following proper washing and again was added incubated for 1 hour at 37 °C. Then a HRP-avidin (1X) to each well and again incubated for 1 hour at 37 °C. The plate was washed 5 times and TMB substrate was added to each well. The plate was incubated again for 30 minutes at 37 °C. Finally, 50 µl of reaction stop solution was added to each well and the absorbance of plate on 450 nm.

### Measurement of seizure control

Seizure control was recorded in the form of reduction

of frequencies or duration of seizures on a standardized proforma in the context of their local language (s).

### Statistical analysis

Changes in plasma levels of vitamin B<sub>6</sub> and homocysteine between baseline and end time of the study were compared using student "t" test. Resistant seizures were expressed in their frequencies and percentages on a Graphpad Prism. The data was analyzed using a Graphpad Prism 6 at 95% CI, P ≤ 0.05.

### Results

#### Demographic characteristics of epileptic patients

Patients' demographic data and types of seizures are expressed in Table 1. It is evident from Table 1 that mean age of both male and female patients were uniform (P>0.05). Generalized tonic clonic seizures were most prevalent (69.6 %) at time of incorporation into the study. 17 patients were dropped from the study because they either did not report to the epilepsy care clinic or lacked interest in the study.

**Table 1: Demographic data and types of epileptic patients**

Demographic data	Patients (n=79) on Baseline	Patient (n=62) on 6 <sup>th</sup> months
Age (year) , mean ± SD (range)	18.1±8.2 (1-42)	18.08±7.5 (1-42)
Male, n (%)	37 (47)	28 (45.2)
Female, n (%)	42 (53)	34 (54.8)
Generalized Tonic Clonic Seizure, n (%)	50 (70.9)	44 (77.4)
Generalized Tonic Seizure, n (%)	4 (5.1)	1 (1.6)
Atonic Seizure, n (%)	3 (3.8)	2 (3.2)
Simple Partial Seizure, n (%)	3 (3.8)	3 (4.8)
Complex Partial Seizure, n (%)	5 (6.3)	3 (4.8)
Secondary Generalized Complex Seizure, n (%)	12 (7.6)	6 (9.7)
Dose (mg/day), mean ± SD (range)	455±133 (200-800)	495±133 (200-800)

Word AEDs used in the discussion mean antiepileptic drugs, while KP means Khyber Pakhtunkhwa

Plasma levels of Vitamin B<sub>6</sub> in different genotypes of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes polymorphisms

Vitamin B<sub>6</sub> plasma levels were significantly (P<0.05) low at end study as compared to base line (Table 2). Fall in mean levels of vitamin B<sub>6</sub> was also significant (P<0.0001) in homozygous and heterozygous variant genotypes of

the target genes except for heterozygous variant of GABRG2 (588CT). However, upon comparison with reference values (30-150 mmole/L) of vitamin B<sub>6</sub>, it is evident that GABRG2 (C588T, C315T) and all its variants had vitamin B<sub>6</sub> levels of the lower limit of the reference range, despite of a statistical significant fall in their levels (Table 2).

**Table 2: Plasma levels of vitamin B<sub>6</sub> in variants of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes polymorphisms of epileptics treated with carbamazepine therapy**

Gene	Genotypes	Vitamin B <sub>6</sub> plasma level (mmole/L), mean ± SD		
		1 <sup>st</sup> phase (n=79)	2 <sup>nd</sup> Phase (n=62)	P values †
MTHFR C677T	CC (n=32, 27)	71.9±20.8	45.2±22.3	< 0.0001***
	CT (n=29, 22)	67.2±20.7	31.0±22.8	0.0001***
	TT (n=18, 13)	62.0±18.0	27.3±7.3	0.0001***
MTHFR A1298C	AA (n=35, 28)	71.2±19.2	45.9±22.4	< 0.0001***
	AC (n=29, 22)	65.1±24.9	20.4±6.6	< 0.0001***
	CC (n=15, 12)	64.3±23.4	23.3±4.9	0.0005***
GABRG2 C588T	CC (n=37, 30)	68.9±20.4	39.4±20.4	< 0.0001***
	CT (n=27, 21)	71.8±25.8	49.4±25.3	0.08
	TT (n=15, 11)	71.3±16.8	31.9±27.7	0.002**
GABRG2 C315T	CC (n=36, 32)	49.4±21.9	39.8±20.8	< 0.0001***
	CT (n=25, 16)	70.5±17.4	41.4±31.2	0.01*
	TT (n=18, 14)	69.1±18.0	37.0±20.9	0.003**

†= T test, Mean level of vitamin B<sub>6</sub> on baseline verses mean levels of vitamin B<sub>6</sub> on sixth month of carbamazepine therapy

**Mean plasma levels of homocysteine in different Genotypes of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) Genes Polymorphisms**

Rise in mean plasma levels of homocysteine at six months

was not statistically significant (P>0.05) as compared to their baseline (Table 3) values. Though, there was a significant difference in vitamin B<sub>6</sub> levels in respective homozygous and heterozygous variants of the target genes.

**Table 3: Plasma levels of homocysteine in variants of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes polymorphisms of epileptics treated with carbamazepine therapy**

Gene	Genotypes	Homocysteine plasma level (mmole/L), mean ± SD		
		1 <sup>st</sup> phase (n=79)	2 <sup>nd</sup> Phase (n=62)	P values †
MTHFR C677T	CC (n=32, 27)	6.7±2.3	7.2±2.0	0.20
	CT (n=29, 22)	9.8±3.2	10.1±3.9	0.83
	TT (n=18, 13)	10.6±3.4	11.2±2.7	0.75
MTHFR A1298C	AA (n=35, 28)	7.3±2.8	7.7±2.6	0.42
	AC (n=29, 22)	9.0±2.5	11.3±2.8	0.07
	CC (n=15, 12)	9.1±4.5	8.7±4.0	0.88
GABRG2 C588T	CC (n=37, 30)	7.9±3.0	8.5±3.0	0.37
	CT (n=27, 21)	6.8±2.6	7.2±1.9	0.75
	TT (n=15, 11)	8.2±2.8	10.7±3.1	0.09
GABRG2 C315T	CC (n=36, 32)	7.8±3.2	8.5±3.0	0.28
	CT (n=25, 16)	7.3±2.2	7.9±2.7	0.52
	TT (n=18, 14)	8.0±3.8	8.2±3.6	0.91

†= T test, Mean level of homocysteine on baseline verses mean levels of homocysteine on sixth month of carbamazepine therapy

## Seizure control

There were 34 patients resistant to carbamazepine therapy (Table 4). Vitamin B<sub>6</sub> levels were below the reference

range (30-150 mmole/L) in 21 resistant patients. MTHFR (C677T, A1298C) observed high frequency (42.2%) with low plasma levels of vitamin B<sub>6</sub> in patients that had poor seizure control (Table 4).

**Table 4: Frequencies of different genotypes who were resistant to carbamazepine therapy with their respective plasma vitamin B<sub>6</sub> and plasma homocysteine levels on sixth month of the therapy**

Genes	Genotypes	Genotypes in poor seizure control (n=34), n (%)	Genotypes in low B6 patients (n=21), n (%)	Vitamin B6 (mmole/L) †	Homocysteine (mmole/L) ‡
MTHFR (C677T)	CC	9 (26.5)	6 (28.6)	22.6±6.7	8.1±2.6
	CT	15 (44.1)	8 (38.1)	20.8±6.4	10.7±3.7
	TT	10 (29.4)	7 (33.3)	24.0±5.1	11.2±2.8
MTHFR (A1298C)	AA	11 (32.4)	8 (38.1)	23.8±6.2	9.0±3.2
	AC	14 (41.2)	9 (42.8)	20.4±6.6	11.3±2.7
	CC	9 (26.4)	4 (19.1)	23.2±4.9	8.9±4.0
GABRG2 (C588T)	CC	12 (35.3)	8 (38.1)	22.9±5.8	9.7±3.2
	CT	16 (47.1)	10 (47.6)	21.6±1.7	8.6±2.7
	TT	6 (17.6)	3 (14.3)	22.5±8.0	9.1±4.1
GABRG2 (C315T)	CC	12 (35.3)	10 (47.6)	23.0±5.9	10.0±3.9
	CT	13 (38.2)	7 (33.3)	24.2±5.7	7.3±2.1
	TT	9 (26.5)	4 (19.1)	18.8±8.1	11.5±4.4

†= 30-150 mmole/L, ‡= 4-9 mmole/L

## Discussion

Seizure control with carbamazepine is affected by different factors. However, gene polymorphisms and nutrition are also considered as contributing factors in resistant epilepsy. We observed that plasma levels of vitamin B<sub>6</sub> were low after six months of the therapy once we compared with their baseline levels. As mentioned earlier, some of the variants, particularly heterozygous variant of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes were relatively more resistant to their respective homozygous variants. Other studies suggest that vitamin B<sub>6</sub> levels can be affected by various factors like carbamazepine and some anti-tuberculous drugs<sup>6</sup>. Recent studies have shown that duration of antiepileptic drugs (AEDs) is also significantly associated with low plasma levels of vitamin B<sub>6</sub> and elevated levels of homocysteine<sup>12</sup>. Hence, our findings suggest that hyperhomocysteinemia is mostly prevalent in heterozygous variant genotypes of MTHFR (C677T and A1298C) gene which appear in the 6<sup>th</sup> month of the carbamazepine therapy. An interesting part of the study was that resistant patients to carbamazepine therapy had low level of vitamin B<sub>6</sub>, despite their mean plasma level of carbamazepine was within therapeutic range (4-12 mg/L in the resistant patients (data not shown) this suggests

possible changes at drug receptors sites or poor affinity for receptors, which still need to be discovered using modern techniques. Thus we agree with Ebaid et al.<sup>13</sup> who reported that therapeutic steady state did not guarantee effective clinical response<sup>13</sup>. The resistance in response may be due to MTHFR (C677T and A1298C) gene polymorphisms that may alter the individual response to a drug despite having patients' plasma levels in therapeutic range. As MTHFR gene polymorphisms affect DNA methylation leading to differential gene expression that can influence the drug response, hence, poor response may be attributed to low level of vitamin B<sub>6</sub> as well<sup>14-18</sup>. On the other hand, vitamin B<sub>6</sub> is responsible for synthesis of GABA which regulates the functions of alpha, beta and gamma subunits of GABA receptors<sup>19</sup>. Therefore, alteration in receptor site changes its sensitivity to administered antiepileptic drugs (AEDs) despite plasma levels of AEDs falling within the therapeutic range. The above mentioned facts supported the evidence that genetic variations in genes has a role in multidrug resistance. However, heterozygous variants of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes are more resistant to carbamazepine therapy as compared to wild genotypes in the population of Khyber Pakhtunkhwa (KP).

## Conclusion

Following six months of carbamazepine therapy in resistant epilepsy, we observed a significant fall in vitamin B<sub>6</sub> level in heterozygous variant genotypes of MTHFR (677CT and 1298AC) and GABRG2 (588CT and 315CT) genes. Conversely, their plasma homocysteine levels started rising in the 6<sup>th</sup> month of the carbamazepine therapy in resistant heterozygous variants.

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## Recommendations

Vitamin B<sub>6</sub> supplements may be advised in heterozygous variants of MTHFR (C677T, A1298C) and GABRG2 (C588T, C315T) genes if clinicians opt for carbamazepine therapy that might help for better management of epilepsy.

## Limitations of the study

We studied only vitamin B<sub>6</sub> and homocysteine levels. Further studies are required to study levels of folic acid and vitamin B<sub>12</sub> in epileptics as these are related to hyperhomocysteinemia.

**Abbreviations:** Anti-epileptic drugs (AEDs); Deoxyribonucleic Acid (DNA); Gamma-Aminobutyric Acid Receptor (GABRG2); Horseradish peroxidase (HRP); Methylene Tetrahydrofolate Reductase (MTHFR) and Single Nucleotide Polymorphisms (SNPs); Tetramethylbenzidine (TMB)

## Conflict of interest

The authors have no conflict of interest.

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