Apolipoprotein E in temporal lobe epilepsy: A case-control study

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Abstract. *Purpose:* To investigate the relationship of apolipoprotein E (apoE) genotype, plasma levels of apoE and lipids in temporal lobe epilepsy (TLE) patients in Asian Indians. Status of plasma levels of Apo E in epilepsy patients has not been reported till date.

Methods: ApoE gene polymorphism was analyzed in 58 patients with temporal lobe epilepsy (TLE) and 57 age and sex approximated controls using Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). Levels of plasma apoE and lipids were measured using ELISA and enzymatic kits respectively.

Results: The distribution of ApoE genotype in epilepsy patients and controls was comparable. Higher levels of plasma ApoE were observed in TLE patients as compared to controls ($p = 0.0001$). Individuals with plasma levels of apoE >190 mg/L were at 20 times higher odds (95%CI $= 2.46$–163.34, $p = 0.005$), while those with levels of apoE between 150–190 mg/L were at 4.9 times higher odds (95% CI $= 1.85$–13.9, $p = 0.001$), to develop TLE.

Conclusions: We have observed for the first time, high levels of plasma apoE in epilepsy patients. The findings of this case-control study suggest that apolipoprotein E may play an important role in epilepsy.

Keywords: Apolipoprotein E, gene polymorphism, plasma apo E levels, temporal lobe epilepsy, asian Indians

1. Introduction

Apolipoprotein E (apo E) is a 32 kD glycoprotein associated with the lipid transport and metabolism. It has three common isoforms, E2, E3 and E4, which are coded by the alleles $\varepsilon2$, $\varepsilon3$ and $\varepsilon4$ respectively. ApoE has special relevance to the lipid metabolism in the nervous tissue. It plays a central role in the mobilization and redistribution of cholesterol and phospholipids, in growth and maintenance of myelin and neuronal membranes during development and in repair after injury [1].

Experiments conducted in animal models indicate that, in the presence of one of the allelic form of ApoE (apo $\varepsilon4$), the brain's ability to forms new synapses is impaired [2]. Recent studies conducted in our laboratory have shown positive association of apo $\varepsilon4$ allele with stroke [3] and the onset of Alzheimer's disease [4]. Several workgroups have reported a less favorable outcome after a traumatic, epileptic or hemorrhagic brain injury in patients with the $\varepsilon4$ allele compared to $\varepsilon3$ or $\varepsilon2$ [5–7].

Several studies have suggested the association of $\varepsilon4$ allele with neurological disorders, however the mechanism by which the apo E isoforms affect neuronal vulnerability *in vivo* is still unknown. *In vitro* investiga-

tions have shown that apoE3 stimulates branching of growing neurons and prevents neuronal death in conditions of oxidative stress while E4 has been found to have opposing effects [8–10]. Overall peroxidation levels are higher in the brain tissues of $\varepsilon 4$ carriers and correlate inversely with apoE concentrations [11]. Experiments in animal models suggest that deficiency of apoE mimics apo $\varepsilon 4$ status [8,12,13].

Gouras et al. [7] reported that apo $\varepsilon 4$ allele promotes the intracerebral accumulation of β-amyloid in the patients with temporal lobe epilepsy (TLE), similar to that observed in Alzheimer's disease (AD) [7,14]. Sheng et al. [8] have found high levels of β-Amyloid precursor protein (β-APP) in epileptic patients and suggested that it could explain the neuritic sprouting characteristic of TLE [8]. Apo $\varepsilon 4$ has also been found to be associated with earlier onset of TLE [15].

The underlying pathology of epilepsy and the factors which bring about the degenerative changes are not yet absolutely clear, but these changes disrupt depolarization and repolarization mechanisms and result in development of aberrant neuronal network causing abnormally increased excitability and synchronization, leading to epilepsy. Keeping in view the role of apoE alleles and its isoforms in neurodegeneration [16], one could hypothesize a potential epileptogenic role for apoE, by altering production, metabolism and clearance of the β-amyloid, affecting the repair mechanisms in the neuronal tissues resulting in an epileptogenic focus [14, 17–19].

Few workgroups have reported an association of apoE4 genotype with epilepsy [7,15,20]. On the other hand, recent studies conducted in Turkish [21] and other epileptic patients [22] reveal no such association of the apoE4 genotype with the onset or the severity of disease. The findings are contradictory and the available data is far from satisfactory [7,15,20–26].

This study is designed to assess the status of apoE gene polymorphism and plasma levels of apo E in the Asian Indians where epilepsy is quite prevalent.

2. Methods

A total of fifty-eight temporal lobe epilepsy (TLE) cases were recruited from the epilepsy clinic, department of Neurology, All India Institute of Medical Sciences, New Delhi, India. Patients were diagnosed to have TLE on the basis of a detailed history; physical examination and clinical evaluations including EEG, brain imaging (MRI) and perfusion scans (SPECT).

Patients were categorized as with or without lateralized seizure features, based on laterized EEG discharge. EEG was obtained in awake as well as sleep state. MRI was done in all patients to detect the presence or absence of any temporal lobe lesion. TLE was classified as lesional or nonlesional on the basis of MRI findings.

Fifty-seven age and sex approximated healthy controls were randomly selected from the general population and included in the study. Selected controls were ruled out for any evidence of neurological or cardiovascular disease or any past history of seizures.

2.1. Collection and storage of Samples

10 ml fasting blood was collected from the cases and controls in 15 ml poly-vinyl tubes containing dry EDTA (2 mg/ml of blood). It was centrifuged at $500 \times$ g rcf (2000 rpm) for 15 minutes to separate the plasma and the blood cells. Plasma was divided into two parts and kept frozen at $-20°$. One part was used to determine the lipid profile and the other part was used to estimate Apolipoprotein E levels using apoE kit (MBL, Japan). Blood cells separated after centrifugation, were used for genomic DNA isolation. DNA Samples were stored at $-20°C$.

2.2. Apolipoprotein E genotyping

ApoE genotyping was done by restriction isotyping. Genomic DNA was isolated from the leukocytes of the subjects by high salt precipitation method [27–29]. The exon 4 of ApoE gene was amplified by one stage PCR [28]. The amplified portion of apoE gene was then digested by restriction enzyme, Hha I [30]. Apo E allelic variations result in the polymorphic restriction sites that on digestion results in different sized DNA fragments (Table 1, Fig. 1A). The DNA fragments were resolved on a 10% polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining (Fig. 1B).

2.3. Estimation of lipid profile

Plasma lipids and lipoproteins, total cholesterol (TC), Triglycerides (TG) and High Density Lipoprotein cholesterol (HDL-C) were estimated for all the cases and controls using commercially available enzymatic kits from Randox (India).

Low Density Lipoprotein cholesterol (LDL-C) and Very Low Density Lipoprotein cholesterol (VLDL-C) were estimated using Friedwald's formula. All the values for plasma levels of lipids were expressed in mg/dl.

Table 1
Fragment size obtained after restriction digestion (for Hha I) of the amplified portion of apo ε in various genotypes namely $\varepsilon2$, $\varepsilon3$ and $\varepsilon.4$

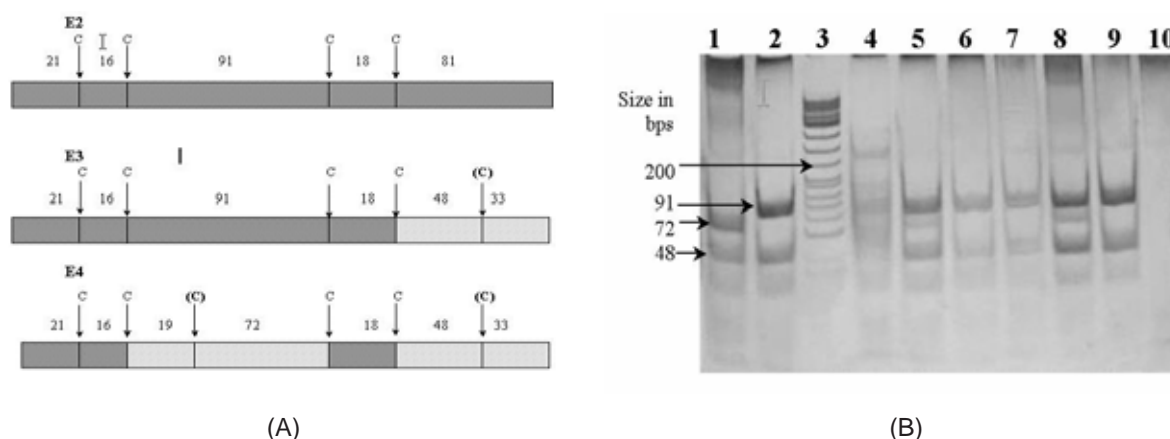|	E2/E2	E2/E3	E3/E3	E2/E4	E3/E4	E4/E4	Undigested
Fragment Size in bps	91	91	91	91	91	–	227
	81	81	–	81	–	–	
Size in pbs	–	–	–	72	72	72	
	–	48	48	48	48	48	

(A)   (B)

Fig. 1. (A): Restriction map (for Hha I) of the amplified portion of apo ε in various genotypes namely $\varepsilon2$, $\varepsilon3$ and $\varepsilon.4$. (B): Silver stained DNA fragments of amplified portion of apoE gene after restriction digestion on 10% PAGE.

2.4. Estimation of plasma apolipoprotein E levels

Apolipoprotein E levels were estimated in plasma samples with commercially available apoE ELISA kit (from MBL Co. Ltd, Japan) using manufacturer's guidelines. Values for ApoE plasma levels were expressed in mg/L. The kit detects both free and lipid bound apoE in human serum, plasma, CSF and tissue culture supernatants and is capable of accurate quantitation from fresh and frozen samples. Assay is based on sandwich ELISA and uses affinity purified polyclonal antibody against apoE.

2.5. Statistical analysis

Data was recorded on a pre-designed worksheet and managed with Microsoft Excel. Data Entry was double-checked. After confirming the normality aspects of the quantitative variables, descriptive data were computed using mean and standard deviation and qualitative variables were summarized by proportions. Student's t-test was applied to compare the mean values of quantitative variables among the epilepsy cases and the healthy controls. Associations of categorical variables with epilepsy were assessed by Chi square (χ^2) test. Correlation between the quantitative variables was an-

alyzed using Pearson's correlation coefficient. In the study, $p < 0.05$ was considered significant. Statistical analysis was done using the intercooled STATA version 8.0 (STATA Corp. Houston Texas, USA).

3. Results

At the time of study the age of the Epilepsy (TLE) patients ranged from 2.5 to 61 years. The mean age of the TLE patients at seizure onset was 11.72 yrs. The mean duration of disease was 11.53 years. Of the epilepsy cases 28.13% had right sided TLE, and 43.75% had left sided TLE whereas 28.13% had bilateral temporal paroxysmal activity. MRI abnormality indicative of mesial temporal sclerosis was observed in 25.86% patients. Patients with drug resistant epilepsy were not included in the study. Most of the patients were on single drug therapy with valproate or carbamazapine.

3.1. Age and gender distribution

The mean age of epilepsy (TLE) cases included in the study was 22.7 ± 12.1 years. The mean age of seizure onset in the epilepsy (TLE) cases was 11.7 years. A total of fifty-seven age and sex approximated controls

Table 2
Frequency (%) distribution of apoE alleles and genotypes in epilepsy cases and controls

ApoE Alleles/genotypes	$\varepsilon2$	$\varepsilon3$	$\varepsilon4$	$\varepsilon2/\varepsilon2$	$\varepsilon2/\varepsilon3$	$\varepsilon2/\varepsilon4$	$\varepsilon3/\varepsilon3$	$\varepsilon3/\varepsilon4$	$\varepsilon4/\varepsilon4$
Cases $n = 58$	1 (0.86)	102 (87.9)	13 (11.2)	0 (0)	1 (1.7)	0 (0)	46 (79.3)	9 (15.5)	2 (3.4)
Controls $n = 57$	3 (2.6)	102 (89.5)	9 (7.9)	0 (0)	3 (5.3)	0 (0)	46 (80.7)	7 (12.3)	1 (1.8)

n = Total number of subjects.
No. in parentheses indicate % frequency.

were taken. None of the controls had any history of any neurological and cardiovascular disease. The mean age of controls was 20.9 ± 9.2 years. The gender distribution was also comparable among the cases and the controls (Total of 43 males and 15 females in the cases, 40 males and 17 females in control group).

3.2. Genotyping of Apolipoprotein E

ApoE Genotyping was performed in the cases and the controls and the frequency distribution of apoE allele and genotype is shown in (Table 2). $\varepsilon3$ was the most common allele observed in both the groups i.e. epilepsy (TLE) cases (87.9%) as well as the healthy controls (89.5%). Though $\varepsilon2$ was higher in the control group, the frequency of $\varepsilon2$ allele was very low in both the cases (0.83%) and the controls (2.63%). $\varepsilon4$ allele was frequent in cases (11.2%) as compared to the controls (7.9%) but this difference was not significant. Among the genotypes $\varepsilon3/\varepsilon3$ was the most common genotype in both the groups i.e. epilepsy (TLE) cases (79.3%) and the controls and (80.7%) with comparable frequencies. $\varepsilon3/\varepsilon4$ was observed at a frequency of 15.5% in epilepsy cases and 12.91% in the controls. $\varepsilon4/\varepsilon4$ and $\varepsilon2/\varepsilon3$ were infrequent in both the groups with no significant difference while $\varepsilon2/\varepsilon2$ and $\varepsilon2/\varepsilon4$ genotypes were not observed in any of the groups.

3.3. Correlation of $\varepsilon4$ allele in epilepsy cases and controls

Both the groups, epilepsy (TLE) cases and the controls were divided into further subgroups depending upon the presence of even a single $\varepsilon4$ ($\varepsilon4$+ve) or complete absence of $\varepsilon4$ ($\varepsilon4$-ve) allele. Presence of $\varepsilon4$ allele was analyzed for any possible association with the disease process, age of onset and other quantitative variables namely plasma levels of apoE, total cholesterol (TC), triglycerides (TG), high dendisty lipoprotein- cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and very low density lipoprotein- cholesterol (VLDL-C). In both the groups no significant correlation of $\varepsilon4$ was found with any of quantitative variable (*Data not shown*).

3.4. Plasma levels of apoE

Significantly high plasma levels of apoE protein were found in the epilepsy (TLE) cases with a mean value of 149.0 ± 51.1 mg/L as compared to 111.9 ± 36.5 mg/L in controls ($p = 0.0001$) (Table 3, Fig. 2A). Since apoE levels were high in cases, Odds of apoE levels for having temporal lobe epilepsy was calculated. Subjects were divided into groups having plasma apo E levels <150 mg/L of, 150–190 mg/L and >190 mg/L. Frequency distribution of cases and controls falling under the three groups was compared (Fig. 2B) and it was observed that out of twelve subjects having plasma Apo E levels > 190 mg/L, eleven were having epilepsy. Odds of individuals who have plasma apo E levels >190 mg/L were 20.04 (95%CI = 2.46–163.34, $p = 0.005$). Odds of individuals having 150–190 mg/L of plasma apo E were 4.94 (95% CI = 1.85–13.9, $p = 0.001$), when compared to individuals having plasma Apo E < 150 mg/L (Fig. 2C).

3.5. Lipid profile of the subjects

Higher levels of TC and LDL-C were observed in epilepsy (TLE) cases compared to controls. Difference in LDL-cholesterol levels was significant ($p = 0.03$) whereas difference in total cholesterol levels was approaching significance ($p = 0.07$).

Other lipid parameters were comparable in both groups (Table 3).

4. Discussion

Results of the genotyping revealed that $\varepsilon3$ allele and the $\varepsilon3/\varepsilon3$ genotype was commonest in the cases as well as the controls and there was no difference in the prevalence of $\varepsilon3$ in both the groups. This finding is consistent with the earlier international reports and also with the previous findings of our group in studies conducted in normal population [31]. $\varepsilon2$ Allele was found to be infrequent but had three fold higher frequency in controls as compared to cases in this study. The difference in frequency of $\varepsilon2$ allele between the two groups was
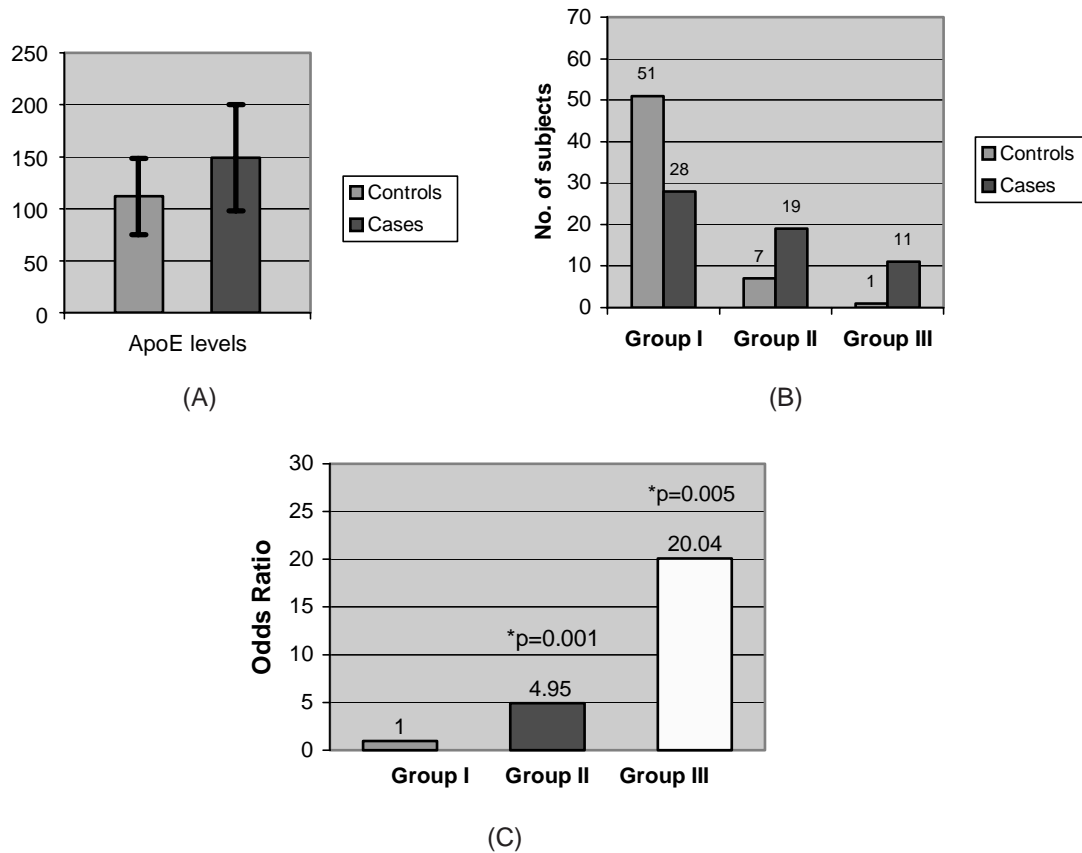
Fig. 2. (A): Plasma levels of apoE (mg/L) in epilepsy cases and controls. Error bars represents ± 2 SD. (B): Distribution of cases and controls in the three groups based on levels of plasma Apo E. (C): Odds of having epilepsy in the three groups based on plasma levels of Apo E. Cases and controls were divided in three groups according to the plasma Apo E levels.
Group I- Subjects having plasma ApoE levels of < 150 mg/L.
Group II- Subjects having plasma ApoE levels of 150–190 mg/L.
Group III- Subjects having plasma ApoE levels of > 190 mg/L.

evident with $\varepsilon 2/\varepsilon 3$ genotype being 5.26% in controls and 1.72% in the cases, however statistical analyses lacked the required degree of freedom to signify this difference due to the small number of $\varepsilon 2$ positive genotypes in the subjects. There have been contradictory reports regarding the association of apo $\varepsilon 4$ with epilepsy. Some studies suggest a positive association of Apo $\varepsilon 4$ genotype with epilepsy and others show no such association. The findings of the present study reveals no significant association of apoE alleles or genotypes with epilepsy (TLE) and is in accordance to the recent reports from Italian and Turkish populations [21,26].

Significantly higher plasma levels of ApoE were observed in epilepsy (TLE) cases as compared to the controls. The comparable frequency distribution of the apoE alleles and genotypes observed in the epilepsy (TLE) and the control groups suggest that the difference in the plasma levels of apoE is not due to any

genetic variation. In addition, a positive correlation between plasma apoE and total cholesterol was observed in these subjects. Our result is in accordance with earlier report [32] correlating high concentrations of apoE with high levels of total and LDL cholesterol. Elevated plasma levels of LDL-cholesterol and triglycerides were also observed in the epilepsy (TLE) patients. Increased levels of serum cholesterol have been shown to alter the cholesterol levels in the frontal cortex of mice fed atherogenic diets although total brain cholesterol levels remain stable [33]. The resulting regional cholesterol imbalance could alter neuronal synaptic membrane properties [34] and predispose to epileptogenesis.

It is established that apoE is produced in abundance in the brain. It is synthesized by astrocytes and microglia and enters into neurons through LDL, and VLDL receptors [35]. It serves as the principal trans-

Table 3
Lipid and lipoprotein profile in epilepsy cases and the controls

	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL Cholesterol (mg/dl)	LDL Cholesterol (mg/dl)	VLDL Cholesterol (mg/dl)	ApoE serum levels (mg/L)
Cases ($n = 58$) (Mean $\pm$ SD)	172.3 $\pm$ 37.0	111.2 $\pm$ 56.2	43.8 $\pm$ 9.7	106.3 $\pm$ 32	22.2 $\pm$ 11.3	149.2 $\pm$ 51.1
Controls ($n = 57$) (Mean $\pm$ SD)	159.9 $\pm$ 36.2	117.1 $\pm$ 50.0	40.9 $\pm$ 10.2	92.3 $\pm$ 36.7	22.6 $\pm$ 10.7	111.9 $\pm$ 36.5
p-value	0.07	0.55	0.13	0.03	0.85	0.0001

port vehicle in the CNS for cholesterol and phospholipids.

Apart from lipid transport, apo E is thought to be particularly important for repair mechanisms in the CNS and also in regulation of innate and adaptive immune responses [16,36–38]. In animal studies, apoE is reported to be induced at high concentration in peripheral nerve injury and suggested to play a key role in repair by redistribution of lipids to regenerating areas and to schwann cells during remyelination [9,12].

Several studies have shown $\varepsilon 4$ allele to be inefficient at neural repair [9,39], whereas apo $\varepsilon 3$ has been reported to have a neuroprotective role in injury and repair [9,40]. In cultured dorsal root ganglion neuron, apoE3 stimulates neural growth by stabilizing the cytoskeleton. Association of apo $\varepsilon 4$ allele with stroke and Alzheimer's disease has been well established in a number of studies [4,41,42]. Christen et al., 2000 have reported higher peroxidation levels in the brain tissue of $\varepsilon 4$ carriers which correlate inversely with apoE concentrations in Alzheimer disease patients [11]. It is postulated that neurons respond to limit the neurodegenerative damage, by synthesizing more amount of apoE3 protein [16]. Higher levels of ApoE protein have been reported with the $\varepsilon 3/\varepsilon 3$ genotype [18,43,44] and is found to be protective against neurodegenerative disorders.

Additionally, studies indicate that ApoE may play a role in regulating calcium homeostasis, and therefore in turn can affect neuronal regulation of various ion-dependant receptors [45,46]. Studies also report a role for ApoE in modulation of neurotransmitter release/sequestration, including the ability of ApoE to enhance the rate of glutamate uptake and prevent excitotoxicity [38,47,48]. There is also evidence of ApoE enhancing the effects of some growth factors, such as ciliary neurotrophic factor (CNTF) thus promoting neuron survival and sprouting [46,49]. ApoE may also salvage neurons from oxidative stress following injury. Miyata and Smith have shown that ApoE reduces the toxicity of hydrogen peroxide in a glial cell line [46, 48].

In light of the role of apo E in CNS, our finding of higher levels of plasma apoE in the epileptics, suggests that the high levels of apoE observed in the epileptic (TLE) patients may be a response to limit the neurodegenerative damage. Determination of the levels of specific apolipoprotein E3 isoform could further substantiate the above finding.

To conclude, significantly high plasma apolipoprotein E levels have been observed in the epilepsy (TLE) patients for the first time. Another salient observation of this case control study is that the elevated levels of plasma apoE in epilepsy (TLE) patients is probably not due to a genetic variation since the distribution of Apo ε alleles and genotypes were very much comparable in the cases and the controls. Further studies on the status of apoE levels in a large number of epilepsy patients with regular follow-ups would enable us to assess the possible role of apoE in Epilepsy.

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