Author's response to reviews

**Title:** Clinical significance of serological biomarkers and neuropsychological performances in patients with temporal lobe epilepsy

**Authors:**

Chiung-Chih Chang (neur099@adm.cgmh.org.tw)
Chun-Chung Lui (lchung@adm.cgmh.org.tw)
Chen-Chang Lee (lcsc@adm.cgmh.org.tw)
Shang-Der Chen (chensd@adm.cgmh.org.tw)
Wen-Neng Chang (cwenneng@adm.cgmh.org.tw)
Cheng-Hsien Lu (chlu99@adm.cgmh.org.tw)
Nai-Ching Chen (naiging@yahoo.com.tw)
Alice Y.W. Chang (cgmf.kmc@gmail.com)
Samuel H.H. Chan (shhchan@adm.cgmh.org.tw)
Yao-Chung Chuang (ycchuang@adm.cgmh.org.tw)

**Version:** 2  **Date:** 2 February 2012

**Author's response to reviews:**

Response to the editors and reviewers:

Dear Professor Garg:

Thank you for considering our manuscript, titled "Clinical significance of serological biomarkers and neuropsychological performances in patients with temporal lobe epilepsy" for publication in BMC Neurology. We appreciate the suggestions and comments from the editor and reviewers. In this revision, we have made a substantial effort to clarify the issues raised and to make the manuscript more concise. These changes are explained in detail, item by item, in the response letter to the reviewers. In this revision, we have also highlighted the changes by using red coloured text.

Correspondence should be addressed to:
Yao-Chung Chuang, M.D., Ph.D.,
Department of Neurology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 83301, Taiwan
Tel: +886-7-731-7123, Fax: +886-7-731-8762,
E-mail: ycchuang@adm.cgmh.org.tw

We hope that the editorial board will agree on the interest of this study.

Sincerely yours,

Yao-Chung Chuang on behalf of the authors.
Response to Reviewer 1:

Comments: Well-design study; Interesting and attracting results; Editing and proofing is needed

Response: We would like to thank you for the comments. We have revised, edited and proofed the manuscript according to the editors and reviewer’s suggestions. The style has also been edited according to BMC Neurology and the final version had been read and approved by all of the authors. We have also enlisted the help of a colleague who is a native English speaker to identify grammatical mistakes in our manuscript.

Response to Reviewer 2:

1) Due to its cross-sectional design, this study cannot differentiate between reactive and pathogenic changes. In other words, it is unclear whether the changes in the serum biomarkers identified are implicated in the process of epileptogenesis and epileptogenicity or represent the outcome of other, under-recognized nosogenic processes. Unless a longitudinal follow-up study design is implemented to characterize the evolution of these biomarkers with the clinical and radiological endpoints of interest, safe conclusions cannot be drawn.

Response:

We appreciate the reviewer’s comments. Indeed, the study design here could not answer the queries raised by the reviewer, such as the evolution of the biomarkers as the disease progressed or the nosogenic process between epileptogenicity and epileptogenesis. A major reason is that the study was not designed to investigate the pathogenic mechanisms occurring during seizure propagation in these TLE patients. We focused instead on the clinically feasible tools (i.e. serological biomarkers and MRI) and their clinical significance in relation with the cognitive outcomes in TLE patients after a long duration of epilepsy. Therefore, the conclusions drawn by this study were mainly made based on the observations thereof. We agree with the reviewer that a longitudinal study could extend the understanding of these serum biomarkers in terms of disease processes and we will continue to follow up these patients in the future. We have elaborated our discussion and future directions in the last two paragraphs of the discussion.

To explain further, we suggest that serum biomarkers such as HSP70 can only reflect reactive nosogenic changes in TLE rather than a direct reflection of epileptogenesis, since HSP70 is a constitutive protein which is expressed after cell stress. Additionally, the biomarkers were collected during the interictal state as we have stated in the Methods section (page 12, paragraph 2, line 1-2). Therefore, the elevated serum levels of HSP70 can only be considered as a stress biomarker reflecting neuronal cell damage (page 20, paragraph 1, line 10-11). Compared with HSP70, the elevated S100ßP and NSE levels found in this study could be regarded as indirect evidence for blood brain barrier
disruption (page 22, paragraph 1, line 4-5).

In this revision, the future direction such as a longitudinal follow-up for these patients has been included in the Discussion section: Conclusion and future direction. The limitation listed by the reviewer has also been included in the Discussion section (page 23 paragraph 3 and page 24.).

2) The current study cannot differentiate between biomarkers of epileptogenesis vs epileptogenicity. The population under study has established TLE and it is unclear how early in the disease genesis or course the biomarker and related abnormalities took place. Risk factors of epileptogenesis (e.g. genetic predisposition, history of childhood febrile seizures, etc) both in the disease and control group that may be linked to the abnormalities identified have not been explored.

Response:

These serum biomarkers were collected during an interictal state as evidenced by routine EEG prior to blood collection. We did not perform physiological monitoring on these patients during the ictal state.

To answer the reviewer's question regarding how early in the disease genesis or course the biomarker and related abnormalities took place, we consider that the study was conducted in a relatively chronic stage of the disease. This is based on the following reasons: 1. The TLE patients had a mean disease duration of 18.9 ± 11.9 years. 2. The quantitative measurements of brain MRI was analyzed by gray matter (GM) atrophy, which is often regarded as delayed damage parameter after chronic insults.

For the analysis of epileptogenesis risk factors, as pointed out by the reviewer, there were no genetic predispositions based on the family history review. Although childhood febrile seizures were common in TLE, none of the study population had history of febrile seizure history. Careful MRI selection (i.e. no lesions other than increased T2 signals and/or atrophy in hippocampal formation identified) was arranged to avoid migrational disorder or symptomatic epilepsy in these patients. These statements have been included in the material and method section (page 9, first paragraph, line 1-4).

3) As mentioned in the limitations, the duration, type and dosing of antiepileptic treatment was not adequately taken into account. That on its own may have a significant impact not only in the levels of the measured biomarkers but also in the neuropsychological and even radiological outcomes. Similarly, comorbidities other than overt psychiatric illnesses that were controlled for may indirectly affect these biomarkers.

Response:

Since all of the patients in this study had at least one AED treatment, it is possible that the use of AEDs alone could affect the levels of biomarkers in this study. However, because the small case numbers, this has been considered as a major confounder and has been included in the manuscript in the Limitations section (page 24, paragraph 1, line 3-9).
As the reviewer pointed out, comorbidities other than overt psychiatric illnesses that were controlled for may indirectly affect the interpretations of the biomarker results. Since this study looked at a TLE population with a longer duration of epilepsy, a developmental process or psychiatric illnesses (with or without concomitant antipsychotic medications) posed greater difficulties for the interpretation of the cognitive test results because they may have borderline or lower intelligence quotients at the baseline which was not directly related to the seizure risk characteristics. This has been discussed during the research enrollment stage, and we have also listed it in our Limitations section (page 23, paragraph 4, line 1-5).

4) The biomarker measurements and neuropsychological tests were performed presumably during the interictal state but without concomitant EEG monitoring it is impossible to ascertain that. Moreover, despite certain tools utilized such as the MMSE or the CDR may not necessarily be the most suitable for the epilepsy population.

Response:

We apologize for the confusion. We did not state clearly enough the status of the patients. All of these cognitive tests were performed during the interictal state as evidenced by the EEG recording before the cognitive tests. We have added this statement in our manuscript (page 12, line 7-8).

The rationale of using CDR was because it evaluates the general daily functional capacity of the subject in 6 independent cognitive domains and that the MMSE is easily applied for general intellectual function. We agreed with the reviewer that these tools may not be universally accepted in the epilepsy literature, and we have therefore deleted these two neuropsychological tools in this revision. Thank you for the suggestion.

5) The segregation of disease severity in two groups, while useful, cannot be always representative of the reality since rare but prolonged seizures (i.e. status) may have more impact than more, short lasting events. A biomarker in epilepsy can be useful if it can predict which risk factors would materialize in epilepsy down the road, if it can predict the course of the disease and response to various treatments and if it can help identify potential surgical candidates and localize their epileptogenic zones. The currently proposed biomarkers do not seem to serve any of these purposes yet. However they constitute important contributions to our current knowledge in that field, setting the basis for future longitudinal studies to address the paramount issues of epileptogenesis and epileptogenicity.

On the whole, this paper should be accepted and an effort should be undertaken to address some of the aforementioned limitations, acknowledge the rest and discuss about the future perspectives on the topic.

Response:

We appreciate the reviewer's suggestions. In this revision, we have listed all of the limitations as suggested by the reviewer including the confounding effects of AEDs on the biomarkers, a preselected study population as our study might not applied to the general TLE population, the study design here was not able to
address the epileptogenesis versus epileptogenecity issue, and that further longitudinal studies may help to understand the evaluation of these biomarkers as the disease progresses.

Response to Reviewer 3

Reviewer's report:

Major Compulsory Revisions:

1. The implication of the relation between changes in these biomarkers and atrophy in a limited region (i.e., hippocampus ipsilateral to the seizure focus) of the brain is unclear. Even though the biomarker is known to vary with CNS damages and widespread extra-temporal lobe atrophy associated with TLE, why was the correlation found significant only in such a small area? Was this correlation truly limited in the hippocampus or would there be a continuum of correlation varying across structures and may have not been found in the current study due to the sample size and/or technical limitation of voxel-based analysis. If assumed so, the authors are strongly recommended to perform a power analysis to evaluate this.

Response:

We apologize for the confusion. The correlation study between morphometric data with biomarkers was based on a hierarchical order as follows: First, we compared the Gray matter (GM) volume between the patient and the controls. As shown in Table 2, GM atrophy in the patients with TLE was found in various regions, including the thalamus, fusiform gyrus, temporal regions (including inferior and medial), caudate nucleus, hippocampus, supplementary motor area, amygdala and putamen. These regions, to be specific, had GM atrophy extending more than 250 voxels between the controls and the patients (as stated in our statistical model). After obtaining these results, morphometric data with smaller GM volumes in the TLE were first extracted from 4 representative regions (i.e. right supplementary motor area, right thalamus, left caudate nucleus and left hippocampus) to investigate the variability of voxel changes between the controls and patients with TLE. These 4 regions were considered to be representative regions because they were bilaterally involved. The morphometric data with atrophic regions were further extracted and checked to investigate the variability of voxel changes between the controls and patients with TLE. The results revealed that voxel variabilities in the TLE group were small in three regions (i.e. supplementary motor area, right thalamus and left caudate nucleus) but large in the hippocampus, although the mean value was still lower than the controls. Lastly, to understand whether the variability of the hippocampus were related with any of our biomarkers, we did a correlation and found that the level of HSP70 was correlated with a lower ipsilateral hippocampal volume after correcting for the effect of age. The represented MNI coordinates were provided for the reader's interest. Since the significant coordinates extended more than 250 voxels as defined, the results were not confined to the voxel where the MNI coordinates were labeled. These has been summarized in the discussion section (page 23, line 1-3).

For TLE, the epileptogenecity focus mainly originated from the hippocampus with
secondary epileptogenesis elsewhere. Based on these results, the GM atrophy was relatively symmetric in the supplementary motor area, thalamus and caudate nucleus, suggestive of common pathways of epileptogenesis through these structures. We did not perform the correlation in the initial manuscript to avoid the possibility of type I errors since we had 5 biomarkers and 3 a priori anatomical (i.e. supplementary motor area, thalamus and caudate nucleus) regions.

2. The 2nd major concern is that the diagnostic value of serological biomarkers in TLE is unclear. Can this type of biomarkers react only upon the disease process of TLE? What about other types of epilepsy or other neurological and neuropsychological conditions? There have been studies that show serum level changes in Alzheimer's disease as well as in healthy elderly. What would be the advantage of using serological biomarkers in specifically diagnosing individual patients with TLE? Can these predict favorable surgical outcome? Can these biomarkers lateralize the side of the seizure focus (i.e., Do these react differently hemispherically according to the seizure focus?)? Such analyses would definitely increase the value of the study.

Response:

This was a cross-sectional study with serum biomarkers collected during the interictal state. Therefore, the biomarker measurements can only reflected an interictal state. To answer whether these types of biomarkers react only upon the disease process of TLE, a longitudinal study should be conducted. This has been included in our Limitations section (Page 25. line 7-9).

The introduction to the biomarkers with Alzheimer dementia, other neurological disorders and epilepsy syndrome has been included in the introduction section (page 5, second paragraph 2, line 2-10).

To answer the question regarding whether these biomarkers lateralize the side of the seizure focus, we performed statistical analysis. Among the patients with epileptic focus, 21 had left temporal origins, and 13 from the right side. The biomarker levels were compared between those with initial left versus right seizure focus, and none of the 5 biomarkers reached statistical significance. This suggests that initial epileptogenecity (whether left or right) did not influence the serum biomarker levels during follow-up. The biomarkers did not react differently according to the seizure focus. This has been added to the Results section (page 16 , line 2-3).

The clinical value of these serum biomarkers was clinically accessible and correlated with cognitive outcomes with a mean duration of epilepsy of 18.9 ± 11.9 years. We found elevated HSP70 levels and considered this to be a stress biomarker based on the neuropsychological and volumetric correlation. Our results suggested that memory scores were inversely associated with serum HSP70 level, and that levels of plasma nuclear DNA were inversely correlated with verbal fluency and a positive correlation was found between plasma nuclear DNA and Trail Making test completion time. In addition, our results also suggest that while levels of HSP70 and S100BP were higher in the patients with TLE, only HSP70 correlated inversely with hippocampal volume.
3. The 3rd major concern is no information of use of intensity inhomogeneity correction or no use of such a correction prior to spatial normalization. This correction is a crucial step which significantly increases the accuracy of subsequent brain tissue segmentation. Relative to lower tesla, in 3T, incorrect tissue classification due to no use of such a correction will highly impact on a morphological analysis and may ultimately lead wrong biological interpretation (for example, location of atrophy/volume growth). The authors should redo the analysis if the correction was not included.

Response:
We apologize for the confusion. A standard protocol of VBM using SPM was performed for intensity inhomogeneity correction during the segmentation step. We understand the importance of intensity inhomogeneity correction since we also performed a morphological analysis later. This has been included in the material and method section (page 13, last line).

4. The final major concern is about patient selection. First, no information of the seizure lateralization was given. In VBM, volume changes in TLE was then analyzed with respect to left/right hemispheres instead of the side of the seizure focus. This approach possibly causes two issues in the results. Given that brain structural damages reflect epileptogenic process and/or seizure spread from the seizure origin, the laterality of findings can be obscured if patients were pooled regardless of the side of the seizure focus. If Left and right focused TLE patients were separately analyzed (not likely in this study as no such information was found), the statistical power will decrease. To solve, the authors could have normalized values at each voxels using z-score normalization based on the mean and SD of controls and then pooled patients with respect to the side of the focus. The authors claimed that they measured 'ipsilateral' hippocampal volume from page 18 without explanation how to switch the 'ipsilateral' from 'left' or 'right' in previous pages. This is also unclear.

The authors mentioned that cognitive abilities in TLE declines over time, meaning cognitive parameters are correlated with epilepsy duration. Indeed, the level of serum biomarkers were correlated with neuropsychological performance as well as duration of epilepsy. I wonder if the correlation with cognitive abilities can remain significant when the duration is included as a covariate.

Response:
For the seizure lateralization, 21 patients had left temporal origins, and 13 from the right side (page 15, paragraph 1, line 12-15). From the original page 18, we described "a higher level of HSP70 was correlated with a lower ipsilateral hippocampal volume after correcting for the effect of age in the linear regression model (R square = 0.22, p = 0.007)." The statistical analysis was performed by extracting morphometric data in the hippocampus corresponding to the initial epileptogenicity (whether left or right).

We understand the advantage of pooling the patients into one group based on the lateralization. A major concern not to pool the patients into one is because the gray matter of the right and left cerebral hemispheres is asymmetric in healthy
subjects, likely reflecting underlying functional specificity. The pathologic contribution from epilepsy to this asymmetry is substantially greater than normal underlying asymmetry. Since the study results were used to correlate with cognitive performances rather than tracing the epileptogenesis circuit, a final decision is to analyze the general GM atrophic pattern of these patients. This has been included in the discussion (page 23, paragraph 3).

For the reviewer's reference, we perform VBM analysis on the left temporal group (n=21) and compared with the age-matched controls (n=21). The anatomical regions showed greater atrophy in the left hemisphere including thalamus, fusiform, caudate, hippocampus, putamen and supplementary regions, consistent with the manuscript results, albeit with lower T values. However, we did not include the result into this revision and would consider enroll more patients for further researches.

As the reviewer suggests, we performed an analysis between serum biomarkers with cognitive test results controlling for the duration of epilepsy. The results showed that while including duration of epilepsy as a covariate, the correlation was not significant. This suggests that the cognitive parameters were correlated with epilepsy duration. We have included the analysis into the Result section (page 16, paragraph 3, line 7-8).

--------------------------------------------------------------------------------------------------

Minor Essential Revisions

In the title, the 'biomarkers' has to be specified as serological biomarkers as a biomarker can be any type measurements from any modality.

Response:

We have revised the title according to the reviewer's suggestion as follows: "Clinical significance of serological biomarkers and neuropsychological performances in patients with temporal lobe epilepsy." Thank you.

P9, 'non-surgical patients with TLE': should specify number of patients who are medically intractable and have gone to surgery after the data were obtained unless all patients are benign (i.e., pharmacologically tractable). The prevalence is important to indicate as severity of brain damages differs between benign and malignant epilepsy (Labate et al., 2011a; Labate et al., 2011b).

Response:

No patients in this study received an operation for epilepsy. According to the consensus paper that defines medically refractory epilepsy as having one or more seizures despite being treated with 2 consecutive first-line antiepileptic medications (AEDs) over 2 years (Epilepsia. 2003;44(6):741-51). We included the information in our result section (page 14, paragraph 1, 12-14).

P13: What was the 'study-specific template'? Is this an individual template or a template averaged from the used samples? The authors should clarify how to create this template and why this was used than the MNI template (there is a nonlinear MNI template). Why was the template smoothed?
Response:

The study-specific template were created from all participants to avoid possible inaccuracy in the spatial normalization and segmentation using SPM default templates. The customized templates were created follows the standard procedure from SPM instruction (http://www.fil.ion.ucl.ac.uk/spm/) as follows: (1) the native T1 image for each subject was segmented into gray, white matter and CSF using the default SPM brain tissue prior probability maps; (2) the segmented gray matter was spatially normalized to the SPM gray matter prior map by using affine registration and nonlinear warping, normalization parameters estimated were then applied to the raw T1 image; (3) the normalized T1 image was segmented into gray, white matter and CSF; and (4) The T1, gray and white matter and CSF maps obtained from step 3 were respectively averaged over all subjects and smoothed with an 10 mm full width at half maximum (FWHM) Gaussian kernel to create the study-specific templates. The purpose of spatial smoothing was to cope with functional anatomical variability that was not compensated for by spatial normalization and to improve the signal to noise ratio.

P14: Age frequently correlates with disease duration. Plus, aging effect inpatients may also differ from that in controls. Thus, the independency between age and duration variables has to be examined before using age as a covariate in the regression. If age in the patient group is correlated with the disease duration, it cannot be used. In this case, controls' (normal) aging or age of seizure onset in patients may be used as an alternative covariate.

Response:

Thank you for the suggestion. In the patient group, the correlation between age and duration of epilepsy was not significant (#=0.313, p=0.07). Therefore, there was in dependency between these two variables during analysis. This has been included in the result section (page 16, paragraph 3, line 1-2).

P14: 'A p value < 0.01 was considered statistically significant'. How was this p-value determined? Why not Bonferroni correction?

Response:

We consulted a statistician at the National Sun Yat-Sen University (Dr. Chang CW) regarding the questions raised by the reviewer. Initially, we used a p value less than 0.01 to avoid a type I error since we considered that the sample number was small. In addition, using serological biomarkers to evaluate the cognitive outcomes is a relatively new method of analysis from a literature search. This was the reason why we used a more stringent p value in the previous section. A "Two Means Power Analysis using Simulation" was performed using the Mann-Whitney test which achieved 99% power either in #=0.01 or 0.05. Therefore, after full consideration, we used p<0.01.

P14: ' the whole brain (false discovery rate) with an extended threshold of 250voxels': This 250 voxels sounds like to control small-size clusters as another type of false positive. But, based on what was '250' chosen?

Response:
Since the general linear model performs a univariate t-test for every voxel, which leads to a mass-univariate testing, there will be a lot of false positives (voxels activated when there is no true effect in the data) on a given alpha value of, for instance, 0.05. FDR controls for the proportion of false positives committed out of all significant comparisons. The use of 250 voxels has been traditionally used in VBM analysis because for a cluster size of 1/4 cm³, it will be 250 voxels when the voxel size is 1 X 1 X 1 mm.

P20: 'the high morphometric variabilities from the left hippocampus indicated the relatively lateralized nature of TLE.' This conclusion is most likely to be modified after reanalysis given that major body of previous studies have reported ipsilateral hippocampal atrophy.

Response:
We apologize for the confusion. We have rephrased the sentence as follows: "...the high variabilities in the hippocampal volume suggest the relatively lateralized nature of TLE..."

P24: 'Therefore, the interpretation presented here might represent the results related to the study group only.' This statement is poor and unnecessary as it limits the significance and reproducibility of the study. Please improve it.

Response:
Thank you for the comment. We have revised the sentence as follows: "Whether the results of this study can only be applied to this unique epilepsy syndrome or whether they can be applied to the general population of epilepsy patients still requires larger study cohorts for verification."

There are a few typos.
P23, 1st line: between TLE with the controls -> TLE and controls
P24 the 2nd last line in the discussion: there are two 'changes', omit one of either.
Response:
Thank you. The typo errors have been corrected accordingly.