PEER REVIEW HISTORY

This paper was submitted to the JNNP but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Open. The paper was subsequently accepted for publication at BMJ Open.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Differential motor neuron involvement in progressive muscular atrophy: a comparative study with amyotrophic lateral sclerosis
AUTHORS	Riku, Yuichi; Atsuta, Naoki; Yoshida, Mari; Tatsumi, Shinsui; Iwasaki, Yasushi; Mimuro, Maya; Watanabe, Hirohisa; Ito, Mizuki; Senda, Jo; Nakamura, Ryoichi; Koike, Haruki; Sobue, Gen

VERSION 1 - REVIEW

REVIEWER	Swash, Michael The Royal London Hospital
REVIEW RETURNED	15-Jan-2014

GENERAL COMMENTS	The authors do not state whether or not they explained to the patients that their clinical findings and autopsy data would be used for research publication. They should have done this, but I don't think the absence of such permission would invalidate publication and it may be that in Japan such strict ethical guidelines are not standard practice? The results are not exactly earth-shattering and the paper could be greatly shortened with benefit in terms of readability. The last sentence of the Results into the Summary should be shifted into the Conclusions, or the latter should be shifted in to the Results! The conclusion really is that met PMA is really ALS, although the UMN signs are difficult to detect? The dominance of TDP pathology over FUS pathology is already well-known - although I (perhaps we) don't understand why yet. The sensitivity of the Lusex AP system, and its limitations, should be spelled out. What staining technique is used for this method of counting? What size fibres were accepted? What does the method do about degenerate axons? What is the test-retest results like? What histological techniques were used on the paraffin-embedded sections for pathological studies? Do the authors consider paraffin-embedded methods reliable in assessing "large" or small" fibres in the corticospinal tracts? Why was there such marked variability if the fibre size mean

	measures (large SD's). Did the authors consider construction fibre size histograms? The two case reports could be deleted - they are irrelevant did all the PMA cases have negative sum gene studies? The authors state that UMN signs were "probably masked by LMN signs" bu this i soberly simplistic; the authors could simply refer to a review that seeks are more physiological explanation (Why are upper motor neuron signs difficult to elicit in amyotrophic lateral sclerosis? Swash M. JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY. 2012;83:659-662) The concluding paragraph is perhaps the main message, but this is stated three times in the manuscript - I suggest it be deleted, but allowed to remain in the Summary. The frequency of the observed differences should be explained more fully, so that readers will quickly grape it. Are there any implication with regard to clinical diagnosis? The authors appear to shy away from this, yet it is the most important point for most neurologists. This somewhat small point is the only message of the paper that will endure.

REVIEWER	Eisen, Andrew University of British Columbia, Neurology
REVIEW RETURNED	15-Jan-2014

GENERAL COMMENTS	This study sets out to answer the question "are patients presenting with pure LMN signs a clinical phenotype of ALS?. This was tackled by comparing clinical and autopsy pathology in ALS and PMA. 107 patients met criteria for inclusion of which 93 had classic ALS and 14 clinical PMA. Other causes of LMN disease were excluded in the PMA group. The numbers are confusing as throughout the text the authors refer to 13 PMA patients and 29 clinical ALS patients. The data is interesting and well presented, the figures are good. The pathological profile of both PMA and ALS was similar as was disease progression, however in the discussion and final paragraphs the authors do not answer the question they posed - even though they data indicates that they both disease share immuno-patholigical features. There is no attempt to hypothesize as to why, if both disorders are part of a spectrum what determines the clinical phenotype. Some suggest that ALS variants are different conditions with a common end-stage pathology (this is not a view shared by this

	reviewer) but does need comment.

- The manuscript received three reviews at the JNNP but the last referee had declined to make his comments public.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Comments to the Author

Comment) The results are not exactly earth-shattering and the paper could be greatly shortened with benefit in terms of readability. The last sentence of the Results into the Summary should be shifted into the Conclusions, or the latter should be shifted in to the Results! The conclusion really is that met PMA is really ALS, although the UMN signs are difficult to detect?

Response) As this reviewer sharply noted, our data indicated that a larger part of clinical PMA was really “pathologically ALS” than in previous descriptions. In contrast, the UMN involvement was actually mild or sparse in some of PMA patients that were included in this study. This is also important message because it still remains to be elucidated whether the UMN involvement is actually mild in PMA patients even if PMA is included a spectrum of ALS. To improve readability, we have revised the Abstract and the Discussion sections.

Comment) The dominance of TDP pathology over FUS pathology is already well-known - although I (perhaps we) don't understand why yet.

Response) We agree with the reviewer’s comment and have deleted the text about the dominance of TDP pathology over FUS pathology in ALS and PMA.

Comment) The sensitivity of the Lusex AP system, and its limitations, should be spelled out. What staining technique is used for this method of counting? What size fibres were accepted? What does the method do about degenerate axons? What are the test-retest results like?

Response) We have added the information regarding the detailed methodology for axon measurement using Luzex AP to the Materials and Methods section. This software automatically recognizes particles, performs particle counts, and the diameters of these particles on binarized pictures; the software has been used for the morphological evaluation of the corticospinal tract or peripheral nerves. (Ann Neurol 2003;54:19-29, Neurology 1987;37:529-32). We stained all sections using anti-neurofilament immunohistochemistry pigmented with diaminobendizine without any additional stains (i.e., hematoxylin staining). Therefore, only axons are visualized as brown particles. They were then binarized and automatically recognized by this software. An expected limitation of this measurement was the between-test variability of axonal density even in the same patient between tests, due to the digital processing of pathological pictures or heterogeneous distribution of axons in each visual field. To validate the duplicability between tests, we constructed two axon size histograms of 13 ipsilateral control samples. The results from the validation test have been uploaded as supplementary material. Briefly, the variability between the test and re-test was sufficiently small to count the axons.

Comment) What histological techniques were used on the paraffin-embeded sections for pathological studies? Do the authors consider paraffin-embedded methods reliable in assessing "large" or small" fibres in the corticospinal tracts?

Response) As the reviewer noted, the paraffin-embedded tissues may tend to distort, compared with conventional nerve fixation using glutaraldehyde and epon. Unfortunately, epon-embedded samples were not available in a majority of the studied patients. In our assay, the proportions of the axonal sizes from 13 control samples were approximately close to previous reports in human (Anat Anz. 1984;157:97-111) or animal materials (Exp Brain Res. 1986;61:303-310). Our methods may be appropriate to assess the proportional changes in each size of pyramidal axons, but the absolute values that we obtained may vary more than those using other histological techniques. Therefore, we added an explanation of this weak point into the Discussion section (page 20, 2nd paragraph).

Comment) Why was there such marked variability if the fibre size mean measures (large SD's). Did the authors consider construction fibre size histograms?

Response) Prior to this study, we attempted to construct fiber size histograms for each clinical group because there was no established definition for “large axon” to the best of our knowledge. The histogram revealed that the ratio of axons > 1 μm in ALS and PMA were smaller than in the controls, resulting in a relative increase of the percentage of axons that were < 1 μm in the patients. On the basis of this result, we measured the axon fibers greater than 1 μm in diameter in this study. We have added the histogram into Fig. 3 to clarify why we labeled the axons that were > 1 μm as large axon fibers.
As the noted, fiber sizes in ALS and PMA patients showed large SDs. One reason for this may be that the materials were formalin-fixed and paraffin-embedded. Additionally, we consider that the degree of large axon loss may vary between patients, resulting in large variability of patients’ axonal densities > 1 μm. In contrast, the control samples showed evidently smaller variability, supporting the stability of our methods.

Comment) The two case reports could be deleted - they are irrelevant

Response) In terms of compactness of the manuscript, we agree with the reviewer’s comment and have deleted the case presentation section.

Comment) Did all the PMA cases have negative smn gene studies?

Response) The genetical materials (i.e., patients’ blood cells or frozen tissues) were available from only some patients; we were thus not able to perform SMN gene screening.

Comment) The authors state that UMN signs were "probably masked by LMN signs" bu this i soberly simplistic; the authors could simply refer to a review that seeks are more physiological explanation (Why are upper motor neuron signs difficult to elicit in amyotrophic lateral sclerosis? Swash M. JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY. 2012;83:659-662)

Response) As suggested by the reviewer, we have cited this concise review suggested in the Discussion section (page 20, 2nd paragraph).

Comment) The concluding paragraph is perhaps the main message, but this is stated three times in the manuscript - I suggest it be deleted, but allowed to remain in the Summary. The frequency of the observed differences should be explained more fully, so that readers will quickly grape it.

Response) We agree with this suggestion and have revised the concluding paragraph in the Discussion (page 21, last paragraph).

Comment) Are there any implication with regard to clinical diagnosis? The authors appear to shy away from this, yet it is the most important point for most neurologists. This somewhat small point is

the only message of the paper that will endure.

Response) We also consider this to be important. In our patient series, only 71.4% of the clinical PMA patients were correctly diagnosed by the first referred physicians, although 94.6% of the clinical ALS patients were initially diagnosed. We have written the clinical diagnoses of the included patients in the Results section (page 12) and the implications in the Discussion section (page 20, 2nd paragraph).

Reviewer: 2

Comments to the Author
This study sets out to answer the question "are patients presenting with pure LMN signs a clinical phenotype of ALS?. This was tackled by comparing clinical and autopsy pathology in ALS and PMA. 107 patients met criteria for inclusion of which 93 had classic ALS and 14 clinical PMA. Other causes of LMN disease were excluded in the PMA group.

Comment) The numbers are confusing as throughout the text the authors refer to 13 PMA patients and 29 clinical ALS patients.

Response) We enrolled 14 PMA and 93 ALS patients for clinical evaluations, but the pathological assay included 13 PMA and 29 ALS. The reasons for this were that 1 of the PMA patients was invalid for pathological evaluations, and the number of ALS patients was disproportionally large compared with the PMA patients. The 29 ALS cases were consecutively autopsied after 2006 (last 5 years of the study span); this means that they were not conveniently selected. For readability, we have revised the writing about this process in the Material and Method section (page 8, last paragraph).

Comment) The data is interesting and well presented, the figures are good.

Comment) The pathological profile of both PMA and ALS was similar as was disease progression, however in the discussion and final paragraphs the authors do not answer the question they posed - even though they data indicates that they both disease share immuno-pathological features.

Response) Our data revealed that a larger part of clinical PMA was really “pathologically ALS” than in previous descriptions. We have revised the conclusion section in the Abstract and final paragraph of the Discussion section in regard to comments by this reviewer and reviewer 1. (Please also see our response to reviewer 1.)

Comment) There is no attempt to hypothesize as to why, if both disorders are part of a spectrum what determines the clinical phenotype.

Response) Significant pathological evidence has revealed that one half or more of PMA patients had degenerative changes in UMN, TDP-43 pathology in UMN or broad areas in the cerebral cortices and subcortical gray matters. Our study fully evaluated, integrated, and supported these previous results. In conclusion, ALS and PMA may be included into a disease spectrum related with TDP-43 pathology in our patient series. We have added this to the Discussion section (1st paragraph on page 20).

Comment) Some suggest that ALS variants are different conditions with a common end-stage pathology (this is not a view shared by this reviewer) but does need comment.

Response) Several investigators have described variants in pathological changes during end-stage ALS managed using respirators for extended durations. Some of these patients showed widespread TDP-43 pathology throughout CNS that was not restricted to the motor neuron systems (Acta

Neuropathol 2008;116:169-82). Some of the respirator-managed patients in our study contained such patients (Patient 13 in Clinical PMA and Patient 27 and 28 in Clinical ALS). However, we have not distinguished these from other patients because it remains inconclusive whether this pathological phenotype represents another pathogenesis. Thus, we have simply noted that result in the revised manuscript (page 18) but did not intensively discussed this issue.