PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	A retrospective cohort study identifying the principal pathological features useful in the diagnosis of inclusion body myositis
AUTHORS	Brady, Stefen; Squier, Waney; Sewry, Caroline; Hanna, Mike; Hilton-Jones, David; Holton, Janice

VERSION 1 - REVIEW

REVIEWER	Werner Stenzel Department of Neuropathology Charite - Universitaetsmedizin
REVIEW RETURNED	02-Jan-2014

GENERAL COMMENTS	Stefen Brady and co-authors analyze sIBM using a panl of morphological features and stains. They identify MHC class I expression (IHC) and Cox negative SDH positive fibers (as a sign of mitochondrial stress) to differentialte between IBM-RV and PM/DM as being more relevant in terms of sensitivity and sensibility than 'classically' and previously used (GRIGGS) criteria such as congophilic inclusions, partial invasion by inflmmatory lymphocytes or ultrastructural abnormalities. They also identify p62 immunoreactive inclusions as beeing relevant for differentiating between IBM+RV from PAM The ms. is well written, the results are clearly presented and the authors have an important clinical and diagnostical message. Some minor points to consider: On page 8 the authors say: 'the distribution and intensity of the inflammatory infiltrate in IBM-RV and PM/DM was similar' while on page 9 they say:' greater numbers of perimysial T cells ...were observed in PM/DM than in IBM-RV could the authors please clarify this issue- is it a simple quantitative difference? I guess that he infiltrates in DM especially are following a specific distribution (although there are well described variabilities in terms of quantity) I am personally a bit surprised that the authors have never seen any desmin aggregation while they describe myotilin positivity in 10 out of 15 cases. (Although the 'positivity' is certainly unspecific.) Did the authors also test other markers of autophagy like LC3 etc. In the discussion the authors say taht they ...'found no differences in the number of Cox negative SDH + fibers, the degree of MHC class I upregulation, the morphology and distribution of p62 and the pattern of inflammation between IBM+RV and IBM-RV. I would like to ask if the authors know which muscles were biopsied

	in both groups and if they did serial sectioning. and Unfortunately in clinical practice, this observation does not help: If present RVs assure the diagnosis of sIBM but if absent, although we see the other features, diagnostic/morphological uncertainty remains. The authors describe 4 patterns of p62 staining, however they do not explain for which disease they claim these may be specific. suppl Tab 2: IBM-RV: What is a standard diagnostic histological assessment? PAM: the authors group dystrophinopathy in this category? flow chart: Are the authors sure that the'absence of COX- SDH+ fibers effectively rule out a diagnosis of IBM-RV?

REVIEWER	Ingrid Lundberg KArolinska Institutet Sweden
REVIEW RETURNED	07-Jan-2014

GENERAL COMMENTS	This is a carefully performed study using muscle biopsy investigations including both conventional staining and immunohistochemistry staining in order to develop new pathological criteria for diagnosis of inclusion body myositis (IBM). The inclusion criteria of cases for the muscle biopsy investigations are well defined. Adequate controls groups from a clinical perspective have been included. The low number of each diagnostic groups is a limitation, as discussed by the authors, however. The algorithm for clinical diagnostic purpose of the muscle biopsy evaluation is clearly described and may become useful in clinical practice. However, there are some major concerns that need to be addressed by the authors. Firstly, principally I question the use of pathological criteria for diagnosis. Importantly, and as the authors point out in the introduction, the muscle biopsy features are only a complement in the diagnostic work up in the context of clinical features. Furthermore, this study does not take into account early cases, with e.g. less than one year of disease history, these may be missed if specific muscle pathology criteria are required for diagnosis. Therefore I would suggest to change the title to something like “Identification of principal pathological muscle biopsy features in patients with inclusion body myositis- useful in diagnosis” In the suggested diagnostic procedures of muscle biopsies for patients with suspicion of IBM immunohistochemistry staining is suggested. Such stainings may be sensitive to the accurate protocols used. Therefore, more details on the

	immunohistochemistry protocol should be included; e.g. how were the sections fixed; at what time point? Immediately after sectioning, or could stored, unfixed tissue sections be used? Blocking procedures should be described and so on. In Supplementary Table 3, only primary antibodies are presented, which secondary antibodies were applied and which staining was used. For immunohistochemistry staining both positive and negative controls are important. Control tissues have been used, which is a strength of the paper. However, also negative controls for the staining protocol are important for the validity. Which negative controls were used, e.g. species specific isotype antibodies? This information should also be included in the paper and some examples of negative staining sections with the different antibodies corresponding to Figure 1, would be helpful for the clinician, and could be included as a supplementary figure. The authors found that presence of T cells had added value for IBM diagnosis. The authors included three CD markers to identify T cells (CD3+, CD4+ and CD8+). As I understand from the manuscript, this study was a descriptive study and did not include functional T cells studies. Therefore I would avoid naming T cells for function (e.g. helper och cytotoxic) as the T cells subsets using the CD-markers CD4 and CD8 may be more complex and include more subtypes. I would suggest to use just the name the T cells after used CD marker to avoid misunderstanding. Secondly, the authors propose to include CD8+ T cells in the diagnostic work-up to distinguish IBM from other myopathies with rimmed vacuoles. Looking at the data it seems to me that replacing CD8 with CD3+ T cells in the algorithm would be equally clinically useful. This would make the suggested algorithm more useful and easier to implement world-wide. If this is not the case, I suggest that the authors present such comparative data and discuss more clearly why CD8 staining is much more helpful than CD3 in this work-up. Furthermore, do the authors mean to exclude CD3 staining or still propose CD3, CD4 and CD8 as part of routine staining for patients with suspicion of IBM? This is an important aspect as each staining adds costs and time and not all laboratories have these as routine and this limitation of the algorithm should be addressed in the discussion.

VERSION 1 – AUTHOR RESPONSE

Reviewer Name Werner Stenzel
Institution and Country Department of Neuropathology
Charite - Universitaetsmedizin
Germany
Please state any competing interests or state ‘None declared’: none

Stefen Brady and co-authors analyze sIBM using a panl of morphological features and stains. They identify MHC class I expression (IHC) and Cox negative SDH positive fibers (as a sign of mitochondrial stress) to differentialte between IBM-RV and PM/DM as being more relevant in terms of sensitivity and sensibility than 'classically' and previously used (GRIGGS) criteria such as congophilic inclusions, partial invasion by inflmmatory lymphocytes or ultrastructural abnormalities. They also identify p62 immunoreactive inclusions as beeing relevant for differentiating between IBM+RV from PAM The ms. is well written, the results are clearly presented and the authors have an important

clinical and diagnostical message.

Some minor points to consider:
1. On page 8 the authors say: 'the distribution and intensity of the inflammatory infiltrate in IBM-RV and PM/DM was similar'
while on page 9 they say:' greater numbers of perimysial T cells ...were observed in PM/DM than in IBM-RV could the authors please clarify this issue- is it a simple quantitative difference? I guess that he infiltrates in DM especially are following a specific distribution (although there are well described variabilities in terms of quantity)

Response
We thank the reviewer for pointing this out. As the reviewer has suggested the difference is due to a difference in the quantification. The first statement on page 8 refers to the sum of the scores for T-cells, B-cells and macrophages at each site (endomysial, perimysial and perivascular areas) in isolation. The statement on page 9 refers to a more detailed analysis of the inflammatory cell sub-type present at each site. To make this clearer we have made changes to both Results sections on pages 8 and 9.

2. I am personally a bit surprised that the authors have never seen any desmin aggregation while they describe myotilin positivity in 10 out of 15 cases.
(Although the 'positivity' is certainly unspecific.)

Response
We fully agree with the reviewer's comment that the pattern of staining is non-specific and we were surprised at the lack of desmin aggregation in the presence of myotilin aggregates. Increasingly in our routine staining, we have found that although desmin staining highlights abnormal fibres such as atrophic fibres the staining pattern in fibres is typically diffuse and well-defined aggregates are less frequently present than observed with myotilin staining.

3. Did the authors also test other markers of autophagy like LC3 etc.

Response
We are aware of the recent publications using LC3 and NBR1 staining in IBM and although we would have liked to include other markers the detailed nature of this study required that we limit ourselves to the most frequently described markers and widely used markers. We have included two statements in the Discussion to highlight that other autophagic markers have been observed in IBM and a brief discussion about the relevance of altered autophagy in IBM.

4. In the discussion the authors say taht they ...'found no differences in the number of Cox negative SDH + fibers, the degree of MHC class I upregulation, the morphology and distribution of p62 and the pattern of inflammation between IBM+RV and IBM-RV. I would like to ask if the authors know which muscles were biopsied in both groups and if they did serial sectioning.

Response
Serial sections were cut from each case (IBM and controls). Muscle biopsies were taken from deltoid or quadriceps muscles. We agree these are important points to include and to make it clearer we have added statements to the Methods section to indicate that the tissue sections were serial sections and that the biopsies were taken from the deltoid or quadriceps. We have previously audited the pathological findings from deltoid and quadriceps biopsies in our clinically and pathologically defined cohort of IBM patients and found no significant differences in the pathological findings and diagnostic yield between the sites. We have included a further statement to indicate this in the Discussion section.

5. Unfortunately in clinical practice, this observation does not help: If present RVs assure the diagnosis of sIBM but if absent, although we see the other features, diagnostic/morphological uncertainty remains.

Response
We agree with reviewer that rimmed vacuoles were considered to be sensitive and specific for IBM and in their absence the diagnosis of IBM is uncertain despite many of the other pathological features.

Additionally, although the presence of an inflammatory infiltrate is said to differentiate IBM from other PAM the degree of inflammatory change necessary is uncertain, often inflammatory changes are observed in muscular dystrophies. Therefore, albeit a slightly artificial division of IBM into two groups based on the presence or absence of rimmed vacuoles we felt this was the best way to assess the biopsies to produce a clinically useful diagnostic tool. We agree with the reviewer that there is great difficulty in pathologically differentiating IBM without rimmed vacuoles from steroid-responsive determine, but we believe that the presence of characteristic p62 aggregates and an absence of COX-/SDH+ fibres have some diagnostic utility. We have altered the Discussion to clarify that these features are helpful rather than diagnostic in differentiating IBM from disease mimics.

6. The authors describe 4 patterns of p62 staining, however they do not explain for which disease they claim these may be specific.

Response
We thank the reviewer for bringing this to our attention, it is an important point. We have included extra statements in the Results and the Discussion to indicate that we found pattern I staining to be specific for IBM-RV and sensitive for IBM+RV. Although patterns II and III appeared to be associated with myotilinopathy and DM respectively, the small number of cases makes it difficult to draw any conclusion from these findings. We have included a further statement in the Discussion to emphasise this point.

suppl Tab 2:
IBM-RV:
7. What is a standard diagnostic histological assessment?

Response
The study was performed at two separate centres therefore the standard assessment differed slightly, but the following stains are included at both centres for the work-up of an inflammatory myopathy: H&E, GT, Sudan black or oil red O, periodic acid Schiff, nicotinamide adenine dinucleotide dehydrogenase, succinate dehydrogenase, cytochrome c oxidase, combined cytochrome c oxidase and succinate dehydrogenase, phosphorylase, acid and alkaline phosphatase, adenylate deaminase, ATPases at pH 4.2/4.3/9.4 and immunohistochemical staining including neonatal myosin, utrophin, major histocompatibility complex class I, membrane attack complex and a combination of inflammatory cell markers. We have included a statement in the table to clarify what is meant by a standard assessment of an inflammatory myopathy and which stains were included.

PAM:
8. the authors group dystrophinopathy in this category?

Response
We agree with the reviewer’s sentiment that dystrophinopathy is not typically included under this term; however, because of the case’s pathological similarity to the protein accumulation myopathies – the presence of rimmed vacuoles, p62 and myotilin aggregates in morphologically normal fibres – it was included under this term. Additionally, it enabled all the control cases with rimmed vacuoles to be referred to using one term. A statement has been added to the Table to explain that this case is included in the control group and the reason why - its pathological similarities to PAM.

flow chart:
9. Are the authors sure that the'absence of COX- SDH+ fibers effectively rule out a diagnosis of IBM-RV?

Response
We thank the reviewer for pointing out that although the absence of COX-/SDH+ fibres casts doubt on the diagnosis of IBM the statement “effectively rules out a diagnosis” is much too strong. We have altered the figure legend and Discussion to make this clear.

Reviewer Name Ingrid Lundberg
Institution and Country KArolinska Institutet
Sweden
Please state any competing interests or state ‘None declared’: None declared

This is a carefully performed study using muscle biopsy investigations including both conventional staining and immunohistochemistry staining in order to develop new pathological criteria for diagnosis of inclusion body myositis (IBM).

The inclusion criteria of cases for the muscle biopsy investigations are well defined. Adequate controls groups from a clinical perspective have been included. The low number of each diagnostic groups is a limitation, as discussed by the authors, however. The algorithm for clinical diagnostic purpose of the muscle biopsy evaluation is clearly described and may become useful in clinical practice. However, there are some major concerns that need to be addressed by the authors.

1. Firstly, principally I question the use of pathological criteria for diagnosis. Importantly, and as the authors point out in the introduction, the muscle biopsy features are only a complement in the diagnostic work up in the context of clinical features. Furthermore, this study does not take into account early cases, with e.g. less than one year of disease history, these may be missed if specific muscle pathology criteria are required for diagnosis. Therefore I would suggest to change the title to something like “Identification of principal pathological muscle biopsy features in patients with inclusion body myositis- useful in diagnosis”

Response
We fully agree with the reviewer’s sentiments and the need for a complimentary approach to the diagnosis of IBM. We believe that the diagnosis of IBM is predominantly a clinical one with a supportive muscle biopsy. However, in our experience a number of patients do not exhibit the full characteristic clinical findings at initial presentation and previously, we have shown that patients younger at the time of muscle biopsy may lack the diagnostic pathological features. In our clinic we do not usually see patients within the first 12 months of their symptom onset, most probably because of the slowly progressive clinically course of the disease. The inclusion IBM-RV emphasises the spectrum of pathological findings we observe in IBM in routine clinical practice.

We have altered the title to reflect the reviewer’s comments.

2. In the suggested diagnostic procedures of muscle biopsies for patients with suspicion of IBM immunohistochemistry staining is suggested. Such stainings may be sensitive to the accurate protocols used. Therefore, more details on the immunohistochemistry protocol should be included; e.g. how were the sections fixed; at what time point? Immediately after sectioning, or could stored, unfixed tissue sections be used? Blocking procedures should be described and so on. In Supplementary Table 3, only primary antibodies are presented, which secondary antibodies were applied and which staining was used.

Response
We fully agree and have included greater detail on the immunohistochemical staining protocol used in both the Methods section and Supplemental Table 3, including the fixatives used, blocking procedures and the freezing and storage of samples after biopsy.

The secondary and chromagen used to visualise all antigen-primary antibody binding was a Dako REAL (TM) EnVision (TM) Detection System; this system includes horseradish-peroxidase labelled goat anti-rabbit/mouse secondary which is then incubated with a 1:50 solution of 3,3'-diaminobenzidine. We have included greater details on this in the Methods section and within the legend of Supplementary Table 3.

3. For immunohistochemistry staining both positive and negative controls are important. Control tissues have been used, which is a strength of the paper. However, also negative controls for the staining protocol are important for the validity. Which negative controls were used, e.g. species specific isotype antibodies? This information should also be included in the paper and some examples of negative staining sections with the different antibodies corresponding to Figure 1, would be helpful for the clinician, and could be included as a supplementary figure.

Response
‘No primary’ negative controls were included with each staining run performed. We have included this detail in the Methods section. As helpfully suggested by the reviewer we have included a Supplementary Figure1 showing both positive and negative controls for the p62, TDP-43, ubiquitin,α

B-crystallin and myotilin IHC staining and alkalinised congo red.

4. The authors found that presence of T cells had added value for IBM diagnosis. The authors included three CD markers to identify T cells (CD3+, CD4+ and CD8+). As I understand from the manuscript, this study was a descriptive study and did not include functional T cells studies. Therefore I would avoid naming T cells for function (e.g. helper och cytotoxic) as the T cells subsets using the CD-markers CD4 and CD8 may be more complex and include more subtypes. I would suggest to use just the name the T cells after used CD marker to avoid misunderstanding.

Response
We thank the reviewer for raising this point and to avoid misunderstanding we have altered the text throughout the article as suggested.

5. Secondly, the authors propose to include CD8+ T cells in the diagnostic work-up to distinguish IBM from other myopathies with rimmed vacuoles. Looking at the data it seems to me that replacing CD8 with CD3+ T cells in the algorithm would be equally clinically useful. This would make the suggested algorithm more useful and easier to implement world-wide. If this is not the case, I suggest that the authors present such comparative data and discuss more clearly why CD8 staining is much more helpful than CD3 in this work-up.

Furthermore, do the authors mean to exclude CD3 staining or still propose CD3, CD4 and CD8 as part of routine staining for patients with suspicion of IBM? This is an important aspect as each staining adds costs and time and not all laboratories have these as routine and this limitation of the algorithm should be addressed in the discussion.

Response
These are important points and we agree with the reviewer's comments. As she rightly suggests, there are only minor differences in the sensitivity and specificity of immunohistochemical staining for CD3+ T-cells or CD8+ T-cells clinically and using CD3 staining may make the algorithm easier and cheaper than sub-typing the T-cell inflammatory infiltrate. Therefore, we have included a statement to indicate that their diagnostic yield is similar and that CD3 staining may be more widely available and cheaper within the Discussion and we have altered the flow-chart to include both CD3 and CD8 IHC staining.

VERSION 2 – REVIEW

REVIEWER	Werner Stenzel Department of Neuropathology Berlin Charite Universitätsmedizin Germany
REVIEW RETURNED	28-Feb-2014

- The reviewer completed the checklist but made no further comments.

REVIEWER	Ingrid Lundberg Karolinska Institutet
REVIEW RETURNED	02-Mar-2014

GENERAL COMMENTS	The authors have improved the manuscript and clarified some issues raised in the first review. With this additional information I am sorry to say that the immunohistochemistry staining is not adequately described or performed. The staining procedures have been clarified in more detail. However, there is still some concern regarding the control staining for immunohistochemistry:

	As I understand from Supplementary Figure 1 and the Figure legend to Figure 1: As negative control staining the authors omitted the primary antibody. I do not think this is an appropriate negative control to demonstrate the specificity of the antibody used. For an adequate negative control staining for immunohistochemistry the investigators are suggested to use irrelevant isotype-matched control antibodies for all their respective antibodies in muscle tissue from patients with IBM with typical histopathological changes. If such controls were used I would suggest the authors to include the results in a Supplementary Figure.

VERSION 2 – AUTHOR RESPONSE

Reviewer Name Werner Stenzel
Institution and Country Department of Neuropathology
Berlin ChariteUniversitätsmedizin
Germany
Please state any competing interests or state 'None declared': None declared

All concerns were answered satisfactorily

Reviewer Name Ingrid Lundberg
Institution and Country KarolinskaInstitutet
Please state any competing interests or state 'None declared': None declared

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