Author’s response to reviews

Title: Gene expression profiling in patients with polymyalgia rheumatica before and after symptom-abolishing glucocorticoid treatment

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Author’s response to reviews:

Re: BMSD-D-16-01185; ”Gene expression profiling in patients with polymyalgia rheumatica before and after symptom-abolishing glucocorticoid treatment” evaluated for publication by BMC Musculoskeletal Disorders.

Response to Editor and Reviewers.

Editor:

Thank You for a patient and careful handling of our manuscript. We have now revised the paper according to most of the comments raised by the reviewers. Below we have explained why we in a few instances were not able to follow the proposals.

You are right that we have no original specimen left for RNA extraction. However, we had some cDNA, which allowed us to extend the qRT-PCR analysis to include BDNF and MARK4 as proposed by Reviewer 2. Furthermore, Reviewer 3’s request for re-extracting RNA was based on the assumption that RNA samples had been stored at -20°C, which would raise concerns about the integrity of the RNA. However, the samples were, in fact, stored at -80°C, the confusion reflecting that some cDNA samples were stored at -20°C, which is generally accepted for such samples.

All changes to the manuscript are highlighted by use of track changes.

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Reviewer 1:

1. “Good job”. Thank You!

Reviewer 2:

1. Major criticism. “Interpretation of possible cytoskeletal dysregulation in PMR stiffness seems conjectural” and “only microtubule-related gene checked by qRT-PCR was TUBD1”.

We have now used remaining cDNA to also check microtubule affinity-regulating kinase 4 (MARK4) by qRT-PCR. The primer sequence is included in Table 6. The results are presented in Table 5. They agreed with microarray findings as now described in Results, p. 14, l 315 and 319, and in Discussion, p. 17, l 395 and p.19, l 444. Furthermore, we have now used a more cautious wording, substituting “may possibly explain the muscle complaints” with “may possibly contribute to the muscle complaints”, Discussion p.15, l.

2. Abstract. To be more specific about the statement that “Overall, qRT-PCR confirmed the microarray findings”, we have now expanded the text, see p. 2 l. 32 and 42-46.

3. Methods. “The arguments for using the Chuang classification criteria should be moved to the Discussion”. This has now been done (p. 15, l 342-348).

4. Discussion: “BDNF and MARK4 were not studied in qRT-PCR”. The objection has now been repealed, because the warranted analyses have been performed, and the results turned out to be in accordance with the original text. For primer sequences and results, please see tables 5 and 6 and p. 14, l 315 and 319. For corrections in Discussion, see p.17 l 395 and 400 and p. 19 l 444 as well as a new panel in Figure 5.

5. Statistical review: “A bioinformatician or statistician would be required”. The second author Ph.D. Rehannah Borup is, in fact, a bioinformatician.

Reviewer 3:

1. General comments and comments to l. 127 and 133: “The storage of RNA at -20°C questions the integrity of the RNA used for analysis”. This would be true, if the description in the Methods section was correct. Unfortunately, the description was wrong: Both muscle samples (as stated) AND isolated RNA were, in fact, stored at -80°C. The text has now been revised accordingly, p.7 l.132). The confusion reflected that cDNA was sometimes stored
briefly at -20°C, which is a generally accepted procedure. We thank the reviewer for being alert and apologize for the mistake.

2. “Major revision – re-extract RNA and analyze candidate genes by qPCR”. We have no original specimen left for RNA extraction. However, the request for re-extracting RNA is based on the assumption that RNA samples had been stored at -20°C, which, in fact, is not the case (see 1. above). Furthermore, we had some cDNA left, which allowed us to extend the qRT-PCR analysis to include BDNF and MARK4. For primer sequences and results, please see tables 5 and 6 and p. 14, 1315 and 319. For corrections in Discussion, see p.17 l 395 and 400 and p. 19 l 444 as well as a new panel in Figure 5.

Minor comments:

P.2, l.34. The sentence has been changed as proposed.

P.3, l.54 (now p.4, l.57-58). The sentence has been changed as proposed.

P.3, l.56 “check manuscript for consistency of abbreviations”. This has been done.

P.3, l.59 (now p.4, l.63 ). The term “synovial inflammation” has been specified: “in the synovia of bursae, joints and tendon sheaths”.

P.4. “Subject section should not thoroughly describe the information provided in Table 1”. We do not understand this objection, because apart from the number of subjects included in the study, no information from the Table is repeated in the text. The text states that “10 matched (age, sex, and BMI) non-PMR control subjects were studied”. Age, sex and BMI data are given in the Table. So, it is possible that if we delete the parenthesis “(age, sex, and BMI)” from the text, the reader might him/herself guess, which criteria were used for the matching. But he/she could not be sure.

P.4. “Sentence repeated”. Correct, what a mistake! The first of the two identical sentences (former l. 93-95) has now been deleted.

P.4, l.98. The sentence has been changes as proposed (now p.5 l 102).

P.5, l.117 (now p.6, l.122). “Celsius” now substituted by “C”.

P.6, l.127 and 133. “Due to storage at -20°C, there are concerns about the integrity of the RNA samples”. The objection is based on an error in the description of the storage conditions, which has now been corrected (see 1. above; text corrected p.7, l.132).
P.8, l. 178. “The section could be more concise. Not necessary to describe what the functional annotation does; more briefly describe enrichment values”. We think it is appropriate to give the non-expert reader a minimal impression of what functional annotation clustering implies. This together with a summary introduction of the use of enrichment scores in the ranking of clusters is done in only 6 lines. Because we think the understanding of the procedures will suffer from shortening of the paragraph, we hesitate to change it. It may be added that enrichment scores are further explained in the Discussion.

P.8, l. 186. “Small gene lists may result in low enrichment scores, but, nevertheless, be biologically relevant”. We have added the argument proposed by the reviewer: “(e.g. small gene lists do not generally get very high enrichment scores, illustrating that categories with lower scores may still be biologically relevant)”.

P.8, l.198. “de novo” has been deleted.

P.9, l.202. “Table 6 not included”. Mysteriously, the Table apparently had fallen out of the manuscript during submission. We hope that we have now succeeded in including it. This table shows the primer sequences, whereas comparisons between qRT-PCR and microarray findings are shown in Table 5.

P.9, l.207. “qPCR analysis: Were data subjected to relative comparisons, and was RPLP0 used as the internal control?”. In order to answer these questions, the description of the analysis has been extended as follows (p.10, l.215-218): “The Ct values for the samples were converted to relative numbers using the standard curves and normalized to the internal “housekeeping” control, ribosomal protein P0 (RPLP0). Microarray analysis confirmed that the RPLP0 mRNA level is stable under the current conditions and therefore suitable as normalizer.”

P.9, statistics. In order to comply with the Reviewer’s questions and proposals we have now in the section on Statistics (p. 10) specifically mentioned also the statistical treatment of ESR and CRP levels. Furthermore, the fact that we had analyzed data by both parametric and non-parametric methods and achieved identical conclusions is now also mentioned.

P.10, l.226. “This section is unnecessary and can be summarized in the beginning of the following section”. The text has been reorganized as proposed (p.11, l.254-257).

P.10, l.238. “Re-word section headings”. The section headings have been re-written to become more direct (p. 11, l.252; p.12, l.271; p.13, l.287; p.16, l.369; p.18, l.425; p.19, l.454).

P.10, l.241. “Figure 1 difficult to interpret”. The layout of the figure has been changed.

P.10, l.244. “In figures 2-4 GO categories are small”. Unfortunately, a crucial verb is missing in the reviewer’s proposal to solve the problem. To us it seems that the only way to increase the size of the letters would be to increase the number of figures from 3 to 8 (one for each cluster), and that would hardly be accepted.
P.10, l.244. “Entire gene list submitted for analysis?” It has now been clarified p.12, l.262 that all 78 genes differing between patients and controls before treatment were analysed together.

P.10, l.246. We have changed the wording as proposed here (now p.12, l.264), and by analogy also in l. 247 (now l.265) and 249 (now l. 267) and on p.13 l. 302 and 303.

P.12, l.293. “Table 5: tedious to read the description of symbols below the Table”. The layout of the Table is quite traditional. Furthermore, if the heading “Fold changes” should be described in a row adjacent to the heading rather than beneath the Table, then the same should apply to the other heading “Fold differences”. This would take up much space in the Table and would not look nice.

P.14, l. 340. “remove “Insert Fig.1” and others from this section”. The indications of where to place figures were meant to be proposals of possible help during the printing process. They have now been removed.

We hope that after the above described substantial revision, the manuscript is now acceptable for publication.

Sincerely,

Henrik Galbo,

Professor, MD, DMSc