Author’s response to reviews

Title: Inflammation biomarker discovery in Parkinson's disease and atypical parkinsonisms

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Author’s response to reviews:

First of all, we would like to thank you for giving us the opportunity to submit a revised version of our manuscript to be considered for publication in BMC Neurology. In addition, we would like to thank the reviewers for their critical review and helpful comments. Below we provide a point-by-point response to the reviewer comments, and how we addressed these points in the manuscript. In the enclosed manuscript we used Track Changes to pinpoint the modifications that we made based on the reviewer comments. Added references are highlighted in yellow.

Yours sincerely,

Dr. Marcel M. Verbeek

On behalf of all co-authors.

Point by point response to the reviewer and editor

Reviewer 1

Clinical diagnosis of MSA and PD is sometimes difficult and useful biomarkers are required. This article shows possible usefulness of such CSF biomarkers. However I have some concerns.
1. Pathophysiology of PD and MSA begin before the onset of motor symptoms, and prognosis of both disorders differ. Authors should describe the disease duration from symptom onset at baseline assessments.

Response: We thank the reviewer for pointing out this omission in our manuscript. Disease duration has been added to Table 1 where patients’ characteristics are described. Kruskal-Wallis test has been performed to assess possible differences between diagnosis. No differences were observed (p = 0.556).

2. It is known that mean survival duration of MSA is shorter than that of PD. Were all MSA patients alive after 12 years of follow up? If so, patients included in this study could be considered as "milder MSA". I think there is a possibility that pathology of such patients differ from that in typical MSA patients with worse outcome. Were all PD patients in life after the 12 years follow up? Authors should clarify the outcome of patients if patients died within the 12 years. Were there difference in the CSF level of biomarkers according to the survival duration?

Response: We would like to apologize if the text was not clear enough, as well as, for the lack of information. Three and 10 years after inclusion, the clinical condition was re-evaluated by a repeated structured interview and extensive neurological examination, and a clinical diagnosis was established by consensus of 2 movement disorder specialists. In 2018, 12 years after inclusion, all clinical diagnoses were evaluated again and updated according to the most recent clinical criteria (1-6), disease course based on the patients’ medical files and follow-up visits, and neuropathological examination whenever available. A clarification on that regard can be found in line 81 of the main text. We also calculated the mean survival time for each patient in the study taking 2018 as endpoint. The mean survival time of MSA is indeed shorter than that of PD (5.1 versus 11.8 years) and, thus, the patients included in this study cannot be regarded as “milder MSA”. We added the number of patients who were alive or dead after 12 years to Table 1.

We also correlated the survival duration of MSA patients with the levels in CSF of DNER and β-NGF, the two biomarkers that were differentially expressed between PD and MSA. No correlation was found (p = 0.88 and p = 0.65 respectively), pointing out that there are no differences in CSF levels of biomarkers according to the survival duration.

3. Authors evaluated ataxia using ICARS score. Authors should mention number of MSA-C and MSA-P patients respectively.

Response: Thank you for your remark. The number of MSA-C and MSA-P patients are now indicated in Table 1.

4. Is a red blood cell count of 200/μL enough to rule out blood contamination? Do red blood cell include inflammatory proteins?
Response: Blood contamination in cerebrospinal fluid (CSF) can influence biomarker levels. The red blood cell count cut off of 200/µL that we used is based on previous observations on the influence of blood contamination on micro-RNA stability in CSF (7). Other studies have also tested the influence of blood contamination on CSF diagnostics (8) and found that a blood contamination of 2,500 erythrocytes/µL led to false pathological results of total protein levels. This study also noted that a contamination with up to 5,000 erythrocytes/µL is an acceptable number which does not yet lead to false-positive results of intrathecal synthesis of IgG. Altogether, we consider that a cut-off of 200 erythrocytes/µL (i.e. maximal 0.004% blood contamination in the CSF) is enough to rule out effects of blood contamination on inflammatory proteins levels or any other kind.

References have also been added to the main text at line 103.

Reviewer 2

The article entitled "Inflammation biomarker discovery in Parkinson's disease and atypical parkinsonisms" by Santaella et al aimed to find inflammatory biomarkers in CSF to differentiate PD and atypical parkinsonism syndromes. The authors enrolled 25 controls, 46 PD patients, 15 multiple system atrophy (MSA), 9 vascular parkinsonism and 7 patients with PD mixed with vascular parkinsonism. Multiplex Proximity extension assay (PEA) was conducted covering 92 biomarkers.

This work trying to identify disease-related inflammatory CSF biomarkers to differentiate PD from atypical parkinsonism syndromes are commendable. In addition, the study cohort was diagnosed after a 12-year follow up study, which is also a merit. However, I have some concerns for this study.

1. The significance of the statistical analysis should be corrected by Bonferroni correction for multiple comparison, which was not used in the study. The P value would not be 0.05 and will be much lower to reach the significance level. In this case, the difference beta nerve growth factor (β-NGF) and DNER would not be different between MSA and PD as the P values was 0.03 in both group.

Response: Thank you for your comment. As described in the methodology, Bonferroni correction or Games Howell post hoc test were applied. In general, the Bonferroni correction divides the desired alpha-value by the number of comparisons and uses this number to determine significance. However, the SPSS package uses a mathematical equivalent adjustment; it takes the observed (uncorrected) p-value and multiplies it by the number of comparisons made. This corrected p-value is used to conclude significance. If the value is less than 0.05, one can conclude that the difference is significant (https://www.ibm.com/support/pages/calculation-bonferroni-adjusted-p-values). The Games Howell post hoc test is used to compare groups with unequal variances. The test was designed based on Welch’s degrees of freedom correction and
uses Tukey’s studentized range distribution. Altogether, we confirm that the p-value of 0.03 is adjusted and indeed significant.

2. The exact P value in each comparison should be addressed, not only address as &lt;0.05.

Response: We agree with this reviewer that the exact p-value should be noted since it gives extra information. We added the exact p-value when possible but, for clarity of the text, we decided to set a threshold and not give the exact value for the multiple comparisons study.

3. What is the definition for H-Y or UPDRS progression?

Response: We would like to apologize if the text was not clear enough. As stated in line 85 of the introduction and line 124 of the methods, disease progression is defined as the annual change in the score of the respective scale. Hereto, disease progression at 3 years follow-up is calculated by the subtraction of the score of each scale (H-Y, UPDRS, etc.) at follow-up from the score at baseline, and divided by years between baseline and follow-up (Δt = 3 years):

\[
\frac{(\text{FollowUp\_score} - \text{Baseline\_score})}{\Delta t}
\]

4. Is there any correlation between age and the CSF biomarkers, ex: β-NGF, DNER,…

Response: We performed a bivariate Spearman correlation between age and each of the biomarkers. 49 out of 53 biomarkers positively correlated with age. Because age was significantly different between diagnosis, according to Kruskal-Wallis test results, all the analyses were performed taking along age as a covariate.

5. This study did not cover the other atypical parkinsonism syndrome, for example PSP, CBS or DLBD, which should be listed as one of the study limitations.

Response: We agree with the reviewer that this is indeed a limitation of the study. We did not include a large enough number of patients of all the different atypical parkinsonisms into the clinical longitudinal study to warrant inclusion into the current study. We added the limitation to the last paragraph of the discussion.

6. How did the authors diagnose the group of PD combined with vascular parkinsonism syndrome?

Response: Thank you for the question which was not properly addressed in the text. All vascular parkinsonism syndrome (VaP) patients fulfilled the Zijlmans criteria for VaP (6) which include: 1) presence of parkinsonism, 2) evidence of cerebrovascular disease (by brain imaging (CT or MRI) or the presence of focal signs or symptoms that are consistent with stroke), and 3) a likely
relationship between the above two disorders. However, for some patients, the relationship between their parkinsonism and cerebrovascular disease was uncertain, because they also met diagnostic criteria for PD. Based on the updated diagnostic approach for VaP subtypes (5), the diagnosis of neurodegenerative parkinsonism with overlapping VaP, was established according to the following criteria: 1) patients meeting clinical diagnostic criteria for probable neurodegenerative parkinsonism (PD, DLB, PSP, MSA, CBS), and 2) imaging evidence of cerebrovascular disease in location(s) that is/are spatially congruent with the presenting parkinsonism.

References have also been added to the main text at line 81.

Other changes made to the manuscript that were not asked for:

We changed the numbers of patients in the tables of the manuscript, due to an oversight in counting the correct number of patients, we only now discovered. The correct number of patients was included in the analyses, but this did not correspond to the numbers we presented in the original submission. Although, we have now corrected the number of patients in the tables, the results of the study did not change. We apologize for the confusion this may have created.

We also modified the legend of supplementary data 1 to make the scheme more understandable.

References


