**Author’s response to reviews**

**Title:** Selected serum cytokines and nitric oxide as potential multi-marker biosignature panels for Parkinson disease of varying durations: A case-control study

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**Author’s response to reviews:**

RESPONSE TO REVIEWERS

Johannes Schlachetzki (Reviewer 1):

In the present manuscript, Rathnayake et al. determine serum levels of four immune mediators (IFNγ, IL-10, NOx, and TNFa) with the question, whether these markers alone or in combination change according to disease duration. A multicenter, cross-sectional study design was chosen. Altogether, 72 patients with PD were enrolled and placed into three categories according to disease duration (<1 year, 1-3 years, and > 3 years). 26 age-and gender healthy matched controls, and 30 with other neurological diseases were recruited.

There is an extensive effort to identify serum biomarkers in PD. Thus, the study is of interest, however, the data as presented here are inconclusive. Furthermore, statistical analysis is insufficiently described. There are major points the authors need to address.

Major points:

* The first clinical appearance of motor symptoms can be challenging to determine and relies on the patient's or relative's recollection. Was the disease duration based on the clinical history or when the diagnosis was made? Furthermore, what was the rational to divide the PD patients into these three categories? A more objective variable would be Hoehn and Yahr staging. The authors need to additionally use H&Y as categories.

We agree that the most contentious issue in PD diagnosis is determining the onset of disease. All patients recruited into the study were seen by a single neurologist (TC). If the neurologist judged the patient to be a reliable historian, then the onset of disease was recorded based on when the patient noted the first motor symptom. If the patient was an unreliable historian, the onset of disease was recorded as when the clinical diagnosis of PD was made by the attending doctor.
The H-Y scale was used as a tool in classifying the PD patients based on their clinical impairment to establish the stage of the disease. The categorization of PD patients based on their disease durations was an arbitrary division to identify patients with early as opposed to advanced disease. This was correlated with the patient H-Y status. PD duration of < 1 year – H-Y stage 1; 3 – 12 years – H-Y stage 2 and > 12 years H-Y stage 3 or more. The study explored whether there was any abnormalities in the peripheral cytokine profile with the motor manifestations of PD in the three sets of durations, in which < 1year PD and 1-3 years PD are particularly ascertained to mild PD, while 3-12 years of PD was considered to represent moderate-severe PD. (Zhao, Y. J., Wee, H. L., et al. (2010), Progression of Parkinson’s disease as evaluated by Hoehn and Yahr stage transition times. Mov. Disord., 25: 710-716. doi:10.1002/mds.22875)

The H-Y scale was used as an additional category in the context of the levels of serum immune mediators discussed in the study which did not render significant results in relation to the increase in H-Y stage leading to motor impairment (Table 2).

* Figure 1 is difficult to interpret. Instead of a line plot with error bars, the authors need to show their data with a categorical scatter plot with mean segments or box plot. This enables the reader to better interpret the data and demonstrates more clearly the spread of the data. Since this is a cross-sectional and not a longitudinal study, a line plot is misleading.

As suggested, a box plot with mean segments has been included along with a descriptive legend.

* It is unclear, how these biomarkers can be of prognostic value. To address this question, a longitudinal study would be needed. Thus, the statement that these immune mediators may be used as prognostic biomarkers can be discussed but should be removed from the abstract.

This was discussed in limitations for not using a longitudinal study and as suggested the word ‘prognostic’ was removed from the abstract.

* According to Table 1, only eleven patients were included with a disease duration less than one year, resulting in a total of 71 PD patients enrolled. However, 72 PD are reported throughout the manuscript.

This is a typographical error. Thank you for pointing it out. There were twelve patients enrolled in the disease group of less than one year. The typo has been corrected.

* Medication may influence serum levels, did the authors take this into account in their analysis?

All patients were on anti-parkinsonian therapy unless clinically diagnosed for the first time by the neurologist on the date of data collection and blood drawing (n<5 in the individuals with less
than 1 year of PD). Patients on immunosuppressive drugs or anti-inflammatory drugs were excluded from the study. Anti-parkinsonian drugs such as levodopa and dopaminergic drugs are not known to influence inflammatory markers. None of the patients were on MAOB inhibitors. However, a possible effect of medications has been included under limitations of the study.

* It is crucial to report the statistical test used to determine significance. This is missing throughout the manuscript. In addition, within the text, the authors need to report not only significance values, but also mean, standard deviation, F-ratio, etc.

The required information has been provided in the statistical analyses section as well as in figure and table legends as appropriate, in the revised manuscript.

* Figure 2 shows a bar graph displaying a IFNγ:IL-10 ratio. The ratio for healthy controls is given as less than 0.4. According to figure 1, IFNγ is about 20pg/ml, and IL-10 about 5pg/ml resulting in a ratio of about 4 and not 0.5. What was the statistical test used to determine significance?

Each Th1:Th2 ratio was calculated using individual serum concentrations of IFNγ and IL-10 to obtain the mean ratio of each PD patient for statistical analyses. (Mann-Whitney U Test *P<0.0001) – stated under the legend of Figure 2. The overall mean concentration of IFNγ and IL-10 in each group was not taken into account as stated above.

* The legends need to be extended.

The legends have been extended as suggested in the revised manuscript.

* Figure 3 and 4 are difficult to understand and are insufficiently explained in the manuscript.

As suggested, explanations have been included within the manuscript where appropriate and some details are described in the subsequent figure legends as well.

Minor points:

* The authors need to explain what SE/SP is.

SE-true positives to rule-out the disease status

SP=true negative to rule-in the disease status
* Did the authors observe any sex differences with regards to the four measured proteins?

No, the production of cytokines showed negligible and non-significant differences in each test group when investigated in relation to the sex of participants (data not shown)*****

* Did the authors perform another blood draw from the same patients to evaluate how consistent the serum concentrations are?

No. This was not a longitudinal study.

* Levels of these serum proteins may change depending on whether the patients were eating or the time of day blood was drawn or food. Did the authors take this into account?

No. However the neurology clinic was held between 5 pm to 8 pm of the day so that in all individuals, blood was drawn in between this period only, which was before dinner and way past the lunch time. Since the individual food habits were also not taken into account, it has been included as a limitation of the study.

* The authors need to discuss more recent studies aiming to identify serum based biomarkers.

There is one major study done related to the serum based cytokines as biomarkers in PD, which is taken into consideration in the discussion, however it does not deal with clinical performance and validation of these markers with ROC curves.

Reviewer 2:

OBJECTIVE - Full research articles: is there a clear objective that addresses a testable research question(s) (brief or other article types: is there a clear objective)?

Yes - there is a clear objective

DESIGN - Is the current approach (including controls and analysis protocols) appropriate for the objective?

No - there are minor issues

EXECUTION - Are the experiments and analyses performed with technical rigor to allow confidence in the results?
No - there are major issues

Statistics - Is the use of statistics in the manuscript appropriate?
Yes - appropriate statistical analyses have been used in the study

INTERPRETATION - Is the current interpretation/discussion of the results reasonable and not overstated?
Yes - the author’s interpretation is reasonable

OVERALL MANUSCRIPT POTENTIAL - Is the current version of this work technically sound? If not, can revisions be made to make the work technically sound?
Probably - with minor revisions

PEER REVIEWER COMMENTS:

GENERAL COMMENTS: The study addresses a valid research question, and the background information provides a comprehensive overview of the study context, but at the same time there are some flaws in the overall study.

REQUESTED REVISIONS:

The authors should include the race and ethnicity of the patients in table 1.

All patients belong to the same ethnic group and this is justified within text as cited below and thus was not included in table 1.

Methods-Subjects: line 107

Age and gender matched healthy adult volunteers with the same ethnic origin and area of residence served as ‘normal, healthy controls’ while, individuals with other neurological disorders other than PD (i.e. stroke, myasthenia gravis, multiple sclerosis, Guillain-Barre syndrome, etc.), were enlisted as ‘disease controls’.

Results-Participant Characteristics line 192-194

All participants belonged to the same race and ethnic group based on the same ancestry and geographic origin and socio-cultural background respectively [Bopal, 2003, p.444]. (Additionally, three generations of spoken language were employed as a tool of differentiation).
The authors should include the time period when the study was conducted and they should also include when the data analysis was performed.

Methods-Subjects line 107-110

From the period between 1 June to 1 December 2016, we recruited patients with idiopathic PD of varying chronological history; (i) <1-year PD, (ii) 1-3 years PD and (iii) 3-12 years PD, from private neurology clinics of the Colombo district, Sri Lanka.

Data analyses lines 161-164

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, New York, USA) and Graph-Pad Prism (GraphPad Software, Inc, San Diego, California, USA) after data collection and laboratory analyses of serum immune markers.

The data analyses were performed from 1 December, 2016 to 1 January, 2017 while the CombiROC analyses were performed with the availability of the CombiROC online tool in May, 2017. This response is not included in the manuscript because we usually do not include the period of data analysis in a manuscript.

The authors should clearly note the inclusion criteria used for the patients in the study.

Methods-Subjects lines106-115

All patients were examined by a single neurologist (TC). PD was diagnosed based on established clinical criteria [19]. Patients with evidence of atypical parkinsonian syndromes, secondary parkinsonism or with current infections, associated chronic inflammatory diseases including connective tissue diseases and multi-system inflammatory disorders, malignancies or patients on immunosuppressive drugs were excluded from the study.

Age- and gender-matched healthy adult volunteers with the same ethnic origin and area of residence served as ‘normal, healthy controls’ while, individuals with other neurological disorders other than PD (i.e. stroke, myasthenia gravis, multiple sclerosis, Guillain-Barre syndrome, etc.), were enlisted as ‘disease controls’.

The authors should state that the data collectors were trained before collecting the patient data and whether they were blinded or whether randomization was performed during the analysis of the data, as well as during establishment of the scoring model.

The data collectors were trained to perform the MoCA test by the neurologist. All the clinical data was acquired by the neurologist while only the socio-demographic data was collected by the data collector. Interviewer-administered questionnaires and blood collection were performed based in the order of attending the neurology clinic and were later divided into the three sets of groups during data analyses ((i) <1-year PD, (ii) 1-3 years PD and (iii) 3-12 years PD).

Methods Subjects Lines 119-125
For collection of socio-demographic data, we interviewed all the patients in-person. All patients recruited were randomly chosen based on their order of attending the neurology clinic without any bias and were later categorized into the three groups stated above during data analyses.

The authors should include how they determined which statistical test to utilize to analyze their information.

Data analyses lines 161-171

The data was checked for normality distribution prior to statistical analyses. The continuous variables, not normally distributed were expressed as mean ± SD and compared using non-parametric tests. The clinical/demographic variables of the cases and controls were compared as follows; independent-samples T test for continuous variables and chi-square test for categorical variables. The mean serum immune marker levels were compared using Mann-Whitney U/Kruskal-Wallis H tests as appropriate.

They should expand the figure legends by, for example, specifying which statistical tests were applied to each data set, the number of patients included and levels of significance. They should provide details for the Fig. 2 legend.

The suggested corrections have been done in the revised manuscript.

The authors should provide a label for the Y-axis shown in Fig. 2.

This correction has been made in the revised manuscript.

The discussion is too lengthy and not up to the mark. At the end of the discussion, they should also include the limitations of the study.

The discussion has been revised as suggested. Limitations of the study have been included as follows:

lines 364-376

While significant results were found with respect to altered levels of immune mediators in serum of PD patients compared to controls, some of the limitations of our study should be addressed. Since time was a limiting factor, these results are clearly exploratory and preliminary due to small sample size which may explain some of the non-significant differences observed in certain subgroups (<1-year PD [n=12]). Additionally, the levels of these immune mediators may be influenced by a variety of factors other than PD pathology, including individual genetic variations, medication, dietary preferences, etc. which were not broadly addressed in our study. Future longitudinal studies utilizing larger sample sizes could help to better characterize variations of these immune markers observed in serum levels and elucidate their relationship underlying PD severity. Furthermore, since no previous studies have been published on clinical performance of immunologic markers in PD diagnosis and prognosis except for their up-
regulation during disease course, additional studies with a large panel of immune markers are warranted for further validation.

Editor Comments:

1. Please ensure that tables are free of shading and formatted according to BMC Neurology guidelines (https://bmcneurol.biomedcentral.com/submission-guidelines/preparing-your-manuscript#preparing+tables), and placed (together with numbers, titles and legends) at the end of the manuscript following the references.

Done.

2. Please clarify if any patient was unable to provide written informed consent due to their condition and, if so, please detail this and the procedure to obtain consent from legal guardians in the Ethics approval and consent to participate section of the Declarations.

Done. See Declarations Ethics approval and consent to participate: lines 398-401

3. Please state the role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript. This should be declared in the funding section of the Declarations.

Declarations Funding lines 414-418:

This study was supported by funds from the University of Colombo, Sri Lanka, specially provided by the Vice Chancellor for multi-disciplinary undergraduate research, and the funding body had no influence in designing the study, data collection, analyses, interpretation and writing the manuscript.

4. Please remove the boxes surrounding the Figure numbers/titles/legends following the references.

Done.

5. Figure 1, in its current form, is somewhat difficult to interpret due to the double y-axes and multiple parameters. Please reformat for enhanced clarity and/or consider presenting different aspects of the data as separate graphs.

The format of Fig 1 has been changed as suggested in the revised manuscript.
6. Please ensure that the x-axis in Figure 2 is labelled appropriately.

Done.

7. Please replace the labels in the color keys for each plot in Figure 3 with the relevant biomarker name (i.e. ‘Marker 1. with ‘IFN’ in Figure 3a), and remove the larger key associating marker/combo labels with the marker name (bottom right of figure 3), for enhanced clarity. Where possible, please keep the color consistent for each specific marker/combo across plots.

This was attended to, however, with regard to colour consistency, in Figure 3 B and C, the colours were kept consistent, whereas this was not possible with A, as these figure are automatically generated from the CombiROC tool once you feed the data, and the colours cannot be changed.

8. In Figure 3 please remove the figure title and figure numbers (e.g. ‘Fig. 3(a)’) below each plot, and replace with a single letter (e.g. ‘A’).

Done.

9. In Figure 4 please remove the figure numbers (e.g. ‘Fig. 4(a)’) below each plot, and replace with a single letter (e.g. ‘A’).

Done.

10. In addition to Reviewer 1’s comments, please replace instances in the manuscript where significance is reported as P= 0 (or P=0.000) as P<0.001.

Done.

11. Please ensure that all References are formatted correctly.

Done.