Author's response to reviews

**Title:** Reduced rhinovirus-specific antibodies are associated with acute exacerbations of chronic obstructive pulmonary disease requiring hospitalisation

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**Author's response to reviews:** see over
Dear Dr Shipley,

Re Manuscript ID 9739137896826504 “Reduced rhinovirus-specific antibodies are associated with acute exacerbations of chronic obstructive pulmonary disease requiring hospitalisation”

Thank you very much for the opportunity to respond to the reviewer’s comments. Changes to the manuscript are indicated with track changes and specific responses are included below.

**Editorial Comment**

*Please ensure that you revise the manuscript to clarify consent was obtained from patients for participation in this study*

**Our response:** We apologise for omitting this and have now included it in the revised manuscript (Page 5).

**Referee 1 – Patrick Mallia**

1) The authors need to provide more data on the subject characteristics to be able to argue that the low VP1 IgG levels are the cause of frequent exacerbations rather than a marker of a group of patients susceptible to exacerbations. There is already a suspicion of this in that the frequent exacerbation group had worse lung function than the no exacerbation group. In the Discussion the authors have stated that ‘Though exacerbation-prone COPD patients had more severe airflow obstruction than the stable COPD patients, the association between lower anti-VP1 IgG1 antibody concentrations and COPD exacerbations appeared to be independent of FEV1’. What they have shown is that there was no statistically significant correlation between antibody levels and FEV1 which is not the same as showing the association between VP1 IgG and exacerbations was independent of FEV1. The authors should provide data on the number of exacerbations prior to recruitment as, if the frequent exacerbation subjects had more exacerbations previously, then it is likely that they are a group susceptible to exacerbation. This does not exclude low VP1 levels as a mechanism for frequent exacerbations but the authors should provide this data and discuss confounding factors such as lung function and exacerbation frequency.

**Our response:**

To address the possible confounding factor of lung function, a multivariate linear regression analysis was formed. Both reduced lung function and lower anti-VP1 IgG
levels were independent predictors of exacerbation frequency. This has now been included as Table III in the revised manuscript. Unfortunately, data on the number of exacerbations before subjects entered into the study is not available. We agree with the Reviewer that exacerbation prone COPD patients are susceptible to further exacerbations. Whether or not lower anti-VP1 IgG levels are a cause or a consequence of exacerbation frequency will require a much larger and appropriately designed study in which COPD patients with varying VP1 IgG levels are followed over a much longer period. We believe that our findings provide an important justification for such a larger study.

2) An alternative analysis would have been a multivariate analysis that could have included lung function and previous exacerbation history and would have strengthened their hypothesis if it had shown that low VP1 IgG levels were independently related to exacerbation frequency.

Our response:
Please see our response above.

3) To be able to assess the relevance of the data regarding pneumococcal antibodies the pneumococcal vaccine status of the patients should be provided.

Our response:
The proteins that we use to measure the pneumococcal antibodies are not included in the conjugate vaccines. Therefore the measured pneumococcal antibody levels are not affected by the vaccination status of the patients used in this study.

4) Influenza vaccination status may also be relevant and should be compared between the groups.

Our response:
Over 90% subjects in the study had received influenza vaccination within the preceding 12 months, and vaccination rates did not differ between patients who had exacerbations, and those who were stable during the study.

5) No virological sampling was carried out so it is impossible to determine whether the exacerbations were caused by rhinovirus. Evidence of rhinovirus infection would certainly have strengthened the hypothesis. The authors must have data regarding the season of exacerbations. Is there any indirect evidence that these are virus-induced exacerbations by the time of year they occurred? Also from the hospital records can the authors derive any information as to whether the exacerbations were associated with 'viral' symptoms.

Our response:
We agree with the reviewer that we have no direct evidence that the exacerbations were associated with rhinovirus infections. This would require a much larger study in which comprehensive virological sampling was undertaken. As expected, most exacerbations occurred in winter and spring at times when multiple respiratory pathogens (including rhinoviruses) are circulating in the community. This information has been added to the manuscript (Page 8). We do not have information on whether the exacerbations were associated with 'viral' symptoms. Trying to retrospectively
extract this type of information from hospital records is unlikely to provide accurate data.

6) What proportion of exacerbations had evidence of bacterial infection (sputum purulence, raised inflammatory markers, sputum cultures etc).

Our response:
Most acute exacerbations were associated with elevations in white blood cell counts and CRP. Haemophilus and Pseudomonas were isolated from sputum in a small number of subjects, but many subjects did not have sputum cultures collected prior to the administration of antibiotics, so we are unable to give an accurate estimate of the proportion of exacerbations linked to bacterial infection.

Referee 2- Umadevi Sajjan
1. The antibody concentration may change significantly even with non-symptomatic infections. Have authors considered this scenario? This should be at least discussed.

Our response:
The reviewer is correct in stating that the antibody concentrations may vary with infection. This has now been discussed in the revised manuscript (Page 13).

2. The difference in VP1 antibody appears to be due to one patient who show antibodies below the detection limit, and who also happen to have 7 exacerbation in one year. If this one anomalous data point is excluded from the study would the difference still be significant between the two groups? Although authors briefly mention this in result section, does not discuss this anomaly.

Our response:
If the patient with undetectable VP1 antibodies who had 7 exacerbations is excluded from the study, the results remain significant (p=0.036) with differences observed in anti-VP1 antibodies between those with and without frequent exacerbations. This is not surprising and highlights why the statistical tests we performed were based on the assumption of non-normality which uses ranked data rather than the actual values, thereby reducing the effect of outliers.

3. There was also one patient who had high rhinovirus antibody titer, yet had 5 exacerbations. This is neither mentioned nor addressed in the discussion.

Our response:
While this is correct, we would caution against an ‘anecdotal’ approach to analysing individual data points. Statistical analysis of group data is a much more robust way to analyse the data. A number of factors are likely to influence vulnerability to COPD exacerbations, and we have never tried to assert that anti-VP1 IgG levels are the only factor that predicts exacerbations. See our earlier discussion of the multivariate analysis.
Minor Comments 1. Numbers in Y-axis in the figures are too small and are difficult to read. Please increase the font size.

Our response:
We apologise for the hard to read y axis labels. These have now been increased in size.

Referee 3 – Jennifer Quint
1. The numbers in the study are very small and there is no power calculation to suggest that the statistically significant results have occurred for any reason other than chance. Please provide a power calculation for the primary outcome.

Our response:
While the numbers in the study are small, as the reviewer stated in her preamble, this is a hypothesis generating study. We would advise against post-hoc sample size calculations as they offer no further information to the current study and the retrospective assessment of power to make an inference is invalid. We would instead use this data to design a sufficiently powered prospective longitudinal study to assess antibody stability and the relationship to viral infection and exacerbation rates in COPD patients.

2. As the study is very hypothesis generating and more studies are needed before any firm conclusions can be drawn, the findings should be discussed with this in mind.

Our response:
We agree. Our study has shown an association between low VP1 antibody levels and a higher exacerbation frequency, but we cannot conclude that there is a causal link between the two findings. We believe that our findings provide an important justification for such a larger study as discussed above.

3. The introduction of the paper talks about frequent and infrequent exacerbators and yet the results apply to those with or without a hospitalised exacerbation and not FE and IE. The introduction should be adjusted as such.

Our response:
The Discussion (page 13) has been adjusted to address this issue. Hospitalisation defines a subset of severe exacerbations, and we do not know whether low VP1 antibody levels are associated with milder exacerbations that resolve without requiring hospital admission.

4. While the addition of disease severity did not alter the model, the numbers in the study are very small and I do not think you can be sure that the findings are not disease severity related.

Our response:
To address this we formed univariate and multivariate linear modelling (see point 1 of the first Reviewer) which showed that both disease severity (as assessed by lung function) and low anti-VP1 antibodies were independent predictors of exacerbation frequency.
5. The inter-assay and inter-sample variability is not discussed anywhere. As the numbers are small and driven by outliers on the graphs it is important to discuss quality control.

Our response:
The antibody assays are well validated and the intra-assay coefficient of variation (CV) is typically 1-10%. Standards and controls are included in every assay and the inter-assay CV ranges from 5-15%.

6. I do not think you can say p=0.046 is statistically significant.

Our response:
By convention, p=0.046 is less than α=0.05 for assessing significance.

7. Please reference the statement in the methods for measurement of specific antibodies that states "patients who had values below the limit of detection were assigned a value of half the lower limit of detection".

Our response:
Patients who have antibodies below the limit of detection are often given the value of ‘0’. However, this may not be true, as a value less than the limit of detection may be anywhere between the lower limit of detection and zero. However this figure cannot be used when data is transformed on a logarithmic scale for use in parametric hypothesis tests. Therefore it is common practice to assign a value half that of the detection limit. A reference to this has been included in the revised manuscript.

Thank you once again for the opportunity to respond to the reviewer’s comments.

Kind regards,

[Signature]

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