Author's response to reviews

Title: Seasonal variation of serum KL-6 concentrations is greater in patients with hypersensitivity pneumonitis

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Version: 4
Date: 15 July 2014

Author's response to reviews: see over
Dear Dr. Peter Wark,

Thank you very much for the positive reviews of our manuscript and interest in the work carried out. On behalf of all the authors, I would like to resubmit our manuscript entitled “Seasonal variation of serum KL-6 concentrations is greater in patients with hypersensitivity pneumonitis” for publication in the *BMC Pulmonary Medicine* as an original research article. This manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission. This work was supported in part by Grants-in-Aid for Scientific Research of the Japanese Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors report no conflicts of interest.

We changed several parts of the result based on the statistical reanalysis using the Holm’s sequential Bonferonni correction for multiple comparisons. The changes made were highlighted by the underline with blue color in the revised manuscript. However, main result is same as the previous manuscript. Our point-by-point responses to the reviewers’ comments are as follows:

**Comments from the Reviewer: Dr. Peter Wark**
This is an interesting observational series of a cohort of subjects with ILD that tracts variation in serum KL-6 over a period of summer and then winter. The authors demonstrate that greater variation of KL-6 in hypersensitivity pneumonitis, than the other ILDs and attribute this season, with differences in presumed bird HP compared to domestic HP. This is an interesting observation and adds to the field, it brings KL-6 at least a little closer to being an interesting biomarker in ILD and will add to the evidence supporting it becoming so, but by itself this manuscript does not achieve this. One major limitation is the cohort size, with rare diseases this is always a problem, but multi centre trials or national registries are going to be needed to really answer these questions. This will reduce the impact of the external validity of these findings. The authors need to consider the following issues.

**Major**
1. The limited time frame of measurement is somewhat arbitrary, confining this to winter months or summer, would encourage identifying these seasons as accounting for variability in KL-6. Certainly the measures vary in those with HP,
but I think a stronger model would have been to measure levels consistently through the year and capture environmental data (domestic and external) in reference to this change. This is a much greater effort to go to but if the investigators are seeking to establish a direct link then this would be needed. The limited numbers make the differences due to seasons in the figures unconvincing. I think there is too much focus on attributing these differences to season, but compelling that it may reflect variation in antigen exposure. This assertion needs to be reduced in veracity. The other potential causes for variation in antigen exposure should be explored more.

Response) We agreed with your opinion that both consistent measurement of KL-6 and environmental data through the year are the best way to identify the direct relationship between KL-6 and antigen levels. However, we think such prospective study with antigen exposure without treatment intervention is difficult to set up because of ethical issues, which we already mentioned in the study limitation of discussion session. Although we did not collect seasonal environmental data to establish the direct link between seasonal change of KL-6 concentrations and antigen exposure levels, we visited and searched environment of each patients’ house and nearby and found mold spread during summer in the house of patients with House-HP or increased usage of feather products during winter in patients with Bird-HP and therefore speculated that increased antigen exposure could be at least one of the causes of seasonal changes in serum KL-6 concentrations. We added lack of seasonal environmental data collection to the study limitation. We changed conclusion as “Serum KL-6 concentrations exhibited significant seasonal variation presumably in response to seasonal fluctuating antigen exposure levels in patients with HP”.

2. Page 17 of the discussion the authors introduce data on BAL cell counts. This is not described in the methods or the results. While reference is made to how diagnosis is made and presumably these BAL samples were taken at the time this is unclear. If BAL was done it must be described when and how. If this was not done in reference to KL-6 measures I am not sure this will be interpreted. Needs clarification. This should not appear in the discussion alone or should be removed.

Response) BAL samples were obtained at the diagnosis of HP without treatment and BAL was collected by instillation of 50 ml of saline 3 times. There are no correlation between serum KL-6 concentration at same day of BAL and lymphocytosis in BAL fluids. We added these information and data in methods and results section.

Comments from the Reviewer: Dr. Chris Grainge
Many thanks for asking me to review this interesting manuscript examining the seasonal variability of serum KL-6 in patients with interstitial lung disease of varying aetiology. The manuscript has value as it examines for the first time the seasonal variation in KL-6 concentrations and also compares various ILDs of various aetiologies. The manuscript demonstrates that there are larger alterations in serum KL-6 concentrations in cases of HP where antigen load varies seasonally.
There are several points which could be addressed to improve the manuscript;
Major Compulsory revisions;
1. How were the patients identified and classified into each of the groups, and exactly which diagnostic criteria were used? This is important as the criteria have changed between the dates given for the study (mid 2009 to early 2014). The gold standard is multidisciplinary discussion based on the ATS/ERS consensus
statement. Was this performed? What proportions of the patients had tissue diagnoses in each of the groups? Currently it isn’t possible to ascertain, for example, how many NSIP patients had a surgical lung biopsy. Perhaps this data would be best given in a table. This information is vital as when comparing groups of ILD it is essential the groups are correctly identified.

**Response**  Eight patients with NSIP were diagnosed by surgical lung biopsy based on the ATS/ERS multidisciplinary consensus classification of IIPs (*Am J Respir Crit Care Med* 2002; 165: 277-304.). We added this to references. Other 8 patients were clinically diagnosed by the chest HRCT findings compatible with NSIP pattern, increased lymphocytosis in BAL fluid, lack of granuloma in TBLB specimens, and exclusion of IPF, CVD-IP, HP, or CPFE. We made this clearer in the methods and added numbers of BAL/TBLB/SLB (surgical lung biopsy) in table 1.

2. ‘House’ HP is not a disease classification that is recognised in Europe / Australia (and I don’t think in America), what is the antigen (is it the Trichosporon?) or is it a diagnosis used where there is no identifiable antigen found? This needs to be made clearer for an International audience.

**Response**  House-HP refers to HP positive for specific IgG antibody against *Trichosporon asahii*. As mentioned in introduction, such house mold-related HP especially for *Trichosporon* is common type of HP in summer in Japan. We made this clearer in the methods.

3. There were 21 p values presented from a single set of data, were any corrections done to account for multiple analyses? This is important to reduce the possibility of Type I error. The data should be re-analysed using a Bonferonni correction or similar to reduce this possibility, or at least the increased risk of Type I error be acknowledged and discussed.

**Response**  We reanalyzed the data using Holm’s sequential Bonferonni correction for multiple comparisons between each ILD and p<0.0033 was considered statistically significant. Even after the correction, Bird-HP showed lower Smax/Wmax than that the other ILD. Therefore, the main result of this study was similar to the previous manuscript.

4. The differences between groups in terms of variation of KL-6 concentrations is statistically significant, however there is considerable variability within groups and overlap between groups which would make comparing individuals difficult. Do the authors consider this an issue clinically? This should be discussed.

**Response**  We agree with your concern that comparing KL-6 concentrations in each patients is not diagnostic as overlaps in KL-6 concentrations exist among different ILD groups. We demonstrated that greater seasonal variations in KL-6 concentrations in HP group than the other ILD. This finding suggests that the presence of seasonal changes of KL-6 concentrations in each patient, but not comparing KL-6 concentration among patients, is clinically important for suspecting the possibility of HP. We discussed this in the revised manuscript.

5. The authors used non-parametric methods for statistical analysis, presumably as the data were not normally distributed, however the values are presented as mean +/-SD. The data should either be presented as median (IQR) or a reason given for the discrepancy between data presented as if parametric but analysed using non-parametric testing.

**Response**  We changed data presentation as median (interquartile range) throughout text and table.

Minor Essential revisions
1. Figure 2B needs units for the serum KL6, also this is a concentration, not a level (similar comment for figure 2C)
   Response) We changed KL-6 levels to KL-6 concentrations.
2. The variation of KL-6 DURING the summer does not suggest that the KL-6 concentrations were significantly increased in the Summer, just that variability increased (line 18 page 11).
   Response) We deleted that sentence.
3. Throughout the manuscript, KL-6 concentrations are referred to as ‘levels’ this should be changed.
   Response) According to the suggestion, we corrected “levels” to “concentrations” throughout the text and table.

Discretionary revisions
1. The changes in KL-6 concentrations in the House HP group are significant, however examining the figure (2B) there were only 2 from 5 seasons when the difference between summer and winter was apparent visually. Similarly in the Bird HP only 2 of at best 4 seasons showed much change. Presumably these were the most apparent changes between seasons and hence chosen for the figures. In order for this testing to be useful clinically, the change would need to occur in a single year or so reliably. The data do not look like this would be the case. If the data are re-examined in a ‘real world’ manner (ie within a year) are they still helpful?
   Response) The duration when the patient did not refer to the hospital was shown by dot line in Figure 2C. HP did not always exacerbate in each year as exacerbation occurred in response to increased antigen exposure levels, which is highly dependent on the climate and living environment in House-HP or the use of feather products in Bird-HP. We think finding seasonal changes of KL-6 concentrations in each patient in their further clinical course is clinically important for suspecting the possibility of HP in real world manner.

Sincerely,

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