Author's response to reviews

Title: Exhaled 8-isoprostane as a prognostic marker in sarcoidosis. A short term follow-up.

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Author's response to reviews: see over
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Dr Hans Zauner
Scientific Editor
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Re. MS: 5956383031530753

Dear Editor,

Please find below our response to the reviewer’s comments.

Best regards

WJ Piotrowski
Reviewer 1

Major comments:

1. Three patients were treated in the past. All these patients had 8-IC >20 pg/mL and none of them experienced complete remission at V2. 8-IP at V1 and V2 between all and never-treated patients were not different statistically:

<table>
<thead>
<tr>
<th></th>
<th>All (n=40)</th>
<th>Never treated (n=37)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>8.50 (2.50-17.40)</td>
<td>7.60 (2.50-16.90)</td>
<td>0.75</td>
</tr>
<tr>
<td>V2</td>
<td>8.35 (2.50-24.00)</td>
<td>7.90 (2.50-24.00)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

The results of chi square test after exclusion of these patients give the same results:

<table>
<thead>
<tr>
<th>8-IP</th>
<th>&lt;5</th>
<th>5-20</th>
<th>&gt;20</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR+</td>
<td>No</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>% with CR</td>
<td>63.6</td>
<td>18.2</td>
<td>18.2</td>
</tr>
<tr>
<td></td>
<td>% with EBC 8-IP level</td>
<td>50</td>
<td>10.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>p=0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR-</td>
<td>No</td>
<td>7</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>% with CR</td>
<td>26.9</td>
<td>65.4</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>% with EBC 8-IP level</td>
<td>50</td>
<td>89.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I suggest to leave these patients in the study group and add more information in Patients and methods:
Page 4, Line 9 (old version): Three patients had been treated in the past, but all were
treatment-naïve for at least 12 months at the time of initial evaluation.

And Results: Page 8, last sentence in the chapter: As 3 patients had been treated in the
past (the treatment was stopped more than 12 months before initial evaluation), the
possible influence of previous treatment was checked, and finally neglected (data not
shown).

2. It was changed, as suggested by Reviewer.

Background, last sentence: We were especially interested, whether high initial EBC 8-
IP concentrations predispose to more severe disease, low initial concentrations
increase a chance of early remission, and whether remissions are connected with the
decrease of EBC 8-IP concentrations.

3. We consulted this issue with a statistician. Cox regression analysis is used most often
for estimation of survival. If we take a remission as a final point, we still are not be
able to estimate an exact time of remission. We know only a time range (6-12 months)
when remission occurred or that the disease was still ongoing at the time of visit 2.
Therefore we think, this is not possible to evaluate these data by Cox regression.

However, we made an additional calculations with logistic regression (progressive
selection), and we found, that from all analyzed data (8-IP, laboratory markers,
radiological stages, BAL cellularity, disease duration etc.) only radiological stages are
significantly related to lack of remission (p=0.002). It was not a case for EBC  8-IP.
The statistical significance was only found when categories were applied (for the
category below 5 pg/mL, RR = 3.33, p=0.04 by $\chi^2$), as described in the text. As the
influence of other predictive factors (like radiological stages) on early disease
regression vs chronicity was not a subject of this study, we suggest not to change the primary version in that matter. The predictive value of radiological classification is well established in the literature.

4. The Intra-assay reproducibility was provided previously. The Inter-assay reproducibility was estimated on the basis of the data coming from separate collections (4 weeks interval) in 17 control subjects. The following sentences were added:

Page 5, line 9 (old version): The inter-assay reproducibility was estimated on the basis of repeated measurements in 17 control subjects (samples taken over a 4 week interval). The CV was 19.0%. In 2 subjects (11.8%) the one-grade change between 8-IP concentration was found between ranges (from <5 to 5-20 pg/mL).

5. Conclusions have been changed, as suggested (page 10, last paragraph):

Conclusions. This short-term follow-up study does not provide an evidence for negative prognostic value of high levels of EBC 8-IP. A long-term follow up study, involving more numerous and clinically heterogenous group of patients is necessary for conclusive evaluation. However, our data shows, that low concentrations of 8-IP in EBC may predispose to early remission. Complete remissions are not connected with a consistent decrease of EBC 8-IP. Only in patients treated with steroids, regardless the remission was achieved, the decrease of EBC 8-IP was noticed.

6. Following the reviewer’s suggestion other possible explanations were discussed:

Discussion, page 10, after line 8 (old version): On the basis of our results it is difficult to explain why some patients with clinical remission have high concentrations of 8-IP
in exhaled breath at final evaluation. The influence of other clinical states should be considered. Although infection and allergy were exclusion criteria, we were not able to exclude subclinical states, which could have influenced the measurements. In some patients bronchial hyperreactivity may evolve [17], which would give another possible explanation for these outstanding results. Finally, we can not exclude, that in some of these subjects the disease is still active or will result in relapses.

We add a new reference position [17].

Minor revisions: The new manuscript is now corrected by a native speaker. Please, see new acknowledgements.
Reviewer 2

1. The Intra-assay reproducibility was provided previously. The Inter-assay reproducibility was estimated on the basis of the data coming from separate collections (4 weeks interval) in 17 control subjects. The following sentences were added:

Page 5, line 9 (old version): The inter-assay reproducibility was estimated on the basis of repeated measurements in 17 control subjects (samples taken over a 4 week interval). The CV was 19.0 %. In 2 subjects (11.8%) the one-grade change between 8-IP concentration was found between ranges (from <5 to 5-20 pg/mL).

2. More information was added on time of storage and time of collection:

Page 4, line 22-23: EBC was collected using a condensing device (Ecoscreen, Jaeger, Germany), always before bronchoscopy and always on the same time of a day (between 9-11 a.m.).

We have not used internal dilution factor, nor amylase concentration to exclude saliva contamination. The latter may be especially important in case of LTB4, as saliva is a source of this eicosanoid.

3. The correct number of control subjects was 34. Therefore, the following corrections were done:

Results, line 7-8 (old version): In 14 out of 40 patients (35 %) and in 26 out of 34 healthy controls (76 %) 8-isoprostane concentrations were below detection limit (figure 1).
4. Page 4, line 18-19 (old version): Control group: 34 healthy never smokers (19 women, age 45±10), members of a hospital staff, free of respiratory infection in the last 4 weeks.

The age was provided as mean±SD, therefore age for sarcoidosis was changed for 39±11. It was added:

Page 4, line 14: Statistical analysis. Data were expressed as mean ± standard error of means (SEM), with exception of age (mean±standard deviation).

5. The header “Figure 3” was deleted from the top of the figure.

6. The p value generated by Kruskal-Wallis test was 0.03, but the Dunn’s Multiple Comparison Test (post-test) showed significant differences between stage 1 and 3 (p<0.05), and no differences between other stages (p>0.05). When selected groups were compared by t-test (stage 2 vs 3) or Mann-Whitney test (stage 1 vs 2 and stage 1 vs 3), the significant differences were found between 1 vs 3 (p=0.04). There were no differences between and 1 vs 2 (p=0.05) and 2 vs 3 (p=0.28). Regardless the above, we believe, that the proper way of calculating differences between 1, 2 and 3rd stage is by Kruskal-Wallis test, which gives the overall p value describing whether the subgroups differ. We propose to leave the overall p value provided by Kruskal-Wallis test and describe the results of the Dunns post-test (it does not give exact p values) in the figure legend:

Figure legend, Figure 2. Comparison of exhaled breath condensate (EBC) 8-isoprostane (8-IP) concentrations in sarcoidosis patients divided according to radiological stages. There was a significant difference between stages estimated by
Kruskal-Wallis test (p=0.03). The Dunn’s Multiple Comparison post-test showed the significant difference between stage I and III (p<0.05). There were no differences between stage I and II and stage II and III (p>0.05).

7. Some minor typographic errors were found and corrected. The text has been corrected by a native speaker (please, see Acknowledgements).
1. We are aware of this. That’s why we exclude patients with respiratory infection, asthma/allergy, other respiratory inflammatory diseases and smoking, as states known to result in increased EBC concentrations of various inflammatory markers, including 8-IP. It was further discussed as other underdiagnosed states may explain high values of 8-IP at follow-up:

Discussion, page 10, after line 8 (old version): On the basis of our results it is difficult to explain why some patients with clinical remission have high concentrations of 8-IP in exhaled breath at final evaluation. The influence of other clinical states should be considered. Although infection and allergy were exclusion criteria, we were not able to exclude subclinical states, which could have influenced the measurements. In some patients bronchial hyperreactivity may evolve [17], which would give another possible explanation for these outstanding results. Finally, we can not exclude, that in some of these subjects the disease is still active or will result in relapses.

We add a new reference position [17].

2. Please, refer to discussion, where we mention about preliminary character of our results, and the need of further investigation in a long term (>2 years) follow-up study.

See also Conclusions in its new shape:

**Conclusions.** This short-term follow-up study does not provide an evidence for negative prognostic value of high levels of EBC 8-IP. A long-term follow up study, involving more numerous and clinically heterogenous group of patients is necessary for conclusive evaluation. However, our data shows, that low concentrations of 8-IP in
EBC may predispose to early remission. Complete remissions are not connected with a consistent decrease of EBC 8-IP. Only in patients treated with steroids, regardless the remission was achieved, the decrease of EBC 8-IP was noticed.

3. It might be a problem of sensitivity or simply in those patients with undetectable 8-IP an oxidative stress is insignificant. In the context of imperfect reproducibility, the use of categories might be applied, ie grade 1: <5, grade 2: 5-20, grade 3: >20 pg/mL. We believe, that it is not a disadvantage, as it helps to distinguish those, in whom oxidative stress and lipid peroxidation are not clinically significant.

4. The role of TNF is shortly discussed in a new sub-chapter of discussion (Discussion, last paragraph):

   *Oxidative stress in the pathogenesis of sarcoidosis.*

   Our results provide an argument for the possible role of oxidative stress and lipid peroxidation in sarcoidosis. Production of superoxide anion by BAL cells is increased proportionately to the grade of lymphocytic inflammation [18]. Oxidative stress may be involved even in the very early stages of granuloma formation, as oxidants may stimulate macrophages to the production of TNF-α and other cytokines indispensable for granuloma formation. TNF-α may be detectable in EBC, however the concentrations were not elevated in sarcoidosis [19]. Although, we have not measured TNF-α in our patient’s EBC, such data would be interesting in the context of the possible application of anti-TNF agents in the treatment of refractory sarcoidosis [20]. Elevated 8-IP concentrations in EBC may be related to the destruction of cellular membranes, but further studies are required to evaluate whether patients with high EBC 8-IP have a higher risk of lung fibrosis.