Author’s response to reviews

Title: Exhaled nitric oxide is related to atopy, but not asthma in adolescents with bronchiolitis in infancy

Authors:

Ingvild Bruun Mikalsen IBM (miib@sus.no)
Thomas Halvorsen TH (thomas.halvorsen@helse-bergen.no)
Knut Øymar KØ (oykn@sus.no)

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Thank you for the opportunity to submit a revised version of this manuscript. We also wish to express our gratitude for valuable reviews that incited some interesting discussions in our group.

We have considered comments and proposals from the two reviewers, and made alterations accordingly.

The original manuscript included 3 tables and 2 tables as additional files. One of the reviewers suggested to include the additional files in the manuscript, and based on the suggestions we also included a new table of regression analyses in the post-bronchiolitis group. As described in the reply we find it difficult to omit any of the other tables. The manuscript therefor now includes 6 tables. However, if the editor considers this as too many, we suggest adding the revised Table 1 and 2 to “additional files”.

We have used a red font instead of black font to indicate the revised portions of our manuscript.

The work has not been published before and is not being considered for publication elsewhere.

All authors have significantly contributed to the work and have approved the final manuscript.

There are no conflicts of interest for any of the authors.

Sincerely,

Ingvild Bruun Mikalsen
Reply to comments

Referee 1-Christophe Marguet:

This paper aimed to define persistent airway inflammation in teenagers who underwent an acute bronchiolitis during infancy. FeNO, lung function and BHR have been chosen as inflammatory outcomes. A control population has simultaneously been studied. The conclusion of the authors was that FeNO remained an atopic marker as previously described. This long term study was well and successfully conducted and the originality was supported by the specific population that the authors studied and the connection with the debate of post RSV bronchiolitis atopy. Moreover, the authors provide additional data on the weak utility of FeNO in clinical practice. However, this paper could be improved as regards the aim of the study.

Thank you for this comment, and for a thorough review with valuable comments that have incited some interesting discussions in our group and contributed significantly to the quality of this paper.

Major compulsory revision:

1. The strenghts of this study is the post bronchiolitis population that was studied and the discussion on the persistent inflammation. Therefore, the FeNO results should be displayed accounting this feature. The figure displayed the results regarding asthma and atopy status, and those on RSV +ve and RSV -ve should be also appeared somewhere.

   FeNO was associated with RSV negative bronchiolitis by univariate regression analysis (B= 0.335; 95% CI 0.019, 0.651; p-value = 0.038), but not by the multivariate model. Numerically, FeNO in children with RSV positive and negative bronchiolitis was relatively similar. Thus, in our dataset, the association between FeNO and RSV status was either weak or not present. Accordingly, and after thorough discussions in our group, we have opted not to add an additional figure displaying these issues. However, the aforementioned results have been explicitly specified in the text of the revised Results section (page 10). A figure displaying these data can of course be provided, if this is a critical issue.

2. The results of FeNO are displayed as means and 95% CI. However, the number of patients did not exceed 5 or 9 in some groups. This is disturbing, and individual values as dots are required on the figure, and will be more informative.

   FeNO was not normally distributed, but regarded as ln-normally distributed and the results from former Table 2 (revised version renamed Table 4) and Figure 1 are not means and 95% confidence intervals (CI), but back-transformed values given as geometric means with 95% CI. We agree that the numbers of children in the groups are small. However, presenting individual levels as absolute values and geometric group means in the same figure would in our view appear confusing. We would therefor recommend keeping the figure unchanged. As suggested below, we have added the p-values in the figure.
3. Lung function and BHR were also identified as outcome markers, and should also be discussed in this paper. The authors should have a global interpretation by associating FeNO, LF and BHR, all being potent inflammation markers. In fact, the decrease in FEF25-75% was expected to be sequelae of bronchiolitis. The lack of relationship between small airways diseases and FeNO would support that this LF alteration was not related to an active disease and should be added in Table 3.

We acknowledge this line of arguments and have added FEF$_{25-75\%}$ predicted to the regression analyses, and the results are presented in Table 5 (former Table 3) and Table 6. FEF$_{25-75\%}$ predicted was not associated with FeNO, neither in the regression analyses including all participants (Table 5), nor in the separate regression analyses for the post-bronchiolitis group (Table 6). These results are discussed in the revised Discussion section, page 12.

4. The same comments could be applied for BHR, and the results more detailed as
regards the aim of the study (table 3)

We also acknowledge this comment, and have addressed these issues in the revised Discussion section, page 13.

5. We need to have the description of the population, which surprisingly was added as supplementary files. Conversely, p values of table 2 could be displayed on the figure, and significant data of Table 1 could be switch in the text. These Tables 1&2 did not provide additional information, and could be withdrawn.

We agree, and have added the originally additional files1 and 2 to the manuscript, and renamed them Table 1 and 2.

The results from the ANOVA analyses given in former Table 1(revised version Table 3) are however by us considered as major results of the study, according to its primary aim. We have made sincere attempts to include and explain these data in the text, but eventually ended up considering this approach to be too complex and difficult to understand for the reader, especially regarding the statistical issues. Based on this, we find it difficult to withdraw the originally Table 1 from the text, and have kept the tables in the same form as originally submitted, but changed the name to Table 3. The importance of this table may also be underlined by the discussion with reviewer no. 2 regarding the power of the study. The former Table 2 has been renamed Table 4.

The total number of tables is now 6. However, if the editor considers this as to many, we suggest adding the revised Table 1 and 2 to “additional files”.

6. The chapter page 9 "regression analyses...lnFeNo (table3) remains unclear. On which analyses did the first paragraph refer ? table 3 ?

We understand that this may be confusing, and apologize. This chapter contains regression analyses for the whole group of children (control and post-bronchiolitis groups together) as well as separate regression analyses for the post-bronchiolitis group. This has been rewritten to clarify. The results for the whole group together are given in Table 5; the results for the post-bronchiolitis group are given in Table 6.

7. The results of regression analyses in the post bronchiolitis group are confusing; i.e. FeNO was not related to DRS, but was in the above paragraph. in addition, the authors claimed in the discussion that HBR was not related with FeNO.

Again, we understand that this may be confusing, and apologize. We have clarified by separating the results from the two different regression models.

As clarified in the revised manuscript, BHR was associated with FeNO in the regression analyses including all children. Also in the regression analyses that only included children in the post-bronchiolitis group, there was a significant association between BHR and FeNO in the fully adjusted and final model.
8. Because the originality of the paper was supported by this studied population, this part of the results should be improved, structured and made more readable. Univariate and multivariate should be clearly separated and the results displayed. In fact, RSV negative bronchiolitis was related to FeNO and disappeared in the multivariate analyses. Therefore, that means a link with another variable(s). Atopy was expected, but it is not. It would be interesting to understand this part of statistics.

*The results from this separate regression analyses are now given in Table 6. Hopefully, this makes the text more readable and provides more exact information of the statistical analyses that were performed.*

9. The fully adjusted model is not defined.

*We agree that the fully model was not properly defined and that the statistical methods could be more clearly described.*

*We have rewritten to clarify in the paragraph of Statistics in the Material and Method section, page 7-8.*

*In all regression analyses, each variable was entered separately into univariate models. Variables with p-values < 0.1 in univariate analyses, were initially further analyzed in a backward multiple regression model.*

*After discussion with our statisticians, we have concluded that including only variables in the multivariate analysis with p-values <0.1 from the univariate analyses may lead to exclusions of variables with significant effects in the final adjusted model. Therefore, in the revised version, variables with p-values < 0.2 in univariate analyses were included in the multivariate analyses.*

*With this change of statistical approach, ln DRS (BHR) was included in the multivariate model and also remained in the final model in the separate regression analyses for children in the post-bronchiolitis group. This also influenced the other variables in the final model. As shown in Table 6 in the revised paper, we found a positive and significant association between FEV1% and FeNO. An interaction effect also became apparent between the RSV negative vs. positive bronchiolitis variable and atopy. These results are added in the table and text and discussed in the Discussion section page 13 and 14.*

**Discussion**

FeNO is more and more discussed in the medical literature as a potent specific marker of asthma. This part should be added, and related to the interpretation of the results. I don't understand their opinion. The levels that they found are low, and match with our experience. Thus, the lack of RSV related atopy, controlled asthma or the absence of in progress airways disease could explain these results. This could be discussed in accordance with the other markers that they used (FEF25-75, BHR).

*Given that FeNO is considered a measure of eosinophilic inflammation in asthma, we can understand the comments and follow the line of arguments from referee no 1.*
However, conflicting results between FeNO and eosinophil counts in airway biopsies have been reported, both in children and adults [1]. FeNO has been associated with atopy, and there are suggestions that FeNO reflects atopy rather than airway inflammation or asthma, although these findings have been equivocal [1, 2].

In the overall regression analyses with all children included, FeNO was associated with BHR, height and atopy but not asthma, FEV\textsubscript{1} % or FEF\textsubscript{25-75} % predicted. In addition, we did not find differences for FeNO in children with former bronchiolitis and controls. It may be difficult to explain the association between BHR and FeNO, but it is important to be aware of the multifactorial causes behind BHR.

We have rewritten the discussion to clarify these issues, page 13.

RSV vs. no RSV: the lack of PCR diagnosis should be somewhere mentioned, because false negative RSV might be expected.

We agree, and have added a comment in the Discussion, page 14 (strengths and limitations).

FeNO and BHR: this paragraph did not really match with the exposed results (see above)

We have rewritten this paragraph to make it more clear and readable, page 13.

Conclusion is restricted to asthma.

I think that the main results was to demonstrate that persistent inflammation is rare at 11y in a population who suffered from acute bronchiolitis in the infancy, did not depend of the viral aetiology (in opposition with the relationship between RV and early asthma) and FeNo mainly remains an atopy marker.

We agree that the conclusion could be more in agreement with the aim and the conclusion has been rewritten. The aim has been clarified as also requested by referee 2.

Minor essential revision:

Does really reference 15 match with the corresponding text?

In our opinion, this reference (revised version reference number 10) [3] is consistent with the text. This publication refers to a previously published study from the same study population reporting on asthma prevalence, lung function and BHR. In that study we observed higher prevalence of asthma in children with former RSV negative bronchiolitis compared to RSV positive bronchiolitis and lower lung function and higher BHR in children with former bronchiolitis compared to controls.

references 16 and 17: which one was used? one is sufficient.

Reference number 16 (revised version reference number 17) from the American Thoracic Society is a guideline for the performance of spirometry, whereas reference number 17 (revised version number 18) provides reference equations for spirometric values except FEF\textsubscript{25-75} %. Reference number 18 (revised version number 19) provide information regarding reference equations used to calculate FEF\textsubscript{25-75} %. We therefore, find it necessary to refer to
all 3 papers in the reference list. We have rewritten the text in order to clarify which exact reference each lung function variable refers to.

**Level of interest:** An article of outstanding merit and interest in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
'I declare that I have no competing interests
Referee 2: George Konstantinou

Reviewer's report:
In this article the investigators tried to assess the effect of early lower respiratory infections (bronchiolitis) on FeNO, asthma and atopy #11 years after this episode was recorded.

Major Compulsory Revisions:
1. The authors should clearly provide the working hypothesis of their work and the primary and secondary outcomes of interest. The structure of the results should be based on these outcomes.

As presented in the last paragraph of the introduction, the aim of the study was as followed:

"The aim of this study was to investigate the role of FeNO as a biomarker for asthma, atopy and BHR in subjects with a history of severe bronchiolitis in infancy and in an age matched control group with no such history, with focus on differences between the two groups."

In order to describe the aim more clearly, we have rewritten to clarify:

The primary aim of this study was to assess if FeNO was different in children with former hospitalization for bronchiolitis compared to a control group, and secondly to explore whether the role of FeNO as a marker of asthma, atopy or BHR differed between these two groups of children.

We have structured the results based on these outcomes.

2. Atopy is one of the most important parameters examined in this study and was assessed with SPTs. On which basis were the allergens used to assess sensitization selected? Are they representative enough to appropriately assess sensitization in Norway? Please quote appropriately.

The allergens included, are considered the most common allergens causing atopic sensitization in Norwegian children in this age group, and are commonly used in studies evaluating atopy in Norway. Only rarely children will be sensitized to other allergens without being sensitized to at least one of these common allergens [4, 5]. Information about this has been added under the Material and Method section under the heading Skin prick test (page 6-7) and a reference included.

3. In the paragraph “follow-up” please explain what does “As previously described … were slightly younger at the 11-year follow-up” mean?

We agree that this could be more clearly described. Children in the post-bronchiolitis group (11.4 years; 11.0, 11.7) (median; quartiles) were slightly younger than the controls (11.7 years; 11.3, 12.1) at the 11 year follow-up (p<0.001). This should, however, not influence the lung function values as they are presented as percentages of predicted, but could possibly influence the prevalence of atopy and asthma.
We have clarified this in the revised manuscript and added a sentence including the exact ages in the text, page 9 and also made a comment to this in the Discussion section page 14 (strengths and limitations).

4. The unselected age-matched control group plays an important role in all comparisons and regression analyses. This control group was recruited from 190 children that were primary invited. Which were the criteria to invite those 190 children? How sure could the investigators be that there is no selection bias among those that finally consent to be tested? Are there any data to show how representative the finally recruited control sample is?

The control group was unselected; all children from 6 school classes in the city of Stavanger were invited. We invited 191 children and 142 children (74%) responded positively. The control group was recruited from an urban area, and may not be entirely representative for the complete study area [6]. Further, we cannot exclude that there has been a selection bias among those that finally consented to be tested. We agree that this may be a limitation and comments about this are added in the Discussion section page 14 (strengths and limitations).

5. Although it is stated in the discussion that the study is underpowered to perform multiple comparisons and adjust for so many parameters, this doesn’t change the fact that the inferences are indeed very weak and possibly incorrect. A post hoc power analysis could clarify this issue provided that there is a working hypothesis and a primary outcome of interest. For instance, there could be a completely different interpretation of the results presented in Table 2. Namely, bronchiolitis seems to prevent an increase in FeNO in the atopics or those with current atopic asthma (see both significant p-values in Table 2).

We agree that this study is underpowered to perform multiple comparisons and sub-group analyses. However, post hoc power analyses are controversial, particularly if the calculations are done by using the observed effect size and variance, as underlined in a paper from Thomas [7]. As stated in that paper: “Both the p-value and the power are dependent upon the observed effect size and so are inversely related such that tests with high p-values tend to have low power and visa-versa. Therefore calculating power using the observed effect size and variance is simply a way of re-stating the statistical significance of the test”. We assume that the referee means that there seems to be an interaction effect between the 4 subgroups and the control/post-bronchiolitis variable, by studying the geometric means in Table 2 (revised version Table4). There was no such interaction effect between the subgroups and the control/post-bronchiolitis variable (p=0.147). The interaction term was therefore removed from the overall ANOVA model.

We suggest that the best way to estimate the power in this analysis is to study the CIs for the observed differences between the groups, as suggested by Thomas [7]. The CIs are given in the table below (similar to the revised Table 3). In this analysis the CI for the difference between the control and post-bronchiolitis group was (-0.309, 0.070) for ln FeNO, i.e. from 30 % decline to 7 % increase in FeNO. A 30 % decrease in FeNO in these populations, with overall low FeNO values (~20 ppb) does not seem to be of major clinically importance.

To summarize, there were no significant interactions between the four subgroups and the variable control/post-bronchiolitis group, there was no significant difference between the control and post-bronchiolitis groups and finally the numerical difference in FeNO was small and probably of low clinical bearing (as judged from the CIs). Our overall interpretation of this scenario was that there was no major difference in FeNO between children in the post-
bronchiolitis and control groups. Based on this, we have not changed the major conclusions of the text, but refer to the aforementioned discussion regarding power, now included in the revised Discussion section page 14 (strengths and limitations).

Table 3

Analysis of variance for fractional exhaled nitric oxide (FeNO) given as ln FeNO in children hospitalized for bronchiolitis (n=105) during their first year of life and an age matched control group (n = 89) at 11 years of age

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95% CI</th>
<th>P-value †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main groups</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Post-bronchiolitis group</td>
<td>-0.120</td>
<td>-0.309, 0.070</td>
<td>0.214</td>
</tr>
<tr>
<td><strong>Sub-groups by atopy and asthma</strong> status</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Healthy children</td>
<td>0</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Atopic children</td>
<td>0.745</td>
<td>0.522, 0.967</td>
<td></td>
</tr>
<tr>
<td>Current non-atopic asthma</td>
<td>0.013</td>
<td>-0.308, 0.335</td>
<td></td>
</tr>
<tr>
<td>Current atopic asthma</td>
<td>0.651</td>
<td>0.286, 1.102</td>
<td></td>
</tr>
<tr>
<td>Intercept ‡</td>
<td>2.131</td>
<td>1.970, 2.291</td>
<td></td>
</tr>
</tbody>
</table>

No significant interaction effects were observed between the variables post-bronchiolitis/control group and the four subgroups of the study, i.e. the relationships between FeNO values measured in these four subgroups were similar in the post-bronchiolitis and the control group.

* Regression coefficient; represents the amount of change of ln NO induced by a change of 1 unit of the explanatory variable.

† P-values from F test. ‡ Reference group (healthy children in the control group).