Author's response to reviews

Title: Reduced Dicer expression in the cord blood of infants admitted with severe respiratory syncytial virus disease

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Author's response to reviews: see over
To the Editor, BMC Infectious Diseases

Please find enclosed the revised manuscript “Reduced Dicer expression in the cord blood of infants admitted with severe respiratory syncytial virus disease.”

We wish to thank the reviewers for their recommendations, and have amended the manuscript accordingly. Responses to the reviewers’ questions and comments are given point-by-point below, and highlighted in the text using track changes.

We were uncertain as to whether to upload Table 3 separately. After revision, the main body of the table is still under two pages. However, if we count with the footnote, the table is over 2 pages long. We have for now included the table with the rest of the manuscript. Please advise us if you would prefer to have the table uploaded separately.

The manuscript has not been submitted or accepted for publication in any other journals. All authors have been significantly involved in the conception and design of the study, analysis and interpretation of the data, drafting and revision of the manuscript for intellectual content, and consent to the final version of the revised manuscript submitted to BMC Infectious Diseases. We have not received any other writing assistance.

We feel that our manuscript has been considerably improved with this revision. We thank the reviewers and editor for their thorough work and hope the manuscript can now be accepted for publication in BMC Infectious Diseases.

Yours sincerely,

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Initial Comments:

1. In response to both reviewers, we have added a paragraph in the discussion (Pg 13 – 14) on the drawbacks of using retrospective clinical data; the risk of misclassification; the possible effects of misclassification.

2. In response to Dr Noviski, we have removed figure 1 (the Western blot figure) since the results are adequately explained in the text.

3. We have changed the word Primary Care Physician to General Practitioner (GP). The word appears so many times in the text that we wished to abbreviate it, and found the abbreviation GP more familiar that PCP (which I normally associate with pneumocystis carinii pneumonia).

4. We have added and discussed a reference on page 17 (Zamora et al). This was published in September 2010, and we were unaware of it when we submitted our manuscript. We found the results relevant to the discussion and wished to include it.

Reponses to Dr Fabbiani’s report:

Major Compulsory revisions:

1. Page 6 “Patient identification and clinical information”

   a) Definition of healthy controls: We have restructured this section and added some sentences that we hope clarify how we defined patients and controls (see pages 6 and 7).

   b) Why only 17 controls? We conducted a power analysis based on previous microarray results, and found a need for 11 individuals in each group. We have added a sentence on this in the Statistical analysis section (page 12). Of course, increasing the number of controls further would have been ideal, but economical constraints prevented this.

   c) How did we confirm that controls were not admitted elsewhere? Thank you, this was an omission on our part. We did this the same way as for RSV positive patients not referred to our unit, and according to the protocol accepted by the ethics committee: we contacted the archives unit at neighboring hospitals to determine if controls were admitted to these hospitals. We have moved a paragraph from the section on page 7 “Disease definition and classification” to page 7 “patient identification and clinical information,” and expanded it to include controls, and the fact that we contacted the archives.

2. Risk of unrecognized RSV disease amongst healthy controls: We have included a paragraph in the discussion (page 14) addressing this issue. We have also expanded slightly the end of the section on disease definition and classification (page 7) to introduce the uncertainty associated with this issue.
3. Table 2 (page 27):

a) Only results from the 37 patients included in the pPCR analysis, not all 51 patients should be presented in the table: We have redone table 2 and statistics with only the 37 patients and 16 controls included in the Dicer analysis (as stated in the methods section, Dicer mRNA was amplified in only 16 of 17 controls). On re-analysis, we discovered a significant difference in placental weight between mild RSV and control groups (p=0.008). We have therefore included the placental weight and Apgar scores in the tables, and commented on this in the discussion, (pg 13).

b) Age on admission. Is it the median value? We have stated in the table that it is the median age, and included the IQR. We used the median value and IQR because the ages were not normally distributed.

c) Percentages for categorical data: We have added percentages.

d) P-values for non-significant parameters: We have added p-values.

4. Table 3 (page 29)

a) Only results from the 37 patients included in the pPCR analysis, not all 51 patients should be presented in the table: We have redone the table and statistics with only the 37 patients included in the analysis.

b) Total number of patients with mild or severe RSV: We have added the totals.

c) Percentages for categorical data: We have added percentages for categorical data.

d) P-values for non-significant parameters: We have added p-values.

e) What do we mean by primary and secondary diagnosis? In Norway, patients are coded with a main diagnosis, indicating the disease most significantly contributing to the admission, and also secondary diagnoses for other conditions associated with, or in addition to the main diagnosis. “Bronchiolitis with atelectasis” and “bronchiolitis with pneumonia” were the most common combinations. Some also had bronchiolitis or pneumonia with cardiovascular compromise as an additional diagnosis, but we have moved cardiovascular compromise to the clinical findings section of the table in response to reviewer 2 (major revision 1), so this is no longer included in this section.

We appreciate that this distinction is not clearly presented, and may be confusing. Instead, we have listed the total number receiving each diagnosis in each group, and then stated that patients may have received more than one diagnosis in the footnote (page 31).

5. Table 4 (Page 32)

a) and b) Specification of number of controls and patients in the title: We have included these changes in the title
c) **Use of exact p-value:** We have added the p value for the mild disease group vs controls.

d) **Comparison of mild and severe groups:** We have compared the mild and severe groups, included the p-value in the results text (page 13) and commented on it in the discussion (pgs 13 - 14).

6.

a) **Retrospective patient selection** and b) **Low number of patients; limited power:** We have addressed both these points in the 2nd paragraph of the discussion (Pgs 13 - 14).

**Minor Essential Revisions:**

1. **Abstract; criteria for control group:** We have specified in the abstract that controls did not present with RSV disease. We chose this wording because, as you have pointed out, we cannot be certain that they did not have disease, and we have discussed in our revision that there is a fair chance that they were infected at some point (pg 14). We are interested in discovering why some infants become very sick from RSV, whilst others do not. Our aim is thus to separate those with severe disease from those without disease and we consider that this wording best summarizes this.

2. **Clinical difference between 37 included and 14 not included in qPCR experiment; “Data not shown:”** We have specified this according to your suggestion (Pg 12; results)

**Discretionary Revisions:**

1. **Introduction. Rephrasing of sentence “although research the last 10 years has provided important clues:”** We have adopted your suggestion. (Pg 4)

2. **Identification of RSV infection; rephrasing of sentence “If negative the NPA was for RSV analyzed…”** Thank you, we have adopted your suggestion (Pg 6).
Responses to Dr Noviski’s Report:

Major Compulsory Revisions:

1. Definition of severe RSV disease; were those with ventilatory support excluded? Thank you for highlighting this. None of the infants in our cohort were so ill that they needed mechanical ventilation or CPAP. We have revised the discussion section to make this clearer (pg 13). Nobody was excluded because of use of a ventilator or CPAP. We have also expanded table 3 (pg 29) to include an interventions section and moved number of patients with cardiovascular compromise to the clinical features section of the table, below “significant dyspnea” and “apnea.”

Minor Essential Revisions:

General Comments:

1. Dicer protein level; is the protein more sensitive to degradation than mRNA? We have tried to address this issue in a little more detail in the paragraph on protein levels in the discussion (pg 15), and hope that it is clearer to readers. Ultimately, we do not know why we could not see dicer in our blots. In order to try to exclude faults with our Western blot analysis or with our samples, we tried 2 different antibodies testing a variety of splice variants; took new cord blood and adult blood samples; and showed our method to be sensitive for dicer in a different cell type.

Specific Comments:

1a) Introduction. Clear statement of the hypothesis: We have altered the last sentence of the introduction so that it is presented as a hypothesis rather than a statement of intention (Pg 5).

b) Alteration of sentence “This mechanism is called RNA interference…” and change of “whilst” to “while.” We have revised the two sentences as suggested. (pgs 4 and 5)

2a: What is a journal? We have altered the word “journal” to “medical record” to be more specific (pgs 6 and 7).

2b: Were investigators reviewing medical records blinded to Dicer status? The patient classification was done before the qPCR experiment, so investigators were blinded to Dicer status. We have added a sentence explaining this in the section on Disease definition and classification (pg 7).

2c: Table 1; Definition of dyspnea; use of a validated symptom score: We agree that a validated system would have been best. Most systems for assessment of severity of bronchiolitis described in the literature are intended for prospective studies comparing effects of inhaled medications. These are not, to our knowledge, validated, and different trials have used very varying systems. In addition, the prospective nature of such trials means that detailed information is available for classification of patients. We had to construct an algorithm based on the data available. Unfortunately respiratory rate was inconsistently recorded in
our patients’ medical journals, and missing from approximately 10 – 20%, so some patients may not have been classifiable if this was included in such a score. The pediatrician’s overall assessment of the degree of respiratory effort was always recorded, including the severity and extent of retractions. In future studies we plan to gather information prospectively, and this will improve the classification of patients.

We have discussed the drawbacks of using retrospective data gathering in a new paragraph in the discussion (pg 14) and stated that there may be inter-observer variations in the assessment of respiratory effort. In the first drafts of the paper we included the following sentences in the section on Disease definition and classification, but removed them to save space. If you feel it is necessary, then we can include them again:

“Several scoring methods for infantile bronchiolitis have been utilized in the last decade to define the severity of bronchiolitis. These scores have primarily been used in clinical trial settings to determine immediate patient response to treatment with nebulized medicines, and require detailed, prospective data gathering to be effective. Because of the retrospective nature of our data gathering, it was not possible to use such a score.”

3. **Figure 1; is it necessary to demonstrate Dicer via Western Blot?** We believe that you question whether it is necessary to include the Western Blot figure when we have stated the results in the text. We are happy to remove figure 1, and have done so.

3a: **Did any patients without sufficient RNA for analysis have documented RSV infection in the first year of life?** We had 52 patients with documented RSV infection in the first year of life. Of these, one had cleft palate and was excluded. 14 did not have sufficient RNA for analysis, leaving us with 37 in the final experiment. This is included in the results section (page 12). So all the patients without sufficient RNA for analysis had documented RSV infection before 1 year of age.

3b: **Table 3: pCO2, oxygen saturation, respiratory rate and pulse. Value on admission, mean or most severe values?** Thank you, this is an omission on our part. We wanted to include the values most descriptive of the patient’s clinical condition, including potential worsening after admission. Therefore the highest pCO2, lowest oxygen saturation and highest respiratory rate during the admission were recorded. Heart rates generally increase after inhaled salbutamol/ albuterol or racemic adrenaline, therefore the pulse on admission, before inhalations, was recorded. We have added this information to the table (Pg 29).

3c: **Figure 2 (Now figure 1): Dicer values appear higher for severe patients:** We are sorry to say that we are not quite sure if we understand your comment. In panel A the control group has a median value of 0; the severe group has a median value of approximately minus 0.4 (lower than control; a negative value denoting reduced expression). In panel B we have only presented results from the severe group, compared to the control group (who have a median value of 0). Also here,
18 of the severe patients have a negative value, that is to say a lower value than the control group, also denoting reduced expression.

When conducting qPCR experiments, a positive ddCT normally means that there is less mRNA (more amplification cycles are necessary to obtain the threshold value). This can appear counter-intuitive on a graph if one has not seen qPCR data before. We have therefore assigned negative values to the positive values and vice-versa to aid interpretation, and also explained in the footnote that a negative value on the graph denotes downregulation. Please ask again, if this does not make things clearer.

4. Discussion. Further discussion of the inability to detect Dicer protein in subjects and controls: We hope that the expanded discussion of the protein levels on pages 14-15 makes this a little clearer.

4a: Remove or elaborate “We can hypothesise that Dicer dowregulation would similarly result in greater RSV load, potentially promoting a negative pro-inflammatory viral effect.” We wish to keep this reference because it is one of few investigations in human tissue demonstrating how artificial Dicer downregulation to 30% functional level affects tissue response to an airways pathogen (influenza in this case). It seems relevant to our findings. We have therefore rewritten our comment on this article in order to further highlight the problems with comparing blood leukocytes to human alveolar cells, and described what we mean by a “negative pro-inflammatory viral effect” (pg 17).