Author's response to reviews

Title: Assessment of Surfactant Protein A (SP-A) dependent agglutination

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

Thank you for reviewing our manuscript; we have addressed your editorial comments and the comments of the reviewers step by step in the letter below and made the appropriate changes in the manuscript.

Editorial comments:
Written informed consent from the subjects was obtained and the experimental research reported in the manuscript was approved by our institutional ethics committee (EK65/96). This is acknowledged in the manuscript in the methods section.
We have attempted to improve the style of written English of the paper and the revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals ).

Referee 1:
Major
1. While this finding is reproducible, it is not clear what it means. This is an important issue as there is no clear indication of when or why this assay should be used. Indeed the authors state "it will be important to investigate for correlations of this assay for SP-A function with lung function and meaningful lung disease outcome variables to completely appreciate its value" (second to last paragraph of discussion).

Response:
As suggested we have now stated more precisely what the meaning of this novel variable is (text on page 15): “The data presented clearly show a local pulmonary deficiency of SP-A dependent agglutination with the pulmonary diseases investigated, whereas no abnormalities were seen in systemic circulation. In future studies it will be important to investigate the correlation of this assay with other functional properties of SP-A and more importantly the correlation with lung function and long term disease outcome variables, in order to determine its role as a surrogate marker and potential modulator of the clinical course.”
2. Reduced agglutinate size in disease BAL is interpreted as a change in SP-A function. In fact a link between SP-A function and agglutinate size is not investigated in this study. Decreased agglutinate size could reflect a qualitative change (e.g. altered SP-A1:SP-A2 ratio) or a quantitative change (there is less SP-A in disease BAL compared to control).

Response:
Quantitative changes in SP-A amount were not responsible for the observed differences, as the SP-A amount was adjusted to the same level for each sample (legend Fig. 4), so the influence of the actual SP-A amount in BAL or serum was excluded in the assay. Furthermore the SP-A level were not significantly different between the 3 study populations (Tab 1).
Qualitative changes within the SP-A from a specific patient were addressed by isolating specific fractions of SP-A present in different patients at different levels, adjusting their concentrations to comparable levels and testing for their capacity to agglutinate; no differences were found. This makes it likely that not different compositions of the fractions are responsible for their activity, but their respective amount. This information is included in the revised manuscript on page 13. We defined the agglutination activity of the SP-A isolated as its function and found that it was very clearly related to the structural composition of SP-A, as separated by gel chromatography. How this may compare to other functional activities of SP-A may be investigated in future studies.

3. There is some confusion regarding the dose-dependence of the assay. BAL from controls contains 391X more SP-A than serum (table 1) yet forms the same size agglutinates (~ 500 pixels, Fig 4).

Response:
The size of the agglutinates was similar, as BAL and serum were analysed at the same final concentration of SP-A, because the assay was adjusted for concentration to obtain comparable results (Page 12, end of 1st para and Methods).

Minor
1. What is the evidence for the antiparallel association of CRD domains in Fig
The scheme attempted to only illustrate the components of the reactants bound to the beads. Indeed there is some parallel assembled SP-A depicted, but generally the reviewer is right, we have no knowledge how the molecules bind exactly. This is stated in the figure legend more clearly now: “The scheme attempts to illustrate the components of the reactants, but does not render how the molecules bind exactly”.

2. The order of presentation in the methods section should be revised e.g. in the description of the assay (second paragraph of methods) the statement "final concentration of SP-A derived from the peak fractions was 100 ng/ml SP-A" is very confusing because SP-A purification is discussed much later.

Response:
The order of the methods was changed as suggested.

Referee 2:
1. Its a very interesting, clearly written manuscript.
2. The abstract could be shorten, in particular, the "Background".

Response:
The abstract was shortened from 263 to 227 words.

3. The pictures obtained by light microscopy need a scale bar.
Scale bars were inserted in every light microscopy picture.

4. The english language should be checked.
Done, as suggested above.

Referee 3:
Major:
1. It is not completely clear to me what the agglutination assay is measuring. Is it the ability of SP-A molecules to aggregate as an active process induced in the assay, or is it simply a reflection of the oligomeric/aggregation state that the SP-A molecules within the samples are in. Experiments to address this question would enhance the manuscript.

Response:
The agglutination assay is measuring the ability of SP-A molecules to aggregate as an active process induced in the assay. This ability was mainly a reflection of the oligomeric aggregation state in which the SP-A molecules within the samples were and also of the origin of the samples and the disease state. This was measured by the experiments and is now clarified in the text.

2. Some of the quantification and assessment of oligomerization are very vague. How was the concentration of SP-A measured (was it the slot blots)? The evidence for the different oligomeric structures after the gel filtration procedure is limited and not clear idea of the specific oligomers that are obtained in the samples is presented.

Response
Yes, as written in the method paragraph “gel chromatography” the amount of SP-A in a fraction was measured by Slot-Blot. This is now clarified in the manuscript more clearly: “A slot blot was used to quantify SP-A concentration.”
The oligomeric structure of SP-A was analysed by the gel chromatography method with a superose 6 column as described. This is stated in the manuscript and detailed in reference 27.

3. Ultimately, the take home message is a bit vague. The authors need to convince me a bit more that their study adds new information to the current literature. Their assay is consistent with everything that is known about SP-A. The measurements of patient samples indicates that maybe the samples have different oligomeric structures and/or aggregating properties. But I still wonder if the same information have been obtained by running non-denaturing western blots?

Response:
We present a very simple and fast method to test for the aggregate forming properties of SP-A in serum and broncho-alveolar lavage samples. The strengths of this approach are the specific measurement of binding to the carbohydrate recognition domain (CRD) of SP-A and its application to samples of very small size without purification or isolation steps necessary before the measurements. With non-denaturating Western-blot there are extreme difficulties for the large aggregates that can be easily resolved by gel-chromatography to enter the gel. In addition this paper for the first time describes a method suitable for very small samples to assess the agglutinating ability of SP-A.

Minor comments:
1. Abstract, first sentence: the terminology “organization of biophysical surfactant function” is inaccurate or at least unclear.

Response:
The term was deleted.

2. In general, the authors report almost a lot of background information on roles of SP-A. This information could be more critically assessed, rather than simply listing everything, and the role of self aggregation in these processes could be expanded.

Response:
To keep the focus of the manuscript and to keep it brief we decided not to extent on the details of the background information in this manuscript.

3. Page 15: last paragraph: The statement about an test system to assess functional properties is not true unless self aggregation of SP-A can be directly linked with function, and even then it would only be an indirect measure.

Response:
We assessed the self-aggregation of SP-A. If this truly and directly reflects many or all of the other functions SP-A has, must be addressed in future studies and is beyond the scope of this manuscript. This was clarified in the last but one paragraph on page 15, as detailed above.