Author's response to reviews

Title: Surfactant Replacement or Open Lung Concept? Comparison of two treatment strategies in an experimental model of neonatal ARDS

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Author's response to reviews: see over
Dear Reviewers,

Thank you very much for sending us the constructive comments. We were encouraged that our study was judged to be interesting and potentially important. We have endeavoured to address all your remarks and critical comments.

Enclosed please find a detailed point-by-point reply:

# Reviewer 1:

Major

Ad 1.) The main focus of our study was to investigate, whether one treatment regime or the other reveals lung protective aspects, as it may be reflected by reconstituted surfactant composition or reduced signs of tissue damage as examined by histological strategies. In order to focus on the differences between the different study groups, no “saline control group” has been included. Regarding physiologic parameters (lung function, ventilation parameters) data obtained at T=0h, i.e. before therapeutic intervention, serve as true control values. We, however, agree with the reviewer, that it would have further strengthened the
study, in particular with regard to biochemical and histological analysis, but was not feasible due to limitations in animal numbers.

Ad 2.) We agree with the reviewer, that surfactant treatment is an established therapeutic option in ARDS-like lung injury in neonates, as secondary surfactant deficiency is related to the clinical picture. Nevertheless, surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects and the potential need for repetitive doses. Thus, conventional ventilation regimes are often used as first-line therapies in this patient cohort. If conventional ventilation strategies fail, treatment options are searched for in order to minimize ventilator-induced lung injury. Thus, the present study setting has been used to mimic clinical conditions. This background is now strengthened in the manuscript. As correctly noted by the reviewer, there is still no consensus of ventilation strategy in combination with surfactant treatment until now (see also van Kaam A. et al; Crit Care Med 2007). As it has been shown in the present study and previously by van Kaam et al, applying the OLC strategy is in some way comparable to surfactant administration regarding the effects on lung function. The combination of both treatment strategies allows decreasing both the opening and the closing pressures after some hours. This finding may be explained by a higher alveolar stabilization after recruitment maneuvers in combination with the effect of surfactant administration. The consecutive reduction in shear-stress and further traumatic events to the lung may also contribute to type-II cell integrity as reflected by improved surfactant composition in lung lavage specimen. We agree with the reviewer, that combination of both treatment regimes will gain increasing interest as shown by van Veenendaal et al, Crit Care Med 2006 and Frerichs et al, Am J Respir Crit Care Med 2006. Nevertheless, ventilation following the open lung concept has been used as a rescue therapy in the present study setting and is discussed in this context. This point of view has now been strengthened in the discussion.

Ad 3.) There is only a practical justification for the numbers of animals investigated: the number of laboratory animals was limited and so we were geared to the experiments of other study groups as indicated in the manuscript and discussed the present findings beyond the background of previous studies using a comparable experimental setting.

Ad 4.) We agree with the reviewer, that the problem of differences in lung function variables between the study groups at the beginning of the interventions after induction of lung injury needs to be taken into account when analyzing the results. These differences are common in animal experiments, especially when lung failure is induced through different interventions. In order to meet this problem, the variables were analyzed using a two-way ANOVA, analyzing
the development of the variables over time. Furthermore, the changes of the variables over the whole study period were comprised using the difference between the starting and the end point of the study (T5-T0). These differences were then compared between the study groups. Thus, effects over time were analyzed in spite of sole differences between the groups at different time points. This enables to compare the variables in face of the time course despite differences at the starting point.

Regarding the histologic results, although a tendency towards lower histologic sum scores in the monomeric SP-B group might be assumed, there was no statistical significant difference. This is now further underlined in the manuscript.

Ad 5.) The ventilation following the open lung concept was initiated as a rescue maneuver, as it is often used in clinical practice. Nevertheless, we have performed the statistical analysis again – as suggested by the reviewers – after extracting two time courses (0 to 2 hours and 3 to 5 hours). These analyzes resulted in comparable levels of significance regarding the differences between the study groups.

Ad 6.) The main focus of our study was to investigate, whether one treatment regime or the other may reveal lung protective aspects. Reconstitution of the surfactant system has been discussed as an important measure assessing different treatment regimes in ARDS-like lung disorders in infants and children. Thus, the investigation of different components of the surfactant system has been considered to be important in order to compare outcome between the treatment regimes. Differentiated analyses of surfactant composition provide insight into the mechanism of lung injury on the basis of type-II cell function. Reconstitution of surfactant composition serves as an indicator of type-II cell integrity and metabolism.

We agree with the reviewer, that findings from the BAL analyses could be discussed in more detail. This is now included in the manuscript (see also comments below).

Ad 7.) We agree with the reviewer, that the title does not adequately fit the discussion. It has thus been revised into Surfactant Replacement and Open Lung Concept - Comparison of two treatment strategies in an experimental model of neonatal ARDS

**Minor**

**Abstract**

- Due to the different time points and groups for each of the various variables, the numbers and p-values of the different statistical analyses would not fit adequately in the limited passage of the abstract. We have thus amended the terms ‘significant’ etc. in the text in
order to weight the findings. The full term of the abbreviation VEI is now stated in the abstract.

**Introduction**

- The first sentence of the introduction has been reworded.

- Page 4, end of first paragraph: We agree with the reviewer, that surfactant treatment is an established therapeutic option in ARDS-like lung injury in neonates, as secondary surfactant deficiency is related to the clinical picture. Nevertheless, as outlined above (response to point 2) surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects and the potential need for repetitive doses. Thus, conventional ventilation regimes are often used as first-line therapies in this patient cohort. If conventional ventilation strategies fail, treatment options are searched for in order to minimize ventilator-induced lung injury. Thus, the present study setting has been used to mimic clinical conditions. Ventilation following the open lung concept has been used as a rescue therapy, as it is often used in clinical practice. This background is now strengthened in the manuscript.

As correctly noted by the reviewer, there is still no consensus of ventilation strategy in combination with surfactant treatment until now (see also van Kaam A. et al; Crit Care Med 2007). As it has been shown in the present study and previously by van Kaam et al, applying the OLC strategy is in some way comparable to surfactant administration regarding the effects on lung function. The combination of both treatment strategies allows decreasing both the opening and the closing pressures after some hours. This finding may be explained by a higher alveolar stabilization after recruitment maneuvers in combination with the effect of surfactant administration. The consecutive reduction in shear-stress and further traumatic events to the lung may also contribute to type-II cell integrity as reflected by improved surfactant composition in lung lavage specimen. We agree with the reviewer, that combination of both treatment regimes will gain increasing interest as shown by van Veenendaal et al, Crit Care Med 2006 and Frerichs et al, Am J Respir Crit Care Med 2006. This point of view has now been strengthened in the discussion.

- Page 4, second paragraph, references 11-14: We appreciate the comment of the reviewer. The cited references have been extended in order to add references dealing with the problem of neonatal ARDS like lung injury and the investigation of different surfactant preparations. Reference 16 has been omitted.
Materials and Methods

- Page 5, second paragraph: Different studies investigating the pulmonary response to surfactant administration or different ventilation concepts were using neonatal piglet models including piglets at an age from 1-11 (14) days (see e.g. van Kaam et al., Hilgendorff et al.). According to statements of responsible veterinarians the pulmonary situation does not change significantly in these days. Thus, these animals can be stated as comparable regarding their initial pulmonary situation.

- Page 5, second paragraph: We apologize for this mistake. The term has been corrected for inner diameter.

- Page 5, last paragraph: We appreciate the comment of the reviewer. It is true that this model of lung injury using repeated lavage procedures may cause additional lung damage by non-physiologic stretch. Nevertheless, the model is well introduced and known from previous publications. Its limitations have been discussed previously and are now included in the discussion.

- Page 7, 2nd paragraph: In order to obtain comparable results between the different study groups especially regarding the outcome in terms of lung injury and surfactant composition, homogeneity of the different study groups played an important role regarding the design of the study. Thus, animals showing no recovery from lung lavage procedures, indicated by a paO2 below 60 kPa were excluded as hypoxia played an important role in these subjects. Hypoxia is known to lead to severe hemodynamic compromise and has important impact on outcome variables. As well impairment of lung function as alterations to pulmonary tissue may be expected, especially regarding inflammatory processes and growth hormone regulated alteration of vascular and epithelial processes. The procedure of excluding these animals has been discussed with the statistician in preparation for the study. The in- or exclusion criteria, respectively have now been specified in the methods section.

- Page 7/8: The reviewer is right that increasing PaCO2, decreasing PaO2 and decreasing blood pressure can also indicate alveolar collapse. Interpreting the variables beyond their development over time and the effect of the rescue / recruitment maneuvers, one can see, that the PV loops during the recruitment maneuver reflected an improvement of lung mechanics and function as well as blood gas monitoring showed an increase in oxygenation. Furthermore, levels of PEEP were defined in order to prevent alveolar collapse and lung function improved over time. Thus, the risk of alveolar overdistension can be assumed to be
higher compared to the risk of alveolar collapse. Nevertheless, the latter may not be disregarded.

- Page 8, 2nd paragraph: In order to mimic clinical conditions the implementation of the OLC has begun after one hour with paO2 below 100 kPa according to Lachmann et al. and thus initiated as a rescue therapy.

Surfactant treatment is an established therapeutic option in ARDS-like lung injury in neonates, as secondary surfactant deficiency may be causally related to the clinical picture. Nevertheless, surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects or the need for repetitive doses. Thus, conventional ventilation strategies are often used as first-line management of the disease. If conventional ventilation fails, further treatment regimes are searched for in order to minimize ventilator-induced lung injury. Thus, the present study setting has been used to mimic clinical conditions. This background has now been strengthened in the manuscript. Nevertheless, the induction of lung injury after one hour of conventional ventilation in the PPVOLC group has now also been taken into account and is included in the discussion.

**Results**
- In order to give a concise presentation of the data, the results section gives a description of the results and the p-values whereas the tables clearly lists the numbers. Due to the different time points and groups for each of the various variables, this would not fit adequately in the text.

The formula to calculate the ventilation efficiency index (VEI) is now given in the manuscript in addition to the reference (\(VEI = \frac{3,800}{\Delta P \times R \times P_{a_{CO2}}}\)).

- Page 12, 1st paragraph: Number of lavages performed in the animals as well as mean recovery rate of lavage fluid is now given in the methods section.

- Page 12, 2nd paragraph: Decreasing blood pressure was one prerequisite besides increasing PaCO2 or decreasing PaO2 to decrease PEEP as these variables indicate alveolar overdistension. That none of the animals showed signs of hemodynamic compromise means continuous decrease of blood pressure due to volume problems or cardiac failure. This did not occur and is thus stated in the results section.

- Page 12, 1st paragraph, Table 2a: In order to meet the problem of differences of lung function variables at the beginning of the interventions after induction of lung injury between
the study groups, the development of the variables over time was analyzed as well as the
differences between the starting and the end point of the study, respectively (T5-T0). Thus,
effects over time were discussed in spite of sole differences between the groups at different
time points.
Furthermore, we agree with the reviewer that in the present study relatively high tidal
volumes have been applied in the OLC group. This point has now also been focussed in the
discussion as the formerly described concept of OLC ventilation comprised lower tidal
volumes. Nevertheless, the PIP has been decreased following a standardized protocol. Thus,
the lower tidal volumes applicated in one group can be attributed to a better recovery
regarding blood gases and thus lung function.

- We apologize for this open question regarding data presentation: Yes, the asterisks
  present the results of the post-hoc tests.

- Page 12, 5th paragraph: This finding is supported by the 2-way-ANOVA test over the
  indicated time points, that showed an effect over time without significant interaction. This is
  know further clarified in the manuscript.

- Page 12, last paragraph: The time point T=0 has been included in the ANOVA analysis as
  the ventilation following the open lung concept was initiated as a rescue maneuver, as it is
  often used in clinical practice. Nevertheless, the statistical analysis has now also been
  performed in a different manner – as suggested by the reviewers – after extracting two time
  courses (0 to 2 hours and 3 to 5 hours). These analyses resulted in comparable levels of
  significance regarding the differences beween the study groups.

- Page 13, 2nd paragraph: Significances over the time course comparing the different study
  groups are given in the text in order to keep the table clearly arranged. The significances in
  the table are quoting the comparison of lavaged versus healthy values.

- Page 13, 3rd paragraph: See comment above

- Page 13, data presentation: The tables list the detailed information about the values. The
  graphs give an overview of the development over time, which can not be made as clear in
  the table as in the figure. Furthermore, the results and levels of significance from the two-way
  ANOVA with respect to time are more clear when displayed in the graph as this refers to the
  whole time course and not to single time points.
Four tissue slides were analysed from both the upper and lower lung lobe and two slides from the middle lung lobe, respectively. Blinded to the animal’s group assignment, lung histology was scored according to a previously described procedure (Mrozek et al.). Variables scored for histologic evaluation were atelectasis, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, alveolar and interstitial edema, necrosis and overdistension. The variables were scored using a four-point scale with no injury corresponding to 0 points and 4 points indicating maximum injury.

Regarding the different therapeutic interventions, their effects may show an inhomogenous distributional pattern. This is a well known fact for exogenous surfactant administration and is as well true for different ventilation concepts. Furthermore, the degree of lung injury following the lavage procedures can as well be suggested to show differences between the different lung lobes, as this is also a consequence of inhomogenous distribution. Distributional problems are now addressed in the manuscript.

The bar graph has been omitted from the figure.

We agree with the reviewer, that figure 4 and table 3 are redundant, giving the same values. Thus, figure 4 has been omitted.

We apologize for this mistake. The text has been corrected for phospholipid-to-protein ratio as given in the tables (see footnote).

We agree with the reviewer, that a „saline control group“ would have strengthened the manuscript but has not been feasible due to limited animal numbers. Nevertheless, pulmonary surfactant is highly conserved and data from previous experiments may help to interpretate the results of the present study (see table below). The indicated references are given in the manuscript (see discussion).

In healthy individuals, PC accounts for approximately ca 80% and PG for approximately 8-10%. The following table gives a few reference values and the references, accordingly.
<table>
<thead>
<tr>
<th>species</th>
<th>PC [%]</th>
<th>PG [%]</th>
<th>PI [%]</th>
<th>PE [%]</th>
<th>PS [%]</th>
<th>Sph [%]</th>
<th>Lyso-PC [%]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>83.1</td>
<td>8.6</td>
<td>3.2</td>
<td>1.7</td>
<td>1.2</td>
<td>0.8</td>
<td>0.1</td>
<td>[33]</td>
</tr>
<tr>
<td>mouse</td>
<td>72.3</td>
<td>18.1</td>
<td>n.d.</td>
<td>1.9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0</td>
<td>[70]</td>
</tr>
<tr>
<td>rat</td>
<td>82.3</td>
<td>7.5</td>
<td>1.8</td>
<td>5.1</td>
<td>0.1</td>
<td>0.8</td>
<td>0.3</td>
<td>[71]</td>
</tr>
<tr>
<td>rabbit</td>
<td>80.6</td>
<td>7.2</td>
<td>4.0</td>
<td>4.4</td>
<td>1.9</td>
<td>1.5</td>
<td>n.d.</td>
<td>[71]</td>
</tr>
<tr>
<td>bovine</td>
<td>81.7</td>
<td>5.8</td>
<td>3.4</td>
<td>2.9</td>
<td>n.d.</td>
<td>1.3</td>
<td>n.d.</td>
<td>[59]</td>
</tr>
<tr>
<td>ovine</td>
<td>81.0</td>
<td>7.9</td>
<td>2.6</td>
<td>4.8</td>
<td>n.d.</td>
<td>1.7</td>
<td>n.d.</td>
<td>[72]</td>
</tr>
<tr>
<td>canine</td>
<td>86.3</td>
<td>11.1</td>
<td>n.d.</td>
<td>2.0</td>
<td>0.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>[41]</td>
</tr>
</tbody>
</table>

Table 1: Phospholipid composition of extracellular surfactant in selected mammalian species.

The relative content of each PL class is given as percentage of total PL (mean values). PC = phosphatidylycerol, PG = phosphatidylglycerol, PI = phosphatidylinositol, PE = phosphatidylethanolamine, PS = phosphatidylserine, SPH = sphingomyelin, n.d. = not determined.

References:


- The reason, why PS was not detectable in BAL specimen is, that PS is only found in traces (<1%). Thus, these amounts are masked in the surfactant groups, as PC and PG are much more higher concentrated in these BAL specimen after exogenous surfactant administration.
In contrast, the PPV_{olc} group showed a typical surfactant profile indicating disruption of surfactant homeostasis with a consequent loss of PG and an increase in the minor components PE, PS, PI, Sph.

- The increase in polyunsaturated FA indicates lung injury and disruption of surfactant homeostasis. In contrast, normal surfactant composition and BAL specimen after exogenous surfactant administration contain higher percentages of saturated FA (e.g. palmitic acid). Thus, surfactant administration leads to a shift of the FA profile and a reduction of the increase in PUFA after lung injury. This is now amended to the discussion.

- Page 14, 2nd paragraph: We agree with the reviewer that the discussion of this point needs to be done with caution. A possible explanation may be that exogenous surfactant led to the preferential administration of PL in the lung in relation to SP-B. This is due to only small amounts of surfactant proteins in the surfactant preparations (about 0.5-1% of PL in Alveofact). Thus, administration of surfactant leads to a shift in the surfactant protein - PL ratio with the previous increase in the SPB/PL ratio now resulting in a reduction of the SPB/PL quotient.

- Page 15, 1st paragraph: We agree with the reviewer, that figure 5 and table 4 give the same values. Thus, figure 5 has been omitted.

- This finding may be explained by the physiology of the surfactant system. First, there is a latency between mRNA synthesis / expression and the translational processes leading to the formation of the protein. Furthermore, SP-B, as synthesized in the type II cell undergoes extensive processing and proteolytic cleavage as well as final deposition in lamellar bodies before it is secreted by the type II cell and thus detectable in BAL fluid.

**Discussion**

- The first paragraph of the discussion has been rewritten in order to meet the remarks of the reviewer.

-Page 17, 2nd paragraph: The statistical analysis has now also been performed in a different manner – as assumed by the reviewers – after extracting two time courses (0 to 2 hours and 3 to 5 hours). These analyzes resulted in comparable levels of significance regarding the differences between the study groups.
- Page 17, 2nd paragraph: We agree with the reviewer that in the present study relatively high tidal volumes have been applied in the OLC group and – in part – in the surfactant groups. This point has now also been focussed in the discussion, especially as the formerly described concept of OLC ventilation comprised lower tidal volumes. Nevertheless, the PIP has been decreased following a standardized protocol. Thus, the lower tidal volumes applied in one group can be attributed to a better recovery regarding blood gases and thus lung function.

- Page 17, last paragraph: The discussion regarding the effect of mon SP-B on lung histology has been rewritten as recommended.

- Page 18, 2nd paragraph: The discussion of the results on the surfactant system has now been revised and extended according to the reviewers comments.

- Page 18, last paragraph: We agree with the reviewer, that surfactant treatment is an established therapeutic option in ARDS-like lung injury in neonates, as secondary surfactant deficiency is related to the clinical picture. Nevertheless, surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects and the potential need for repetitive doses. Thus, conventional ventilation regimes are often used as first-line therapies in this patient cohort. If conventional ventilation strategies fail, treatment options are searched for in order to minimize ventilator-induced lung injury. Thus, the present study setting has been used to mimic clinical conditions. This background is now strengthened in the manuscript.

As amended by the reviewer, there is still no consensus of ventilation strategy in combination with surfactant treatment until now (see also van Kaam A. et al; Crit Care Med 2007). As it has been shown in the present study and previously by van Kaam et al, applying the OLC strategy is in some way comparable to surfactant administration regarding the effects on lung function. The combination of both treatment strategies allows decreasing both the opening and the closing pressures after some hours. This finding may be explained by a higher alveolar stabilization after recruitment maneuvers in combination with the effect of surfactant administration. The consecutive reduction in shear-stress and further traumatic events to the lung may also contribute to type-II cell integrity as reflected by improved surfactant composition in lung lavage specimen. We agree with the reviewer, that combination of both treatment regimes will gain increasing interest as shown by van Veenendaal et al, Crit Care Med 2006 and Frerichs et al, Am J Respir Crit Care Med 2006. This point of view has now been strengthened in the discussion.
- Page 19, 1st paragraph: We agree with the reviewer that these findings need to be discussed in two ways. Nevertheless, the results may also be a consequence of a shift in the surfactant phospholipid/protein ratio after administration of relevant amounts of PL. The discussion has been rewritten accordingly.

- Page 20, 1st paragraph: The open lung concept aims at alveolar recruitment via application of an increased $\Delta P$ while reducing lung injury mediated by high tidal volumes. As administration of higher tidal volumes may result in higher degrees of lung injury, this fact needs to be taken into account when comparing the present study to previous investigations. This has been the background for discussing this methodical characteristic as a limitation of the study.

# Reviewer 2

Ad 1.) We agree with the reviewer that the age of study animals is an important point. Different studies investigating the pulmonary response to surfactant administration or different ventilation concepts were using neonatal piglet models including piglets at an age from 1-11 (14) days (see e.g. van Kaam et al., Hilgendorff et al.,). According to statements of responsible veterinarians the pulmonary situation does not change significantly in these days. Thus, these animals can be stated as comparable regarding their initial pulmonary situation.

Ad 2.) The main focus of our study was to investigate, whether one treatment regime or the other reveals lung protective aspects, as it may be reflected by reconstituted surfactant composition or reduced signs of tissue damage as examined by histological strategies. In order to focus on the differences between the different study groups, no “saline control group” has been included. Regarding physiologic parameters (lung function, ventilation parameters) data obtained at T=0h, i.e. before therapeutic intervention, serve as true control values. We, however, agree with the reviewer, that this would have further strengthened the study, in particular with regard to biochemical and histological analysis, but was not feasible due to limitations in animal numbers.

Ad 3.) In order to make the results between the different study groups comparable especially regarding the outcome in terms of lung injury and surfactant composition homogeneity of the groups played an important role regarding the design of the study. Thus, animals showing no recovery from lung lavage procedures, indicated by a paO2 below 60 kPa were excluded as
hypoxia played an important role in these subjects. Hypoxia is known to lead to severe hemodynamic compromise and has important impact on outcome variables. As well impairment of lung function as alterations to pulmonary tissue may be expected, especially regarding inflammatory processes and growth hormon regulated alteration of vascular and epithelial processes.

The procedure of excluding these animals has been discussed with the statistician in preparation for the study. The in- or exclusion criteria, respectively have now been specified in the methods section.

Ad 4.) Surfactant treatment is an established therapeutic option in ARDS-like lung injury in neonates, as secondary surfactant deficiency may be causally related to the clinical picture. Nevertheless, surfactant treatment is a very cost-intensive therapy and surely has its limitations due to adverse effects or the need for repetitive doses. Thus, conventional ventilation strategies are often used as first-line management of the disease. If conventional ventilation fails, further treatment regimes are searched for in order to minimize ventilator-induced lung injury. Thus, the present study setting has been used to mimic clinical conditions. This background has now been strengthened in the manuscript. Nevertheless, the induction of lung injury after one hour of conventional ventilation in the PPVOLC group has now also been taken into account and is included in the discussion. Furthermore, we agree with the reviewer that in the present study relatively high tidal volumes have been applied in the OLC group. This point has now also been focused in the discussion as the formerly described concept of OLC ventilation comprised lower tidal volumes.

Ad 5.) There is no statistical justification, only a practical one: the number of laboratory animals was limited and so we were geared to the experiments of other study groups as indicated in the manuscript.

Ad 6.) Continuous chest movements have been monitored during the whole procedure of surfactant administration as described in the methods section. Both surfactant preparations were provided as lyophilized powder and resuspended in sterile saline 0.9% (Braun) to a final concentration of 60 mg phospholipids/mL. These concentrations and thus administered volumes are in accordance with the manufacturers instructions and hold true as well for clinical as experimental conditions.

Ad 7.) The figure title and legends have been rewritten as recommended by the reviewer.
Ad 8.) The differences in the histologic pattern tend to result in a difference between the groups without reaching statistical significance after standardized scoring of different fields as described in the methods.

Ad 9.) The reviewer is right, that different studies tried no „normalize“ absolute concentrations in BAL specimen using the urea balance. Nevertheless, this procedure never proved to be reliable. As the lavage procedure was performed in a highly standardized manner and the recovery rates were found to be very similar, this procedure does not lead to a specific benefit. The data on lavage procedures and recovery rates are now given in the manuscript.

Ad 10.) The discussion has been rewritten in order to meet the remarks of the reviewer.