Patients with ARDS show improvement but not normalisation of alveolar surface activity with surfactant treatment: putative role of neutral lipids

Philipp Markart, Clemens Ruppert, Malgorzata Wygrecka, Thorsten Colaris, Bhola Dahal, Dieter Walmrath, Heinz Harbach, Jochen Wilhelm, Werner Seeger, Reinhold Schmidt, Andreas Guenther

Background: Extensive biochemical and biophysical changes of the pulmonary surfactant system occur in the acute respiratory distress syndrome (ARDS).

Methods: The effect of intrabronchial administration of a recombinant surfactant protein C-based surfactant preparation (Venticute) on gas exchange, surfactant composition and function was investigated in 31 patients with ARDS in a randomised controlled phase I/II clinical pilot trial. Bronchoalveolar lavage fluids for surfactant analysis were obtained 3 h before and 48 and 120 h after the first surfactant application. Potentially deleterious effects of surfactant neutral lipids in patients with ARDS were also identified.

Results: Before treatment all patients had marked abnormalities in the surfactant phospholipid and protein composition. In response to surfactant treatment, gas exchange improved and surfactant phospholipid and protein content were almost normalised. Alveolar surface activity was dramatically improved before treatment and only partially improved after surfactant administration. Further analysis of the bronchoalveolar lavage fluids revealed a twofold increase in neutral lipid content and altered neutral lipid profile in patients with ARDS compared with healthy controls. These differences persisted even after administration of large amounts of Venticute. Supplementation of Venticute or natural surfactant with a synthetic neutral lipid preparation, mimicking the profile in ARDS, caused a dose-dependent deterioration of surface activity in vitro.

Conclusion: Intrabronchial surfactant treatment improves gas exchange in ARDS, but the efficacy may be limited by increased concentration and altered neutral lipid profile in surfactant under these conditions.

Pulmonary surfactant is a lipoprotein complex consisting of lipids (90%) and proteins (10%). Surfactant reduces the alveolar surface tension to near 0 mN/m, preventing alveolar collapse, and enables gas exchange and alveolar ventilation at physiological transpulmonary pressure gradients. Approximately 80–90% of the lipids are phospholipids. Four surfactant proteins (SP) have been identified (SP-A, SP-B, SP-C and SP-D). Surfactant obtained by bronchoalveolar lavage (BAL) can be separated into different subfractions by buoyant density gradient or differential centrifugation. The large surfactant aggregate (LA) fraction, consisting of lamellar bodies, tubular myelin and large multilamellar vesicles, displays excellent biophysical activity in vitro and in vivo and represents the precursor fraction of the interfacial surface film. In patients with the acute respiratory distress syndrome (ARDS), extensive alterations of biochemical and biophysical surfactant properties have been described. In addition, intrabronchial surfactant administration was shown to be beneficial in different ARDS models. Impaired surfactant function may therefore play a role in the development of gas exchange abnormalities in clinical ARDS. Accordingly, in some recent phase II and phase III clinical trials, administration of surfactant in subjects with ARDS significantly improved arterial oxygenation and surfactant composition.

In the present study we investigated surfactant abnormalities in the BAL fluids of patients with ARDS participating in a randomised controlled phase I/II study in Europe/South Africa addressing the safety and efficacy of a recombinant SP-C-based surfactant (Venticute). Before randomisation, profound surfactant abnormalities were encountered, with a marked loss of surface tension-lowering properties and severe alterations in phospholipid, fatty acid and surfactant protein composition. In response to surfactant treatment, gas exchange was improved and phospholipid, fatty acid profiles and SP-C content were nearly normalised, but surface activity was only partially restored. To investigate the underlying mechanism for this partial restoration, we further analysed the LA fraction from the BAL fluids. Interestingly, we observed a twofold increase in the relative amount of neutral lipids in the LA fraction of patients with ARDS. Physiologically, neutral lipids represent 10–20% of the BAL fluid lipids and about 5% of the LA lipids. Within the LA fraction, the neutral lipids consist of cholesterol (30–40% of total neutral lipids), free fatty acids (25–30%), cholesterol ester (~12%), monoglycerides (10–15%), diglycerides (~14%) and triglycerides (~8%). The increased concentration of neutral lipids in the LA fraction of subjects with ARDS was still present even after administration of large amounts of Venticute. Moreover, the composition of the neutral lipid fraction was markedly different between subjects with ARDS and healthy controls. Reconstitution of Venticute or a natural surfactant preparation with a neutral lipid preparation, mimicking the profile in subjects with ARDS, provoked a dose-dependent inhibition of surfactant function in vitro. This effect was already evident at a relatively neutral lipid dose found in patients with ARDS.

Abbreviations: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; PaO₂, fractional inspired oxygen; γads, surface tension after 1/2 s of film adsorption; γmin, surface tension values after 5 min of film oscillation at minimum bubble radius; HPTLC, high-performance thin-layer chromatography; LA, large surfactant aggregate; PaCO₂, arterial oxygen tension; SP, surfactant protein

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Changes in quantity and composition of the surfactant neutral lipids may thus contribute to the impairment of surface activity and gas exchange abnormalities in ARDS.

METHODS
Study design
Thirty-one patients with ARDS as defined by the American-European Consensus criteria for a maximum of 120 h since diagnosis (early ARDS) were investigated. All patients with ARDS participated in a randomised multicentre controlled phase I/II pilot study investigating the safety and efficacy of an intrabronchial administration of a recombinant SP-C-based surfactant (Venticute). Efficacy was assessed by calculating (1) the excess area under the arterial oxygen tension/fractional inspired oxygen (PaO2/FIO2) curve during the 24 h after administration of the first drug dose and (2) the number of days with unassisted breathing within the 28 day observation period and comparing these variables among groups. Another objective of the study was to assess BAL fluid recovered from the patients for surfactant components and function.

During a 6 h baseline period, clinical and respiratory parameters were recorded and the first BAL fluid sample for surfactant analyses was obtained. Baseline demographic and physiological data for each of the patient groups are shown in table 1. No significant differences were detected between groups. Values for the modified Acute Physiology and Chronic Health Evaluation (APACHE) II score and for baseline positive end-expiratory pressure and PaO2/FIO2 values indicate a similar severity of illness among the groups.

Immediately after the conclusion of the baseline period, patients were prospectively randomised to receive either standard care (ARDS-standard care, n = 17) or standard care + 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight given up to four times in 24 h (ARDS-surfactant; n = 14). BAL fluid was obtained from previously unlavaged lung segments at 48 and 120 h after the first treatment. Patients were subsequently observed for up to 28 days or until discharge from hospital. Additional information on study methods is provided in the online data supplement available at http://thorax.bmj.com/supplemental.

As a control group, 11 healthy volunteers without any history of cardiac or lung disease and with normal pulmonary function were studied. To investigate the effect of ventilation on the neutral lipid content in the BAL fluid, the relative neutral lipid content in the BAL fluid was also determined in four mechanically ventilated patients suffering from cardiogenic pulmonary oedema in the absence of ARDS and lung infection. A comparable disturbance in gas exchange was observed in patients with ARDS and in those with cardiogenic pulmonary oedema (PaO2/FIO2 ratio 126 mm Hg vs 152 mm Hg).

In vitro experiments with neutral lipids and surfactant preparations
Preparation of a rabbit BAL fluid pool
Healthy rabbits of either sex were killed by intravenous application of a lethal dose of pentobarbital/ketanest. A catheter was placed into the trachea and the lungs were lavaged three times with 50 ml 0.9% NaCl. After filtration of the lavage fluid through sterile gauze and sedimentation of cells (200 g, 4°C, 10 min), supernatants originating from 20 rabbits were pooled and then centrifuged at 48 000 g (4°C, 1 h). The pellet represents the LA fraction and was resuspended in a small volume of 0.9% NaCl/3 mM CaCl2 and adjusted to a stock concentration of 2 mg/ml phospholipids.

Recombinant SP-C-based surfactant preparation
The recombinant SP-C-based surfactant preparation used for the in vitro experiments was identical to the study drug material and was donated by ALTANA Pharma AG, Konstanz, Germany. The recombinant SP-C-based surfactant preparation contains dipalmitylophosphatidylcholine and phosphatidylglycerol (7:3 wt/wt), enriched with 5% wt/wt palmitic acid and 2% (wt/wt) recombinant human SP-C. A stock preparation containing 2 mg/ml phospholipids was used.

Neutral lipid preparation
The neutral lipid preparations used for the in vitro experiments were designed to mimic the neutral lipid profile in patients with ARDS and consisted either of 100% or 50% unsaturated fatty acids. Increasing amounts of a 1 mg/ml stock solution were added to liposomes containing dipalmitylophosphatidylcholine and phosphatidylglycerol (7:3 wt/wt), enriched with 5% wt/wt palmitic acid and 2% (wt/wt) recombinant human SP-C. A stock preparation containing 2 mg/ml phospholipids was used.

Surfactant analysis
Isolation of large surfactant aggregates (LA)
BAL fluid from patients with ARDS, those with cardiogenic pulmonary oedema or healthy volunteers was centrifuged at 48 000 g (4°C, 1 h). The resulting pellet containing the LA fraction was resuspended in a small volume of 0.9% NaCl/3 mM CaCl2 and adjusted to a phospholipid concentration of 2 mg/ml. In parallel experiments, the LA fraction from seven patients with ARDS was further purified by sucrose gradient centrifugation as described in detail previously.

Phospholipid and fatty acid analysis
Lipids were extracted with chloroform/methanol according to method of Bligh and Dyer and the phospholipid content was determined by spectrophotometric measurement of phosphorus. For separation and analysis of phospholipid classes,
high-performance thin-layer chromatography (HPTLC) was used as previously described. Similarly, the fatty acid profile of phosphatidylcholine was assessed by gas-liquid chromatography as outlined previously.

**Determination of neutral lipid content and profile**

The amount and relative distribution of neutral lipids was measured by HPTLC and densitometric scanning as previously described, with the following modifications: plates were pre-run with chloroform before application of the lipid standards and samples and the staining procedure was performed by incubation of the plates for 30 min with a 0.1% (wt/v) aqueous solution of 8-anilino-1-naphthalenesulfonic acid (8-ANS, Merck, Darmstadt, Germany).

**Quantification of surfactant proteins**

The content of the surfactant proteins in the BAL fluid was determined by recently described ELISA techniques. Determination of surface activity

Surface tension measurements were performed by means of a pulsating bubble surfactometer (Electronetics, New York, USA) at a phospholipid concentration of 2 mg/ml. Surface tension after 12 s of film adsorption (yads) and after 5 min of film oscillation at minimum bubble radius (ymin) were recorded.

**Analysis of data**

Data are given as median and interquartile range. The box and whisker plots indicate the median, 1st and 3rd quartiles; the whiskers are extended to the most extreme value inside the 1.5-whisker plots indicate the median, 1st and 3rd quartiles; the data are given as median and interquartile range. The box and whisker plots indicate the median, 1st and 3rd quartiles; the whiskers are extended to the most extreme value inside the 1.5-

**RESULTS**

Gas exchange, biochemical and biophysical surfactant properties on surfactant treatment

Patients with early ARDS were treated up to four times by intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight. A significant improvement in \( \text{PaO}_2/\text{FiO}_2 \) ratios at 48 h (\( p=0.008 \)) and 120 h (\( p=0.0005 \)) after initial treatment was observed (table 2). The value for the excess area under the \( \text{PaO}_2/\text{FiO}_2 \) curve during the 24 h after administration of the first drug dose was also higher in the surfactant treatment group than in patients receiving standard care only (402 vs 220 mm Hg/h; see table S2 in the online data supplement available at http://thorax.bmj.com/supplemental); however, this difference did not reach statistical significance, probably due to small sample sizes. Further study results are summarised in table S2 in the online data supplement (http://thorax.bmj.com/supplemental). In addition to the improvements in gas exchange, surfactant treatment produced a far-reaching restoration of key biochemical surfactant properties (table 2). In detail, the concentration of phospholipids was markedly increased in total BAL fluid, and the relative amount of the LA fraction was nearly normalised. Moreover, the relative amount of phosphatidylglycerol and the degree of palmitoylation in phosphatidylcholine were both fully normalised upon surfactant application, and the concentration of SP-C in total BAL fluid was greatly increased (table 2). No significant differences in BAL fluid concentrations of SP-A, SP-B or SP-D were detected between the standard care group and the surfactant treatment group (data not shown).

In contrast to the complete normalisation of the phospholipid and fatty acid profiles, surface tension values after 5 min of film oscillation (ymin, fig 1) and after 12 s of film adsorption (yads, data not shown) were significantly improved in the patients receiving surfactant treatment (\( p=0.02 \) at 48 h, \( p=0.04 \) at 120 h), although not to values observed for healthy subjects. This observation was made in LA obtained by either high speed centrifugation (as depicted in fig 1) or by sucrose gradient centrifugation (data not shown).

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**Table 2** Biochemical surfactant properties and gas exchange in healthy controls and patients with acute respiratory distress syndrome (ARDS) receiving either standard care or additional surfactant treatment

<table>
<thead>
<tr>
<th>ARDS</th>
<th>Control</th>
<th>Baseline</th>
<th>+48 h</th>
<th>+120 h</th>
<th>Surfactant treatment</th>
</tr>
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<tbody>
<tr>
<td><strong>LA (% of PL)</strong></td>
<td>79.1 (70.5–86.5)</td>
<td>45.4 (28.8–53.2)</td>
<td>27.4 (23.2–52.2)</td>
<td>30.3 (25.3–31.10)</td>
<td>73.3 (68.3–77.2)***</td>
</tr>
<tr>
<td><strong>PL (ug/ml)</strong></td>
<td>23.5 (17.2–29.8)</td>
<td>23.6 (14.5–44.6)</td>
<td>30.4 (15.2–42.1)</td>
<td>17.0 (9.6–25.4)</td>
<td>185.0 (44.8–230.0)**</td>
</tr>
<tr>
<td><strong>PC (% of total PL)</strong></td>
<td>80.0 (79.0–82.3)</td>
<td>77.9 (70.3–81.6)</td>
<td>81.1 (73.2–83.1)</td>
<td>81.9 (80.6–85.3)</td>
<td>77.6 (68.1–83.5)</td>
</tr>
<tr>
<td><strong>PG (% of total PL)</strong></td>
<td>11.8 (11.3–12.7)</td>
<td>1.6 (1.2–2.6)</td>
<td>1.9 (0.7–5.5)</td>
<td>2.1 (0.8–2.3)</td>
<td>1.57 (8.9–21.8)**</td>
</tr>
<tr>
<td><strong>Palmitic acid</strong></td>
<td>74.2 (71.4–77.1)</td>
<td>53.6 (44.8–59.2)</td>
<td>52.6 (50.3–57.4)</td>
<td>57.8 (56.3–59.2)</td>
<td>85.6 (77.8–89.4)***</td>
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<tr>
<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
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<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
</tr>
<tr>
<td><strong>PC (%) fatty acids</strong></td>
<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
<td><strong>(as % of total PL)</strong></td>
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<tr>
<td><strong>SP-C (mg/ml)</strong></td>
<td>494.9 (397.0–601.0)</td>
<td>350.2 (212.4–724.3)</td>
<td>437.0 (250.0–657.0)</td>
<td>436.0 (284.0–760.0)</td>
<td>1970.0 (770.0–2490.0)**</td>
</tr>
<tr>
<td><strong>Paco₂/FiO₂ (mm Hg)</strong></td>
<td>461.0 (446.0–469.0)</td>
<td>126.5 (103–149.7)</td>
<td>156.0 (85.0–215.0)</td>
<td>143.0 (138.0–182.0)</td>
<td>212.0 (156.0–250.0)**</td>
</tr>
</tbody>
</table>

Data are presented as median (25th–75th percentiles).  
LA, large surfactant aggregate fraction; PL, phospholipids; PC, phosphatidylcholine; PG, phosphatidylglycerol; SP-C, surfactant protein C; PaO₂/FiO₂, arterial oxygen tension/ fractional alveolar oxygen inspiration.  
Data are presented as median (25th–75th percentiles).  
LA, large surfactant aggregate fraction; PL, phospholipids; PC, phosphatidylcholine; PG, phosphatidylglycerol; SP-C, surfactant protein C; PaO₂/FiO₂, arterial oxygen tension/ fractional alveolar oxygen inspiration.  

**p<0.05; **p<0.01; ***p<0.001** pre-treatment vs post-treatment.  
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Neutral lipid content and profile of the LA fraction

To investigate the underlying mechanism for the partial restoration of surface activity, we further analysed the LA fraction from the BAL fluids for neutral lipid levels and profiles. Compared with healthy controls, the relative amount of neutral lipids within the LA fraction was significantly increased in the initial BAL fluid samples of the patients with ARDS at baseline (p = 0.0005), and no major changes were noted over the subsequent 120 h observation period, regardless of whether the patients received standard care only (p=0.001 at 48 h) or standard care + Venticute (p = 0.013 at 48 h, p<0.001 at 120 h; fig 2). Taking into consideration the absolute amount of phospholipids in the BAL fluid and the relative LA content, the median concentration of LA-related neutral lipids was 11.1 μg/ml (interquartile range 2.1–19.2) 48 h after surfactant treatment and 2.7 μg/ml (interquartile range 1.4–6.5) 120 h after treatment, and thus several fold higher in the ARDS-surfactant group compared with the pre-treatment data (baseline) and the ARDS-standard care group. Analogous data were obtained when the LA fraction was analysed after isolation by sucrose gradient centrifugation (data not shown). In contrast, in mechanically ventilated patients with cardiogenic pulmonary oedema, the relative amount of neutral lipids within the LA fraction (% of phospholipids) was unaltered compared with healthy controls (median (interquartile range) 6.1% (5.4–7.6%) vs 5.9% (5.2–7.1%)).

In addition to the increased levels of neutral lipids in patients with ARDS, changes were also observed in the neutral lipid profile. At baseline the relative contents of cholesterol, diglycerides and triglycerides were significantly increased (all p<0.0001), whereas free fatty acids and cholesterol esters were decreased (all p<0.0001; fig 3). Forty-eight hours after randomisation, the percentages of cholesterol and free fatty acids approached those in healthy controls whereas other neutral lipids remained at baseline concentrations (fig 3). There was no major difference in the neutral lipid composition between the ARDS-surfactant group and the ARDS-standard care group, with the exception of a slightly higher amount of free fatty acids (putatively based on the application of free palmitic acid contained in the recombinant SP-C surfactant) at the expense of the cholesterol fraction in the ARDS-surfactant group.

Impact of neutral lipids on the surface activity of different surfactant preparations in vitro

The potential impact of neutral lipid mixtures—mimicking the neutral lipid profile in patients with ARDS—on the surface activity of different surfactant preparations was then investigated. Addition of increasing amounts of a completely unsaturated neutral lipid mixture to the recombinant SP-C surfactant or to a natural rabbit LA preparation resulted in a dose-dependent increase of γm in almost 20 mN/m in both surfactant preparations (fig 4). γm values were only slightly increased in the presence of increasing amounts of neutral lipids (see fig S1 in online data supplement available at http://thorax.bmj.com/supplemental). A neutral lipid mixture consisting of 50% unsaturated fatty acids exhibited a similar inhibitory effect on the biophysical activity of the recombinant SP-C surfactant (fig 4 and fig S1 of the online data supplement available at http://thorax.bmj.com/supplemental).

In order to define the putative surfactant inhibitory effect of each component of the neutral lipid mixtures more precisely, we investigated the influence of increasing amounts of single neutral lipids on the surface activity of the recombinant SP-C surfactant. Diglycerides and, to a lesser degree, free fatty acids showed a marked inhibitory effect on the γm values (fig 5). Interestingly, two different neutral lipids—monoglycerides and cholesterol—were the most effective in inhibiting the γm values (fig S2 of the online data supplement available at http://thorax.bmj.com/supplemental).
DISCUSSION

In this study we have investigated the effect of intrabronchial administration of a recombinant SP-C-based surfactant (Venticute) on gas exchange, surfactant composition and function of 31 patients with ARDS. All patients participated in a randomised controlled multicentre phase I/II study in Europe/South Africa addressing the safety and efficacy of this surfactant preparation in ARDS. The study was performed in parallel to an almost identical trial in North America. In accordance with some former studies, a significant improvement was seen in gas exchange in the early period after surfactant administration. At baseline the BAL fluid concentrations of total phospholipids, phosphatidylglycerol and SP-C, the relative amount of the LA fraction and the degree of palmitoylation in phosphatidylcholine were all significantly decreased in patients with ARDS. Following administration of the recombinant SP-C surfactant all parameters were almost fully normalised, which paralleled the improvement in gas exchange. Surfactant treatment favourably decreased the risk of ARDS surfactant (48 hrs) baseline (3 hrs) and from patients with ARDS receiving standard care or additional treatment with 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight given up to four times at 48 h after initial treatment is given. The box-and-whisker plots indicate the median, 1st and 3rd quartiles; the whiskers are extended to the most extreme value inside the given. The significance level is indicated by *p<0.05; **p<0.01; ***p<0.001 (patients with ARDS pre or post standard or surfactant treatment vs healthy controls). MG, monoglycerides; DG, diglycerides; Chol, cholesterol; FFA, free fatty acids; TG, triglycerides; C-E, cholesterol ester; NL, neutral lipids.

Figure 3 Neutral lipid profile of the large surfactant aggregate (LA) fraction of healthy controls and patients with acute respiratory distress syndrome (ARDS). The content of different neutral lipid classes (% of total neutral lipids, wt/wt) in the LA fraction obtained by high speed centrifugation from healthy controls, from patients with ARDS before treatment (–3 h = baseline) and from patients with ARDS receiving standard care or additional treatment with 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight given up to four times at 48 h after initial treatment is given.

Figure 4 Influence of neutral lipids on the surface activity of a recombinant surfactant protein (SP)-C-based surfactant preparation and of the large surfactant aggregate (LA) fraction of natural rabbit lung surfactant. A neutral lipid mixture containing 100% unsaturated neutral lipids and mimicking the neutral lipid profile in patients with acute respiratory distress syndrome (ARDS) was added in increasing amounts (0–25% of phospholipids, wt/wt) to 2 mg/ml recombinant SP-C surfactant (squares) and to 2 mg/ml of the LA fraction of natural rabbit lung surfactant (circles). A neutral lipid mixture containing 50% unsaturated neutral lipids was also added in increasing amounts to recombinant SP-C surfactant (triangles). The surface tension values after 5 min of film oscillation at minimum bubble radius (min) are given. Data are given as median values (n = 8 for each concentration). NL, neutral lipids; PL, phospholipids.

Fluid, thereby separating the LA fraction from proteinaceous material and other inhibitors. Protein recovery in the LA fraction was 2.7% with centrifugation at 48 000 g and <0.5% with sucrose gradient centrifugation (data not shown). As the surface activity of the LA fraction obtained by the two methods deteriorated to the same extent and as there was no appreciable contamination of the lipid fraction with lysoPC, platelet-activating factor or proteins, we think that inhibitory phenomena are unlikely to explain the incomplete restoration of surface activity of the LA fraction. Nevertheless, we would anticipate that the “true” alveolar surface tension of patients with ARDS, both before and after surfactant treatment, is higher as a result of the presence of these inhibitors, as suggested in previous reports. Second, it might be argued that the lack of SP-B was responsible for the incomplete improvement in surface activity. However, numerous studies have shown that SP-C-based surfactants such as the recombinant SP-C surfactant achieve similar low surface tension values in vitro and similar efficacy in vivo to that of natural surfactant extracts containing both hydrophobic surfactant proteins. Nevertheless, this issue may be of some importance in the presence of inhibitors where SP-B-based surfactants have been shown to display better surface activity than SP-C-based surfactants. However, as discussed above, the isolated LA fractions were almost completely separated from potential inhibitors. We therefore think that this issue is not of major importance.

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values after 5 min of film oscillation at minimum bubble radius (wt/wt) to 2 mg/ml of the recombinant SP-C surfactant. The surface tension neutral lipids were added in increasing amounts (0–15% of phospholipids, recombinant surfactant protein (SP)-C-based surfactant preparation. Single Figure 5

Influence of single neutral lipids on the surface activity of a recombinant surfactant protein (SP)-C-based surfactant preparation. Single neutral lipids were added in increasing amounts (0–15% of phospholipids, wt/wt) to 2 mg/ml of the recombinant SP-C surfactant. The surface tension values after 5 min of film oscillation at minimum bubble radius (\( \mu \)m) are given. Data are given as median values (n = 8 for each concentration). Monoglycerides (diamonds); diglycerides (upward triangles); cholesterol (circles); free fatty acids (downward triangles); triglycerides (stars); cholesterolester (squares); NL, neutral lipids; PL, phospholipids.

Neutral lipids have been known for a long time to be an integral component of the pulmonary surfactant system. They are actively secreted within the lamellar bodies from type II cells, but their precise role within the surfactant system is still unclear. In particular, data are scarce in view of the regulation and role of neutral lipids in acute respiratory failure. Alveolar cholesterol was found to be increased in some animal models of acute lung injury but neutral lipids have never been investigated in patients with ARDS. It has recently been shown that cholesterol plays a critical role in promoting the lateral organisation of bilayer membranes made of native pulmonary surfactant. Furthermore, neutral lipids such as cholesterol enhance adsorption at the air-water interface and also improve film respreading of dipalmitoylphosphatidylcholine or more complex phospholipid films, thus improving surface activity. However, neutral lipids were much less effective in enhancing adsorption than the hydrophobic surfactant proteins SP-B and SP-C. In oscillating bubble studies, in contrast to mixtures of phospholipids and hydrophobic surfactant proteins, a protein- free mixture consisting of phospholipids and neutral lipids never reached a minimum surface tension of <20 mN/m. Furthermore, investigations with the pulsating bubble surfactometer showed that the addition of cholesterol to different surfactant preparations can impair the surface tension-lowering ability on bubble pulsation and may destabilise surface films on maximum compression. Finally, all of the preceding studies have been undertaken with “physiological” neutral lipid concentrations (~5% neutral lipids wt/wt of phospholipids). Higher neutral lipid concentrations, as reported for the first time in this study of patients with ARDS, have not yet been investigated in detail. The present findings suggest that an increased and “non-physiologically” composed neutral lipid fraction within the LA fraction may cause instability of the interfacial surfactant film on lateral film compression, with increased minimum surface tension values. This interpretation is reinforced by our finding that, in particular, the minimum surface tension values obtained under cyclic surface area changes were increased, whereas adsorption remained largely unchanged with neutral lipid supplementation.

The source of increased neutral lipids and altered composition in ARDS is presently unknown. One possibility is altered synthesis or secretion by type II cells. Alternatively, changes may be due to spillover of neutral lipids from the systemic circulation via a leaky endothelial and epithelial barrier. However, the plasma neutral lipid profile is characterised by a large amount of cholesterol ester (46%) and triglycerides (30%), whereas only traces of free fatty acids, monoglycerides and diglycerides can be detected. The plasma neutral lipid profile is thus strikingly different from the neutral lipid profile in the LA fraction, regardless of whether the LA fraction was obtained by high speed centrifugation or further purified from blood contaminants by sucrose gradient centrifugation. Thus, “containment” by plasma-derived neutral lipids seems unlikely. Moreover, if the type II cell is indeed the main source of increased neutral lipids in ARDS, our observation of a persistently raised neutral lipid content despite application of large amounts of exogenous surfactant material would suggest the existence of a mechanism that maintains a particular neutral lipid/phospholipid ratio in the acutely injured lung.

We conclude that increased levels and an altered neutral lipid profile may contribute to the impairment of surface activity in ARDS. Remarkably, neutral lipid abnormalities of the LA fraction were found to persist even after intrabronchial surfactant administration and may prevent full normalisation of the alveolar surface tension by surfactant treatment. Further studies are needed to address the mechanisms underlying the disturbances in surfactant neutral lipid homeostasis in ARDS.
One implication of this study would be to test if neutral lipid-free exogenous surfactant preparations are more suitable for the treatment of ARDS.

ACKNOWLEDGEMENTS

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Additional information is provided in the online data supplement available at http://thorax.bmj.com/ supplemental.

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This study was approved by local institutional review boards/ethics committees at each participating institution and informed consent was obtained from all patients or their legal representatives.

REFERENCES

Online data supplement

Patients with ARDS show incomplete restoration of alveolar surface activity upon recombinant SP-C-based surfactant treatment: putative role of neutral lipids

Philipp Markart, Clemens Ruppert, Malgorzata Wygrecka, Thorsten Colaris, Bhola Dahal, Dieter Walmrath, Heinz Harbach, Jochen Wilhelm, Werner Seeger, Reinhold Schmidt, and Andreas Guenther
METHODS

Study synopsis

All ARDS patients participated in a randomized, multicenter, controlled, phase I/II pilot study, investigating the safety and efficacy of an intrabronchial administration of a recombinant SP-C-based surfactant (Venticute). Table 1 shows the study synopsis of the Venticute trial.

Table 1

<table>
<thead>
<tr>
<th>Investigational drug:</th>
<th>recombinant surfactant protein C (rSP-C) based surfactant (Venticute; in the pharmaceutical formulation 1 mg rSP-C is applied with 50 mg phospholipids)</th>
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<tr>
<td>Phase:</td>
<td>I/II</td>
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</table>
| Objectives:           | - to assess the safety and efficacy of Venticute (maximum total dose 4 mg rSP-C plus 200 mg phospholipids per kilogram lean body weight) in the treatment of adults with the Acute Respiratory Distress Syndrome in comparison to standard therapy  
- to assess the composition and function of surfactant recovered from bronchoalveolar lavage (BAL) fluid |
| Design:               | randomized, multicenter, parallel group, controlled pilot study                                  |
| Study population:     | Subjects diagnosed with ARDS with one or more identifiable ARDS risk factors (e.g. sepsis, aspiration, trauma or surgery, multiple blood transfusions, pancreatitis, pneumonia)  
Patients must have been diagnosed for ARDS within 120 hours prior to study entry |
| Study groups:         | Group 1 (ARDS-standard care): receives standard ARDS treatment but no Venticute  
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| Dosing scheme:        | Patients receive Venticute by intratracheal instillation. Patients are alternately positioned in the left or right lateral decubitus position, and the calculated drug-volume is given in aliquots of up to 25 ml via intratracheal catheter during a brief pause of the mechanical ventilation. |
Treatment overview: Patients receive an initial dose of Venticute. Up to 3 additional doses may be given. Allowed time points of administration are 4, 8, 12, 16, and 20 hours after the initial dose. Subsequent doses will be given every four hours up to a maximum of 4 doses in total. If a patient is not retreated, he/she should be retreated at the next possible time point provided the retreatment criteria are still met.

Treatment period: 24 hours

Observation period; up to 28 days

Primary variables:
- Primary efficacy variable: excess-area under the $PaO_2/FlO_2$ curve during 24 hours after study time $t = 0$ (start of first administration)
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Study design

The study commenced in April 1998 at the following European/South African Clinical Centers: University Hospital of Geneva, Switzerland; University Hospital Zurich, Switzerland; Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; University Hospital, Tubingen, Germany; University Hospital of Regensburg, Regensburg, Germany; Medical Clinic II, Justus-Liebig-University, Giessen, Germany; Klinikum Grosshadern, Ludwig-Maximilians-University, Munich, Germany; University Hospital RWTH Aachen, Germany; Charite, Campus Virchow-Klinikum, Humboldt-University, Berlin, Germany; Hannover Medical School, Hannover, Germany; University Hospital of Mannheim, Germany; University Hospital of Bonn, Germany; University Hospital Carl Gustav Carus, Technical University, Dresden, Germany; Henri Mondor Hospital, Creteil, France; Hopital Sainte Marguerite, Marseille, France; Guy's and St. Thomas' Hospital, London, United Kingdom; Milano-Bicocca University, San Gerardo Hospital, Monza, Milano, Italy; Erasmus MC, University Medical Center Rotterdam, The Netherlands; St. Clara Hospital, Rotterdam, The
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During a 6-hour baseline period, clinical and respiratory parameters were recorded. At three hour intervals, three sets of physiological parameters (ventilator settings, arterial blood gases, vital signs) were obtained. Volume-controlled ventilation was adjusted to tidal volumes of 6-10 ml/kg body weight and employed throughout the study. Permissive hypercapnia was allowed. A modified APACHE II score was calculated. Due to the use of sedatives, neurologic evaluation could not be performed consistently and was therefore omitted from this modified score. The first BAL (- 3 hours) for surfactant analyses was obtained. Immediately after the conclusion of the baseline period (within 30 minutes), patients were prospectively randomized (by means of a computer generated randomization list) to receive either standard care (ARDS-standard care; $n = 17$) or standard care plus surfactant treatment (ARDS-surfactant; $n=14$). Treatment with the first dose of surfactant (for treated patients) had to start within 2 hours after randomization. Up to three additional doses were administered during the 24 hours after initial treatment (treatment period; see below). During the treatment period, physiological parameters (ventilator settings, arterial blood gases, vital signs) were
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RESULTS

Clinical results of the Venticute trial

Table 2

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Table 2

Clinical Results

Data are presented as median (25-75 percentile)

\(\text{AUC}_{0-24}\) hours = area under the \(\text{PaO}_2/\text{FI}_2\)-versus-time curve from 0 to 24 hours

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Significance level is indicated by * \(p = 0.016\) (ARDS surfactant treatment versus standard care)
Impact of neutral lipids on the surface activity of different surfactant preparations in vitro

We investigated the potential impact of neutral lipid mixtures, mimicking the neutral lipid profile in ARDS patients, on the surface activity of different surfactant preparations. $\gamma_{ads}$ values of the recombinant SP-C surfactant or a natural rabbit LA preparation were only slightly increased in presence of increasing amounts of neutral lipids (Figure 1). Next, we investigated the influence of increasing amounts of single neutral lipids on the surface activity of the recombinant SP-C surfactant. Monoglycerides and cholesterol, were the most effective in inhibiting the $\gamma_{ads}$ values (Figure 2).
FIGURE LEGENDS

Figure 1
Influence of neutral lipids on the surface activity of a recombinant SP-C-based surfactant preparation and of the large surfactant aggregate (LA) fraction of natural rabbit lung surfactant

A neutral lipid mixture containing 100 % unsaturated neutral lipids and mimicking the neutral lipid profile in ARDS patients was added in increasing amounts (0-25 % of phospholipids, wt/wt) to 2 mg/ml of a recombinant SP-C surfactant (squares) and to 2 mg/ml of the LA fraction of natural rabbit lung surfactant (circles). Furthermore, a neutral lipid mixture containing 50 % unsaturated neutral lipids was added in increasing amounts to recombinant SP-C surfactant (triangles). The surface tension values after 12 seconds film adsorption ($\gamma_{ads}$) are given. Data are given as median. n = 8 for each concentration. NL = neutral lipids; PL = phospholipids.

Figure 2
Influence of single neutral lipids on the surface activity of a recombinant SP-C-based surfactant preparation

Single neutral lipids were added in increasing amounts (0-15 % of phospholipids, wt/wt) to 2 mg/ml of the recombinant SP-C surfactant. The surface tension values after 12 seconds film adsorption ($\gamma_{ads}$) are given. Data are given as median. n = 8 for each concentration

Monoglycerides (diamonds); diglycerides (up triangles); cholesterol (circles); free fatty acids (down triangles); triglycerides (stars); cholesterolester (squares); NL = neutral lipids; PL = phospholipids.
Online data supplement

Patients with ARDS show incomplete restoration of alveolar surface activity upon recombinant SP-C-based surfactant treatment: putative role of neutral lipids

Philipp Markart, Clemens Ruppert, Malgorzata Wygrecka, Thorsten Colaris, Bhola Dahal, Dieter Walmrath, Heinz Harbach, Jochen Wilhelm, Werner Seeger, Reinhold Schmidt, and Andreas Guenther
METHODS

Study synopsis

All ARDS patients participated in a randomized, multicenter, controlled, phase I/II pilot study, investigating the safety and efficacy of an intrabronchial administration of a recombinant SP-C-based surfactant (Venticute). Table 1 shows the study synopsis of the Venticute trial.

Table 1

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<th>Investigational drug:</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Phase:</td>
<td>I/II</td>
</tr>
<tr>
<td>Objectives:</td>
<td>- to assess the safety and efficacy of Venticute (maximum total dose 4 mg rSP-C plus 200 mg phospholipids per kilogram lean body weight) in the treatment of adults with the Acute Respiratory Distress Syndrome in comparison to standard therapy</td>
</tr>
<tr>
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</tr>
<tr>
<td>Design:</td>
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Treatment overview: Patients receive an initial dose of Venticute. Up to 3 additional doses may be given. Allowed time points of administration are 4, 8, 12, 16, and 20 hours after the initial dose. Subsequent doses will be given every four hours up to a maximum of 4 doses in total. If a patient is not retreated, he/she should be retreated at the next possible time point provided the retreatment criteria are still met.

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Observation period: up to 28 days

Primary variables:
- Primary efficacy variable: excess-area under the PaO2/FIO2 curve during 24 hours after study time t = 0 (start of first administration)
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**Figure 2**

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Single neutral lipids were added in increasing amounts (0-15 % of phospholipids, wt/wt) to 2 mg/ml of the recombinant SP-C surfactant. The surface tension values after 12 seconds film adsorption ($\gamma_{ads}$) are given. Data are given as median. n = 8 for each concentration. Monoglycerides (diamonds); diglycerides (up triangles); cholesterol (circles); free fatty acids (down triangles); triglycerides (stars); cholesterolester (squares); NL = neutral lipids; PL = phospholipids.
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Philipp Markart¹, Clemens Ruppert¹, Malgorzata Wygrecka², Thorsten Colaris³, Bholadahal¹, Dieter Walmrath¹, Heinz Harbach³, Jochen Wilhelm¹, Werner Seeger¹, Reinhold Schmidt¹, and Andreas Guenther¹

Departments of ¹Internal Medicine, ²Biochemistry and ³Anaesthesiology, Intensive Care Medicine and Pain Therapy, Faculty of Medicine, University of Giessen Lung Center, Giessen, Germany

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Keywords: acute respiratory distress syndrome, acute lung injury, cholesterol, lipids, pulmonary surfactant

Word count 4369
ABSTRACT

Background: Extensive biochemical and biophysical changes of the pulmonary surfactant system occur in the acute respiratory distress syndrome (ARDS).

Methods: In the present study, we investigated the effect of intrabronchial administration of a recombinant surfactant protein C-based surfactant preparation (Venticute) on gas exchange, surfactant composition and function of 31 ARDS patients, participating in a randomized, controlled, phase I/II clinical pilot trial. Additionally, we identified the potentially deleterious effects of surfactant neutral lipids in ARDS subjects.

Results: Prior to treatment, all ARDS patients displayed marked abnormalities in the surfactant phospholipid and protein composition and possessed dramatically impaired surface activity. In response to surfactant treatment, gas exchange improved and surfactant phospholipid and protein content were virtually normalized, however, surface activity was only partially improved. Further analysis of the bronchoalveolar lavage fluids revealed a two fold increase in neutral lipid content and altered neutral lipid profile in ARDS patients compared to healthy controls. These differences persisted even after administration of large amounts of Venticute. Supplementation of Venticute or natural surfactant with a synthetic neutral lipid preparation, mimicking the profile in ARDS, caused dose-dependent deterioration of surface activity in vitro.

Conclusion: Intrabronchial surfactant treatment improves gas exchange in ARDS, but the efficacy may be limited by increased concentration and altered neutral lipid profile in surfactant under these conditions.
INTRODUCTION

Pulmonary surfactant is a lipoprotein complex consisting of lipids (90 %) and proteins (10 %). Surfactant reduces the alveolar surface tension to near zero mN/m, preventing alveolar collapse and enables gas exchange and alveolar ventilation at physiological transpulmonary pressure gradients.[1] 80-90 % of the lipids are phospholipids. Four surfactant proteins (SP), SP-A, SP-B, SP-C and SP-D, have been identified.[2] Surfactant obtained by bronchoalveolar lavage (BAL) can be separated into different subfractions by buoyant density gradient or differential centrifugation. The large surfactant aggregate (LA) fraction, consisting of lamellar bodies, tubular myelin and large multilamellar vesicles, displays excellent biophysical activity in vitro and in vivo, and represents the precursor fraction of the interfacial surface film. [3-5]

In patients with the acute respiratory distress syndrome (ARDS), extensive alterations of biochemical and biophysical surfactant properties have been described.[6-8] Additionally, intrabronchial surfactant administration was shown to be beneficial in different ARDS models.[9] Therefore, impaired surfactant function may play a role in the development of gas exchange abnormalities in clinical ARDS. Accordingly, in some recent phase II and phase III clinical trials, surfactant administration in ARDS subjects significantly improved arterial oxygenation [10-12] and surfactant composition.[13-14]

In the present study, we investigated surfactant abnormalities in the BAL fluids of ARDS patients, participating in a randomized, controlled, phase I/II study in Europe/South Africa, addressing the safety and efficacy of a recombinant SP-C-based surfactant (Venticute). Before randomization, profound surfactant abnormalities were encountered, with a marked loss of surface tension lowering properties and severe alterations in phospholipid, fatty acid and surfactant protein composition. In response to surfactant treatment, gas exchange was improved, phospholipid, fatty acid profiles and SP-C content were nearly normalized, however, surface activity was only partially restored. To investigate the underlying
mechanism for this partial restoration, we further analyzed the LA fraction from the BAL fluids. Interestingly, we observed a two-fold increase in the relative amount of neutral lipids in the LA fraction of ARDS patients. Physiologically, neutral lipids represent 10-20 % of the BAL fluid lipids, and about 5 % of the LA lipids. Within the LA fraction, the neutral lipids consist of cholesterol (30-40% of total neutral lipids), free fatty acids (25-30%), cholesterolester (~12 %), monoglycerides (10-15 %), diglycerides (~14 %), and triglycerides (~8 %).[6, 15] The increased concentration of neutral lipids in the LA fraction of ARDS subjects was still present even after administration of large amounts of Venticute. Moreover, the composition of the neutral lipid fraction was markedly different between ARDS and healthy controls. Reconstitution of Venticute or a natural surfactant preparation with a neutral lipid preparation, mimicking the profile in ARDS subjects, provoked a dose-dependent inhibition of surfactant function in vitro. This effect was already evident at a relative neutral lipid dose found in ARDS patients.

Changes in quantity and composition of the surfactant neutral lipids may thus contribute to the impairment of surface activity and gas exchange abnormalities in ARDS.
METHODS

Study design

Thirty-one patients with ARDS as defined by the American-European Consensus Criteria [16] for a maximum of 120 hours since diagnosis (early ARDS) were investigated. All ARDS patients participated in a randomized, multicenter, controlled, phase I/II pilot study, investigating the safety and efficacy of an intrabronchial administration of a recombinant SP-C-based surfactant (Venticute). Efficacy was assessed by calculating (1) the excess area under the PaO2/FIO2 curve during the 24 hours after administration of the first drug dose and (2) the number of days with unassisted breathing within the 28-day observation period and comparing these variables among groups. Another objective of the study was to assess BAL recovered from the patients for surfactant components and function.

During a 6-hour baseline period, clinical and respiratory parameters were recorded, and the first BAL (-3 hours) for surfactant analyses was obtained. Baseline demographic and physiological data for each of the patient groups are shown in Table 1. No significant differences among groups were detected. Values for the modified Acute Physiology and Chronic Health Evaluation (APACHE) II score and for baseline positive end-expiratory pressure (PEEP) and PaO2/FIO2 values indicate a similar severity of illness among groups.
Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>ARDS-standard care</th>
<th>ARDS-surfactant treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Age, years</td>
<td>50.5 (43.8-64.0)</td>
<td>50.0 (36.0-58.2)</td>
</tr>
<tr>
<td>Sex, m/f, n</td>
<td>12/5</td>
<td>11/3</td>
</tr>
<tr>
<td>Ethnic origin, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>16 (94.1)</td>
<td>12 (85.7)</td>
</tr>
<tr>
<td>African</td>
<td>1 (5.9)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Predisposing events, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma/Surgery</td>
<td>7 (41.2)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Sepsis syndrome</td>
<td>7 (41.2)</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5 (29.4)</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>2 (11.8)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Gastric Aspiration</td>
<td>2 (11.8)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Polytransfusion</td>
<td>1 (5.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Burn</td>
<td>1 (5.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Others</td>
<td>1 (5.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hours from diagnosis to initial treatment</td>
<td>38.0 (21.0-74.0)</td>
<td>23.2 (9.0-53.5)</td>
</tr>
<tr>
<td>Modified APACHE II</td>
<td>15.0 (10.8-21.2)</td>
<td>13.0 (10.2-15.8)</td>
</tr>
<tr>
<td>PEEP, cm H\textsubscript{2}O</td>
<td>12 (12-14)</td>
<td>12 (12-12)</td>
</tr>
<tr>
<td>(P_{A\text{O}_2}/F\text{I}_O_2)</td>
<td>122.5 (96.5-147.8)</td>
<td>126.8 (111.8-152.2)</td>
</tr>
</tbody>
</table>

Table 1

Baseline demographic and physiological data

Data are presented as median (25-75 percentile). APACHE = Acute Physiology and Chronic Health Evaluation.

PEEP = positive end-expiratory pressure. \(P_{A\text{O}_2}/F\text{I}_O_2\) and PEEP values represent the average of 3 readings over the 6 hour baseline period.
Immediately after the conclusion of the baseline period, patients were prospectively randomized to receive either standard care (ARDS-standard care; n = 17) or standard care plus 1 ml of recombinant SP-C surfactant (containing 1 mg of recombinant SP-C + 50 mg of phospholipid) per kilogram lean body weight given up to four times in 24 hours (ARDS-surfactant; n=14).

BAL was obtained from previously unlavaged lung segments at 48 and 120 hours after the first treatment. Patients were subsequently observed for up to 28 days or until hospital discharge. Additional information of study methods is provided in the online data supplement. As control, 11 healthy volunteers without any history of cardiac or lung disease and with normal pulmonary function were investigated. To investigate the impact of ventilation on the neutral lipid content in the BAL fluid, the relative neutral lipid content in the BAL fluid was also determined in four mechanically ventilated patients suffering from cardiogenic pulmonary edema in the absence of ARDS and lung infection. A comparable disturbance in gas exchange was observed in both, ARDS patients and patients with cardiogenic pulmonary edema (PaO2/FIO2 ratio: 126 mm Hg in ARDS versus 152 mm Hg in patients with cardiogenic pulmonary edema).

**In vitro experiments with neutral lipids and surfactant preparations**

*Preparation of a rabbit BAL fluid pool*

Healthy rabbits of either sex were killed by intravenous application of a lethal dose of pentobarbital/ketanest. A catheter was placed into the trachea and the lungs were lavaged three times with 50 ml 0.9 % NaCl. After filtration of the lavage fluid through sterile gauze and sedimentation of cells (200 x g, 4°C, 10 minutes), supernatants originating from 20 rabbits were pooled and then centrifuged at 48,000 x g (4 °C, 1 hour). The pellet represents the LA fraction and was resuspended in a small volume of 0.9 % NaCl/3 mM CaCl2 and adjusted to a stock concentration of 2 mg/ml phospholipids.
Recombinant SP-C based surfactant preparation

The recombinant SP-C-based surfactant preparation used for the in vitro experiments was identical to the study drug material and was generously placed at our disposal by ALTANA Pharma AG, Konstanz, Germany. The recombinant SP-C-based surfactant preparation contains dipalmitoylphosphatidylcholine and phosphatidylglycerol (7:3 wt/wt), enriched with 5 % (wt/wt) palmitic acid and 2 % (wt/wt) recombinant human SP-C. A stock preparation containing 2 mg/ml phospholipids was used.

Neutral lipid preparation

The neutral lipid preparations used for the in vitro experiments were designed to mimic the neutral lipid-profile in ARDS patients and consisted either of 100 % or 50 % unsaturated fatty acids. Increasing amounts of a 1 mg/ml stock solution were dried under nitrogen and resuspended with the rabbit LA pool or the recombinant SP-C surfactant. Details are outlined in the online data supplement.

Surfactant analysis

Isolation of large surfactant aggregates (LA)

BAL fluid from ARDS patients, patients with cardiogenic pulmonary edema or healthy volunteers was centrifuged at 48,000 x g (4 °C, 1 hour). The resulting pellet, containing the LA fraction, was resuspended in a small volume of 0.9 % NaCl/3 mM CaCl\(_2\) and adjusted to a phospholipid concentration of 2 mg/ml. In parallel experiments, the LA fraction from 7 ARDS patients was further purified by sucrose gradient centrifugation as described in detail previously.[17]

Phospholipid and fatty acid analysis
Lipids were extracted with chloroform/methanol according to Bligh and Dyer,[18] and the phospholipid content was determined by spectrophotometric measurement of phosphorus.[19] For separation and analysis of phospholipid classes, high-performance thin-layer chromatography (HPTLC) was used as previously described.[7] Similarly, fatty acid profile of phosphatidylcholine was assessed by gas-liquid chromatography as outlined previously.[20]

**Determination of neutral lipid content and profile**

The amount and relative distribution of neutral lipids was measured by HPTLC and densitometric scanning as previously described,[6] with the following modifications: plates were prerun with chloroform before application of the lipid standards and samples and the staining procedure was performed by 30 minutes incubation of the plates with a 0.1 % (wt/v) aqueous solution of 8-anilino-1-naphthalenesulfonic acid (8-ANSA, Merck, Darmstadt, Germany).

**Quantification of surfactant proteins**

The content of the surfactant proteins in the BAL fluid was determined by recently described ELISA techniques.[7, 21-23]

**Determination of surface activity**

Surface tension measurements were performed by means of a pulsating bubble surfactometer (Electronetics, New York, USA) at a phospholipid concentration of 2 mg/ml. Surface tension after 12 seconds of film adsorption ($\gamma_{ads}$) and after 5 minutes of film oscillation at minimum bubble radius ($\gamma_{min}$) were recorded.
Statistics

Data are given as median and interquartile range. The box-and-whisker-plots indicate the Median, 1st and 3rd Quartile; the whiskers are extended to the most extreme value inside the 1.5-fold interquartile range. Values outside (potential outliers) are indicated by circles. The statistical analyses were performed in R, Version 2.3.1.[24] Deviations from the normal distribution were tested using the Shapiro-Wilk-test. The comparability of data sets to justify a combination was tested with the F-test or multiway-Anova and Wilcoxon rank sum tests. Differences between two groups were tested with the Student's t- and Wilcoxon rank sum test, according to the distribution of the data. Before/after treatment comparisons were performed with paired t-tests and Wilcoxon sign rank tests, respectively. The p-values of multiple comparisons were corrected with the algorithm of Benjamini-Hochberg [25] to control the false-discovery rate. Categorial data was tested for independence in contingency tables employing Fisher's exact test. The distribution of the outcomes between the groups (alive, weaned and alive) was tested using the \( \chi^2 \)–goodness-of-fit test against the null hypothesis of a uniform distribution. All tests were performed with an undirected hypothesis (two-sided). The level of statistical significance was set at 5%.

Deleted: Before-after-treatment tests were performed unpaired using groupwise-combined values for the baseline to increase the power.
RESULTS

Gas exchange, biochemical and biophysical surfactant properties upon surfactant treatment

Patients with early ARDS were treated up to 4 times by intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg of recombinant SP-C + 50 mg of phospholipid) per kilogram lean body weight. A significant improvement of $\text{PaO}_2/\text{FiO}_2$ ratios at 48 ($p = 0.008$) and 120 hours ($p = 0.0005$) after initial treatment was observed (see Table 2). Also the value for the excess area under the $\text{PaO}_2/\text{FiO}_2$ curve during the 24 hours after administration of the first drug dose was higher in the surfactant treatment group as compared to the patients receiving standard care only (402 versus 220 mm Hg · hr; see Table 2 of the online data supplement), however, this difference did not reach statistical significance, probably due to small sample sizes. Further study results are summarized in Table 2 of the online data supplement. In addition to the improvements in gas exchange, surfactant treatment produced a far-reaching restoration of key biochemical surfactant properties (Table 2). In detail, the concentration of phospholipids was markedly increased in total BAL fluid, and the relative amount of the LA fraction was nearly normalized. Moreover, the relative amount of phosphatidylglycerol and the degree of palmitoylation in phosphatidylcholine were both fully normalized upon surfactant application, and the concentration of SP-C in total BAL fluid was highly increased (Table 2). No significant differences in BAL fluid concentrations of SP-A, SP-B or SP-D were detected between the standard care group and the surfactant treatment group (data not shown).
<table>
<thead>
<tr>
<th></th>
<th>Control baseline</th>
<th>Control +48 hours</th>
<th>Control +120 hours</th>
<th>ARDS baseline</th>
<th>ARDS +48 hours</th>
<th>ARDS +120 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA [% of PL]</td>
<td>79.1 (70.5-86.5)</td>
<td>45.4 (28.8-53.2)</td>
<td>27.4 (23.2-52.2)</td>
<td>73.5 (25.8-31.10)</td>
<td>64.2 (68.3-77.2)**</td>
<td>(55.3-75.2)</td>
</tr>
<tr>
<td>PL [µg/ml]</td>
<td>23.5 (17.2-29.8)</td>
<td>25.6 (14.5-44.6)</td>
<td>30.4 (15.2-42.1)</td>
<td>17.0 (9.6-25.4)</td>
<td>185.0 (44.8-230.0)**</td>
<td>(31.6-78.1)</td>
</tr>
<tr>
<td>PC [% of total PL]</td>
<td>80.0 (79.0-82.3)</td>
<td>77.9 (70.3-81.6)</td>
<td>81.1 (73.2-83.1)</td>
<td>81.9 (80.6-85.3)</td>
<td>77.6 (68.1-83.5)</td>
<td>72.9 (66.6-78.1)</td>
</tr>
<tr>
<td>PG [% of total PL]</td>
<td>11.8 (11.3-12.7)</td>
<td>1.6 (1.2-2.6)</td>
<td>1.9 (0.7-5.5)</td>
<td>2.1 (0.8-2.3)</td>
<td>15.7 (8.9-21.8)**</td>
<td>(5.3-13.7)*</td>
</tr>
<tr>
<td>Palmitic acid [% of PC fatty acids]</td>
<td>74.2 (71.4-77.1)</td>
<td>53.6 (44.8-59.2)</td>
<td>52.6 (50.3-57.4)</td>
<td>57.8 (56.3-59.2)</td>
<td>85.6 (77.8-89.4)**</td>
<td>(78.5-84.2)**</td>
</tr>
<tr>
<td>SP-C [ng/ml]</td>
<td>494.9 (397.0-601.0)</td>
<td>350.2 (216.4-724.3)</td>
<td>437.0 (250.0-657.0)</td>
<td>436.0 (284.0-760.0)</td>
<td>1970.0 (770.0-2490.0)**</td>
<td>(290.0-1160.0)</td>
</tr>
<tr>
<td>PaO₂/FIO₂ [mmHG]</td>
<td>461.0 (446.0-469.0)</td>
<td>126.5 (103-149.7)</td>
<td>134.0 (94.1-164.0)</td>
<td>156.0 (85.0-215.0)</td>
<td>143.0 (138.0-182.0)**</td>
<td>(156.0-250.0)***</td>
</tr>
</tbody>
</table>

Table 2

Biochemical surfactant properties and gas exchange in healthy controls and ARDS patients receiving either standard care or additional surfactant treatment
Data are given for healthy controls and for ARDS patients before (baseline; -3 hours), 48 and 120 hours after intrabronchial administration of recombinant SP-C-based surfactant (n=14), and for ARDS patients receiving standard care only (n= 17). Biochemical data are given for the total lavage fluid. The baseline PaO₂/FI_O₂ value represents the average of three readings over the 6 hour baseline period. LA=large surfactant aggregate fraction; PL = phospholipids; PC = phosphatidylcholine; PG = phosphatidylglycerol; SP-C = surfactant protein C. Data are presented as median (25-75 percentile). Significance level is indicated by * p < 0.05; ** p < 0.01; *** p < 0.001 (pre versus post treatment)

In contrast to the complete normalization of the phospholipid and fatty acid profiles, surface tension values after 5 minutes film oscillation (γ_min, Figure 1) and after 12 seconds film adsorption (γ_ads, data not shown) were significantly improved in the patients receiving surfactant treatment (p= 0.02, at 48 hours, p= 0.04 at 120 hours), however, not to values observed for healthy subjects. This observation was made in LA obtained by either high speed centrifugation (as depicted in Fig. 1) or by sucrose gradient centrifugation (data not shown).

Neutral lipid content and profile of the LA fraction

To investigate the underlying mechanism for the partial restoration of surface activity, we further analyzed the LA fraction from the BAL fluids for neutral lipid levels and profiles. Compared to healthy controls, the relative amount of neutral lipids within the LA fraction was significantly increased in the initial BAL fluid samples of the ARDS patients at baseline (p = 0.0005), and no major changes were noted over the subsequent 120 hour observation period, regardless of whether the patients received standard care only (p = 0.0007 at 48 hours) or standard care plus Venticute (p = 0.013 at 48 hours, p = 0.0007 at 120 hours; Figure 2). Taking into consideration the absolute amount of phospholipids in the BAL fluid and the relative LA content, the concentration of LA-related neutral lipids was 11.1 (2.1-19.2) μg/ml (median and interquartile range) 48 hours after surfactant treatment and 2.7 (1.4-6.5) μg/ml
120 hours after treatment and thus several-fold elevated in the ARDS-surfactant group as compared to the pre-treatment data (baseline) and the ARDS-standard care group. Analogous data were obtained when the LA fraction was analyzed after isolation by sucrose gradient centrifugation (data not depicted). In contrast, in mechanically ventilated patients with cardiogenic pulmonary edema, the relative amount of neutral lipids within the LA fraction (% of phospholipids) was unaltered compared to healthy controls (6.1 (5.4-7.6) % versus 5.9 (5.2-7.1) %; median and interquartile range). In addition to the increased neutral lipid levels in ARDS patients, changes in the neutral lipid profile were also observed. At baseline, the relative contents of cholesterol, diglycerides and triglycerides were significantly increased (all \( p < 0.001 \)), whereas free fatty acids and cholesterolesters were decreased (all \( p < 0.001 \); Figure 3). Forty-eight hours after randomization, the percentages of cholesterol and free fatty acids approached those in healthy controls, whereas other neutral lipids remained at baseline concentrations (Figure 3). There was no major difference in the neutral lipid composition between the ARDS-surfactant group and the ARDS-standard care group, with the exception of a slightly higher amount of free fatty acids (putatively based on the application of free palmitic acid contained in the recombinant SP-C surfactant) at the expense of the cholesterol fraction in the ARDS-surfactant group.

**Impact of neutral lipids on the surface activity of different surfactant preparations in vitro**

Next, we investigated the potential impact of neutral lipid mixtures, mimicking the neutral lipid profile in ARDS patients, on the surface activity of different surfactant preparations. Addition of increasing amounts of a completely unsaturated neutral lipid mixture to the recombinant SP-C surfactant or to a natural rabbit LA preparation resulted in a dose-dependent increase of \( \gamma_{\text{min}} \) to almost 20 mN/m in both surfactant preparations (Figure 4).
γ\textsubscript{ads} values were only slightly increased in presence of increasing amounts of neutral lipids (Figure 1 of the online data supplement). A neutral lipid mixture consisting of 50% unsaturated fatty acids exhibited a similar inhibitory effect on the biophysical activity of the recombinant SP-C surfactant (Figure 4 and Figure 1 of the online data supplement). In order to more precisely define the putative surfactant inhibitory effect of each component of the neutral lipid mixtures, we investigated the influence of increasing amounts of single neutral lipids on the surface activity of the recombinant SP-C surfactant. Diglycerides and to a lesser degree free fatty acids showed a marked inhibitory effect on the γ\textsubscript{min} values (Figure 5). Interestingly, two different neutral lipids, monoglycerides and cholesterol, were the most effective in inhibiting the γ\textsubscript{ads} values (Figure 2 of the online data supplement).
DISCUSSION

In the present study, we investigated the effect of intrabronchial administration of a recombinant SP-C-based surfactant (Venticute) on gas exchange, surfactant composition and function of 31 ARDS patients. All patients participated in a randomized, controlled, multicenter phase I/II study in Europe/South Africa, addressing the safety and efficacy of this surfactant preparation in ARDS. This study was performed in parallel to an almost identical trial in North America.[13] In full accordance with some former studies, a significant improvement of gas exchange in the early time course after surfactant administration was encountered. At baseline, the BAL fluid concentrations of total phospholipids, phosphatidylglycerol and SP-C, the relative amount of the LA fraction and the degree of palmitoylation in phophatidylecholine were all significantly decreased in ARDS patients. Following administration of the recombinant SP-C surfactant all parameters were virtually fully normalized, which paralleled the improvement of gas exchange. Surfactant treatment favorably decreased the highly elevated minimum surface tension values of the LA fraction, however, values were still clearly higher than the ranges normally observed in healthy controls. As the applied material *per se* is known to possess excellent surface activity and additionally the far-reaching normalization of biochemical parameters implies that the exogenous surfactant material successfully merged with the endogenous pool, there are only a few possible explanations for the limited improvement of surface activity in response to surfactant administration:

*Firstly*, it could be argued that inhibitors of surfactant function, in particular plasma proteins such as fibrin(ogen),[26] are largely responsible for the incomplete restoration of surface activity upon surfactant treatment. However, we have employed two different techniques, high speed centrifugation and sucrose gradient centrifugation, to isolate the LA fraction from
BAL fluid, thereby separating the LA fraction from proteinaceous material and other inhibitors. Protein recovery in the LA fraction was 2.7% upon 48,000 x g centrifugation [7] and < 0.5% in case of sucrose gradient centrifugation [data not depicted]. As the surface activity of the LA fraction obtained from both methods was identically deteriorated and as there was no appreciable contamination of the lipid fraction with lysoPC, platelet-activating factor (PAF) or proteins, we think that inhibitory phenomena are not likely to explain the incomplete restoration of surface activity of the LA fraction. Nevertheless, we would anticipate that the “true” alveolar surface tension of ARDS patients, both, before and after surfactant treatment, is higher due to the presence of these inhibitors, as suggested in previous reports.[7, 14]

**Secondly,** it may be argued that lack of SP-B was responsible for the incomplete improvement of surface activity. Against this assumption are numerous studies that have shown SP-C-based surfactants such as the recombinant SP-C surfactant reach similar low surface tension values in vitro and similar efficacy in vivo as compared to natural surfactant extracts containing both hydrophobic surfactant proteins.[27-29] Nevertheless, this issue may be of some importance in the presence of inhibitors, where SP-B-based surfactants have been shown to display better surface activity as compared to SP-C-based surfactants.[27-28] However, as discussed above, the isolated LA fractions were almost completely separated from potential inhibitors. Therefore, we think that this issue is not of major importance.

**Thirdly,** direct damage to surfactant-specific proteins, probably by proteolysis, has been previously demonstrated in lungs of ARDS patients.[30] We have demonstrated a significant increase in SP-C levels in the BAL fluids of ARDS patients upon surfactant treatment (p = 0.003 at 48 hours), nevertheless it can not be completely excluded that some of this exogenously administered protein is degraded due to the action of activated
polymorphonuclear neutrophils [30], and this may contribute to the limited improvement of surface activity in response to surfactant treatment.

**Fourthly**, in the LA fraction of ARDS subjects there was a marked increased in neutral lipids. In contrast, no significant difference between the neutral lipid content in the BAL fluids of mechanically ventilated patients with cardiogenic pulmonary edema and healthy controls was observed. These findings indicate that mechanical ventilation per se is not responsible for the observed changes in neutral lipid content in the lungs of ARDS patients. The recombinant SP-C surfactant used in this study contains free palmitic acid at a relative concentration of 5%. Assuming that most of the material reached the distal lung after administration, one may anticipate a reduction of the neutral lipid content of the LA fraction to values close to 5%. Instead, the neutral lipid content remained elevated after surfactant treatment. Additionally, the neutral lipid profile, which was significantly altered at baseline, was only marginally affected by treatment with the recombinant SP-C surfactant. As such an increase in neutral lipids may have disadvantageous effects on surface tension, we analyzed changes in surface activity of different surfactant preparations when supplemented with a synthetic neutral lipid mixture mimicking the neutral lipid profile of ARDS patients. This resulted in a dose-dependent impairment of surface activity above a neutral lipid to phospholipid ratio of 0.05.

Neutral lipids have been known for a long time to be an integral component of the pulmonary surfactant system. They are actively secreted within the lamellar bodies from type II cells,[1] but their precise role within the surfactant system is still unclear. In particular, data are scarce in view of the regulation and role of neutral lipids in acute respiratory failure. Alveolar cholesterol was found to be increased in some animal models of acute lung injury,[31] but neutral lipids have never been investigated in ARDS patients. Recently, it has been demonstrated that cholesterol plays a critical role in promoting the lateral organization of
bilayer membranes made of native pulmonary surfactant.[32] Furthermore, neutral lipids such as cholesterol enhance adsorption at the air-water interface and also improve film repreading of dipalmitoylphosphatidylcholine or more complex phospholipid films, thus improving surface activity.[33-35] However, neutral lipids were much less effective in enhancing adsorption compared to the hydrophobic surfactant proteins SP-B and SP-C.[33] In oscillating bubble studies, in contrast to mixtures of phospholipids and hydrophobic surfactant proteins, a protein-free mixture consisting of phospholipids and neutral lipids never reached a minimum surface tension of less than 20 mN/m.[34] Furthermore, investigations with the pulsating bubble surfactometer showed that the addition of cholesterol to different surfactant preparations can impair the surface tension lowering ability upon bubble pulsation and may destabilize surface films upon maximum compression.[36] Finally, all of the preceding studies have been undertaken employing "physiological" neutral lipid concentrations (~5% neutral lipids wt/wt of phospholipids). Higher neutral lipid concentrations, as reported for the first time in this study of ARDS patients, have not yet been investigated in detail. The present findings suggest that an increased and "non-physiologically" composed neutral lipid fraction within the LA fraction may cause instability of the interfacial surfactant film upon lateral film compression, with increased minimum surface tension values. This interpretation is reinforced by our finding that especially the minimum surface tension values obtained under cyclic surface area changes were increased, whereas adsorption remained largely unchanged upon neutral lipid supplementation.

The source of increased neutral lipids and altered composition in ARDS is presently unknown. One possibility is due to altered synthesis or secretion by type II cells. Alternatively, changes may be due to spillover of neutral lipids from the systemic circulation via a leaky endothelial and epithelial barrier. However, the plasma neutral lipid profile is characterized by a large amount of cholesterol ester (46%) and triglycerides (30%), whereas only traces of free fatty acids, monoglycerides and diglycerides can be detected.[37] The
plasma neutral lipid profile is thus strikingly different from the neutral lipid profile in the LA fraction, regardless of whether the LA fraction was obtained by high speed centrifugation or further purified from blood contaminants by sucrose gradient centrifugation. Thus, “contamination” by plasma derived neutral lipids seems to be unlikely. Moreover, if the type II cell is indeed the main source of increased neutral lipids in ARDS, our observation of a persistently elevated neutral lipid content despite application of large amounts of exogenous surfactant material would suggest the existence of a mechanism that maintains a particular neutral lipid /phospholipid ratio in the acutely injured lung.

We conclude that increased levels and an altered neutral lipid profile may contribute to the impairment of surface activity in ARDS. Remarkably, neutral lipid abnormalities of the LA fraction were found to persist even after intrabronchial surfactant administration and may prevent full normalization of the alveolar surface tension upon surfactant treatment. Further studies are necessary to address the mechanisms underlying the disturbances of surfactant neutral lipid homeostasis in ARDS. One implication of this study would be to test if neutral lipid -free exogenous surfactant preparations are more suitable for the treatment of ARDS.
COMPETING INTERESTS

W. Seeger receives grant and contract support and fees for consulting services by the following companies: Schering AG, Pfizer Ltd., Altana Pharma AG, Lung Rx, Myogen. None of the other authors has any financial relationship with a commercial entity that has an interest in the subject matter or materials discussed in the manuscript.
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ETHICS APPROVAL

The study was approved by local institutional review boards/ethics committees at each participating institution and informed consent was obtained from all patients or their legal representatives.
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REFERENCES


FIGURE LEGENDS

Figure 1
Influence of intrabronchial surfactant administration on biophysical surfactant properties in ARDS patients

The surface tension after 5 minutes of film oscillation at minimum bubble radius (γ min) of the LA fraction obtained by high speed centrifugation of healthy controls and ARDS patients at a phospholipid concentration of 2 mg/ml is displayed. Data are given for ARDS patients before randomization (-3 hours = baseline), 48 and 120 hours after intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg of recombinant SP-C + 50 mg of phospholipid) per kilogram lean body weight given up to 4 times (surfactant), and for patients receiving standard care only (standard). The box-and-whisker-plots indicate the Median, 1st and 3rd Quartile; the whiskers are extended to the most extreme value inside the 1.5-fold interquartile range. Values outside (potential outliers) are indicated by circles. Significance level is indicated by * p = 0.02 (at 48 hours) and p = 0.04 (at 120 hours; all versus baseline values).

Figure 2
Neutral lipid content of the large surfactant aggregate (LA) fraction of healthy controls and ARDS patients

The content of neutral lipids (% of phospholipids, wt/wt) in the LA fraction obtained by high speed centrifugation of healthy controls, of ARDS patients before (-3 hours = baseline) and after intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg of recombinant SP-C + 50 mg of phospholipid) per kilogram lean body weight given up to 4 times, and of ARDS patients receiving standard care only is given. The box-and-whisker-plots indicate the Median, 1st and 3rd Quartile; the whiskers are extended to the most extreme value.
inside the 1.5-fold interquartile range. Significance level is indicated by * $p = 0.013$; *** $p = 0.0005$ (baseline) and $p = 0.0007$ (standard treatment at 48 hours and surfactant treatment at 120 hours; all versus healthy controls). NL = neutral lipids; PL = phospholipids

**Figure 3**

Neutral lipid profile of the large surfactant aggregate (LA) fraction of healthy controls and ARDS patients

The content of different neutral lipid classes (% of total neutral lipids, wt/wt) in the LA fraction obtained by high speed centrifugation of healthy controls, of ARDS patients before treatment (-3 hours = baseline) and of ARDS patients receiving standard care or additional treatment with 1 ml recombinant SP-C surfactant (containing 1 mg of recombinant SP-C + 50 mg of phospholipid) per kilogram lean body weight given up to 4 times at 48 hours after initial treatment is given. The box-and-whisker-plots indicate the Median, 1st and 3rd Quartile; the whiskers are extended to the most extreme value inside the 1.5-fold interquartile range. Significance level is indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; (ARDS patients pre or post standard or surfactant treatment versus healthy controls). MG=monoglycerides; DG=diglycerides; Chol= cholesterol; FFA=fatty acids; TG=triglycerides ; C-E=cholesterol ester, NL = neutral lipids

**Figure 4**

Influence of neutral lipids on the surface activity of a recombinant SP-C-based surfactant preparation and of the large surfactant aggregate (LA) fraction of natural rabbit lung surfactant
A neutral lipid mixture containing 100 % unsaturated neutral lipids and mimicking the neutral lipid profile in ARDS patients was added in increasing amounts (0-25 % of phospholipids, wt/wt) to 2 mg/ml of a recombinant SP-C surfactant (squares) and to 2 mg/ml of the LA fraction of natural rabbit lung surfactant (circles). Furthermore, a neutral lipid mixture containing 50 % unsaturated neutral lipids was added in increasing amounts to recombinant SP-C surfactant (triangles). The surface tension values after 5 minutes of film oscillation at minimum bubble radius (γ\textsubscript{min}) are given. Data are given as median. n = 8 for each concentration. NL = neutral lipids; PL = phospholipids.

**Figure 5**

**Influence of single neutral lipids on the surface activity of a recombinant SP-C-based surfactant preparation**

Single neutral lipids were added in increasing amounts (0-15 % of phospholipids, wt/wt) to 2 mg/ml of the recombinant SP-C surfactant. The surface tension values after 5 minutes of film oscillation at minimum bubble radius (γ\textsubscript{min}) are given. Data are given as median. n = 8 for each concentration.

Monoglycerides (diamonds); diglycerides (up triangles); cholesterol (circles); free fatty acids (down triangles); triglycerides (stars); cholesteroles (squares); NL = neutral lipids; PL = phospholipids.