

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

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**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 impaired 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.<sup>1</sup> Approximately 80–90% of the lipids are phospholipids. Four surfactant proteins (SP) have been identified (SP-A, SP-B, SP-C and SP-D).<sup>2</sup> 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.<sup>3–5</sup> In patients with the acute respiratory distress syndrome (ARDS), extensive alterations of biochemical and biophysical surfactant properties have been described.<sup>6–8</sup> In addition, intrabronchial surfactant administration was shown to be beneficial in different ARDS models.<sup>9</sup> 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<sup>10–12</sup> and surfactant composition.<sup>13 14</sup>

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%).<sup>6 15</sup> 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;  $\text{FI}_{\text{O}_2}$ , fractional inspired oxygen;  $\gamma_{\text{ads}}$ , surface tension after 12 s of film adsorption;  $\gamma_{\text{min}}$ , surface tension values after 5 min of film oscillation at minimum bubble radius; HPTLC, high-performance thin-layer chromatography; LA, large surfactant aggregate;  $\text{PaO}_2$ , arterial oxygen tension; SP, surfactant protein

**Table 1** Baseline demographic and physiological data

Variable	ARDS group	
	Standard care (n = 17)	Surfactant treatment (n = 14)
Age (years)	50.5 (43.8–64.0)	50.0 (36.0–58.2)
Sex (M/F)	12/5	11/3
Ethnic origin, n (%)		
Caucasian	16 (94.1)	12 (85.7)
African	1 (5.9)	1 (7.1)
Asian	0 (0)	1 (7.1)
Predisposing events, n (%)		
Trauma/surgery	7 (41.2)	7 (50)
Sepsis syndrome	7 (41.2)	6 (42.9)
Pneumonia	5 (29.4)	6 (42.9)
Pancreatitis	2 (11.8)	1 (7.1)
Gastric aspiration	2 (11.8)	1 (7.1)
Polytransfusion	1 (5.9)	0 (0)
Burn	1 (5.9)	0 (0)
Others	1 (5.9)	0 (0)
Hours from diagnosis to initial treatment	38.0 (21.0–74.0)	23.2 (9.0–53.5)
Modified APACHE II score	15.0 (10.8–21.2)	13.0 (10.2–15.8)
PEEP (cm H <sub>2</sub> O)	12 (12–14)	12 (12–12)
PaO <sub>2</sub> /F <sub>i</sub> O <sub>2</sub>	122.5 (96.5–147.8)	126.8 (111.8–152.2)

Data are presented as median (25–75th percentile). ARS, acute respiratory respiratory syndrome; APACHE, Acute Physiology and Chronic Health Evaluation; PEEP, positive end-expiratory pressure; PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub>, arterial oxygen tension/fractional inspired oxygen. PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub> and PEEP values represent the mean of three readings over the 6 h baseline period.

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<sup>16</sup> 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 (PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub>) 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 (–3 h) 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 PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub> 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 (PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub> 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/ketane. 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 CaCl<sub>2</sub> 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 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 patients with ARDS 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 available at <http://thorax.bmj.com/supplemental>.

**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 CaCl<sub>2</sub> 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.<sup>17</sup>

**Phospholipid and fatty acid analysis**

Lipids were extracted with chloroform/methanol according to method of Bligh and Dyer<sup>18</sup> and the phospholipid content was determined by spectrophotometric measurement of phosphorus.<sup>19</sup> For separation and analysis of phospholipid classes,

high-performance thin-layer chromatography (HPTLC) was used as previously described.<sup>7</sup> Similarly, the fatty acid profile of phosphatidylcholine was assessed by gas-liquid chromatography as outlined previously.<sup>20</sup>

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,<sup>6</sup> 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-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.<sup>7 21–23</sup>

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 ( $\gamma_{ads}$ ) and after 5 min of film oscillation at minimum bubble radius ( $\gamma_{min}$ ) 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-fold interquartile range. Values outside (potential outliers) are indicated by circles. The statistical analyses were performed in R Version 2.3.1.<sup>24</sup> 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* test 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<sup>25</sup> to control the false discovery rate. Categorical data were 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%.

RESULTS

Gas exchange, biochemical and biophysical surfactant properties on surfactant treatment

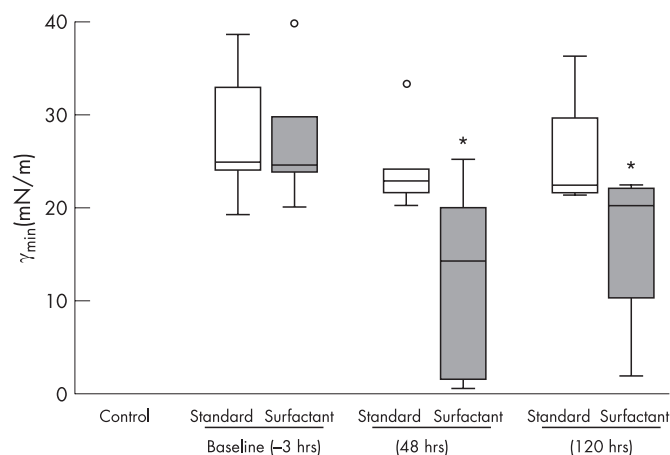
Patients with early ARDS were treated up to four times by intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight. A significant improvement in  $\text{PaO}_2/\text{FiO}_2$  ratios at 48 h ( $p = 0.008$ ) and 120 h ( $p = 0.0005$ ) 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 ( $\gamma_{min}$ , fig 1) and after 12 s of film adsorption ( $\gamma_{ads}$ , 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).

**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

	ARDS					
	Control	Baseline	Standard care		Surfactant treatment	
			+48 h	+120 h	+48 h	+120 h
LA (% of PL)	79.1 (70.5–86.5)	45.4 (28.8–53.2)	27.4 (23.2–52.2)	30.5 (25.8–31.10)	73.5 (68.3–77.2)**	64.2 (55.3–75.2)
PL (µg/ml)	23.5 (17.2–29.8)	25.6 (14.5–44.6)	30.4 (15.2–42.1)	17.0 (9.6–25.4)	185.0 (44.8–230.0)**	48.4 (31.6–78.1)
PC (% of total PL)	80.0 (79.0–82.3)	77.9 (70.3–81.6)	81.1 (73.2–83.1)	81.9 (80.6–85.3)	77.6 (68.1–83.5)	72.9 (66.6–78.1)
PG (% of total PL)	11.8 (11.3–12.7)	1.6 (1.2–2.6)	1.9 (0.7–5.5)	2.1 (0.8–2.3)	15.7 (8.9–21.8)**	6.8 (5.3–13.7)*
Palmitic acid (% of PC fatty acids)	74.2 (71.4–77.1)	53.6 (44.8–59.2)	52.6 (50.3–57.4)	57.8 (56.3–59.2)	85.6 (77.8–89.4)**	84.1 (78.5–84.2)**
SP-C (ng/ml)	494.9 (397.0–601.0)	350.2 (216.4–724.3)	437.0 (250.0–657.0)	436.0 (284.0–760.0)	1970.0 (770.0–2490.0)**	717.0 (290.0–1160.0)
PaO <sub>2</sub> /FiO <sub>2</sub> (mm Hg)	461.0 (446.0–469.0)	126.5 (103–149.7)	134.0 (94.1–164.0)	156.0 (85.0–215.0)	143.0 (138.0–182.0)**	212.0 (156.0–250.0)**

Data are presented as median (25–75th percentile).  
LA, large surfactant aggregate fraction; PL, phospholipids; PC, phosphatidylcholine; PG, phosphatidylglycerol; SP-C, surfactant protein C; PaO<sub>2</sub>/FiO<sub>2</sub>, arterial oxygen tension/fractional oxygen inspiration.  
Data are given for healthy controls and for patients with ARDS before (baseline; –3 h), 48 h and 120 h after intrabronchial administration of recombinant SP-C-based surfactant (n = 14) and for patients with ARDS receiving standard care only (n = 17). Biochemical data are given for the total lavage fluid. The baseline PaO<sub>2</sub>/FiO<sub>2</sub> value represents the mean of three readings over the 6 h baseline period.  
\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 pre-treatment vs post-treatment.

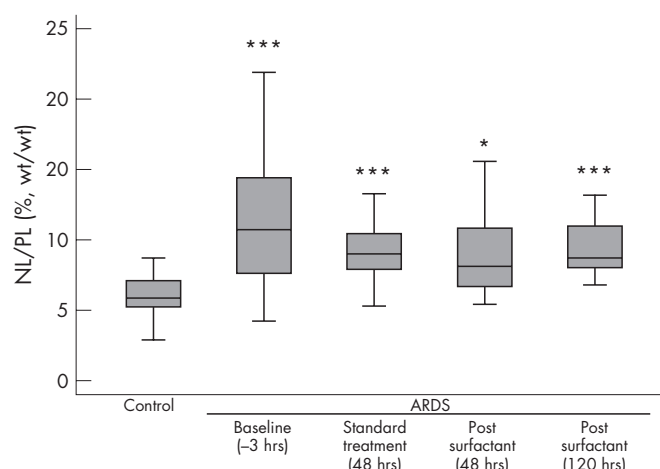


**Figure 1** Influence of intrabronchial surfactant administration on biophysical surfactant properties in patients with acute respiratory distress syndrome (ARDS). The surface tension after 5 min of film oscillation at minimum bubble radius ( $\gamma_{\min}$ ) of the large surface aggregate (LA) fraction obtained by high speed centrifugation in healthy controls and patients with ARDS at a phospholipid concentration of 2 mg/ml is displayed. Data are given for patients with ARDS before randomisation ( $\sim 3$  hours = baseline) and 48 h and 120 h after intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight given up to four times (surfactant) and for patients receiving standard care only (standard). 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-fold interquartile range. Values outside (potential outliers) are indicated by circles. The significance level is indicated by \* $p=0.02$  (at 48 h) and  $p=0.04$  (at 120 h; all versus baseline values).

### 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  $\mu\text{g/ml}$  (interquartile range 2.1–19.2) 48 h after surfactant treatment and 2.7  $\mu\text{g/ml}$  (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  $p<0.001$ ), whereas free fatty acids and cholesterol esters were decreased (all  $p<0.001$ ; 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



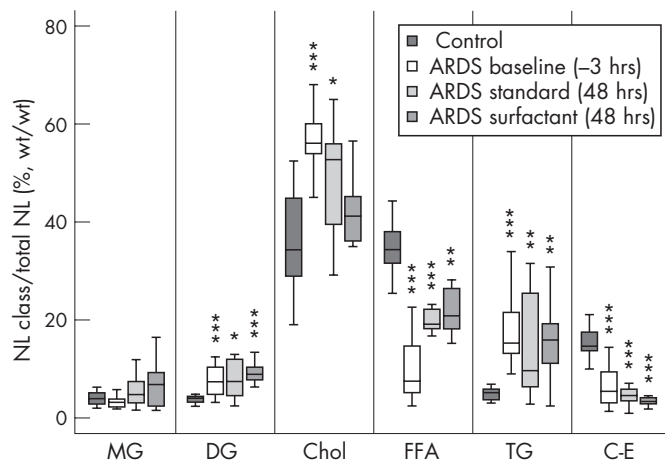
**Figure 2** Neutral lipid content of the large surfactant aggregate (LA) fraction of healthy controls and patients with acute respiratory distress syndrome (ARDS). The content of neutral lipids (% of phospholipids, wt/wt) in the LA fraction obtained by high speed centrifugation from healthy controls, patients with ARDS before ( $\sim 3$  h = baseline) and after intrabronchial administration of 1 ml recombinant SP-C surfactant (containing 1 mg recombinant SP-C + 50 mg phospholipid)/kg lean body weight given up to four times, and from patients with ARDS receiving standard care only 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 1.5-fold interquartile range. The significance level is indicated by \* $p=0.013$ ; \*\*\* $p<0.001$  (baseline) and  $p<0.001$  (standard treatment at 48 h and surfactant treatment at 120 h; all versus healthy controls). NL, neutral lipids; PL, phospholipids.

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  $\gamma_{\min}$  to almost 20 mN/m in both surfactant preparations (fig 4).  $\gamma_{\text{ads}}$  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  $\gamma_{\min}$  values (fig 5). Interestingly, two different neutral lipids—monoglycerides and cholesterol—were the most effective in inhibiting the  $\gamma_{\text{ads}}$  values (fig S2 of the online data supplement available at <http://thorax.bmj.com/supplemental>).

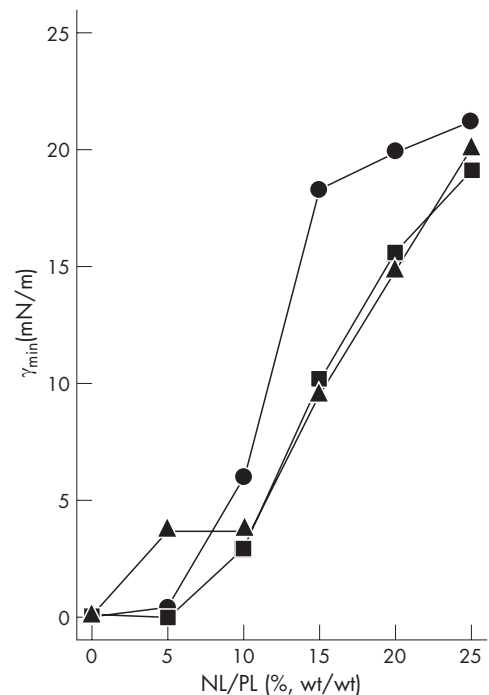


**Figure 3** Neutral lipid profile of the large surfactant aggregate (LA) fraction of healthy controls and patients with acute respiratory distress syndrome (ARDS). The content of different neutral lipid classes (% of total neutral lipids, wt/wt) in the LA fraction obtained by high speed centrifugation 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. 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-fold interquartile range. 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.

## 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.<sup>13</sup> 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 raised minimum surface tension values of the LA fraction, although the values were still considerably higher than the ranges normally observed in healthy controls. As the applied material itself is known to possess excellent surface activity and, in addition, the far-reaching normalisation 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 in surface activity in response to surfactant administration:

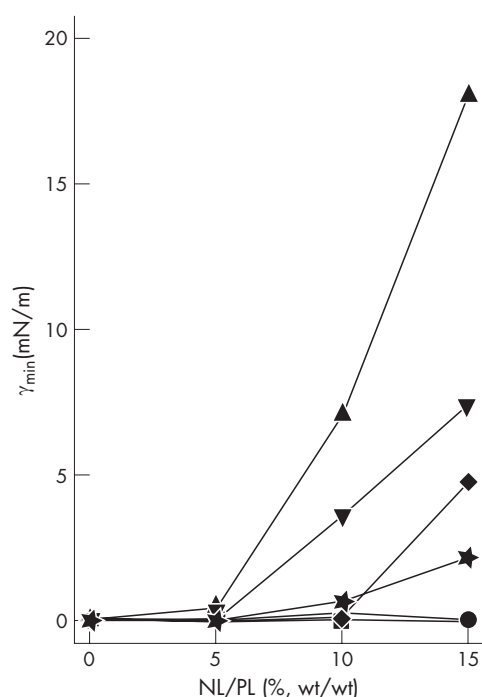
First, it could be argued that inhibitors of surfactant function (particularly plasma proteins such as fibrin(ogen)<sup>26</sup>) are largely responsible for the incomplete restoration of surface activity with surfactant treatment. However, we have used two different techniques—high speed centrifugation and sucrose gradient centrifugation—to isolate the LA fraction from BAL



**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 ( $\gamma_{\min}$ ) 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).<sup>7</sup> 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.<sup>7, 14</sup>

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.<sup>27–29</sup> 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.<sup>27, 28</sup> 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.



**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 ( $\gamma_{\min}$ ) 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); cholesterol ester (squares); NL, neutral lipids; PL, phospholipids.

Third, direct damage to surfactant-specific proteins, probably by proteolysis, has previously been demonstrated in the lungs of patients with ARDS.<sup>30</sup> We found a significant increase in SP-C levels in the BAL fluids of patients with ARDS following surfactant treatment ( $p=0.003$  at 48 h). However, it is possible that some of this exogenously administered protein is degraded by the action of activated polymorphonuclear neutrophils<sup>30</sup> and this may contribute to the limited improvement in surface activity in response to surfactant treatment.

Fourth, there was a marked increase in neutral lipids in the LA fraction of subjects with ARDS, but no significant difference between the neutral lipid content in the BAL fluids of mechanically ventilated patients with cardiogenic pulmonary oedema and healthy controls. These findings indicate that mechanical ventilation itself is not responsible for the observed changes in the neutral lipid content of the lungs of patients with ARDS. 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 in the neutral lipid content of the LA fraction to values close to 5%. Instead, the neutral lipid content remained raised after surfactant treatment. In addition, 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 analysed changes in surface activity of different surfactant preparations when supplemented with a synthetic neutral lipid mixture mimicking the neutral lipid profile of patients with ARDS. 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,<sup>1</sup> 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<sup>31</sup> 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.<sup>32</sup> 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.<sup>33–35</sup> However, neutral lipids were much less effective in enhancing adsorption than the hydrophobic surfactant proteins SP-B and SP-C.<sup>33</sup> 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.<sup>34</sup> 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.<sup>36</sup> Finally, all of the preceding studies have been undertaken with “physiological” neutral lipid concentrations ( $\sim 5\%$  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.<sup>37</sup> 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 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.

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