

RESEARCH ARTICLE

Transforming Growth Factor- β 1 in predicting early lung fibroproliferation in patients with acute respiratory distress syndrome

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Abstract

Background

Fibroproliferative repair phase of the acute respiratory distress syndrome (ARDS) is followed by a *restitutio ad integrum* of lung parenchyma or by an irreversible lung fibrosis and patients' death. Transforming Growth Factor- β 1 (TGF- β 1) is involved in collagen production and lung repair. We investigated whether alveolar TGF- β 1 was associated with the presence of fibroproliferation and the outcome of ARDS patients.

Methods

Sixty-two patients were included the first day of moderate-to-severe ARDS. Bronchoalveolar lavage fluid (BALF) was collected at day 3 (and day 7 when the patients were still receiving invasive mechanical ventilation) from the onset of ARDS. Survival was evaluated at day 60. TGF- β 1 was measured by immunoassay. The patients were classified as having lung fibroproliferation when the alveolar N-terminal peptide for type III procollagen (NT-PCP-III) measured on day 3 was $> 9 \mu\text{g/L}$ as recently reported. The main objective of this study was to compare the alveolar levels of total TGF- β 1 according to the presence or not a lung fibroproliferation at day 3.

Results

Forty-three patients (30.6%) presented a fibroproliferation at day 3. BALF levels of total TGF- β 1 were not statistically different at day 3 (and at day 7) according to the presence or not lung fibroproliferation. Mortality at day 60 was higher in the group of patients with fibroproliferation as compared with patients with no fibroproliferation (68.4% vs. 18.6% respectively; $p < 0.001$). Total TGF- β 1 measured on BALF at day 3 was not associated with the outcome. Multiple

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: ARDS, Acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; FIO₂, fraction of inspired oxygen; ICU, Intensive Care Unit; LIS, lung injury severity score; NT-PCP-III, Procollagen Type III-N-terminal peptide; PaO₂/FIO₂, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; PBW, predicted body weight; PEEP, positive end-expiratory pressure; SOFA, sepsis-related organ failure assessment score; TGF-β1, Transforming Growth Factor-β1; VFD, Ventilator-free days.

logistic regression showed that the presence of lung fibroproliferation was associated with death. In contrast, TGF-β1 was not independently associated with death.

Conclusions

Pulmonary levels of TGF-β1 during the first week of ARDS were not associated nor with the presence of fibroproliferation neither with death. TGF-β1 should not be used as a biomarker to direct anti-fibrotic therapies.

Introduction

Acute respiratory distress syndrome (ARDS) is associated with a mortality rate of 30–60%. [1, 2] Results from histopathological studies classically show two successive periods during ARDS: an early inflammatory phase followed by a fibroproliferative repair phase, with cell proliferation and deposition of matrix proteins leading to the resolution of ARDS or to irreversible lung fibrosis and death. [3, 4, 2]

Transforming Growth Factor-β1 (TGF-β1) plays a pivotal role in lung repair and fibroproliferative processes characterized by the collagen synthesis. [5, 6] TGF-β1 is secreted as a latent complex and must be released from its propeptide to acquire its biological activity (active TGF-β1). [7] Active TGF-β1 is a potent direct stimulator of collagen production. [8–10] TGF-β1 overexpression results in fibroblast migration and proliferation with increased deposition of extracellular matrix. [11, 12] Budinger *et al.* showed that bronchoalveolar lavage fluid (BALF) from ARDS patients, as well as exogenous TGF-β1, activate the human procollagen I promoter. [13] Experimental studies suggest that TGF-β1 is involved early in the course of acute lung injury. [14–19] The inflammatory properties of TGF-β1 could participate in processes observed early in the course of ARDS such as lung cells recruitment, alveolar flooding and cytokine release. [15, 17, 20, 21] However, little is known about the early changes in TGF-β1 levels and their prognostic values in human ARDS.

Procollagen Type III-N-terminal peptide (NT-PCP-III), a peptide released during the conversion of type III procollagen to type III collagen, is a marker of fibroblast activity, collagen synthesis and lung fibrosis. [22, 23] NT-PCP-III is increased in alveolar fluid and serum during lung fibrosis and fibroproliferative-established ARDS. [24–27] Pulmonary levels of NT-PCP-III are also elevated from the onset of ARDS, suggesting that fibroproliferation is involved early in the lung repair process and occurs simultaneously with the inflammatory phase of ARDS. [28–31] Early fibroproliferation in ARDS patients with higher pulmonary levels of NT-PCP-III has been shown to be independently associated with death. [27, 31, 32, 33] It has also been recently reported that NT-PCP-III is a valid biomarker of lung fibroproliferation [34].

Because TGF-β1 could play a central role in the pathophysiology of ARDS during the early phase, the objectives of the present study were to evaluate if alveolar TGF-β1 obtained from BALF analysis was associated with fibroproliferation (assessed by lung production of NT-PCP-III) and with the outcome. The identification of reliable biomarkers is of paramount importance to guide anti-inflammatory treatments and/or therapeutics that modulate fibroproliferation such as corticosteroids.

Materials and methods

The study was approved by the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) de Marseille 1) and was registered

(ClinicalTrials.gov NCT00440882). Written informed consent was obtained from patients and/or next of kin prior to enrollment.

Patients

Patients over 18 years of age were included within the first 24 h of the onset of moderate-to-severe ARDS if they presented a partial pressure of arterial oxygen/fraction of inspired oxygen ratio ($\text{PaO}_2/\text{FIO}_2$) < 200 mmHg at a positive end-expiratory pressure (PEEP) ≥ 5 cm H_2O [35]. Some of these patients ($n = 9$) have also been included in the validation cohort of NT-PCP-III previously reported [34]. The exclusion criteria were as follows: pregnancy, chronic interstitial or fibrotic lung diseases, liver cirrhosis, neutropenia ≤ 1 G/L, corticosteroid (> 200 mg/day of hydrocortisone or equivalent beginning at least 2 weeks before inclusion), immunosuppressive therapy within the last 30 days, presence of an advanced directive to withhold life-sustaining treatment, persistent (more than 4 h) $\text{PaO}_2/\text{FIO}_2 < 70$ mmHg despite maximal treatment (safety criteria for BAL). Written informed consent was obtained from patients and/or next of kin prior to enrollment.

Study protocol

ARDS patients were ventilated according to a lung-protective strategy ($4 \leq$ tidal volume ≤ 8 mL/Kg predicted body weight and plateau pressure ≤ 30 cm H_2O). PEEP and FIO_2 were set according to the algorithm of the National Institutes of Health ARDS Network.[1] Patients with septic shock were treated with 200 mg/day of hydrocortisone for at least the first 5 days. Clinical data, respiratory parameters, and sepsis-related organ failure assessment score (SOFA)[36] were evaluated on days 1 (inclusion), 3 and 7. Survival was evaluated on day 60. Ventilator-free days and alive were evaluated both at day 28 and day 60.

Blood and bronchoalveolar lavage fluid collection

BALF samples were collected on day 3 and 7 from ARDS onset if the patients were still receiving invasive mechanical ventilation. BALF was always obtained from the same territory, corresponding to the most infiltrated lung area on chest X-rays. Sterile saline was instilled in an aliquot of 50 mL. A second aliquot of 50 mL was used if the recovered lavage fluid was lower than 10 mL. After centrifugation, BALF aliquots were stored at -80°C until their analysis.

Biological analyses

TGF- β 1 was measured using a specific ELISA kit (Quantikine, R&D Systems, MN, USA). This immunoassay was performed directly on the BALF samples to measure the spontaneous free TGF- β 1 (free TGF- β 1). The latent form of TGF- β 1 was measured following an acid activation and neutralization protocols in agreement with the manufacturer's instructions. This acidification induced an activation of the latent form of TGF- β 1 and allowed the measure of the both latent and spontaneous free forms (total TGF- β 1) in BALF. Assay sensitivity limits was less than 2 pg/mL in BALF. BALF levels of NT-PCP-III were measured by radioimmunoassay (Orion Diagnostica, Finland). This kit is based on a competitive technique and detects intact NT-PCP-III. The assays were performed in agreement with the manufacturer's instructions. The detection limit was 0.3 $\mu\text{g}/\text{L}$.

Definition of lung fibroproliferation

The patients were classified as having lung fibroproliferation when the NT-PCP-III measured on day 3 was > 9 $\mu\text{g}/\text{L}$ on BALF as recently reported [34].

Objectives of the study

The main objective of this study was to compare the alveolar levels of total TGF- β 1 according to the presence or not a lung fibroproliferation at day 3. Secondary objectives included the diagnostic value of BALF levels of free TGF- β 1 in predicting lung fibroproliferation at day 3, and the association between BALF levels of total TGF- β 1 and the outcome.

Statistics

Descriptive variables of the population were compared by the Fisher's exact test for categorical variables and the Student's *t* test for normally distributed continuous variables. We used the Mann-Whitney test for the non-normally distributed variables (Kolmogorov-Smirnov approach) as total and free TGF- β 1 levels. Two-tailed tests were used. Non-normally distributed variables were reported as median values [interquartile range] and normally distributed variables are reported as mean (SD). A multiple logistic regression analysis was done in order to identify the factors related to the outcome. A *p* value of ≤ 0.05 was considered to be statistically significant. Statistics were performed using SPSS 20.0 software (SPSS Inc., IL, USA).

Results

Patients

Eighty-one consecutive ARDS patients were screened in three intensive care units (34 beds) (Fig 1). The BALF samples were obtained from all 62 prospectively included patients. The baseline characteristics of these 62 patients are shown in Tables 1 and 2. Direct lung injury (such as pneumonia and aspiration pneumonitis) was the main mechanism of ARDS (85.5% of the patients). Forty-three patients (30.6%) presented a fibroproliferation on day 3. In Table 2 are presented the respiratory parameters and ventilator settings on days 1, 3 and 7 of ARDS according to the presence/absence of fibroproliferation. Interestingly, driving pressure and plateau pressure were higher in the fibroproliferation group on days 3 and 7, but not on day 1. Overall mortality at day 60 was 33.9% ($n = 21$). Mortality at day 60 was higher in the group of patients from the fibroproliferation group (68.4% vs. 18.6% in the no fibroproliferation group; $p < 0.001$).

Transforming growth factor- β 1 levels in predicting early lung fibroproliferation

BALF levels of total TGF- β 1 were not statistically different between patients presenting or not lung fibroproliferation both at day 3 and at day 7 (Fig 2). The ROC curves (S1 Fig) showed that the diagnostic performance of BALF levels of TGF- β 1 for the identification of lung fibroproliferation was poor. Furthermore, there was no difference regarding BALF free TGF- β 1 levels in patients with and without lung fibroproliferation both at day 3 and day 7 (S1 Table).

In the specific subgroup analysis of the 9 patients which have been included in the validation cohort of PCIII previously reported [34] with an available open lung biopsy, 5 patients with histologically documented lung fibrosis were reported. No significant difference was observed between BALF total TGF- β 1 levels measured on days 3 and 7 (S2 Fig).

Transforming growth factor- β 1 levels in ARDS according to the outcome

Total TGF- β 1 measured on BALF on day 3 was not associated with the outcome (Table 3 and S3 Fig). In contrast, the presence of lung fibroproliferation as assessed by NT-PCP-III determined on BALF obtained at day 3 was strongly and independently associated with day-60

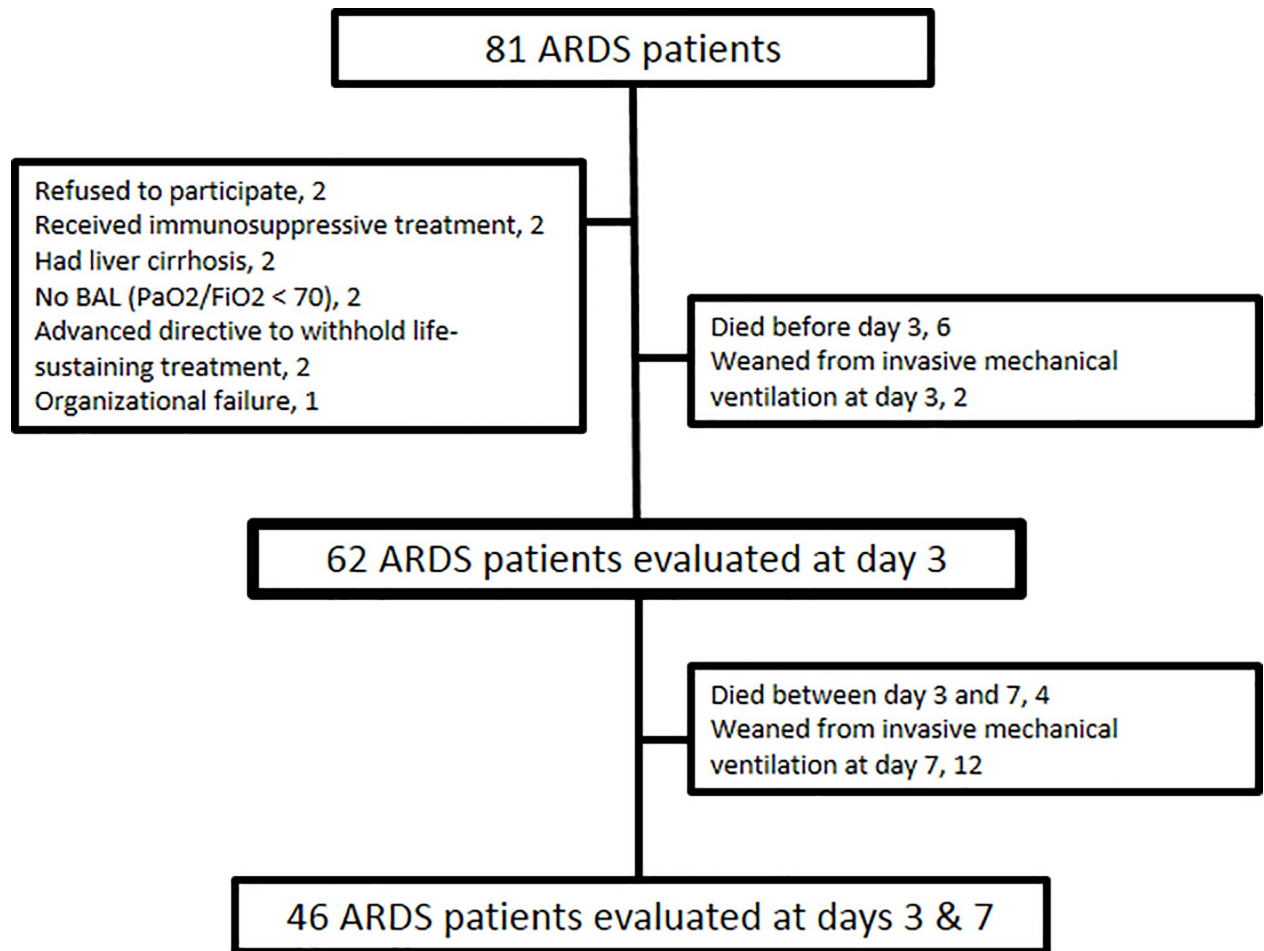


Fig 1. Flow chart of the study. Day 1 is the day of inclusion; ARDS: acute respiratory distress syndrome; PaO₂/FIO₂, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; BALF: bronchoalveolar lavage.

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mortality (Table 3). The SOFA score evaluated on day 3 was also independently associated with day-60 mortality while other factors such as driving pressure or age were not (Table 3).

Discussion

In this prospective clinical study, we observed that alveolar fluid levels of total TGF-β1 were not associated with the presence of lung fibroproliferation early in the course of ARDS patients. Pulmonary levels of TGF-β1 were not different between ARDS survivors and nonsurvivors. In contrast, the presence of lung fibroproliferation as assessed by an elevated level of NT-PCP-III determined on BALF early in the course of ARDS was strongly associated with mortality.

We did not observe any difference in alveolar levels of free and total TGF-β1 between survivors and nonsurvivors in this group of 62 ARDS patients. In a previous study about the prognostic value of free TGF-β1 levels in BALF performed in 29 ARDS patients, Budinger *et al.* found a trend to higher BALF levels of TGF-β1 in ARDS nonsurvivors as compared to survivors but the difference did not reach the significance ($p = 0.14$). [13] TGF-β1 mediates far-ranging biological processes including cell growth, inflammation, angiogenesis, tumorigenesis, morphogenesis, fibrogenesis and tissue repair. [5, 37, 38] The exact biological role of TGF-

Table 1. Characteristics on inclusion and outcome of the patients.

	All (n = 62)	No fibroproliferation (n = 43)	Fibroproliferation (n = 19)	p value
Age, years	59 ± 15	56 ± 14	65 ± 14	0.025
Men, n (%)	47 (75.8)	26 (78.8)	12 (92.3)	0.276
SAPS II score	47.4 ± 14.7	47.8 ± 15.9	46.5 ± 11.9	0.754
SOFA score	9.5 ± 3.2	9.6 ± 3.5	9.3 ± 2.4	0.698
Cause of ARDS, n (%)				0.117
Pneumonia	37 (59.7)	21 (48.8)	16 (84.2)	
Aspiration	14 (22.6)	13 (30.2)	1 (5.3)	
Extra-pulmonary infection	5 (8.1)	4 (9.3)	1 (5.3)	
Pancreatitis	1 (1.6)	1 (2.3)	0 (0)	
Miscellaneous	5 (8.1)	4 (9.3)	1 (5.3)	
ARDS with direct lung injury, n (%)	53 (85.5)	36 (87.7)	17 (89.5)	0.553
Lung Injury Severity Score	2.86 ± 0.48	2.85 ± 0.49	2.88 ± 0.48	0.834
Hydrocortisone, n (%)	48 (77.4)	30 (69.8)	18 (94.7)	0.046
Ventilator free day 28, days	1 [0–12]	4 [0–18]	0 [0–0]	0.006
Ventilator free day 60, days	32 [0–44]	36 [0–50]	0 [0–0]	0.002
ICU-free day 60, days	26 [0–39]	32 [0–43]	0 [0–0]	0.001
Death at day 60, n (%)	21 (33.9)	8 (18.6)	13 (68.4)	< 0.001

Values are expressed as means ± SD, number of cases (%) or median [IQR]

ARDS, acute respiratory distress syndrome; SAPS II, simplified acute physiology score; SOFA, sepsis-related organ failure assessment score; Hydrocortisone, septic shock patients treated by hydrocortisone ≥ 200 mg/kg/day during at least 5 days after inclusion

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β1 in lung pathophysiology has not been clearly identified. A beneficial role is observed in experimental studies where TGF-β1 is expressed at high levels during normal lung development.[5, 39] Moreover, TGF-β1 is also involved in normal tissue repair following lung injury.[40, 41] Nevertheless, a harmful role of TGF-β1 in the development of acute lung injury has been reported.[17, 15] These pleiotropic functions of TGF-β1 and its complex regulation could limit its prognostic value. The relation between TGF-β1, collagen synthesis and ARDS outcome remains complex and the weak relationships between alveolar levels of TGF-β1 and NT-PCP-III, observed in the current study, seem to suggest that the NT-PCP-III synthesis depends of other mediators than TGF-β1.

Study limitations

In our study, the main underlying conditions responsible for ARDS were pneumonia (59.7%) and gastric aspiration pneumonitis (22.6%). This could have influenced the TGF-β1 levels observed in our population because Buhling *et al.* showed that bacterial pneumonia increased TGF-β1 levels.[42] Conclusions could have been different in patients with ARDS from extra-pulmonary causes.

TGF-β exists in three isoforms (β1, 2 and 3)[5, 37] but in the present study, TGF-β2 and 3 were not measured. There is evidence that TGF-β2 and β3 exert significant *in vitro* pro-fibrotic activity and could also drive fibrogenesis in the lung.[43] Further studies are needed to determine if TGF-β2 and β3 could be associated with fibroproliferation and mortality in ARDS patients. Moreover, the detection of TGF-β1 used an immunoassay measuring the level of free TGF-β1 and, after *in vitro* activation of latent (complexed) forms of TGF-β1, the total TGF-β1. This assay quantified the amount of TGF-β1 but did not measure the biological activity of the free TGF-β1. The interpretation of the present results must consider this limitation.

Table 2. Evolution of respiratory parameters and ventilator settings according to the presence/absence of lung fibroproliferation (alveolar N-terminal peptide for type III procollagen > 9 µg/L).

	ARDS Day 1		ARDS Day 3		ARDS Day 7	
	No fibroproliferation (N = 43)	Fibroproliferation (N = 19)	No fibroproliferation (n = 43)	Fibroproliferation (n = 19)	No fibroproliferation (N = 33)	Fibroproliferation (N = 13)
Tidal volume, mL/kg PBW	6.6 ± 0.9	6.6 ± 0.8	6.8 ± 1.0	6.9 ± 1.3	7.4 ± 1.8	6.9 ± 2.3
Respiratory rate, c/min	21.3 ± 5.7	25.5 ± 6.5 *	22.3 ± 6.2	26.4 ± 5.7 #	23.8 ± 8.1	30.8 ± 8.1 †
Total PEEP, cmH ₂ O	12.6 ± 3.1	12.1 ± 2.6	11.8 ± 3.5	11.7 ± 2.4	9.9 ± 3.9	11.5 ± 3.7
Plateau pressure, cmH ₂ O	24.9 ± 5.0	26.7 ± 4.3	24.4 ± 5.3	27.7 ± 5.1 #	22.7 ± 7.0	28.9 ± 6.6 †
Driving pressure, cmH ₂ O	12.3 ± 3.9	14.6 ± 4.9	12.4 ± 4.1	16.1 ± 5.2 #	12.5 ± 5.5	17.1 ± 5.6 †
Tidal compliance rs, mL/cmH ₂ O	39.2 ± 18.5	32.8 ± 11.3	38.9 ± 14.4	31.4 ± 12.9	45.8 ± 27.8	33.6 ± 27.7
FiO ₂	0.68 ± 0.10	0.76 ± 0.19	0.55 ± 0.15	0.61 ± 0.16	0.48 ± 0.14	0.68 ± 0.22 †
PaO ₂ /FiO ₂ , mmHg	122.9 ± 33.3	112.1 ± 29.0	170.6 ± 67.9	135.9 ± 38.6 #	200.8 ± 77.3	127.6 ± 57.6 †
PaCO ₂ , mmHg	46.9 ± 13.3	53 ± 14	42.6 ± 8.1	48.2 ± 9.9 #	43.1 ± 10.1	52.7 ± 15.4 †
pH	7.33 ± 0.10	7.28 ± 0.10	7.39 ± 0.07	7.34 ± 0.09 #	7.43 ± 0.08	7.37 ± 0.11

Values are expressed as means ± SD.

*: p < 0.05 for comparison on day 1

#: p < 0.05 for comparison of ventilator parameters on day 3

†: p < 0.05 for comparison of ventilator parameters on day 7

ARDS, acute respiratory distress syndrome; PaO₂/FiO₂: partial pressure of arterial oxygen/fraction of inspired oxygen ratio; PBW: predicted body weight; rs: respiratory system; plateau pressure was measured during a 1 second end-inspiratory pause; Total PEEP: total positive end-expiratory pressure was measured during a 5 seconds end-expiratory pause.

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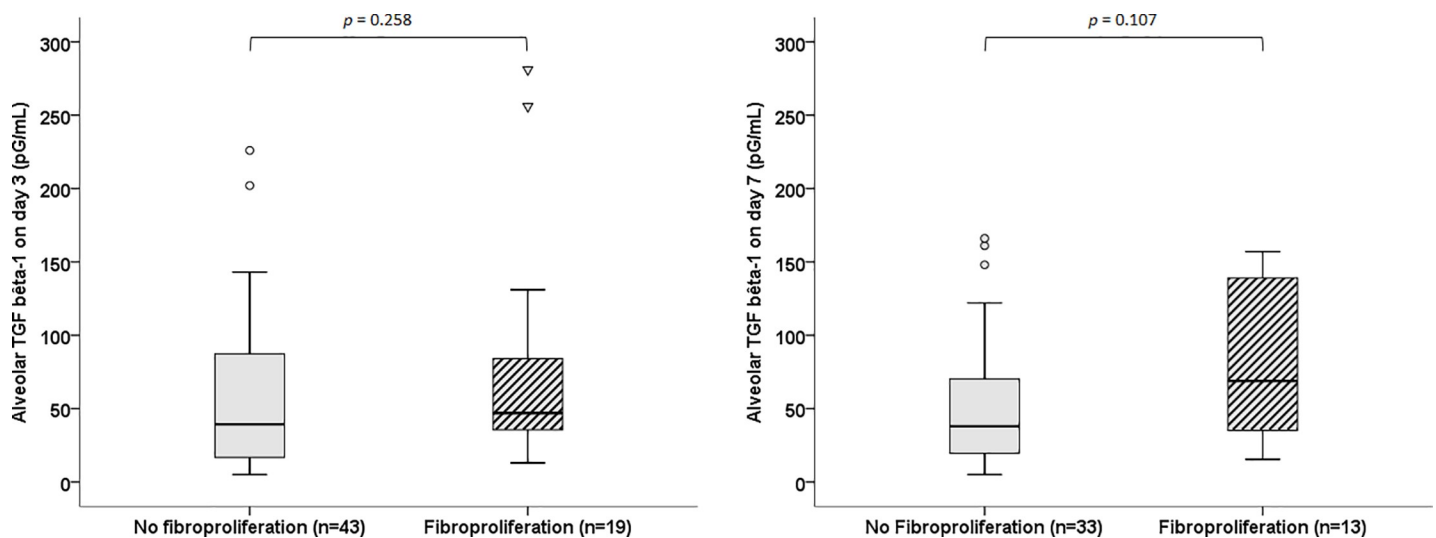


Fig 2. Bronchoalveolar lavage fluid levels of total TGF-β1 on day 3 (left panel) and day 7 (right panel) in ARDS patients according to lung fibroproliferation evaluated on day 3.

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Table 3. Factors evaluated on ARDS day 3 and associated with mortality at day 60.

	Survivors (N = 41)	Non-survivors (N = 21)	Univariate	HR (95% CI)	Multivariate
Age, years	55.9 ± 14.8	65.6 ± 11.9	0.012	1.022 (0.929–1.124)	0.661
Men, n (%)	31 (75.6)	16 (76.2)	0.960	-	-
SOFA score	7.3 ± 3.5	11.0 ± 3.3	<0.001	1.463 (1.064–2.012)	0.019
LIS Score	2.43 ± 0.56	2.72 ± 0.68	0.086	0.442 (0.043–4.506)	0.491
Tidal volume, mL/kg PBW	6.8 ± 1.0	6.9 ± 1.2	0.597	-	-
Respiratory rate, c/min	21.5 ± 5.7	28.0 ± 5.4	<0.001	-	-
Total PEEP, cmH ₂ O	11.7 ± 3.4	12.1 ± 2.7	0.619	-	-
Plateau pressure, cmH ₂ O	24.4 ± 5.2	28.3 ± 5.2	0.018	-	-
Driving pressure, cmH ₂ O	12.7 ± 4.3	16.3 ± 5.1	0.013	1.163 (0.927–1.457)	0.192
Tidal compliance rs, mL/ cmH ₂ O	38.9 ± 13.9	29.9 ± 13.4	0.041	-	-
PaO ₂ /FiO ₂ , mmHg	171.6 ± 65.9	131.7 ± 41.2	0.021	-	-
PaCO ₂ , mmHg	43.9 ± 8.7	45.5 ± 9.8	0.519	-	-
pH	7.39 ± 0.06	7.32 ± 0.09	0.001	0.001 (0.0001–350.827)	0.170
Lung Fibroproliferation, n (%)	6 (14.6)	13 (61.9)	<0.001	24.236 (1.684–349.482)	0.019
Total TGF-β1, pg/L	60.6 ± 52.6	76.6 ± 75.5	0.332	1.003 (0.986–1.019)	0.768

Values are expressed as means ± SD or number of cases (%). ARDS, acute respiratory distress syndrome; SOFA, sepsis-related organ failure assessment score; PaO₂/FIO₂, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; PBW, predicted body weight; plateau pressure measured during a 1-second end-inspiratory pause; Total PEEP, total positive end-expiratory pressure was measured during a 5-second end-expiratory pause. Tidal compliance rs, Plateau Pressure and Driving Pressure were collinear. Driving Pressure was introduced in the multiple logistic regression. Respiratory Rate and pH were collinear. pH was introduced in the model. SOFA score and PaO₂/FiO₂ were collinear. SOFA score was introduced in the model

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Fibroproliferation was based on a NT-PCP-III threshold at 9 µg/L. This threshold has been evaluated in a recently published study where 32 ARDS patients were included [34]. A threshold at 9 µg/L was compared with histological analysis of lung parenchyma. The diagnostic accuracy of a NT-PCP-III higher than 9 µg/L to predict the presence of lung fibroproliferation was 90.6 (95% CI, 75.8–96.8)%. In the present study, we showed that our results were reliable by comparing biopsy results with BALF NT-PCP-III and TGF-β determinations in a subset of 9 patients. Moreover, we reported here that TGF-β was not associated with the outcome, whereas the presence of lung fibroproliferation was linked to mortality.

Conclusions

We showed that alveolar fluid levels of both total and free TGF-β1 evaluated early in the course of ARDS were not associated with the presence of lung fibroproliferation. Moreover, the alveolar fluid levels of total and free TGF-β1 were not associated with an increased risk of death in ARDS patients. We confirmed that the presence of fibroproliferation assessed by elevated pulmonary levels of NT-PCP-III measured within the first 7 days of the onset of ARDS was independently associated with death.

Ethics approval and consent to participate

The study was approved by the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) de Marseille 1) and was registered (ClinicalTrials.gov NCT00440882).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Supporting information

S1 Fig. ROC curves for BALF levels of TGF- β 1 for the identification of lung fibroproliferation.

(PDF)

S2 Fig. Bronchoalveolar lavage fluid levels of TGF- β 1 in ARDS patients according to lung fibroproliferation evaluated by the level of alveolar NT-PCP-III (left panel) and histology (right panel).

(PDF)

S3 Fig. Bronchoalveolar lavage fluid levels of Transforming Growth Factor- β 1 levels in ARDS according to the outcome.

(PDF)

S1 Table. Alveolar Free TGF β -1 according to the presence or not of lung fibroproliferation.

(DOCX)

S1 Database.

(XLSX)

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Author Contributions

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Resources: Laurent Papazian.

Software: Marc Gannier, Anderson Loundou.

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