**Supplemental digital content**

**Alveolar neutrophil extracellular traps in patients with pneumonia-related acute respiratory distress syndrome**

**Material and Methods**

**Patients and data collection**

All patients with severe or moderate pneumonia-related ARDS (Berlin definition 1) were included consecutively with the following inclusion criteria: tracheal intubation and mechanical ventilation since less than 72 hours; pulmonary infection diagnosed less than 7 days before; bilateral pulmonary infiltrates on chest x-ray consistent with pulmonary edema; a PaO2/FiO2 ratio ≤ 200 mm Hg with a positive end-expiratory pressure (PEEP) ≥ 5 cm H2O. Non-inclusion criteria were as follows: age <18 years; pregnancy; chronic respiratory failure requiring long-term oxygen therapy; Child-Pugh C liver cirrhosis; lung fibrosis; immunosuppression (*i.e.*, HIV infection, active hematological malignancy or solid cancer receiving chemotherapy, corticosteroids therapy for more than 0.5 mg/kg/day since more than 4 weeks, organ transplant patients), SAPS II (Simplified Acute Physiology II score) > 90, irreversible neurological disorders, patients with withholding/withdrawing of life-sustaining therapies and profound hypoxaemia (PaO2/FiO2 <75 mm Hg).

Control patients, (*i.e.*, patients free of ARDS, any active infection, diffuse interstitial pneumonia or immunosuppression; n=4) undergoing a bronchoscopy with broncho-alveolar lavage and blood sampling as part of routine care, were also included (see eTable 1 for further more details on control patients).

ARDS patients received mechanical ventilation using a standardized protective ventilation strategy 2,3. Other treatments, including neuromuscular blocking agents 4, nitric oxide inhalation 5, prone positioning 6 and venovenous extra-corporeal membrane oxygenation were administered depending on the severity of ARDS 7. The prevention of ventilator-associated pneumonias followed a multifaceted program 8; Sedation and mechanical ventilation weaning followed standardized protocols 9.

Demographics, clinical and laboratory variables were recorded upon ICU admission, at samples collection time points (see below) and during ICU stay. Patients’ severity of illness was assessed using SAPS II 10 and sequential organ failure assessment (SOFA) scores 11. Other recorded variables included the use of adjuvant therapies for ARDS (*i.e.*, neuromuscular blocking agents, nitric oxide inhalation, prone positioning, extracorporeal membrane oxygenation), the need for hemodialysis or vasopressors, corticosteroids administration, the number of ventilator- and organ failure-free days at day 28, the duration of mechanical ventilation and of intensive care unit stay in all patients and in survivors only and intensive care unit mortality.

**BAL fluid processing**

During a standard flexible bronchoscopy, the bronchoscope was wedged within a bronchopulmonary segment. Four aliquots of normal saline (50 mL each) were instilled through the bronchoscope within the selected bronchopulmonary segment. After each aliquot was instilled, saline was retrieved using a negative suction pressure. Broncho-alveolar lavage fluid cytology was performed by direct microscopy after centrifuging broncho-alveolar lavage fluid samples (12 000 revolutions for 10 min) and dying under the May-Grünwald-Giemsa staining. Total (quantified in cells/mL) and differential (*i.e.*, percent of neutrophils, macrophages and lymphocytes) cell counts were measured as recommended 12.

**Measurements of BAL fluid and blood NETs and cytokines levels**

Broncho-alveolar lavage fluid was collected from all ARDS patients during a bronchoscopy within 48 hours of ARDS onset (day 1-2 sample) and, for patients who were alive and still had ARDS criteria five days thereafter (day 4-5 sample). Broncho-alveolar lavage fluid samples were also collected from controls during bronchoscopy. A blood sample (4 ml) for measurement of serum neutrophil extracellular traps and serum cytokines concentrations was obtained in the same time as part of routine care (see online supplementary data for further details).

**NETs quantification**

Neutrophil extracellular traps were specifically quantified by measuring myeloperoxidase (MPO)-DNA complexes in serum and broncho-alveolar lavage samples using a previously described capture ELISA 13 considered to be the best way to monitor NETosis *in vivo* 14. Briefly, 20 μg/ml of rabbit anti-human MPO antibody (Millipore, AB 1224) was coated overnight to 96-well microtiter plates. After blocking with 2% BSA, samples were added together with a peroxidase-labeled anti-DNA monoclonal antibody (component 2 of the Cell Death ELISA kit, Roche). After incubation, the peroxidase substrate was added for 40 min at 37 °C. Optical density was measured at 405 nm. A standard curve was obtained by dilution of a strongly positive serum. Samples were interpolated from the standard curve using the sigmoidal dose-response (variable slope) equation and results were expressed in arbitrary units (a.u.). The detection range was 4-1000 a.u..

**Quantification of cytokines and biomarkers of alveolar epithelial injury**

Serum and Broncho-alveolar lavage fluid concentrations of the main pro- and anti-inflammatory cytokines involved in neutrophil chemotaxis and activation (IL-6, IL-8, IL10, TNF-α 15), as well as lung epithelium injury markers (surfactant protein D, RAGE16)were measured by a single operator (MS) blinded to the clinical data using a Human Magnetic Luminex Assay (Biotechne – R&D Systems, Abingdon, UK) and expressed in ng/mL.

**Statistical analysis**

Continuous variables are reported as median [25–75th percentiles] or mean (±standard deviation, SD), as appropriate, and compared using Student t test or the Mann-Whitney test. Categorical variables are reported as number and percentages (95 % confidence interval) and compared using the chi2 or Fischer test, as appropriate. Because no clinically relevant neutrophil extracellular traps concentration threshold value was previously reported in Broncho-alveolar lavage fluid of ARDS patients, we categorized patients according to median broncho-alveolar lavage neutrophil extracellular traps (DNA-myeloperoxidase) levels (*i.e.*, patients with “higher” versus patients with “lower” broncho-alveolar lavage neutrophil extracellular traps levels). A p value <0.05 was considered significant. Analyses were conducted using the SPSS Base 21.0 statistical software package (SPSS Inc., Chicago, IL).

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**SUPPLEMENTAL TABLES**

eTable 1. Characteristics of control patients (n=4) included in the study

|  |  |
| --- | --- |
| **Variables** | **Control patientsa**  |
| Age | 40 | 72 | 30 | 25 |
| Gender | Male | Male | Male | Male |
| Immunosuppression | No | No | No | No |
| Comorbidities | None | Prostate cancer (2005, in remission) | None | None |
| Smoker | No | Yes | Yes | No |
| Clinical indication for bronchoscopy | Micronodules in right upper lobe | Ground glass lesions in right upper lobe + nodular lesion | Suspicion of tuberculosis | Hilar adenopathy |
| **Characteristics upon BAL sampling** |
| White blood cell counts, 103/mm3NeutrophilsLymphocytesMonocytes | 6.43.52.40.4 | 17.414.42.20.3 | 5.02.41.50.6 | 6.03.71.30.8 |
| BAL cytologyTotal cell counts, 103/mLMacrophages, %Neutrophils, %Lymphocytes, % | 7787112 | 7084151 | 19766130 | 13066133 |

aAll control patients were free of ARDS, active pulmonary infection, infiltrative lung disease, and immunosuppression

**eTable 2. Microbiological documentation of patients (n=35) with pneumonia-related ARDS**

|  |  |
| --- | --- |
|  | **N (%)** |
| **Bacteria** | 18 (51.4) |
| *Enterobacteriaceae* | 6 |
| *Streptococcus pneumoniae* | 5 |
| *Staphylococcus aureus* | 2 |
| *Legionella pneumophila* | 2 |
| *Mycoplasma pneumoniae* | 2 |
| Group A streptococcus | 1 |
| **Virus** | 11 (31.4) |
| *Influenza H1N1* | 7 |
| *Rhinovirus* | 2 |
| *Respiratory syncytial virus* | 1 |
| *Metapneumovirus* | 1 |
| **No documentation** | 6 (17.1) |

eTable 3. Baseline characteristics of ARDS patients (n=35) having lower (n=18) or higher (n=17) broncho-alveolar lavage fluid neutrophil extracellular traps concentrations within 72 hours of intubation

|  |  |  |  |
| --- | --- | --- | --- |
| Variables\* | **Lower BAL NETs****(n=18)** | **Higher BAL NETs****(n=17)** | **P value** |
| Age, years | 52.5±16.9 | 55.3±17.2 | 0.631 |
| Gender, male | 13 (72) | 11 (65) | 0.604 |
| ICU admission to intubation, days | 0 [0-1] | 0 [0-1] | 0.742 |
| **Comorbidities (%)** |
| Diabetes mellitus | 3 (17) | 5 (29) | 0.443 |
| COPD | 1 (5) | 3 (18) | 0.338 |
| Chronic heart failure | 2 (11) | 2 (12) | >0.99 |
| Obesity | 4 (22) | 2 (12) | 0.658 |
| Liver cirrhosis | 1(5) | 2 (12) | 0.603 |
| Sickle cell disease | 2 (11) | 2 (12) | >0.99 |
| Smoker | 9 (50) | 3 (18) | 0.075 |
| BMI, kg/cm2 | 30.0±8.9 | 27.8±4.7 | 0.369 |
| **Within 24 hours of study inclusion\*\* (moderate/severe ARDS criteria)** |
| SAPS II | 39±14 | 42±18 | 0.657 |
| SOFA | 8±3 | 8±4 | 0.991 |
| Temperature, Celsius degree | 38.6 [37.9-39.3] | 38.7 [38.0-40.0] | 0.518 |
| Shock | 4 (22) | 3 (18) | >0.99 |
| ARDS severity (Berlin classification)*moderate**severe* | 9 (50.0)9 (50.0) | 9 (52.9)8 (47.1) | >0.99 |
| Lung injury score | 2.9±0.7 | 2.6±0.3 | 0.081 |
| CRS, mL/cmH2O | 24 [19-26] | 25 [21-28] | 0.389 |
| Driving pressure, mmHg | 17.8±6.0 | 16.1±4.0 | 0.311 |
| PaO2/FiO2 ratio, mmHg | 99 [81-160] | 130 [69-186] | 0.654 |
| Tidal volume, mL/kg PBW | 6.1 [5.5-6.3] | 6.2 [5.9-7.1] | 0.131 |
| Plateau pressure, cmH2O | 28±6 | 25±3 | 0.152 |
| PEEP, cmH2O | 10 [8-15] | 10 [6-12] | 0.248 |
| Neuromuscular blocking agents | 17 (94) | 11 (65) | **0.041** |
| Prone position | 6 (33) | 6 (35) | >0.99 |
| Arterial lactate levels, mmol/L | 1.0 [0.8-2.9] | 1.5 [1.0-2.1] | 0.226 |
| Arterial pH | 7.35±0.10 | 7.36±0.12 | 0.943 |
| Arterial PCO2, mmHg | 47±9 | 43±9 | 0.195 |
| Serum creatinine, µmol/L | 84 [67-156] | 82 [68-142] | 0.961 |
| White blood cells, 103/mm3 | 13.2±8.8 | 14.7±7.5 | 0.592 |
| Blood neutrophils, 103/mm3 | 12.3±7.3 | 11.1±6.0 | 0.603 |
| Blood platelets, 103/mm3 | 207±94 | 257±138 | 0.212 |
| Blood hemoglobin level, g/dL | 11.6±2.7 | 10.3±1.5 | 0.106 |
| **Upon BAL 1 sampling** |
| Intubation to 1st BAL, days | 1 [1-1] | 1 [0-2] | 0.902 |
| SOFA | 8±4 | 8±3 | 0.952 |
| Temperature, Celsius degree | 38.5 [37.5-39.0] | 38.3 [37.9-39.7] | 0.831 |
| Shock | 4 (22) | 2 (12) | >0.99 |
| Microbiological documentation*Bacteria**Virus**Bacteria + virus**None* | 9 (50.0)6 (33.3)1 (5.5)2 (11.1) | 8 (47.0)5 (29.4)0 (0.0)4 (23.5) | 0.621 |
| Lung injury score | 2.7±0.5 | 2.4±0.5 | 0.076 |
| CRS, mL/cmH2O | 28 [24-37] | 32 [24-43] | 0.262 |
| Driving pressure, mmHg | 13.7±2.7 | 11.9±3.1 | 0.073 |
| PaO2/FiO2 ratio, mmHg | 142 [103-193] | 242 [149-350] | **0.006** |
| Tidal volume, mL/kg PBW | 5.8 [5.0-6.2] | 6.2 [5.9-6.7] | **0.021** |
| Plateau pressure, cmH2O | 24±3 | 23±4 | 0.434 |
| PEEP, cmH2O | 10 [8-14] | 12 [9-14] | 0.849 |
| Neuromuscular blocking agents | 13 (72.2) | 11 (64.7) | 0.725 |
| Prone position | 6 (33) | 8 (47) | 0.500 |
| Arterial lactate levels, mmol/L | 1.1 [0.8-2.3] | 1.4 [1.0-1.7] | 0.987 |
| Arterial pH | 7.37±0.11 | 7.38±0.08 | 0.796 |
| Arterial PCO2, mmHg | 49±13 | 42±7 | 0.132 |
| Serum creatinine, µmol/L | 105 [62-155] | 81 [62-225] | 0.902 |
| White blood cells, 103/mm3 | 11.7±7.8 | 15.6±8.2 | 0.150 |
| Blood neutrophils, 103/mm3 | 9.3±6.6 | 13.6±7.4 | 0.091 |
| Blood platelets, 103/mm3 | 193±104 | 240±113 | 0.116 |
| Shock dose steroids | 3 (17) | 1 (6) | >0.99 |

Continuous variables are presented as mean ± standard deviation when normally distributed and median [1st-3rd quartiles] otherwise; P values come from the unpaired t-test or the Mann-Whitney test, as appropriate; Categorical variables are shown as n (%); P values come from the chi2 or the Fisher exact test, as appropriate; \* None of the data pertaining to this table was missing; \*\* Worst values recorded; ARDS, acute respiratory distress syndrome; BAL: bronchoalveolar lavage fluid; NETs, neutrophil extracellular traps; ICU, intensive care unit; COPD, chronic obstructive pulmonary disease; BMI, body mass index; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Assessment of Organ Failures; PEEP, positive end-expiratory pressure; PBW, predicted body weight;

**eTable 4. Measurement of neutrophil extracellular traps concentrationsin the alveolar and blood compartments in ARDS patients (n=35) having lower (n=18) or higher (n=17) broncho-alveolar lavage fluid neutrophil extracellular traps concentrations within 72 hours of intubation**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Lower BAL NETs****(n=18)** | **Higher BAL NETs****(n=17)** | **P value** |
| **Alveolar compartment** |
| BAL Fluid NETs levels, AU | 74 [52-104] | 1000 [533-1000] | **<0.0001** |
| **Blood compartment** |
| Serum NETs levels\*, AU | 25 [4-58] | 30 [8-93] | 0.39 |

AU: arbitrary units, BAL: bronchoalveolar lavage fluid; NETs, neutrophil extracellular traps; \* Serum NETs levels value was missing for one patient; P values come from the Mann-Whitney test.

eTable 5. Correlation matrix exploring the relationship between serum neutrophil extracellular traps concentrations, serum cytokines, and serum alveolar epithelium injury biomarkers levels.

|  |
| --- |
|  |
| **BAL biomarkers**  | **NETs (o.d.)** | **IL-6** | **IL-8** | **IL-10** | **TNF-α** | **SP-D** | **RAGE** |
|  | **NETs (o.d.)** | Rho | 1.000 | 0.151 | -0.126 | -0.135 | -0.001 | 0.167 | 0.005 |
| P value | - | 0.402 | 0.486 | 0.452 | 0.996 | 0.352 | 0.980 |
| N | 34 | 33 | 33 | 33 | 33 | 33 | 33 |
| **IL-6** | Rho |  | 1.000 | **0.722** | **0.495** | **0.485** | **-0.472** | 0.239 |
| P value |  | - | **<0.0001** | **0.003** | **0.004** | **0.005** | 0.173 |
| N |  | 34 | 34 | 34 | 34 | 34 | 34 |
| **IL-8** | Rho |  |  | 1.000 | **0.364** | **0.405** | -0.212 | **0.425** |
| P value |  |  | - | **0.034** | **0.017** | 0.229 | **0.012** |
| N |  |  | 34 | 34 | 34 | 34 | 34 |
| **IL-10** | Rho |  |  |  | 1.000 | **0.658** | **-0.527** | **0.349** |
| P value |  |  |  | - | **<0.0001** | **0.001** | **0.043** |
| N |  |  |  | 34 | 34 | 34 | 34 |
| **TNF-α** | Rho |  |  |  |  | 1.000 | **-0.456** | **0.511** |
| P value |  |  |  |  | - | **0.007** | **0.002** |
| N |  |  |  |  | 34 | 34 | 34 |
| **SP-D** | Rho |  |  |  |  |  | 1.000 | **-0.099** |
| P value |  |  |  |  |  | - | **0.577** |
| N |  |  |  |  |  | 34 | 34 |
| **RAGE** | Rho |  |  |  |  |  |  | 1.000 |
| P value |  |  |  |  |  |  | - |
| N |  |  |  |  |  |  | 34 |

P values and correlation coefficients (rho) were computed using the Spearman’s test; NETs, neutrophil extracellular traps; NETs concentrations were expressed in optical densities (o.d.); N indicates the number of samples available for each biomarker.

eTable 6. Exploratory uni- and multiple regression analysis using live ventilator-free days at day 28 as the dependent variable.

|  |  |  |
| --- | --- | --- |
|  | **Univariable analysis** | **Multivariable analysis** |
| **Independent variable** | **β coefficient** | **95% CI of the β coefficient** | **P value** | **β coefficient** | **95% CI of the β coefficient** | **P value** |
| NET BAL levels (o.d.) | 3.70 | -0.87 ; 8.30 | 0.084 | 2.40 | -2.13 ; 6.92 | 0.288 |
| PaO2/FiO2 ratio \*  | 0.03 | -0.01 ; 0.06 | 0.097 | 0.01 | -0.33 ; 0.36 | 0.934 |
| Tidal volume \*  | 3.66 | 1.24 ; 6.09 | **0.004** | 3.31 | 0.48 ; 6.13 | **0.023** |

\* Upon BAL 1 collection; CI, confidence interval; p values come from uni- and multiple linear regression

**SUPPLEMENTAL FIGURES**

**eFigure 1. Flow chart of patients with pneumonia-related moderate/severe acute respiratory distress syndrome (ARDS) included in the study.** \* Withholding/Withdrawal of life-sustaining therapies; BAL, broncho-alveolar lavage; NETs, neutrophil extracellular traps; \*\* Reasons for not performing the second BAL included deceased patients (n=5), extubated patients (n=10) or patient mechanically ventilated but not meeting ARDS criteria at day 5-7 (n=2).



**eFigure 2. Linear regression of BAL fluid neutrophil extracellular traps (NETs, DNA-MPO) levels on alveolar macrophages.** There was a significant invert relationship between BAL fluid NETs, expressed as optical density (o.d.) values and alveolar macrophages, as a percentage of total BAL cell numbers (y=-0.016x + 2.027; r2=0.26; p<0.0001), from ARDS patients sampled at days 1-2 (n=35; red circles) and days 5-7 (n=18; blue circles) and controls (n=4; black circles). The grayed area comprised between the dashed curves depicts the 95% confidence interval of the regression line.

