Angiopoietin-2, permeability oedema, occurrence and severity of ALI/ARDS in septic and non-septic critically ill patients

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ABSTRACT
Background: Angiopoietin-2 and vascular endothelial growth factor (VEGF) may impair vascular barrier function while angiopoietin-1 may protect it. It was hypothesised that circulating angiopoietin-2 is associated with pulmonary permeability oedema and severity of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) during septic or non-septic critical illness.

Methods: Plasma levels of angiopoietin-1 and angiopoietin-2 were measured in mechanically ventilated patients (24 with sepsis, 88 without sepsis), together with the pulmonary leak index (PLI) for 67-gallium-labelled transferrin and extravascular lung water (EVLW) by transpulmonary thermal-dye dilution as measures of pulmonary permeability and oedema, respectively. ALI/ARDS was characterised by consensus criteria and the lung injury score (LIS). Plasma VEGF and von Willebrand factor (VWF) levels were assayed.

Results: Angiopoietin-2, VWF, PLI, EVLW and LIS were higher in patients with sepsis than in those without sepsis and higher in patients with ALI/ARDS (n = 10/12 in sepsis, n = 19/8 in non-sepsis) than in those without. VEGF was also higher in patients with sepsis than in those without. Patients with high PLI, regardless of EVLW, had higher angiopoietin-2 levels than patients with normal PLI and EVLW. Angiopoietin-2 correlated with the PLI, LIS and VWF levels (minimum r = 0.34, p < 0.001) but not with EVLW. Angiopoietin-2 and VWF were predictive for ARDS in receiver operating characteristic curves (minimum area under the curve = 0.69, p = 0.006). Angiopoietin-1 and VEGF did not relate to the permeability oedema of ALI/ARDS.

Conclusion: Circulating angiopoietin-2 is associated with pulmonary permeability oedema, occurrence and severity of ALI/ARDS in patients with and without sepsis. The correlation of angiopoietin-2 with VWF suggests activated endothelium as a common source.

Sepsis and major surgery are important risk factors for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).1 These syndromes are characterised by increased pulmonary permeability oedema,1 the cause of which is still incompletely understood. Novel mediators that may be involved include vascular endothelial growth factor (VEGF) and the angiopoietins.2–15

In experimental models, administration of angiopoietin (Ang)-2 can provoke microvascular leakage in the lung and in other organs;2–7 while Ang-1 can protect against microvascular leakage induced by VEGF, Ang-2 or inflammatory agents.7–9 Ang-2 is released from endothelium,11 16 while Ang-1 is produced by various other tissue cells.11 Both Ang-1 and Ang-2 bind to the Tie2 receptor which is abundantly present in lung endothelium.15 Ang-1-induced activation of the Tie2 receptor enforces endothelial barrier function via activation of Rac1, inhibition of RhoA and consequently organisation of the cytoskeleton into a junction-fortifying arrangement,15 inhibition of VEGF-induced calcium influx15 and reduced VEGF-stimulated leucocyte adhesion.35 In contrast, Ang-2 counteracts Tie2 receptor activation in many studies, although there are some exceptions.2,11,21,22 This results in impairment of endothelial barrier function23 and an increase in the adhesion and migration of inflammatory cells.2,25

In patients, circulating Ang-2, VEGF and von Willebrand factor (VWF) levels are increased during ALI/ARDS or sepsis.2,4–6,10,12–15,24,25 A high Ang-2 level correlated with impaired pulmonary gas exchange.2 Plasma VEGF levels correlated in some (but not all) studies with surrogate indicators of systemic permeability.13–15 However, a direct correlation between Ang-2 or VEGF levels and pulmonary permeability oedema in ALI/ARDS of septic or non-septic origin has not yet been demonstrated. VWF is a marker of endothelial activation and injury and is associated with the development and clinical outcomes of ALI/ARDS.24,25 Although it may not be directly involved in permeability, Ang-2 and VWF reside in the same secretory organelle of endothelial cells—namely, the Weibel Palade body—and may be released together after activation of the endothelium.16

Radionuclide techniques26,27 such as the pulmonary leak index (PLI) which reflects pulmonary permeability can be used to differentiate between hydrostatic and permeability oedema.26 The PLI represents the transvascular transport rate of 67-gallium (67Ga)-labelled transferrin and predicts and tracks the clinical course of ALI/ARDS.26 Oedema is reflected by extravascular lung water (EVLW), which can be determined by transpulmonary thermal-dye dilution.26–31

We hypothesised that circulating Ang-2 levels are associated with (1) pulmonary permeability oedema, (2) occurrence and severity of ALI/ARDS, and that (3) Ang-2 and VWF levels are raised simultaneously. To test these hypotheses we measured the Ang-1, Ang-2, VEGF and VWF levels in plasma together with the PLI, EVLW and the lung injury score (LIS) in critically ill, mechanically ventilated patients with or without sepsis, with or at risk of ALI/ARDS.

METHODS

This prospective observational study, approved by the ethical committee of the VU University Medical Centre, Amsterdam, The Netherlands, involved 112 consecutive critically ill patients with or at risk of ALI/ARDS (24 with sepsis and 88 without sepsis). Patients were taking part in a prospective randomised clinical trial on the effect of resuscitation with various fluids in predefined and stratified groups of mechanically ventilated patients with and without sepsis in the intensive care unit (ICU). The data of this trial in cardiovascular surgery have been published elsewhere. The inclusion criteria, judged when the patient was enrolled, were absence of overhydration, defined as a pulmonary capillary wedge pressure (PCWP) < 15 mm Hg in the presence of a pulmonary artery catheter (n = 38) and proper wedging, or a central venous pressure (CVP) < 12 mm Hg at positive end-expiratory pressure (PEEP) < 15 cm H_2O and < 16 mm Hg when PEEP > 15 cm H_2O in the presence of a central venous catheter (n = 74), since PEEP elevates atmospheric pressure referenced intrathoracic filling pressures, and a systolic arterial pressure < 110 mm Hg in the absence of vasopressor therapy. Exclusion criteria were age > 78 years, pregnancy, known anaphylactoid reaction to colloid fluids and a life expectancy < 24 h.

Sepsis was defined by two or more of the following: abnormal body temperature (≥ 38°C, < 36°C), tachycardia (≥ 90/min), tachypnoea (≥ 20/min or arterial carbon dioxide tension (PaCO_2) ≤ 32 mm Hg), abnormal white blood cell counts (< 4 or > 12 x 10^9/l) and a microbiologically proven or clinically evident source of infection. ICU-acquired sepsis was defined as sepsis developing after 2 days in the ICU. The origin of the sepsis was defined by clinical signs and symptoms and positive local and/or blood cultures. Non-sepsis involved cardiac surgery, major vascular surgery, other major surgery and gastrointestinal bleeding. Cardiac surgery was defined as surgery for coronary artery bypass grafting (n = 32), aortic valve replacement (n = 8) or atrial septal defect (n = 2). Major vascular surgery was defined as surgery for thoracoabdominal aortic (n = 4), abdominal aortic (n = 18) or mesenteric vascular disease (n = 4). Other major surgery involved abdominal surgery (n = 14), neurosurgery (n = 4) and surgery for polytrauma (injury severity score > 15, n = 5). Patients were pressure-controlled (during sepsis) or volume-controlled (during non-sepsis) ventilated with an Evita 3 ventilator (Dräger; Lübeck, Germany). Patients with ALI/ARDS were pressure-controlled ventilated with a tidal volume (VT) aiming not to exceed 8 ml/kg and resulting in an end-tidal CO_2 concentration between 4% and 5% using an oxygen-air mixture with an inspiratory oxygen fraction (FiO_2) of 40% and a PEEP of 5 cm H_2O (inspiration:expiration 1:2) or more when physicians not involved in the study according to institutional committee of the VU University Medical Centre, Amsterdam, The Netherlands, involved 112 consecutive critically ill patients with or at risk of ALI/ARDS (24 with sepsis and 88 without sepsis). Patients were taking part in a prospective randomised clinical trial on the effect of resuscitation with various fluids in predefined and stratified groups of mechanically ventilated patients with and without sepsis in the intensive care unit (ICU). The data of this trial in cardiovascular surgery have been published elsewhere. The inclusion criteria, judged when the patient was enrolled, were absence of overhydration, defined as a pulmonary capillary wedge pressure (PCWP) < 15 mm Hg in the presence of a pulmonary artery catheter (n = 38) and proper wedging, or a central venous pressure (CVP) < 12 mm Hg at positive end-expiratory pressure (PEEP) < 15 cm H_2O and < 16 mm Hg when PEEP > 15 cm H_2O in the presence of a central venous catheter (n = 74), since PEEP elevates atmospheric pressure referenced intrathoracic filling pressures, and a systolic arterial pressure < 110 mm Hg in the absence of vasopressor therapy. Exclusion criteria were age > 78 years, pregnancy, known anaphylactoid reaction to colloid fluids and a life expectancy < 24 h.

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We characterised pulmonary dysfunction by the American-European Consensus Conference (AECC) criteria and the LIS, which is common practice, since the definition for ALI/ARDS is not unequivocal. According to AECC criteria, ALI and ARDS are characterised by PaCO_2/FiO_2 < 300 or < 200 mm Hg, respectively (regardless of PEEP), bilateral infiltrations on the frontal chest radiograph and PCWP < 18 mm Hg or no clinical evidence of left atrial hypertension and congestive heart failure. The LIS ranges from 0 to 4, with values ≥ 1 and < 2.5, and ≥ 2.5 used as cut-off values for ALI and ARDS, respectively. The LIS was also used to characterise the severity of the syndromes.

Study protocol

Patients were included within 3 h after major surgery or gastrointestinal bleeding and 12 h after meeting sepsis criteria. Demographic and clinical data were recorded, including the Acute Physiology and Chronic Health Evaluation (APACHE) II score. The FiO_2, VT, plateau inspiratory pressure and PEEP were taken from the ventilator. Total respiratory dynamic compliance was calculated from VT/(plateau pressure-PEEP). Arterial blood samples were obtained for determination of PaO_2, PaCO_2 and oxygen saturations (Rapidlab 865, Bayer Diagnostics, Tarrytown, New York, USA) at 37°C. Mixed or central venous blood, when a pulmonary artery catheter was in place, was taken simultaneously for measurement of gas pressures and saturations. Venous admixture was calculated according to standard formulae, with central venous substituting for mixed venous blood if unavailable. At the time of measurements, we also recorded central venous pressure and inotropic/vasopressor treatment. Plasma samples were collected and stored at −80°C until assayed.

A mobile probe system was used at the bedside to measure the PLI of 67Ga-transferin protein as a measure of pulmonary permeability (n = 108), as previously described.25 26 28 33 Technical problems precluded the PLI measurement in four patients. The reproducibility is within 10%.26 36 39 In brief, autologous erythrocytes were labelled with 99mTc-Nanocolloid (99mTc, 11 MBq, physical half-life 6 h, Mallinkrodt Diagnostica, Petten, The Netherlands). Transferin was labelled in vivo following intravenous injection of 67Ga-citrate (4.5 MBq, physical half-life 78 h; Mallinkrodt Diagnostica). Patients were in the supine position and two scintillation detection probes (Eurorad CTT, Strasbourg, France) were positioned over the right and left lung apices. Starting at the time of 67Ga-citrate injection, radioactivity was detected during 30 min and blood samples (2 ml aliquots) were taken every 4 min for 30 min. The 99mTc and 67Ga counts were corrected for background radioactivity, physical half-life, spill-over and expressed as counts per minute (CPM) per lung field. Each blood sample was weighted and 99mTc and 67Ga counts were determined with a single-well well counter (LKB Wallac 1480 WIZARD, Perkin Elmer, Life Science, Zaventem, Belgium), corrected for background radioactivity, physical half-life and spill-over. The results were expressed as CPM/g. For each blood sample a time-matched CFM per lung field was taken. A radioactivity ratio was calculated (67Ga lung/99mTc lung)/(67Ga blood/99mTc blood) and plotted against time. The PLI was calculated from the slope of the increase of radioactivity ratio divided by the intercept to correct for physical factors in radioactivity detection. The values for both lung fields were averaged. We chose PLI ≥ 14.7 x 10^-3/min and ≥ 50.0 x 10^-3/min as cut-off values for ALI and ARDS, respectively, since the former is the upper limit of normal and PLI is typically raised twofold or more in ARDS.26 35

In order to judge oedema, EVLW was measured with the help of transpulmonary thermal-dye dilution (n = 94), as previously described.28 31 34 36 The reproducibility of EVLW measurements is within 10%.26

An anteroposterior chest radiograph was obtained with patients in the supine position in order to calculate the LIS.26 35 The duration of mechanical ventilation was defined as the interval from intubation to extubation. Patients were followed until death or discharge from the ICU to record the ICU mortality rate.
Assays
Ang-1 and Ang-2 were measured in duplicate in ethylenediaminetetraacetic acid (EDTA) plasma samples from 112 patients and 15 healthy volunteers (controls, mean age 56 years (range 45–75), 12 men, 5 women) using the human Ang-1 and Ang-2 DuoSet ELISA Development kits (R&D Systems, Minneapolis, Minnesota, USA). TheAng-2/Ang-1 ratio was calculated. VEGF was measured in duplicate in EDTA plasma samples from all patients with sepsis, 12 without sepsis (four cardiac surgery, four major vascular surgery and four other major surgery patients were randomly chosen) and four healthy volunteers (randomly chosen) using the human VEGF Quantikine ELISA kit (R&D Systems).

VWF was measured in duplicate in EDTA plasma samples from 112 patients and 15 healthy volunteers using rabbit anti-human VWF polyclonal antiserum and rabbit anti-human VWF peroxidase conjugate (Dako Netherlands BV, Heverlee, Belgium). VWF is expressed as the percentage of a pooled plasma control reference which is standardised against the 1st International VWF Standard. The detection limits for Ang-1, Ang-2 and VEGF were 0.02 ng/ml, 0.03 ng/ml and 3.9 pg/ml, respectively. The intra-assay coefficients of variation were <5%, <5% and <7% for Ang-1, Ang-2, VEGF and VWF, respectively. The inter-assay coefficients of variation were <7%, <5%, <7% and <8% for Ang-1, Ang-2, VEGF and VWF, respectively. The intra-assay coefficients of variation were <5%, <8% and <8% for Ang-1, Ang-2 and VWF, respectively.

Statistical analysis
All data except the APACHE II scores, tidal volumes, PaO2/FIO2 ratios and VWF levels were non-Gaussian distributed. To obtain Gaussian distributions, data were logarithmically transformed before the statistical analysis. The data were then transformed back to the original scale. One-way analysis of variance (ANOVA) was used to evaluate whether continuous variables were different and to evaluate whether the predictor variables Ang-2 and VEGF correlated with the outcome variable PLI. Areas under the receiver operating characteristic (ROC) curve (AUC) (95% CI) were calculated to evaluate the predictive values of the variables. The χ2 test or Fisher exact test were used to compare categorical variables. A p value of <0.05 was considered statistically significant. Data are expressed as mean (95% CI) or as number of patients (percentage). Differences are expressed as arithmetic mean difference (95% CI), p value (Gaussian distributed data) or geometric mean ratio (95% CI), p value (non-Gaussian distributed data). Exact p values are given if p>0.001.

Table 1  Respiratory characteristics and mediators in patients with and without sepsis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sepsis (n = 24)</th>
<th>Non-sepsis (n = 88)</th>
<th>Mean difference or ratio (95% CI), p value</th>
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<tbody>
<tr>
<td><strong>Respiratory characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALI*</td>
<td>10 (42)</td>
<td>19 (22)</td>
<td>61 (46 to 76), &lt;0.001</td>
</tr>
<tr>
<td>ARDS*</td>
<td>12 (50)</td>
<td>8 (9)</td>
<td></td>
</tr>
<tr>
<td>LIS†</td>
<td>2.14 (1.04 to 4.41)</td>
<td>0.93 (0.28 to 3.05)</td>
<td>2.30 (1.89 to 2.79), &lt;0.001</td>
</tr>
<tr>
<td>PaO2/FIO2 (mm Hg)*</td>
<td>207 (104 to 311)</td>
<td>310 (88 to 533)</td>
<td>103 (71 to 135), &lt;0.001</td>
</tr>
<tr>
<td>PLI (&gt;10 ml/kg)*</td>
<td>59 (15 to 228)</td>
<td>20 (5 to 87)</td>
<td>2.83 (2.08 to 4.01), &lt;0.001</td>
</tr>
<tr>
<td>EVLW (ml/kg)*</td>
<td>4.1 (0.6 to 27.3)</td>
<td>17 (9)</td>
<td>68 (53 to 84), &lt;0.001</td>
</tr>
<tr>
<td>VWF (% of reference)*</td>
<td>8.6 (2.3 to 31.7)</td>
<td>6.1 (2.6 to 14.2)</td>
<td>1.41 (1.04 to 1.91), 0.027</td>
</tr>
<tr>
<td>Duration of mechanical ventilation (h)†</td>
<td>281 (50 to 1578)</td>
<td>19 (1 to 295)</td>
<td>14.44 (7.90 to 26.41), &lt;0.001</td>
</tr>
<tr>
<td><strong>Mediators</strong></td>
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<tr>
<td>Ang-1 (ng/ml)</td>
<td>0.5 (0.0 to 58.1)</td>
<td>0.4 (0.0 to 32.4)</td>
<td>1.17 (0.42 to 3.30), 0.759</td>
</tr>
<tr>
<td>Ang-2 (ng/ml)</td>
<td>4.1 (0.6 to 27.3)</td>
<td>0.4 (0.0 to 10.4)</td>
<td>10.66 (6.31 to 18.03), &lt;0.001</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>63.6 (5.0 to 807.2), n = 24</td>
<td>20.3 (2.7 to 160.1), n = 12</td>
<td>3.07 (1.30 to 7.24), 0.012</td>
</tr>
<tr>
<td>VWF (% of reference)</td>
<td>271 (124 to 418)</td>
<td>130 (1 to 258)</td>
<td>141 (111 to 172), &lt;0.001</td>
</tr>
</tbody>
</table>

Variables are expressed as arithmetic or geometric mean† (95% confidence interval (CI)) or as number of patients (percentage) with arithmetic mean difference or geometric mean ratio (95% CI), p value. Control levels of mediators were mean (95% CI) 1.8 (0.3 to 11.3) ng/ml Ang-1, 0.1 (0.0 to 1.2) ng/ml Ang-2, 13.6 (5.0 to 36.9) pg/ml VEGF, 95% (62 to 128) of reference VWF.

RESULTS
Characteristics of patients with and without sepsis
Patients with and without sepsis were comparable with respect to age and sex (see table 1A in online supplement). The APACHE II scores, use of vasopressor/inotropic therapy and the mortality rates were higher in patients with sepsis than in non-septic patients. Patients with sepsis also had a higher occurrence of ALI/ARDS, higher LIS, lower PaO2/FIO2 ratio, higher PLI and EVLW, more often a PLI >30.0 x 10^{-3}/min and EVLW >10 ml/kg, and longer duration of mechanical ventilation (table 1).

Mediators in controls and patients with and without sepsis
Ang-1 levels were lower in patients than in controls (1.8 (95% CI 0.3 to 11.3) ng/ml Ang-1 in controls; geometric mean ratio 4.55 (95% CI 2.40 to 8.64), p<0.001) and comparable between patients with and without sepsis. Ang-2 and VWF levels were

EVLW, as determined by PLI <14.7 x 10^{-3}/min to PLI >=30.0 x 10^{-3}/min, and by PLI <30.0 x 10^{-3}/min and EVLW <10 ml/kg to PLI >=30.0 x 10^{-3}/min and EVLW >10 ml/kg, respectively. If the one-way ANOVA indicated significance, the Student t test was used to further evaluate differences. The Bonferroni Holm method was used to adjust for multiple testing. The Pearson correlation coefficient (r) (95% confidence interval (CI)) was used to express relations. Standard multiple linear regression was performed to evaluate whether the predictor variables Ang-2 and VEGF correlated with the outcome variable PLI. Areas under the receiver operating characteristic (ROC) curve (AUC) (95% CI) were calculated to evaluate the predictive values of the variables. The χ2 test or Fisher exact test were used to compare categorical variables. A p value of <0.05 was considered statistically significant. Data are expressed as mean (95% CI) or as number of patients (percentage). Differences are expressed as arithmetic mean difference (95% CI), p value (Gaussian distributed data) or geometric mean ratio (95% CI), p value (non-Gaussian distributed data). Exact p values are given if p>0.001.
Figure 1  Ang-2 (ng/ml) in controls and patients with non-acute lung injury (ALI), ALI or acute respiratory distress syndrome (ARDS) according to (A) AECC or (B) LIS criteria, (C) in patients grouped according to pulmonary lung index (PLI), or (D) the combination of PLI and extravascular lung water (EVLW). Bars represent mean, whiskers represent standard error of the mean, *significant vs (A) control, (B) non-ALI, (C) PLI <14.7 x 10^{-3}/min, or (D) PLI >30.0 x 10^{-3}/min and EVLW <10 ml/kg; †significant vs (A) non-ALI, (B) ALI or (C) PLI >14.7 and <30.0 x 10^{-3}/min. Differences between means are expressed as mean ratio (95% confidence interval), p value. (A) For non-ALI, ALI and ARDS vs controls: 0.28 (0.11 to 0.72), p = 0.009; 0.07 (0.03 to 0.19), p < 0.001; 0.08 (0.03 to 0.23), p < 0.001, respectively. For ALI and ARDS vs non-ALI: 0.26 (0.12 to 0.55), p = 0.001; 0.28 (0.12 to 0.67), p = 0.005, respectively. For ARDS vs ALI: 1.08 (0.40 to 2.90), p = 0.873. (B) For ALI (lung injury score (LIS) >1 and <2.5) and ARDS (LIS >2.5) vs non-ALI (LIS <1): 0.44 (0.21 to 0.89), p = 0.023; 0.09 (0.05 to 0.17), p < 0.001, respectively. For ARDS vs ALI: 0.21 (0.11 to 0.39), p < 0.001. (C) For PLI >30.0 x 10^{-3}/min vs PLI >14.7 and <30.0 x 10^{-3}/min and PLI <14.7 x 10^{-3}/min: 0.23 (0.10 to 0.52), p = 0.001; 0.28 (0.12 to 0.62), p = 0.002, respectively. For PLI >14.7 and <30.0 x 10^{-3}/min vs PLI >14.7 x 10^{-3}/min: 1.23 (0.55 to 2.73), p = 0.607. (D) For PLI >30.0 x 10^{-3}/min and EVLW >10 ml/kg, PLI >30.0 x 10^{-3}/min and EVLW <10 ml/kg and PLI <30.0 x 10^{-3}/min and EVLW >10 ml/kg vs PLI <30.0 x 10^{-3}/min and EVLW <10 ml/kg: 0.18 (0.06 to 0.57), p = 0.004; 0.18 (0.08 to 0.41), p < 0.001; 0.51 (0.15 to 1.82), p = 0.286. For PLI >30.0 x 10^{-3}/min and EVLW >10 ml/kg and PLI >30.0 x 10^{-3}/min and EVLW <10 ml/kg vs PLI <30.0 x 10^{-3}/min and EVLW >10 ml/kg: 0.36 (0.05 to 2.39), p = 0.269; 0.35 (0.08 to 1.54), p = 0.159. For PLI >30.0 x 10^{-3}/min and EVLW >10 ml/kg vs PLI >30.0 x 10^{-3}/min and EVLW <10 ml/kg: 1.02 (0.27 to 3.84), p = 0.972.

**Acute lung injury**

**Characteristics of non-ALI, ALI and ARDS patients according to the AECC criteria**

Patients with ALI and ARDS had higher plateau pressure, lower total respiratory dynamic compliance, lower PaO2, higher FiO2, lower PaO2/FiO2 ratio, higher LIS and longer duration of mechanical ventilation than non-ALI patients. The PLI was higher in ARDS than in non-ALI patients. The percentage of patients with PLI >30.0 x 10^{-3}/min and EVLW >10 ml/kg increased from non-ALI to ALI and ARDS (p = 0.048 and p = 0.050, respectively, χ² test). Patients with ALI and ARDS also had higher venous admixture and higher CVP associated with higher PEEP levels than non-ALI patients. Patients with ARDS had lower PaO2, higher FiO2, lower PaO2/FiO2 ratio, higher LIS and higher venous admixture than patients with ALI (table 2 and online table 2A).

**Mediators in patients with ALI and ARDS and in non-ALI patients**

Ang-1 levels did not differ between non-ALI, ALI and ARDS patients according to AECC criteria (table 2 and online table 2A). Ang-2 levels and Ang-2/Ang-1 ratios were higher in patients with ALI and ARDS than in non-ALI patients. The Ang-2 level showed a per-category trend from control to ARDS (p < 0.001) with a mean increase of 1.52 ng/ml Ang-2 per category (fig 1A and online fig 2A). Ang-2 levels were also higher in patients with ALI and ARDS than in non-ALI patients and higher in patients with ARDS than in those with ALI according to the LIS criteria. Furthermore, the Ang-2 level showed a per-category trend from non-ALI to ARDS according to the LIS criteria (p < 0.001) with a mean increase of 0.52 ng/ml Ang-2 per category (fig 1B). VEGF levels tended to be higher in patients with ARDS than in those with ALI.

Plots A and D show a per-category trend from control to ARDS (p < 0.001) with a mean increase of 0.39 ng/ml Ang-2 per category (fig 1A and online fig 2A).
with ALI and ARDS than in non-ALI patients (geometric mean ratio 0.10 (95% CI 0.18 to 1.28), p = 0.137 for non-ALI vs ALI/ARDS). VWF levels were higher in patients with ALI and ARDS than in non-ALI patients (table 2 and online table 2A).

### Mediators, pulmonary permeability oedema and severity of ALI/ARDS

Ang-2 levels increased in parallel with a rise in PLI and showed a per-category trend from PLI <14.7×10^{-3}min to PLI >30.0×10^{-3}min (p = 0.002) with a mean increase of 0.57 ng/ml per category (fig 1C). Ang-1 levels did not correlate with the PLI in the smaller group of 36 patients in whom VEGF was measured (r = 0.44 (95% CI 0.13 to 0.67), p = 0.008), while VEGF did not (r = 0.20 (95% CI 0.13 to 0.35), p = 0.253). Indeed, multiple linear regression analysis with PLI as outcome and Ang-2 and VEGF as predictor variables showed that Ang-2 predicted PLI (regression coefficient = 0.41 (95% CI 0.30 to 0.52), p = 0.001) while VEGF did not (regression coefficient = 0.12 (95% CI 0.10 to 0.24), p = 0.682).

To further study clinical significance we evaluated whether Ang-1, Ang-2, VEGF and VWF levels correlated with the LIS, PaO2/FIO2 ratios, compliance, duration of mechanical ventilation and ICU mortality. Ang-2, VEGF and VWF levels positively correlated with the LIS (r = 0.43 (95% CI 0.26 to 0.57), p<0.001; r = 0.33 (95% CI 0.00 to 0.60), p = 0.049 and r = 0.55 (95% CI 0.39 to 0.66), p<0.001, respectively). Ang-2 and VWF inversely correlated with PaO2/FIO2 ratios (r = 0.22 (95% CI 0.05 to 0.39), p = 0.022 and r = 0.43 (95% CI 0.26 to 0.67), p<0.001, respectively). Ang-2, VEGF and VWF inversely correlated with compliance (r = 0.44 (95% CI 0.28 to 0.58), p<0.001; r = 0.40 (95% CI 0.08 to 0.65), p = 0.017 and r = 0.46 (95% CI 0.50 to 0.60), p<0.001, respectively). Ang-2 and VWF levels were higher in non-survivors than in survivors (geometric mean ratio 0.15 (95% CI 0.07 to 0.36), p = 0.006, respectively). Ang-2 levels correlated with Ang-1 and VWF levels (r = 0.29 (95% CI 0.11 to 0.45), p = 0.002 and r = 0.46 (95% CI 0.29 to 0.59), p<0.001, respectively).

#### Predictive value of Ang-2 levels for permeability oedema and ARDS

The ROC curves for ARDS versus ALI and non-ALI characterised by the AECC criteria (online fig 5A) and the LIS criteria
Severity in their study, suggested by higher APACHE II scores. This discrepancy may be explained by a higher disease severity, which antagonises the protective role of Ang-1, is one of the mediators involved in the increase in pulmonary permeability leading to ALI/ARDS. Moreover, Ang-2 was specifically associated with pulmonary permeability as it correlated with the PLI and not with the EVLW. Ang-2 levels also related to the severity of ALI/ARDS as reflected by the LIS and by the predictive value for ARDS in the ROC curve. These associations suggest a contributory role of Ang-2 in the pathogenesis of ALI/ARDS, but further studies (including serial plasma and alveolar compartment measurements of Ang-2 and blocking studies) are necessary to confirm this.

The Ang-2 levels in our patients with ARDS are comparable to those reported in severe sepsis and septic shock by Orfanos et al and mild sepsis in the study of Parikh et al. However, Parikh et al reported higher Ang-2 levels in their patients with severe sepsis. This discrepancy may be explained by a higher disease severity in their study, suggested by higher APACHE II scores. Our study extends the observations of Parikh et al by showing that circulating Ang-2 is associated with the independently measured pulmonary permeability oedema and severity of ALI/ARDS, regardless of the origin.

VEGF levels were higher in patients with sepsis than in those without sepsis and tended to be higher in patients with ALI and ARDS than in non-ALI patients, in agreement with the literature. We could not establish an association between circulating VEGF and directly measured pulmonary permeability, which agrees with some but not all studies using surrogate indicators of systemic permeability. This suggests that circulating Ang-2 may better reflect pulmonary permeability oedema of ALI/ARDS than circulating VEGF.

Ang-2 and VWF were simultaneously enhanced in the plasma of patients with ALI/ARDS or sepsis. Although a common effect on their clearance cannot yet be excluded, it is likely that both are released together from the Weibel Palade body after activation of the endothelium. This may explain why VWF is a marker of endothelial activation and injury resulting in an association with pulmonary permeability, occurrence and severity of ALI/ARDS and subsequent ICU mortality, while it may not be directly involved in permeability.

Our study has some limitations. We cannot exclude the possibility that the relatively high tidal volumes used in some of our patients potentially harmed the lungs during ALI/ARDS. Furthermore, it is uncertain whether the source of Ang-2 is the systemic or pulmonary endothelium. If it is true that Ang-2 mainly acts in an autocrine manner, a pulmonary source is favoured. Finally, the observation that some patients had high PLI and a normal EVLW can be explained in part by inaccessibility of the thermal indicator to injured lung areas, thereby underestimating lung oedema, or to compensatory Starling forces and increased lymph flow, removing excess EVLW in spite of increased permeability.

In conclusion, Ang-2 contributes to the pulmonary permeability oedema of ALI/ARDS in patients with and without sepsis, with or at risk of these syndromes. VWF and Ang-2 may be simultaneously released from the endothelium, thereby explaining the previously reported marker function of VWF for endothelial activation associated with ALI/ARDS.

**REFERENCES**