Sphingosine Kinase 1 Regulates Inflammation and Contributes to Acute Lung Injury in Pneumococcal Pneumonia via the Sphingosine-1-Phosphate Receptor 2

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Prognostic and pathogenetic role of Angiopoietin-1 and -2 in pneumonia

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At a Glance Commentary

Scientific Knowledge on the Subject:

Pneumonia may evoke disruption of pulmonary endothelial barrier integrity resulting in acute lung injury despite antibiotic therapy. Treatment of acute lung injury is mainly supportive, since key elements of inflammation-induced barrier disruption remain undetermined. The Angiopoietins (Ang)-1 and -2 and their receptor Tie2 are involved in the regulation of vascular permeability and inflammation, but their role in pneumonia is unknown.

What This Study Adds to the Field:

Barrier-stabilizing Ang-1 decreased, and its counterpart Ang-2 increased in serum of pneumonia patients. Ang-2 serum levels predicted mortality and length of hospital stay in pneumonia patients, improving predictive value of CURB-65 score. In mice, pulmonary Ang-1 decreased and Ang-2 increased, with Ang-2 contributing to lung barrier dysfunction in pneumococcal pneumonia. Ang-1 therapy reduced lung permeability and inflammation.

Targeting the Ang-/Tie2-system may provide a therapeutic perspective for prevention of acute lung injury in pneumonia.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: During pneumonia, pathogen-host interaction evokes inflammation and lung barrier dysfunction. Tie2-activation by Angiopoietin-1 reduced, while Tie2-blockade by Angiopoietin-2 increased inflammation and permeability during sepsis. The role of Angiopoietin-1/-2 in pneumonia remains unidentified.

Objectives: To investigate the prognostic and pathogenetic impact of Angiopoietins in regulating pulmonary vascular barrier function and inflammation in bacterial pneumonia.

Methods: Serum Angiopoietin levels were quantified in pneumonia patients of independent cohorts (n=67, n=148 and n=395). Human *post mortem* lung tissue, pneumolysin- or Angiopoietin-2-stimulated endothelial cells, isolated perfused and ventilated mouse lungs, and mice with pneumococcal pneumonia were investigated.

Measurements and Main Results: In pneumonia patients, decreased serum Angiopoietin-1 and increased Angiopoietin-2 levels were observed as compared to healthy subjects. Higher Angiopoietin-2 serum levels were found in CAP patients who died within 28 days after diagnosis compared to survivors. ROC analysis revealed improved prognostic accuracy of CURB-65 for 28-day survival, intensive care treatment and length of hospital stay by adding Angiopoietin-2 serum levels. *In vitro*, pneumolysin enhanced endothelial Angiopoietin-2 release, Angiopoietin-2 increased endothelial permeability, and Angiopoietin-1 reduced pneumolysin-evoked endothelial permeability. Ventilated and perfused lungs of mice with Angiopoietin-2-knockdown showed reduced permeability upon pneumolysin stimulation. Increased pulmonary Angiopoietin-2 and reduced Angiopoietin-1 mRNA expression were observed in *S. pneumoniae* infected mice. Finally, Angiopoietin-1 therapy reduced inflammation and permeability in murine pneumonia.

Conclusions: These data suggest a central role of Angiopoietin-1/-2 in pneumonia-evoked inflammation and permeability. Increased Angiopoietin-2 serum levels predicted mortality

and length of hospital stay, and Angiopoietin-1 may provide a therapeutic target for severe pneumonia.

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Keywords: Streptococcus pneumoniae; pneumolysin; endothelial permeability; ARDS

INTRODUCTION

Community-acquired pneumonia (CAP) is a significant cause of morbidity and mortality worldwide, and *Streptococcus pneumoniae* is the most prevalent causal pathogen identified in CAP (1, 2). Despite effective antibiotic therapy, pathogen-host interaction in pneumonia may evoke pulmonary endothelial permeability leading to protein exudation and edema formation, and finally to life-threatening lung failure (3).

Pneumonia-induced pulmonary hyperpermeability can be attributed to both direct effects of pathogenic factors and an uncontrolled host response. Among host factors, recruitment of neutrophils (PMN), increased release of pro-inflammatory cytokines and other, partly unidentified factors may contribute to the pathogenesis (3). The Angiopoietins (Ang-1 – Ang-4) are ligands for the receptor tyrosine kinase Tie2 (4). Ang-1 and Ang-2 are well-known contributors to angiogenesis (5) and regulators of inflammation and vascular leakage (6-8). Constitutively released Ang-1 activates Tie2, resulting in reduction of NFκB translocation, VCAM-1 presentation, Rho-kinase inhibition, and activation of Rac1, PI3K/Akt and KLF-2, amongst others (5, 9-11). Consequently, inflammation and apoptosis are reduced and endothelial barrier function is stabilized (8, 10, 12). In contrast, Ang-2 augments inflammation and permeability by acting as Ang-1 antagonist at Tie2 (6, 13).

In the systemic circulation, Ang-2 is produced and stored in endothelial cells (14). Exogenous inflammatory stimuli induce rapid Ang-2 release (15), and Ang-2 enhances properties of specific pro-inflammatory mediators including TNF-α (6). Further, direct impairment of endothelial integrity by Ang-2 has been suggested (7). In sepsis patients, Ang-2 serum levels were higher than in healthy volunteers and even further increased in patients with sepsis-associated lung injury (7). Previous studies support usage of Ang-2 as biomarker for risk evaluation in sepsis and ARDS (16-19). However, little is known about the expression and

function of Ang-1 and Ang-2 in the pulmonary circulation, and the role of Ang-1 and Ang-2 in pneumonia has not been investigated so far.

In order to comprehensively investigate the role of Ang-1 and Ang-2 in pneumonia, we used several approaches on different levels of complexity. (1) Ang-1 and Ang-2 were quantified in serum of CAP patients in different independent and prospectively collected cohorts, and the predictive value of Ang-2 regarding mortality, ICU treatment and length of hospital stay was analyzed. (2) Expression and regulation of Ang-1 and Ang-2 was analyzed in human post mortem sampled lung tissue from patients who died with pneumonia and in murine lungs with pneumonia. (3) Endothelial regulation of Ang-2 upon stimulation with the pneumococcal exotoxin pneumolysin and its impact on endothelial permeability was analyzed in human and murine cells *in vitro*, as well as *ex vivo* in isolated perfused and ventilated mouse lungs. (4) Therapeutic application of Ang-1 was performed in mice with severe pneumococcal pneumonia.

Some of the results presented here have been previously reported in the form of abstracts (20, 21).

METHODS

For detailed description see online data supplement.

Clinical study

This was an ancillary study of CAPNETZ (22, 23) and PROGRESS (24), both being observational, prospective studies enrolling consecutive patients with CAP as approved by central and local ethics committees. Data and laboratory samples were obtained as controls

from healthy volunteers, as approved by the local ethics committee. Written informed consent was obtained from each patient and volunteer, or each patient's legal representative.

As first cohort, CAPNETZ provided samples of consecutive pneumonia patients with CRB-65 score 2-4. As second cohort, CAPNETZ patients not surviving the first 28 days after pneumonia diagnosis were matched with CAPNETZ survivors in a 1:1 ratio based on age, sex and CRB-65 score. As third cohort, PROGRESS provided samples of consecutive CAP patients enrolled within 48 hours of hospitalization (day 0) and followed for three days thereafter (days 1-3).

Serum Ang-1 and Ang-2 levels were quantified using ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Human post mortem immunohistochemistry

Lungs with and without histologically diagnosed acute pneumonia were examined. All patients had given informed consent at admission. Immunohistochemical staining is detailed in the online supplement.

Endothelial cell experiments

Human pulmonary microvascular endothelial cells (EC) (Promocell, Heidelberg, Germany), human umbilical vein EC (25) and murine lung EC were grown to confluence on evaporated gold microelectrodes connected to an *Electrical cell-substrate impedance sensing* system (Applied Biophysics, Troy, NY) (26) to continuously monitor transcellular electrical resistance (TER). Cells were treated with pneumolysin (PLY) (27) or Ang-2 (R&D). Further, cells pretreated with Ang-1 (R&D) or solvent for 30 min were exposed to PLY. Ang-2 was determined in supernatants 3h after PLY-stimulation.

Angiopoietin-2 knockdown

On four consecutive days, mice were intravenously treated with stabilized liposomal siRNA (AtuPLEX) against Ang-2, warranting suppression of endothelial gene expression (28). Lungs were isolated perfused and ventilated 24h thereafter, human serum albumin (HSA) admixed to perfusate, PLY infused and HSA measured in bronchoalveolar lavage fluid (BALF) (29).

Angiopoietin-1 therapy in murine pneumonia

Animal procedures were approved by institutional and governmental (LAGeSo Berlin) authorities. Mice were anesthetized with ketamine/xylazine and transnasally inoculated with $5x10^6$ cfu *S. pneumoniae* (NCTC7978) (29). Body weight and rectal temperature were measured. Lungs were quick-frozen for qRT-PCR 12h, 24h, 36h and 48h after infection (p.i.) (28). Mice received Ang-1 or solvent intravenously 22h, 34h and 46h p.i. Pulmonary arterial flushing and BAL were performed 48h p.i., leukocytes differentiated by FACS (Calibur; BD, Heidelberg, Germany), and cytokines quantified (BioRad, Hercules, CA). To determine permeability, HSA was infused 47h p.i. intravenously and BALF/serum ratio calculated 48h p.i. (29).

Statistical analyses

Patient data analysis is detailed in the online repository. Experimental data are expressed as mean + SEM. Comparisons were performed using Mann-Whitney U-Tests followed by Bonferroni-Holm corrections where appropriate. For comparison of time points in the third patient cohort, pairwise t-tests were performed and a linear mixed model was used for global tests. Correlation analyses were performed using Spearman correlation coefficients. *P*-values <0.05 were considered significant. Analyses were performed using GraphPad Prism 6.05 (San Diego, CA).

RESULTS

Ang-1 and Ang-2 levels in serum of CAP patients

The first study cohort comprised 67 CAP patients with 35.8% dying within 28 days of CAP diagnosis. The median length of hospital stay (LOS) of the survivors was 12 days (Table 1). Characteristics of patients and volunteers of the first study cohort are summarized in Table E1. In serum of these patients with severe CAP, increased Ang-2 and decreased Ang-1 protein serum levels, and consequently, an elevated Ang-2/Ang-1-ratio were observed compared to healthy volunteers (Fig. 1 A-C).

To re-evaluate these initial findings focusing on Ang-2, an independent study cohort of 74 patients who did not survive the first 28 days after CAP diagnosis, and matched survivors was analyzed. Patient characteristics of this second study cohort are summarized in Table E2. The median LOS of the survivors of this second study cohort was 11 days (Table 2). Higher Ang-2 serum levels in CAP patients compared to healthy volunteers were detected with increased Ang-2 levels in non-survivors compared to survivors (Fig. 1D, Table 2).

In both CAPNETZ study cohorts, CAP patients were older than volunteers (Tables E1 and E2); however, serum levels of Ang-1 and Ang-2 in CAP patients did not correlate with age (Table E3).

To further evaluate the association between Ang-2 serum levels and clinical outcome, a third study cohort was included that comprised 395 hospitalized CAP patients with 4.8% dying within 28 days after study enrollment. The median LOS of the survivors was 8 days (Table 3). Patient characteristics of the third study cohort are summarized in Table E4. Higher Ang-2

serum levels in hospitalized CAP patients who died before day 28 (8.7 [5.8-19.1]; median [25-75% interquartile range]) compared to survivors (4.9 [3.2-8.1]) were confirmed in the third patient cohort at day 0 (enrollment into PROGRESS study). Further, there was a general trend that Ang-2 serum levels decrease in the course of hospitalization (Beta=-0.045, p=5.2x10^{-7}) with non-survivors having significantly greater values (Beta=0.68, p=3.9x10^{-5}) (Fig. 2A).

Predictive value of Ang-2 for 28-day mortality, hard endpoint and length of hospital stay in CAP patients

To investigate whether Ang-2 alone or in combination with other clinical/laboratory parameters is useful to predict 28-day mortality, hard endpoint (HEP), length of hospital stay over 7 days (LOS>7) or 14 days (LOS>14), ROC analysis of the third study cohort was conducted. HEP was defined as death by any cause within 28 days of enrollment or specific treatment on an intensive care unit. As specific treatment, the following were considered: substantial respiratory support (ventilation, extracorporeal oxygenation, oxygen supplementation of at least 6 l per minute, except for patients who were already on ventilation at home), treatment with catecholamines, or dialysis (except for patients with chronic kidney disease). For non-survivors, the endpoints LOS>7 or LOS>14 were set equal to LOS>7 or LOS>14, respectively.

We contrasted the predictive power of Ang-2 against CURB-65 and PCT. We also considered the combination of Ang-2 with CURB-65 and PCT for further predictive advantage. The combination of Ang-2 and CURB-65 showed the highest values of area under the ROC curve (AUC), equivalent to the best predictive power, for all investigated endpoints, 28-day mortality (0.797; 95% CI, 0.703-0.891), HEP (0.809; 95% CI, 0.747-0.871), LOS>7 (0.692; 95% CI, 0.64-0.744) and LOS>14 (0.727; 95% CI, 0.658-0.795). The AUC for the

combination of Ang-2 and CURB-65 was significantly higher than that for CURB-65 alone regarding 28-day mortality (p=0.0198), HEP (p=0.0021) and LOS>14 (p=0.0001). Tables 4 and 5 show detailed AUC values, and ROCs for predicting hard endpoint are displayed at Figure 2B.

In the first consecutive study cohort, Ang-2 serum levels on admission were strongly associated with PCT in serum (Spearman's ρ=0.5171, p=0.0002) and LOS of the survivors (ρ =0.5918, p<0.0001). Correlation analysis of Ang-2 serum levels and clinical and laboratory outcome parameters at day 0 in the third patient cohort revealed strong correlations with the oxygenation index (ρ=-0.37), bilirubin (ρ=0.28), creatinine (ρ=0.35), PCT (ρ=0.43) and CRP (ρ=0.41) values (all p<0.0001) in serum.

Human post mortem immunohistochemistry

Examination of human *post mortem* lung tissue revealed expression of Ang-1, Ang-2 and their receptor Tie2 in lungs of pneumonia patients. Ang-1 protein expression was found in different cell types including endothelial and epithelial cells, smooth muscle cells of arteries and bronchi, macrophages and PMN, whereas Ang-2 and Tie2 protein were exclusively expressed in endothelial cells (Fig. 3). Alterations of Ang-1, Ang-2 or Tie2 protein expression in human lungs with or without pneumonia could not be determined by semi-quantitative analysis.

Endothelial cell experiments

To investigate in greater detail the possible contribution of Ang-2 to pneumonia pathophysiology, Ang-2 release from endothelial cells was analyzed upon stimulation with pneumolysin (PLY). Confluent human umbilical vein endothelial cell (HUVEC) monolayers stimulated for 3h with recombinant PLY dose-dependently liberated Ang-2 (Fig. 4A). Further,

Ang-2 impaired endothelial barrier integrity in HUVEC (Fig. 4B) and human pulmonary microvascular endothelial cell (hPMVEC) monolayers (Fig. 4C) reversibly, and in murine lung EC (mLEC) monolayers in part reversibly (Fig. 4D).

Angiopoietin-2 contributed to pneumolysin-evoked lung injury in mouse lungs

To analyze the contribution of Ang-2 in PLY-induced lung barrier destabilization on a whole organ level, isolated perfused and ventilated mouse lungs were employed. In this model, lungs with suppressed endothelial Ang-2 expression due to RNAi ($siRNA^{Ang2}$) showed reduced PLY-evoked permeability in comparison to control-treated lungs (luciferase-specific siRNA, $siRNA^{Luc}$; Fig. 4E). Reduction of pulmonary Ang-2 mRNA expression by intravenous Ang-2 siRNA-lipoplex was approximately 50% (supplemental Figure E1).

Angiopoietin-1 reduced lung barrier failure

We evaluated whether Ang-1 was able to reduce pneumonia-related barrier failure. PLY-stimulation evoked a dose-dependent decrease in transcellular electrical resistance (TER) of HUVEC monolayers, displaying loss of endothelial integrity (Fig. 5A). Incubation of confluent HUVEC and hPMVEC monolayers with Ang-1 attenuated the PLY-induced TER decrease compared to solvent (Fig. 5B-C).

Further, pulmonary regulation of Ang-1 and Ang-2 was investigated in pneumonia *in vivo*. Mice infected with *S. pneumoniae* showed decreased body weight and temperature (Fig. 6A-B) as signs of disease. Following infection, pulmonary mRNA expression of Ang-1 was decreased after 24h and at later time points (Fig. 6C). Pulmonary Ang-2 mRNA was increased by trend compared to control mice 24, 36 and 48h p.i. (Fig. 6D).

Given the protective effect of Ang-1 *in vitro* and the regulation of Ang-1 and Ang-2 *in vivo*, the therapeutic potential of Ang-1 *in vivo* was investigated. Ang-1 therapy of established

severe pneumonia partly prevented decrease of body weight and temperature observed in solvent treated mice (Fig. 7A-B). Lung hyperpermeability was significantly reduced by Ang-1 (Fig. 7C). Moreover, neutrophil recruitment (Fig. 7D) and inflammatory cytokines (Fig. 7E) in BALF were efficiently reduced by Ang-1 in pneumonic mice.

DISCUSSION

Current management of severe pneumonia could be improved by severity scores that include specific biomarkers to better predict risk of ARDS and death, and by treatment options in addition to antibiotics that reduce the risk for ARDS. The CRB-65 and CURB-65 scores are recommended in international guidelines (30) and CRB-65 is widely used as a predictor of death in newly diagnosed pneumonia patients in Europe (23, 31). Both scores are comparable for mortality prediction (23, 30, 32). However, evidence from previous studies suggests that these scores alone provide insufficient power to predict 30-day mortality (CURB-65 ≥2: positive likelihood ratio 1.7; CRB-65 ≥2: positive likelihood ratio 2.4) or ICU admission (CURB-65 ≥2: positive likelihood ratio 1.58; CRB-65 ≥2: positive likelihood ratio 1.62) (32-34). Addition of specific biomarkers may possibly improve accuracy of CURB-65/CRB-65. As the use of Ang-2 for risk evaluation in sepsis, sepsis-induced ARDS and other critical illness has been discussed (7, 16-19, 35-37) we evaluated serum Angiopoietin levels in patients with pneumonia. Of note, a NHLBI ARDS network study reported that plasma Ang-2 levels measured in 931 subjects with acute lung injury did not have prognostic value for mortality in the subgroup of infection-related ALI (n=653) (35). Calfee et al. recently compared plasma biomarkers between two studies, a single-center and a multicenter study and observed that Ang-2 was lower in plasma of patients with direct ARDS compared to

patients with indirect ARDS (due to non-pulmonary sepsis) (38). In contrast to the present work, the ARDS network studies did not analyze pneumonia patients without ARDS.

The presented analysis revealed decreased serum Ang-1 levels and increased Ang-2 levels in patients with severe pneumonia as compared to healthy controls. As Ang-levels could be influenced by age, we controlled for age and found no association between serum levels of Ang-1 or Ang-2 and age. We showed that Ang-2 serum levels significantly differed between survivors and non-survivors. Moreover, Ang-2 serum levels were negatively correlated with the oxygenation index and positively correlated with different laboratory outcome parameters such as PCT as well as with length of hospital stay among survivors. We therefore tested whether the combination of Ang-2 serum levels with CURB-65 or PCT predicted mortality, our predefined composite “hard endpoint” and LOS better than each single value. Indeed, ROC analysis revealed significantly improved prognostic accuracy of CURB-65 for 28-day mortality, hard endpoint and length of hospital stay by adding Ang-2 serum levels. Accordingly, the implied prognostic improvement achieved by adding the Ang-2 value to CURB-65 is not restricted to mortality prediction but also helps identify patients who require more than standard monitoring and treatment (39).

To enhance the rationale for a possible prognostic and pathogenetic role of Angiopoietins in pneumonia, particularly in pneumonia-induced endothelial disruption and pulmonary hyperpermeability, we investigated the pulmonary localization, regulation and function of Ang-1 and -2 in pneumonia. Consistently with histological studies of other tissues (4, 40), Ang-2 and Tie2 in human lungs were found to be expressed exclusively in endothelial cells, whereas Ang-1 was expressed in different cell types. Although Ang-2 protein in human serum as well as Ang-2 mRNA in mouse lungs increased in pneumonia, semi-quantitative analysis of human post mortem lung tissue did not reveal significantly increased Ang-2 protein expression in pneumonia, which may partly result from postmortal reduction of tissue quality,

limited precision of histologic quantification and/or Ang-2 liberation from endothelial cells in pneumonia. Of note, we found increased Ang-2 liberation in human endothelial cells upon stimulation with pneumolysin, an important exotoxin of *S. pneumoniae* and central contributor to lung hyperpermeability in pneumococcal pneumonia (29, 41). Previous studies demonstrated Ang-2 mRNA upregulation after stimulation with pro-inflammatory mediators, LPS or hyperoxia in endothelial cells (15, 42, 43), where Ang-2 is stored in Weibel-Palade bodies (WPB) (14) and rapidly released upon stimulation with WPB secretagogues including thrombin, vasopressin and histamine (44). Furthermore, Ang-2 disrupts endothelial architecture (15). Accordingly, in the current electrical cell impedance sensing (ECIS) experiments Ang-2 decreased monolayer integrity of different types of endothelial cells including human pulmonary microvascular endothelial cells (hPMVEC) reversibly. The complex mechanisms of pneumolysin-induced permeability in intact lungs are incompletely understood and may include cellular pore formation and inflammation (29, 45, 46). Hypothesizing that Ang-2 might be involved in pneumolysin-induced hyperpermeability, we applied RNAi *in vivo* by treating mice intravenously with liposomal, stabilized Ang-2 siRNA prior to stimulating their isolated perfused and ventilated lungs with pneumolysin. Lungs of mice treated with Ang-2 specific siRNA showed reduced permeability upon pneumolysin stimulation compared to lungs of mice treated with control siRNA, suggesting that Ang-2 contributed to pneumolysin-induced lung permeability.

Increase of Ang-2 mRNA expression in mouse lungs after LPS-stimulation has been reported (15). In lungs of *S. pneumoniae* infected mice we also observed a time-dependent increase of Ang-2 mRNA expression, while Ang-1 expression decreased. Body weight and temperature decrease reflected disease progression.

Hashimoto et al. suggested that the ratio between Ang-1 and Ang-2 is more important for the Ang-/Tie2-effect on endothelial vascular leakage than the absolute Ang-2 concentration, with

a decreased Ang-2/Ang-1 ratio stabilizing endothelial barrier function (47). Indeed, preventive adenoviral Ang-1 gene transfer or administration of mesenchymal stem cells overexpressing Ang-1 as well as prophylactic application of recombinant Ang-1 protein diminished pulmonary hyperpermeability induced by subsequent LPS injection in mice (10, 48, 49). However, therapeutic use of Ang-1 in pneumonia has not been examined so far. Thus, we first investigated whether Ang-1 protein application is capable to reduce pneumolysin-evoked endothelial barrier disruption *in vitro*. Indeed, Ang-1 diminished pneumolysin-induced injury of HUVEC and hPMVEC monolayers. Then, we treated mice with recombinant Ang-1 protein in an intervening instead of prophylactic setting. Ang-1 treatment of *S. pneumoniae* infected mice started 22h after infection, when severe pneumonia was already established. Subsequently, we observed reduction of lung permeability, pulmonary leukocyte recruitment and inflammatory cytokine secretion, and clinical outcome was clearly improved in Ang-1 treated as compared to untreated mice. The exact contributions of Ang-1-dependent anti-inflammatory effects on the one hand and direct barrier-stabilizing properties of Ang-1 on the other hand could not be deciphered in the current *in vivo* treatment experiment. Anti-inflammatory effects of Ang-1 have been described previously in murine lung injury (49), as well as in VEGF-stimulated HUVECs and human umbilical artery ECs *in vitro* (12). Moreover, direct stabilizing effects of Ang-1 on endothelial cells were observed in the current and in different previous studies *in vitro* where cells were stimulated with n,n-Dimethylsphingosine (50) or serum of sepsis patients with high Ang-2 levels (7).

In summary, we found that pneumonia is associated with decreased Ang-1 and increased Ang-2 serum levels in humans. Further, Ang-2 serum levels were even more increased in non-survivors compared to survivors. Prognostic accuracy of CURB-65 for 28-day survival, need for ICU treatment and length of hospital stay over 7 or 14 days was improved by adding Ang-2 serum levels. These results suggest that Ang-2 may add to existing or future pneumonia

severity scores. In complementary experimental studies, pneumolysin evoked Ang-2 liberation, and Ang-2 contributed to pneumolysin-induced pulmonary hyperpermeability. Therapeutic treatment with Ang-1 reduced inflammation and permeability in murine pneumococcal pneumonia, thereby protecting mice from development of acute lung injury. Thus, reducing the Ang-2/Ang-1 ratio may provide a therapeutic perspective for pneumonia-induced acute lung failure.

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FIGURE LEGENDS

Figure 1. Increased Ang-2 and reduced Ang-1 serum levels in human pneumonia with highest Ang-2 levels in CAP patients not surviving the first 28 days after pneumonia diagnosis. (A-C) First patient cohort: Ang-2 and Ang-1 were quantified in serum of patients with severe pneumonia (CRB-65 score 2-4, n=67) and in healthy controls (n=45) and the Ang-2/Ang-1 ratio was calculated. (D) Second patient cohort: Admission levels of Ang-2 were quantified in serum of patients with CAP (CRB-65 score 0-3; n=148; 50% non-survivors (28d) matched with survivors in a 1:1 ratio based on age, sex and CRB-65 score) and in healthy controls (n=24). Data are represented as box plots depicting median, quartiles and range excluding outliers (circles). *p<0.05, ****p<0.0001.

Figure 2. Ang-2 serum levels in the course of hospitalization in CAP patients and their predictive power regarding hard endpoint. Third patient cohort: Ang-2 was quantified in serum of consecutive CAP patients enrolled within 48h of hospitalization (day 0) and followed for three days thereafter (days 1-3). (A) Patients not surviving 28-day follow up (n=19) showed higher Ang-2 serum levels at all investigated time points compared to survivors (n=376). Data are represented as box plots depicting median, quartiles and range excluding outliers (circles). *p<0.05, **p<0.01. (B) ROC curves for serum Ang-2, CURB-65 and serum PCT at admission (day 0) alone and in combination in predicting HEP (n=395). HEP was defined as death by any cause within 28 days of enrollment or specific treatment on an intensive care ward. As specific treatment, the following were considered: substantial respiratory support (ventilation, extracorporal oxygenation, oxygen supplementation of at least 6 l per minute, except for patients who were already on ventilation at home), treatment with catecholamines, or dialysis (except for patients with chronic kidney disease). The combination of Ang-2 and CURB-65 had the best predictive power. The AUC for the

combination of Ang-2 and CURB-65 was significantly higher than that for CURB-65 alone (p=0.0021), PCT alone (p=0.0015) and the combination of both (p=0.035).

Figure 3. Immunolocalization of Ang-1, Ang-2 and Tie2 in human *post mortem* lung tissue. *Post mortem* lung tissue samples of pneumonia patients or healthy controls were stained for Ang-1, Ang-2 or Tie2. Ang-1 was abundantly expressed in different cell types including endothelial and epithelial cells, smooth muscle cells of arteries and bronchi, macrophages and granulocytes, whereas Ang-2 and the receptor Tie2 were exclusively expressed by endothelial cells (black arrows). Representative images (n=15 each group) are shown. Scale bars represent 50 µm.

Figure 4. Pneumolysin evoked Ang-2 liberation, and Ang-2 contributed to endothelial barrier dysfunction. (A) HUVECs were exposed to increasing concentrations of pneumolysin (PLY) for 3h and Ang-2 was quantified in supernatants (blank: n=7; solvent: n=5; PLY: n=3-4). (B-D) Transendothelial electrical resistance (TER) of endothelial cell monolayers was monitored. Human umbilical vein endothelial cells (HUVEC; n=5), human pulmonary microvascular endothelial cells (hPMVEC; n=5) or primary murine lung endothelial cells (mLEC; n=3) were exposed to Ang-2 (2 µg/ml). (E) In mice, siRNA specific for Ang-2 or luciferase (Luc, as control) was injected i.v. once daily on four consecutive days (n=6). Twenty-four hours after the last administration, lungs were isolated perfused and ventilated and exposed to pneumolysin (1 µg/ml). In the control group (n=4), neither siRNA molecules nor pneumolysin were applied. Values are given as (A, E) mean + SEM or (B-D) mean; *p<0.05 vs. solvent group (A) or between indicated groups (E).

Figure 5. Ang-1 protected endothelial cell monolayers against PLY-induced permeability. (A) PLY dose-dependently decreased transcellular electrical resistance (TER) in HUVEC monolayers. (B-C) HUVEC or hPMVEC were exposed to PLY (HUVEC:

2.0 µg/ml; hPMVEC: 0.75 µg/ml) 30 minutes after Ang-1 (300 ng/ml) treatment. Values are given as mean (n=4-7).

Figure 6. *S. pneumoniae* infection decreased pulmonary Ang-1 and increased Ang-2 mRNA expression in mice. (A-B) Body weight (n=5-6 per group) and body temperature (n=5-6 per group) of *S. pneumoniae*-infected mice were measured. (C-D) Pulmonary mRNA expressions of Ang-1 and Ang-2 were quantified using the ΔΔCT method (n=6 per group). Expression levels of mRNA of interest are shown relative to the non-infected group at the corresponding time point and normalized to the mRNA level of PTEN. Values are given as mean + SEM. *$p<0.05$, **$p<0.01$ between indicated groups.

Figure 7. Ang-1 treatment prevented lung injury in mice with severe pneumococcal pneumonia. Mice were transnasally infected with *S. pneumoniae* (*S. pn.*), and 22h, 34h and 46h after infection Ang-1 (300 ng/ml) or solvent was injected i.v. Forty-eight hours after infection, body weight (A) and body temperature (B) were measured, the HSA BALF/plasma ratio (C) measured to determine microvascular leakage and BALF leukocytes (D) and cytokines (E) analyzed. Values are given as mean + SEM; n=5 (sham-infected groups); n=10-12 (*S. pn.*-infected groups). *$p<0.05$, **$p<0.01$ between indicated groups. PMN = neutrophils; MAC = macrophages; LYM = lymphocytes; *n.d.* = not detectable.

Tables

Table 1 Median (25-75% interquartile range) levels of Angiopoietins and clinical parameters of the first study cohort

Parameters	**Survivors (28d)** (n = 43 / 64.2%)	**Non-survivors (28d)** (n = 24 / 35.8%)	***P*-value**
Angiopoietin-2, ng/ml	6.08 (3.92-8.90)	7.32 (3.68-18.13)	0.5789
Angiopoietin-1, ng/ml	35.08 (26.73-44.84)	30.34 (26.72-40.18)	0.2164
Angiopoietin-2/-1-ratio	0.18 (0.10-0.32)	0.25 (0.13-0.49)	0.1727
CRP, mg/l	140 (68-242)	125.3 (47.1-385)	0.5968
Leukocytes, cells/nl	15.79 (10.9-20.07)	14.8 (11.3-22.45)	0.9871
Urea, mmol/l	9.8 (7.6-13.99)	9.24 (7.20-18.98)	0.7926
PCT, ng/ml	1.13 (0.21-5.08)	3.52 (0.38-27.34)	0.3008
LOS, days	12 (8-17)	12 (4.5-21.00)	*not tested*

The serum used for Ang-1 and Ang-2 quantification was obtained from patients with severe community-acquired pneumonia (CRB-65 score of 2-4) at admission. Clinical data were not available for all patients: PCT (30/17, survivors/non-survivors), length of hospital stay (LOS; 41/21 survivors/non-survivors).

Table 2 Median (25-75% interquartile range) levels of Ang-2 and clinical parameters of the second study cohort

Parameters	**Survivors (28d)** (n = 74 / 50%)	**Non-survivors (28d)** (n = 74 / 50%)	***P*-value**
Angiopoietin-2, ng/ml	*4.18 (2.72-6.84)	*5.92 (3.48-9.99)	0.0185
CRP, mg/l	113.0 (37.18-216.75)	110.0 (43.85-196.6)	0.8879
Leukocytes, cells/nl	11.8 (7.88-16.46)	13.1 (9.65-17.8)	0.1461
Urea, mmol/l	*7.10 (5.05-11.58)	*9.44 (6.18-16.34)	0.0135
PCT, ng/ml	0.28 (0.12-1.12)	0.58 (0.20-1.10)	0.1148
LOS, days	11 (7.5-14)	10 (6-16)	*not tested*

The serum used for Ang-2 quantification was obtained from patients with community-acquired pneumonia (CRB-65 score of 0-3) at admission. Clinical data were not available from all patients: CRP (72/73, survivors/non-survivors), leukocytes (70/73, survivors/non-survivors), urea (64/70, survivors/non-survivors), PCT (30/48, survivors/non-survivors), length of hospital stay (LOS; 65/70, survivors/non-survivors). Statistically significant differences between survivors and non-survivors are indicated by asterisks (*).

Table 3 Median (25-75% interquartile range) levels of Ang-2 and clinical parameters of the third study cohort

Parameters	**Survivors (28d)** (n = 376 / 95.2%)	**Non-survivors (28d)** (n = 19 / 4.8%)	***P*-value**
Angiopoietin-2, ng/ml	*4.9 (3.2-8.1)	*8.7 (5.8-19.1)	$9.3x10^{-4}$
CRP, mg/l	176 (98-248)	141 (109-216)	0.62
Leukocytes, cells/nl	11.9 (8.9-16.6)	11.2 (9.7-17.2)	0.92
Urea, mmol/l	*5.9 (4.0-8.6)	*9.0 (7.8-18.0)	$7.9x10^{-5}$
PCT, ng/ml	0.28 (0.11-3.18)	0.39 (0.24-2.05)	0.17
LOS, days	*8 (6-12)	*13 (8-20)	*not tested*

The serum used for Ang-2 quantification was obtained from CAP patients enrolled within 48h of hospitalization (day 0). Statistically significant differences between survivors and non-survivors are indicated by asterisks (*).

Table 4 Performance of initial serum Ang-2, CURB-65 and serum PCT in predicting 28-day mortality or hard endpoint

Parameter	**28-day mortality**		**Hard endpoint**	
	AUC (95% CI)	***P*-value**	**AUC (95% CI)**	***P*-value**
Ang-2	0.725 (0.608-0.842)	$9.34x10^{-4}$	0.756 (0.684-0.827)	$4.93x10^{-10}$
CURB-65	0.764 (0.658-0.869)	$5.35x10^{-5}$	0.752 (0.684-0.82)	$1.65x10^{-10}$
PCT	0.593 (0.492-0.695)	0.17	0.688 (0.613-0.762)	$5.01x10^{-6}$
Ang-2 + CURB-65	0.797 (0.703-0.891)	$1.25x10^{-5}$	0.809 (0.747-0.871)	$5.45x10^{-14}$
Ang-2 + PCT	0.725 (0.608-0.842)	$9.24x10^{-4}$	0.761 (0.689-0.832)	$2.21x10^{-10}$
CURB-65 + PCT	0.761 (0.662-0.86)	$1.26x10^{-4}$	0.772 (0.704-0.839)	$3.78x10^{-11}$

Hard endpoint was defined as death by any cause within 28 days of enrollment or specific treatment on an intensive care ward. As specific treatment, the following were considered: substantial respiratory support (ventilation, extracorporal oxygenation, oxygen supplementation of at least 6 l per minute, except for patients who were already on ventilation at home), treatment with catecholamines, or dialysis (except for patients with chronic kidney disease). AUC, area under the curve; CI, confidence interval; PCT, procalcitonin

Table 5 Performance of initial serum Ang-2, CURB-65 and serum PCT in predicting length of hospital stay over 7 days or 14 days

Parameter	**LOS>7**		**LOS>14**	
	AUC (95% CI)	***P*-value**	**AUC (95% CI)**	***P*-value**
Ang-2	0.635 (0.58-0.69)	4.96×10^{-6}	0.723 (0.657-0.789)	4.87×10^{-9}
CURB-65	0.677 (0.626-0.727)	4.79×10^{-10}	0.691 (0.621-0.761)	1.75×10^{-7}
PCT	0.622 (0.566-0.678)	3.70×10^{-5}	0.63 (0.563-0.697)	0.000647
Ang-2 + CURB-65	0.692 (0.64-0.744)	9.23×10^{-11}	0.727 (0.658-0.795)	2.73×10^{-9}
Ang-2 + PCT	0.637 (0.582-0.693)	3.43×10^{-6}	0.71 (0.644-0.776)	3.69×10^{-8}
CURB-65 + PCT	0.663 (0.609-0.717)	3.90×10^{-8}	0.706 (0.636-0.776)	6.64×10^{-8}

For non-survivors, the endpoints LOS>7 or LOS>14 were set equal to LOS>7 or LOS>14, respectively. AUC, area under the curve; CI, confidence interval; LOS>7, length of hospital stay over 7 days; LOS>14, length of hospital stay over 14 days; PCT, procalcitonin