



ORIGINAL RESEARCH

Analysis of the Local and Systemic Cytokine Response Profiles in Patients with Community-Acquired Pneumonia: Relationship with Outcomes and Disease Severity

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Abstract

The goal of this study was to analyze the local and systemic cytokine responses in patients with Community-acquired pneumonia (CAP) and to develop a model to integrate multiple cytokine data in order to investigate the relationships of different cytokine patterns with measures of CAP outcomes and severity. Forty hospitalized patients enrolled through the Community Acquired Pneumonia Inflammatory Study Group (CAPISG) were included. Based on the ranked distribution of the levels of eight different pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, IL-12p40, IL-17A, IFN γ , TNF α and CXCL10) in both plasma and sputum on the day of hospital admission, "pro-inflammatory cytokine scores (PICS)" were calculated for each patient. PICS in plasma were divided by those in sputum in order to calculate a pro-inflammatory cytokine ratio (PICR). Comparison of PICR values for patients grouped by TCS (\leq and >3 days) or by TSI group (classes I-III and IV-V) indicated statistically significant differences. Patients in the TCS >3 d group had significantly higher PICR compared to the TCS ≤ 3 d group ($p=0.009$) and patients in PSI class IV-V had higher PICR values compared to those in PSI classes I-III ($p=0.042$). ROC analysis showed that PICR values have a good accuracy at separating patients into either of the TCS groups, suggesting the potential use as predictive tools, identifying patients at risk to have a delayed TCS or a more severe disease.

DOI: 10.18297/jri/vol1/iss3/5

Received Date: February 10, 2017

Accepted Date: April 19, 2017

Website: <https://ir.library.louisville.edu/jri>

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1 Introduction

Despite the availability and use of antimicrobial therapy, community-acquired pneumonia (CAP) remains one of the major causes of infectious disease-related death in the world^{1,2}. In CAP, a local immune response to the invading microorganisms induces the recruitment and activation of leukocytes to the lungs^{3,4}. Even in the presence of antimicrobial therapy, this response plays a key role in determining whether or not the offending microorganisms escape control and further spread beyond the lungs, eliciting a vigorous systemic inflammatory response^{5,6}.

While the initial inflammatory response has a protective role, excessive or uncontrolled inflammation may result in deleterious

consequences for the host⁶⁻⁸. In fact, an association between an exaggerated systemic inflammatory response (i.e., "cytokine storm") with poor clinical outcomes has been established by several investigators^{6,9,10} and it is generally agreed that failure to control excessive inflammation may lead to organ damage and a higher mortality risk^{6,9-12}. The potentially deleterious role of disproportionate inflammation has prompted several studies to explore the therapeutic use of immunomodulatory corticosteroids in CAP patients. Unfortunately, results have been inconsistent and their real efficacy so far remains controversial¹³⁻¹⁷. While the reasons for the discrepancies are not clear, it has been suggested that although only a subset of CAP patients may actually benefit from corticosteroid use (those with the most severe inflammation), most studies have enrolled patients without consideration of their inflammatory status¹⁸.

Based on previous studies¹⁹ we have hypothesized that patients who are able to mount strong local inflammatory responses might be able to have better clinical outcomes. However, while most studies in CAP patients have focused on the association between

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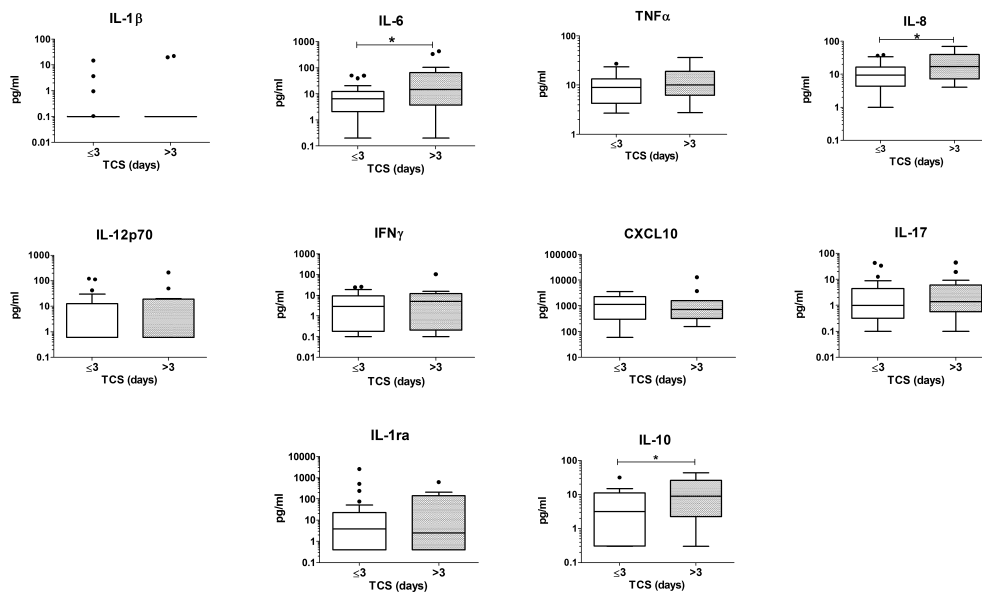


Fig. 1 Plasma cytokine distributions in CAP patient groups relative to the TCS. Blood samples from patients with TCS \leq 3d (n=26) or TCS>3d (n=16) were obtained shortly after admission to the hospital and assayed for the indicated cytokines and chemokines using a bead-based multiplex assay as indicated in Materials and Methods. Box plots depict the 25-75% interquartile range and the horizontal bar depicts the median. Due to non-normal distribution of cytokine scores, groups were compared using the Wilcoxon-Mann-Whitney test. *p < 0.05.

disease severity or clinical outcomes and the systemic inflammatory response (measured in serum or plasma), data on associations with the local inflammatory response, such as measured in bronchoalveolar lavage (BAL) or sputum, are limited. Thus, the aims of this study were to investigate the association of both, local and systemic cytokine response patterns, with the time to clinical stability (TCS) and to explore potential means to integrate the overall cytokine response (plasma and sputum) in the evaluation of CAP patients.

2 Materials and Methods

This was a secondary analysis of data previously collected from the Community Acquired Pneumonia Inflammatory Study Group (CAPISG). The design of our study has been previously described¹⁹. Briefly, this study included 40 patients with CAP hospitalized at the University of Louisville Hospital or the Louisville's Veteran Administration Hospital from 01/04/2011 to 01/08/2012. The study was approved by both the University of Louisville Human Subjects Program Protection Office and the Robley Rex Veterans Affairs Medical Center Institutional Review Boards (Approvals #: 07.0182 and 0009, respectively). The demographic and clinical data of the patient population in this study has already been reported¹⁹.

2.1 Criteria for CAP

CAP was defined as evidence of a new pulmonary infiltrate at chest radiograph associated with at least one of the following: 1) new or increased cough; 2) fever or hypothermia; and 3) leukocytosis, left shift, or leukopenia. Pneumonia was defined as community-acquired if a patient had no history of hospitalization during the 2 weeks prior to admission. The inclusion and

exclusion criteria and full case report forms for this study can be found at the Community Acquired Pneumonia Organization study site at www.caposite.com.

2.2 Microbiologic Analysis

Testing of sputum samples and blood cultures; serology for respiratory viruses and atypical organisms as well as urinary antigens for *Legionella* spp. and *Streptococcus pneumoniae* were performed according to standard clinical practice. The identification of microorganisms and susceptibility testing were performed according to standard methods²⁰. Cases in which no specific etiologic agent was isolated or identified were classified as CAP of unknown etiology.

2.3 Severity of Disease and Time to Clinical Stability

The Pneumonia Severity Index (PSI) was used to define CAP severity²¹. Hospitalized patients were considered to have severe CAP if their PSI was Risk Class IV or V (91 points or higher). Patients with a PSI Risk Class of I-III (<91 points) were defined as having non-severe CAP. Accordingly, 21 patients were classified as having severe and 19 non-severe CAP. The time to clinical stability (TCS) was defined based on the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) 2007 guidelines as improved clinical signs (cough and shortness of breath), lack of fever for at least 8 hours, improving leukocytosis (decreased at least 10% from the previous day), and tolerating oral intake²². For this study, patients were divided into two groups based on a TCS equal or less than 3 days (TCS \leq 3d) and a TCS greater than three days (TCS>3d). Accordingly, 24 patients were classified as having a TCS \leq 3d and 16 patients a TCS>3d.

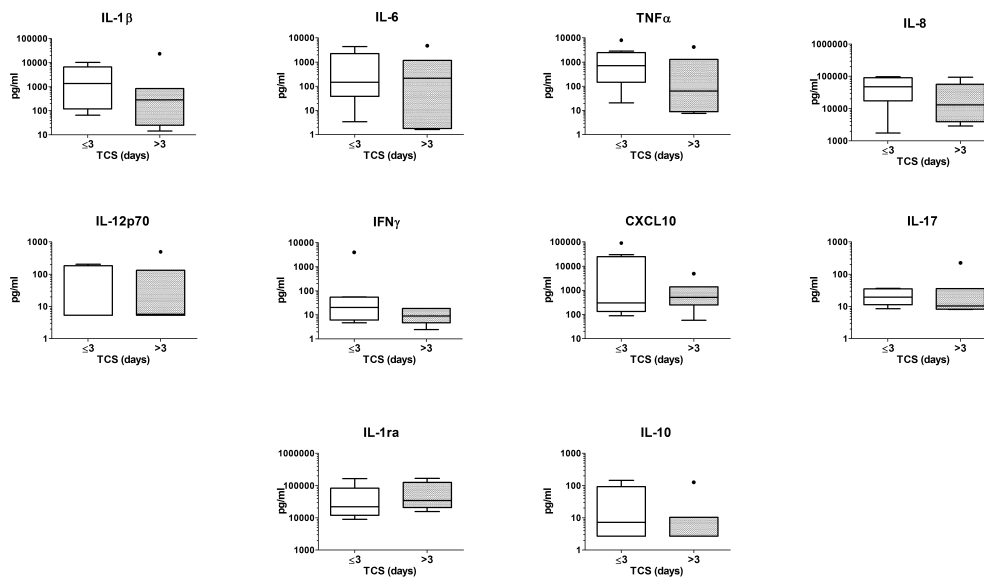


Fig. 2 Sputum cytokine distributions profiles in CAP patient groups relative to the TCS. Sputum samples from patients with TCS \leq 3d (n=8) or TCS>3d (n=7) were obtained shortly after admission to the hospital and processed and assayed for the indicated cytokines and chemokines using a bead-based multiplex assay as indicated in Materials and Methods. Box plots depict the 25-75% interquartile range and the horizontal bar depicts the median. Due to non-normal distribution of cytokine scores, groups were compared using the Wilcoxon-Mann-Whitney test.

2.4 Samples

Plasma samples: Blood samples were obtained on the day of admission at the hospital. Venous blood was collected using sodium citrate Vacutainer tubes. Following centrifugation at 300 x g for 10 min, the plasma was separated by aspiration, aliquoted and stored frozen at -80°C until assayed.

Sputum samples: Sputum samples were collected from a total of 15 CAP patients (7 with non-severe, 8 with severe CAP) on the day of admission to the hospital. Sputum samples were processed following the method described by Pizzichini et al²³. The cell-free supernatants were aliquoted and stored frozen at -80°C until used for the measurement of cytokine levels.

2.5 Cytokine measurements

The concentrations of ten different cytokines and chemokines in plasma and sputum samples were determined using Milliplex MAP High Sensitivity Human Cytokine Magnetic bead kits (HSCYTMAG-60SK, EMD Millipore, Billerica, MA) according to the manufacturer's instructions. The panel included eight important pro-inflammatory cytokines and chemokines: interleukin (IL)-1 β , IL-6, CXCL8 (IL-8), IL-12p40, IL-17A, interferon (IFN) γ , tumor necrosis factor (TNF) α and CXCL10 (IP-10); and two main anti-inflammatory cytokines: IL-1 receptor antagonist (IL-1ra) and IL-10.

2.6 Statistics

Statistical analysis and graphs were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Data distribution was analyzed using the D'Agostino and Pearson omnibus normality test. Due to a non-normal distribution of cytokine data, sta-

tistical comparisons between the groups were performed using the Wilcoxon-Mann-Whitney test. Unpaired t-tests were used to compare normally-distributed data. GraphPad was used to construct ROC curves. The Youden's Index (J) was calculated as $J = (\text{specificity} + \text{sensitivity}) - 1$. p -values of ≤ 0.05 were considered statistically significant.

3 Results

3.1 Cytokine Patterns in Plasma and Sputum and Association with the TCS

In order to associate cytokine patterns with time to clinical stability (TCS), we compared the plasma and sputum cytokine levels in patients divided in two groups based on their TCS (\leq and $>$ 3 days) as indicated in the Methods section. **Figure 1** shows the distribution of plasma concentration values for the ten different cytokines measured. Despite the overlap in values between the two groups, patients in the TCS>3d group had statistically significantly higher plasma levels ($p < 0.05$) of the pro-inflammatory cytokines, IL-6 and IL-8, as well as the anti-inflammatory cytokine, IL-10. There were no statistically significant differences between the two groups for the other cytokines. In the case of cytokine concentrations in sputum (**Figure 2**), there was a tendency for higher cytokine levels in the TCS \leq 3d group in the case of the pro-inflammatory cytokines, particularly IL-1 β ($p = 0.11$), TNF α ($p = 0.06$) and IFN γ ($p = 0.08$), but no statistically significant differences were recorded.

3.2 Pro-Inflammatory Cytokine Ratio (PICR)

In order to integrate systemic and local inflammatory cytokine response profiles in CAP patients, a ratio based on the relative levels of the eight pro-inflammatory cytokines and chemokines

Table 1 Distribution of ranked cytokine values in plasma and sputum

Cytokine	25% percentile	Median	75% percentile
Plasma			
IL-1 β	0.1	0.1	0.1
IL-1Ra	0.4	2.1	37.3
IL-6	2.7	10.2	40.1
IL-8	6.8	14.0	33.7
IL-10	0.9	7.3	17.3
IL-12p70	3.2	3.2	14.0
IL-17	0.5	1.3	8.7
IFN γ	0.2	2.9	9.9
TNF α	5.8	11.5	22.1
CXCL10 (IP-10)	358.8	992.2	2,158.0
Sputum			
IL-1	66.2	289.0	1,738.0
IL-1Ra	16,966.0	26,784.0	124,294.0
IL-6	25.8	163.6	1,212.0
IL-8	4,040.0	29,098.0	91,269.0
IL-10	6.0	6.0	81.9
IL-12p70	2.8	2.8	172.8
IL-17	8.6	12.2	36.1
IFN γ	5.2	9.1	28.3
TNF α	20.7	257.5	1,415.0
CXCL10	190.4	439.7	4,848.0

All cytokine values are expressed in pg/mL. Plasma samples (n=40); Sputum samples (n=15)

Table 2 Characteristics of CAP patients with both Plasma and Sputum Cytokine Data

Patient	Age/Sex	PSI Class	TCS (Days)	Etiologic Agent	Plasma/Sputum (PICS)	PICR
TCS<3d Group						
1	78/M	III	2	<i>Streptococcus pneumoniae</i>	16 / 18	0.88
2	51/F	II	2	<i>Streptococcus pneumoniae</i>	19 / 21	0.90
3	65/M	III	2	<i>Streptococcus pneumoniae</i>	11 / 19	0.57
4	58/M	II	2	<i>Haemophilus influenzae</i>	30 / 24	1.25
5	54/F	IV	2	Influenza A H3	22 / 23	0.95
6	63/M	III	2	None identified	14 / 25	0.56
7	53/F	II	2	None identified	13 / 17	0.76
8	49/M	IV	1	None identified	14 / 19	0.73
TCS>3d Group						
9	40/M	II	7	<i>Pseudomonas aeruginosa</i>	25 / 26	0.96
10	62/M	V	>6*	MRSA	21 / 19	1.10
11	57/F	IV	6	<i>Acinetobacter baumannii</i>	17 / 21	0.80
12	72/F	IV	4	None identified	23 / 14	1.64
13	78/M	V	3	None identified	20 / 13	1.53
14	85/M	V	7	None identified	17 / 13	1.30
15	59/M	IV	>3*	None identified	21 / 12	1.75

*patient died during hospital stay

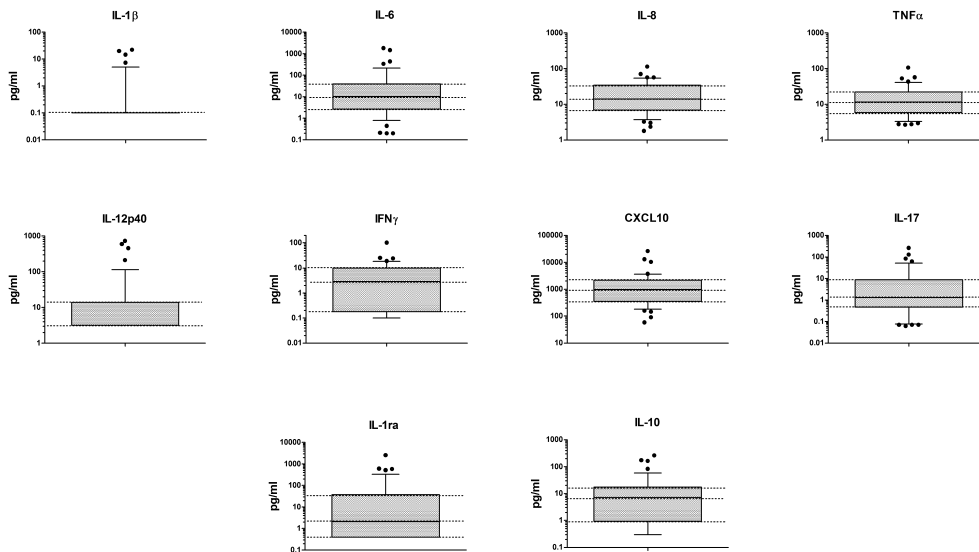
measured in plasma vs. sputum was calculated. As a first step, the distributions of the concentration levels for all cytokines measured in plasma (n=40) and sputum samples (n=15) were analyzed and ranked in quartiles. The cytokine values (in pg/mL) for the 25% percentile, median and 75% percentile for each of the measured cytokines in plasma and sputum are shown in **Table 1** and **Supplementary Figs. 1-2**). Next, for the 15 patients having both plasma and sputum data available, each of the eight pro-inflammatory cytokines was given a score of 1 through 4, according to the quartile it belonged (e.g., 1 for <25%, 2 for 25-50%, 3 for 50-75% and 4 for >75%). Then, the eight cytokine scores in plasma and sputum were added and the total defined as the "Pro-inflammatory cytokine score (PICS)" for the corresponding fluid. Finally, the PICS in plasma was divided by that in the sputum to obtain the Pro-inflammatory cytokine ratio (PICR). The values thus calculated for each of the 15 patients are shown in **Table 2**, along with their relevant clinical information. The TCS \leq 3d group (n=8) included five patients in PSI risk classes I-III and three in PSI risk classes IV-V; whereas the TCS>3d group (n=7) included two patients in PSI risk classes I-III and five in PSI risk classes IV-V.

3.3 Integration of Cytokine Response Profiles

In order to compare the combined cytokine response patterns in plasma and sputum among the patients and to investigate their association with measures of CAP outcomes (TCS) and severity (PSI), we developed an initial model based on a score, the PICR, calculated based on the ranked cytokine scores in plasma divided by sputum. The calculated PICR values for each patient are shown in **Table 2**. In addition, **Figure 3** shows the distribution of PICR for the patients grouped by TCS (\leq and >3 days) or by TSI group (classes I-III and IV-V). Comparison of the groups indicated that there were statistically significant differences in PICR values in both cases. Patients in the TCS>3d group had significantly higher PICR compared to the TCS \leq 3d group ($p=0.009$) and patients in PSI class IV-V had higher PICR values compared to those in PSI classes I-III ($p=0.042$).

3.4 Predictive value of PICR scores

Based on the statistically significant differences found, ROC curves were constructed in order to analyze the ability of PICR scores to predict CAP patient outcomes or severity. As shown in



Supplementary Fig. 1 Distribution of cytokine values in plasma as shown in **Table 1**. Box plots depict the 25-75% interquartile range and the horizontal bar depicts the median. Whiskers represent the 10 and 90 percentiles. Cytokine data from all 40 patients was included.

Figure 4, ROC curves analyzing the ability of PICR to discriminate between those patients with a TCS ≤ 3 d vs. > 3 d showed area under the curve (AUC) values of 0.893 ($p=0.011$), consistent with a very good level of accuracy. The cut-point optimizing specificity and sensitivity (Youden's Index) based on this graph is a PICR of 0.96, which would give a sensitivity of 85.7% and a specificity of 87.5%. PICR values were less accurate, however, at discriminating patients with non-severe vs. severe CAP, as defined by PSI class (AUC: 0.786; $p=0.064$).

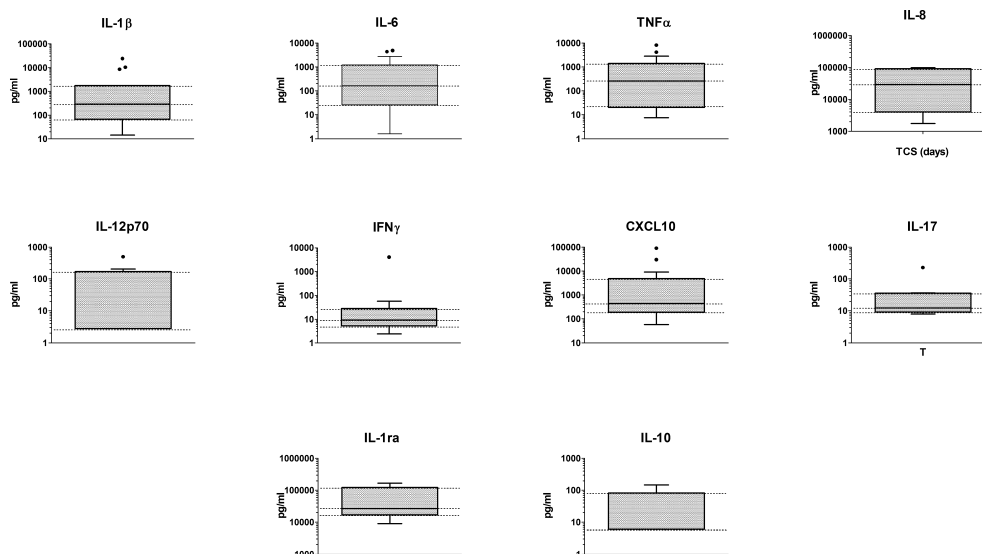
4 Discussion

Results from this study indicate that, similarly to patients severe CAP, patients with a TCS > 3 days have higher median plasma levels of several pro-inflammatory (IL-6, IL-8) and anti-inflammatory (IL-10) cytokines compared to those with shorter TCS. Moreover, our exploratory results suggest that it may be possible to analyze and integrate cytokine response "patterns" in CAP patients based on sputum and plasma cytokine levels assayed at hospital admission. These "patterns" or "scores" (i.e., PICR) may potentially be used as predictive tools for measures of outcomes and disease severity, identifying patients at risk to have a delayed TCS or a more severe disease. With one exception (patient 4), all of the patients in the TCS ≤ 3 d group had PICR values less than 1, suggesting a relatively stronger local compared to systemic response. The only exception was a patient with Haemophilus influenzae who had strong systemic and local cytokine responses, yet had a TCS of 2d. On the other hand, five out of the seven patients with a TCS > 3 d had PICR values > 1.00 , with the two exceptions being patients with infections by Pseudomonas aeruginosa and Acinetobacter baumannii.

Thanks to the current multiplex technology, it is now possible to measure multiple cytokines in the same sample, resulting in the generation of a substantial amount of data. However, it is not yet

clear what the best approach is to integrate all of the cytokine information. Even in our small study, it was evident from comparing the different individual cytokine patterns that considerable variability existed in the cytokine responses of the different patients. Our results suggest, indeed, that analysis of the relationship between the local and systemic cytokine responses may have some value in predicting CAP disease severity and outcomes and that it might help to make predictions and guide potential therapeutic anti-inflammatory and/or immuno-modulatory interventions even upon admission. Much of the evidence linking the deleterious effects of an over-abundant inflammatory response with the severity of CAP has been based on circulating, and thus systemic, cytokine levels⁵⁻⁸. However, local inflammatory responses have been less often studied and assumed to be reflected in the systemic cytokine levels. In a previous study¹⁹, as well as the present study, the distribution of the levels of the different pro-inflammatory cytokines in plasma supports a more pronounced systemic inflammatory response in patients with severe CAP or with a TCS > 3 d. However, cytokine responses in the sputum did not follow the same trend as in plasma, suggesting that these two compartments (lung vs. systemic) do not necessarily mirror each other.

Our model to integrate the local and systemic inflammatory phenotypes in CAP patients was based on the ranked levels of eight pro-inflammatory cytokines/chemokines. When analyzing the data, it was clear that not all cytokines in the same patient followed the same trends nor that all patients had the same cytokine patterns. Given the different etiologies and underlying factors in the different CAP patients, this was not entirely surprising. Indeed, there were differences of inflammatory phenotypes when comparing patients with CAP due to different etiological agents, suggesting that the inflammatory pattern might be influenced by the etiologic agent, in agreement with previous reports²⁴. The



Supplementary Fig. 2 Distribution of cytokine values in sputum as shown in **Table 1**. Box plots depict the 25-75% interquartile range and the horizontal bar depicts the median. Whiskers represent the 10 and 90 percentiles. Cytokine data from all 15 patients with sputum samples was included.

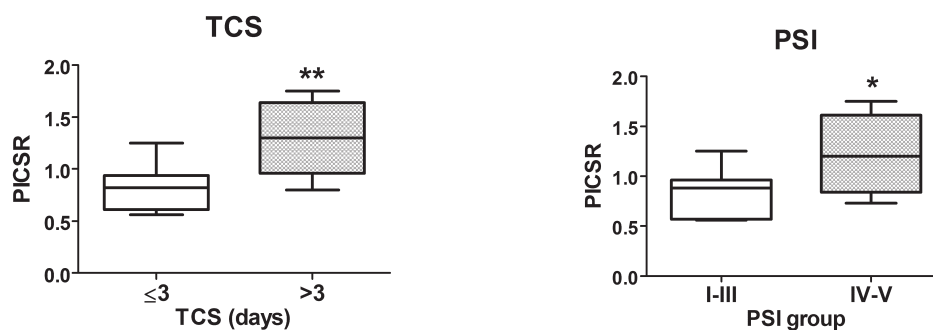


Fig. 3 Distribution of PICR scores in patients grouped by TCS (≤ 3 d or >3 d) or by PSI risk class (I-III and IV-V). PICR scores were calculated by dividing pro-inflammatory cytokine scores for plasma by those in sputum as indicated in Materials and Methods. Box plots depict the 25-75% interquartile range and the horizontal bar depicts the median. Groups were compared using the unpaired t-test. * $p < 0.05$; ** $p < 0.01$

effect of the etiologic agent in our model remains to be further investigated. Moreover, while the pro-inflammatory cytokine response was the initial focus of our model for this study, the involvement of anti-inflammatory cytokine responses needs also to be considered.

Clearly, the main limitation of this study is the sample size. Due to the limited size in this study, our exploratory model needs to be tested and validated against a more substantial number of patients. In this study, we only evaluated eight pro-inflammatory cytokines. Since other cytokines may play an important role in defining inflammatory phenotypes, including anti-inflammatory cytokines, future studies should explore additional pro- and anti-inflammatory cytokines. Additional limitations that may have had affected the measurements include the fact that our model is based on a mathematical analysis of cytokine concentrations.

However, given the potential interrelationships among the different cytokines, their effect *in vivo* is much more difficult, if not impossible, to measure with the current knowledge. Regarding the patients, pre-hospital admission of steroids and/or antibiotics may have influenced their responses and will have to be documented for future studies. Moreover, pre-existing comorbidities, some of which may induce enhanced systemic inflammation, may have also the potential to influence cytokine response patterns. Thus it will be important to characterize how these variables may affect systemic and local cytokine responses and thus disease severity and patient outcomes.

A main strength of this study is the fact that both local and systemic cytokine responses were measured at the time of the patient's admission, allowing the comparison of the two and their correlation with measures of disease severity and clinical out-

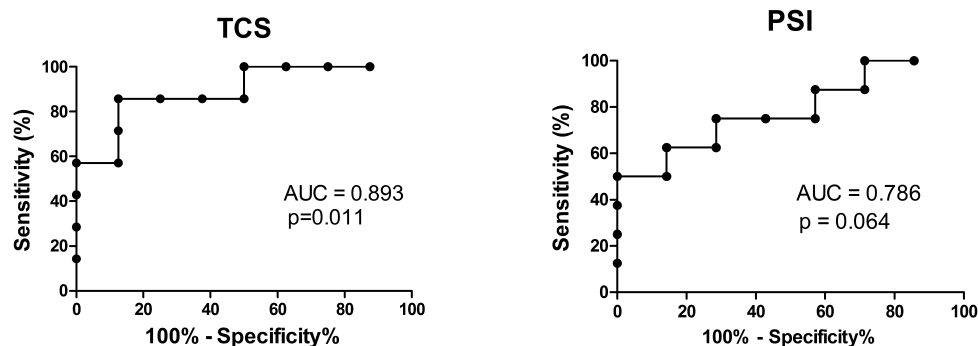


Fig. 4 ROC curves comparing the accuracy of PICR values to separate patients by TCS ($\leq 3d$ or $>3d$) or PSI (I-III and IV-V) group. AUC values are indicated in the graphs.

comes. While still preliminary, our results suggest the feasibility to use cytokine data at hospital admission to help identify those patients at higher risk. After further validation, such an approach may facilitate a better understanding of the relationship between local and systemic inflammatory responses and a more individualized therapeutic approach to modulate the inflammatory response in hospitalized patients with CAP.

Acknowledgments: We are indebted to Dr. Keith Falkner, Department of Pharmacology & Toxicology, University of Louisville, for his help and expertise in the use of the Luminex 100 instrument for the cytokine measurements.

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