ERJ Express. Published on January 11, 2013 as doi: 10.1183/09031936.00060112

Local and systemic cytokine profiles in non-severe and severe community-acquired pneumonia

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Key words: community-acquired pneumonia, cytokines, bronchoalveolar lavage, inflammation

Running title: Cytokine profiles in pneumonia

Total word count for the body of the manuscript: 3151

ABSTRACT

Local inflammatory responses in community-acquired pneumonia (CAP) remain insufficiently elucidated, especially in patients with non-severe CAP. In this study we determined local and systemic cytokine responses in CAP patients and correlated these with disease severity and other clinical parameters.

Levels of interleukin (IL)-6, IL-8, IL-10, IL-1β, tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-22, IL-17A and IL-4 were determined in bronchoalveolar lavage (BAL) fluid and serum of 20 CAP patients upon admission and 10 healthy individuals. Systemic cytokine levels were also measured on days 7 and 30.

In BAL fluid of CAP patients, levels of IL-6, IL-8, and IFNγ were significantly increased compared with healthy individuals, but no correlations with disease severity were found. Systemic levels of IL-6, IL-10 and IFNγ were significantly higher in severe CAP patients than in non-severe CAP patients and healthy individuals. Moreover, these cytokines showed a significant correlation with the pneumonia severity index (PSI). In the total group of CAP patients systemic IL-8 and IL-22 levels were also increased compared with healthy individuals.

We therefore conclude that IL-6, IL-10 and IFNγ are important cytokines in CAP, although differences in disease severity upon admission are only reflected by systemic levels of these cytokines.

Number of words in the abstract: 196

Introduction

Community-acquired pneumonia (CAP) continues to be a common and serious illness. Major gaps remain in the understanding of its pathogenesis: it is not clear why some individuals can easily control bacterial challenges and remain healthy, whereas others develop pneumonia. Several risk factors to calculate the probability of morbidity and mortality among CAP patients are known and described in prediction rules such as the pneumonia severity index (PSI) [1].

The clinical course of CAP is determined by inflammatory responses evoked by the causative pathogen. Studies in mice have shown that survival is associated with a strong inflammatory response early in the course of infection and rapid bacterial clearance [2]. In both mice and humans, regulation of this inflammatory response in pneumonia is dependent on complex interactions between immune cells and pro- and anti-inflammatory cytokines [2-4].

Several cytokines have been studied in relation to severity, aetiology and outcome of CAP [4-15]. Although the number of cytokines identified in the immunopathogenesis of CAP has grown considerably over the years, studies remain focused on well-known cytokines of the innate immune response, including interleukin (IL)-6, IL-10, IL-8, IL-1β, and tumour necrosis factor (TNF)α. But also IL-17A and IL-22, which belong to the novel T helper (Th) 17 subset, have been implicated in CAP [2-3]. Furthermore, interferon (IFN)γ is an important cytokine in both innate and adaptive immunity to respiratory pathogens [16], and IL-4 might be important in the immune response against *Mycoplasma pneumonia* [17]. Further characterization of local and systemic cytokine responses in CAP patients may increase our understanding of the host defence, with the goal of providing prognostic tools for clinicians or identifying potential therapeutic targets.

To date, most studies have focused on systemic inflammatory responses by measuring cytokines in peripheral blood of CAP patients. Those few reports in which local pulmonary cytokines were investigated generally included severe CAP patients on ICU wards [12, 14, 18] or patients with treatment failure [4]. Meanwhile, information on local cytokine responses in non-severe CAP patients is very limited. We hypothesised that the inflammatory response in CAP is compartmentalized and that disease severity correlates better with the local inflammatory response than with the systemic response. In this study we therefore determined both local and systemic cytokine responses in patients with non-severe and severe CAP, and correlated these with severity scores and other clinical parameters.

Material and methods

See the online depository for an extended version of the METHODS.

Study design

A prospective study was performed in twenty CAP patients between January 2009 and May 2011. Patients admitted through the emergency wards of the Erasmus MC and Sint Franciscus Gasthuis, which are both teaching hospitals, were enrolled in the study. The medical ethics committee of both hospitals approved the study. Written informed consent was obtained from the patient or closest relatives.

Inclusion and exclusion criteria are described in the online depository. Ten healthy volunteers matched for age, sex and smoking status and without history of cardiac or pulmonary disease, malignancy or autoimmune disease served as the control group.

Selection of antibiotic treatment was based on national guidelines [19]. The pneumonia severity index (PSI) and CRB65 scores were determined upon admission and patients were classified as non-severe CAP (PSI classes 1-3), or severe CAP (PSI classes 4 or 5).

Obtaining and processing of BAL and blood samples

After written informed consent and within 24 hours after admission, bronchoalveolar lavage (BAL) fluids were collected with a flexible fibre-optic bronchoscope (Olympus) according to recommended guidelines [20]. Venous blood samples were collected directly prior to the BAL procedure. At day 7 and 30 after admission, additional venous blood samples were collected. Methods of processing are described in the online depository.

Cytokine measurements

Levels of IL-6, IL-8, IL-10, IL-1β, TNFα, IFNγ, IL-22, IL-17A and IL-4 were determined by enzyme-linked immunosorbent assay (ELISA), using commercially available assays. Details and detection limits are provided in the online depository.

Statistical analysis

Data are shown as mean values (±SD) in cases of normally distributed data or median values with percentiles if not normally distributed. Cytokine levels were not normally distributed and therefore nonparametric tests were used to compare groups (Kruskal-Wallis test for across group comparison of three or more groups, Mann-Whitney U-test for pair-wise analyses). Normally distributed data were analyzed by unpaired t-tests. Correlations were calculated using Spearman's Rank correlation coefficient. Data analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 and Prism 5.01 (GraphPad). Statistical significance was taken as a p-value <0.05.

Results

Clinical characteristics of study population

Twenty CAP patients and ten healthy individuals matched for age, gender and smoking status were included in this study. Clinical characteristics at baseline of the study population are shown in Table 1.

Thirteen patients (65%) had significant comorbidity (chronic obstructive pulmonary disease, heart disease, neurological disorder, chronic renal disease and diabetes mellitus). Based on the PSI scores upon admission, 10 patients were allocated to the non-severe CAP patient group, and 10 to the severe CAP patient group. In the total patient group, 5 patients (25%) were on statin therapy and 5 patients (25%) reported taking inhalation corticosteroids (ICS). Time from onset of symptoms to hospital admission ranged from 2 to 144 hours, with a median of 48 hours. Eight patients (40%) were included within a time frame of 48 hours and the other 12 (60%) at more than 48 hours from onset of symptoms. Severe CAP patients had a shorter period of time between onset of symptoms and hospital admission compared with the non-severe CAP patients (medians 30 and 48 hours respectively, p=0.01). Six patients (30%) reported taking antibiotics prior to hospital admission. Mean C-reactive protein (CRP) level of the patients upon admission was 227±170 mg/l. No differences in CRP levels were found between the non-severe and severe CAP patients. Furthermore, no significant correlation between CRP concentration and PSI was found. The two patients (5%) who died were both severe CAP patients.

In 14 patients (70%) a microorganism was identified (Table 1). There was no difference in incidence of pathogens found between non-severe and severe CAP patients. The most common pathogen was *S. pneumoniae*, present in seven patients (35%). A viral pathogen was found in 2 patients (10%).

Cytokine levels in BAL fluid

Levels of IL-6, IL-8, IL-1β and IFNγ were detectable in BAL fluid of all CAP patients. BAL fluid levels of IL-10, and IL-22 were only detectable in part of the CAP patients (five and six severe CAP patients, respectively). TNFα was only detectable in one non-severe and three severe CAP patients. In healthy individuals IL-10, TNFα and IL-22 were all below the detection levels. IL-17A and IL-4 were not detectable in BAL fluid of patients or healthy individuals.

When comparing the total group of CAP patients with healthy individuals, we found that IL-6, IL-8 and IFNγ levels in BAL fluid of CAP patients were significantly higher (Figure 1). A separate analysis of the non-severe and severe CAP patient groups showed that in both groups IL-6 was significantly increased, compared with healthy individuals (Figure 1). Levels of IFNγ were also significantly increased in severe CAP patients compared with healthy individuals, but not compared with non-severe CAP patients (Figure 1). For IL-8, IL-10 and IL-22 trends were observed towards increased levels in severe patients versus non-severe patients and healthy individuals, but significance was not reached (Figure 1).

None of the cytokines detectable in BAL fluid of patients upon admission correlated with PSI or CRB65 scores, with the exception of IL-10 for which a weak correlation was found with CRB65 ($p= 0.036$; $\rho= 0.47$; data not shown). Furthermore, cytokine levels in BAL fluid of CAP patients upon admission showed no significant correlations with the causative pathogen found or with time from onset of symptoms to hospital admission. When we compared patients with a pneumococcal etiology (n=7) with patients with bacterial non-pneumococcal etiology (n=5), we did not find significant differences in BAL fluid cytokines. Finally, no correlations with cytokine levels in BAL fluid were observed with other clinical parameters, including COPD comorbidity, mechanical ventilation, mortality, presence of

bacteraemia, prior use of antibiotics, use of statin therapy, smoking habits or CRP levels upon admission. IFNγ levels in BAL fluid of CAP patients were however significantly lower in ICS users compared with non-users (p=0.02).

Systemic cytokine levels

When comparing the total group of CAP patients upon admission with healthy individuals, we found that the concentrations of IL-6, IL-8, IL-10 and IL-22 in serum were significantly increased in patients (Figure 2). TNFα, IL-17A and IL-4 could not be detected in the serum of patients or healthy individuals. For IL-1β, seven CAP patients (35%) had low but detectable serum levels (median 0 pg/ml; 10^{th} and 90^{th} percentiles 0-4.3 pg/ml, data not shown). Serum levels of IL-6, IL-10 and IFNγ upon admission were significantly higher in severe CAP patients than in non-severe patients and healthy individuals (Figure 2). In addition, IL-6 in non-severe CAP patients was significantly increased compared with healthy individuals. IL-8 and IL-22 levels were similar in non-severe and severe CAP, but IL-22 levels of both patient groups were significantly higher than in healthy individuals (Figure 2).

We also investigated changes of serum cytokine levels over time. Seven days after admission, IL-6 and IL-10 already normalized to levels similar to those of healthy individuals. Whereas IL-22 reached normal levels after 30 days, IL-8 remained elevated at day 30 after admission compared with healthy individuals (Figure 3).

When correlating serum cytokine levels with clinical parameters, we found several correlations. First, in contrast to our analyses in BAL, serum concentrations of IL-6, IL-10 and IFNγ upon admission showed strong positive correlations with PSI (Figure 4). Similar positive correlations with CRB65 scores were found for serum concentrations of IL-6 ($p< 0.001$; $\rho= 0.76$) and IL-10 ($p<0.001$; $\rho= 0.80$), but not for serum IFNγ (data not shown). Furthermore, patients with bacteraemia had significantly higher serum levels of IL-6 and IL-

10, compared with non-bacteraemic patients (p=0.005 and p=0.007, respectively, data not shown). Those four patients who required mechanical ventilation had higher serum IL-10 levels upon admission than patients who did not (p=0.02), but other cytokines measured in serum were not significantly higher in these four patients. The two patients (5%) who died within 30 days of hospital admission also had higher serum IL-10 levels, compared with surviving patients upon admission (p=0.03). Although we could identify a causative pathogen in 70% of patients, we did not find correlations between serum cytokine levels and pathogens. In addition, COPD comorbidity, the reported usage of antibiotics prior to hospital admission or the time of onset between symptoms and admission to the hospital had no detectable effect on serum cytokine levels. Statin therapy, which may induce systemic inhibition of pro-inflammatory cytokines [21], and ICS usage did not have any detectable effect on serum cytokine levels in our cohort. Finally, no correlations between serum levels of any of the cytokines tested, including IL-6 and CRP levels upon admission, were found.

Correlations between BAL fluid and serum cytokines

In CAP patients, IL-6, IL-8 and IL-1β levels in BAL fluid were significantly higher than those in serum (p=0.0019, p=<0.0001 and p=0.0007 respectively). In contrast, IL-10 levels were higher in the serum than in BAL fluid (p=0.03), although levels were low in both compartments.

In CAP patients, a positive correlation was found between IL-6 levels in serum and BAL fluid (rho=0.58, p=0.003, data not shown). None of the other cytokines tested, showed a correlation between serum and BAL fluid.

Discussion

The host inflammatory response in CAP is largely compartmentalized to the affected lung [6-7]. Nevertheless, local pulmonary cytokine responses remain insufficiently elucidated, especially in patients with non-severe CAP. To our knowledge, this is the first study investigating local pulmonary and systemic cytokine profiles in both non-severe and severe CAP patients directly upon admission to the hospital. Our most important finding is that although inflammatory cytokine responses in CAP are higher in the lungs than in peripheral blood, disease severity only correlated with systemic IL-6, IL-10 and INFγ levels and not with any of the local cytokines tested. This study showed that in BAL fluid, levels of IL-6, IL-8 and IFNγ were significantly elevated in CAP patients compared with healthy individuals. In serum, IL-6, IL-8, IL-10 and IL-22 levels, but not IFNγ, were significantly increased compared with healthy individuals. However, of these cytokines IL-6, IL-10 and IFNγ in serum could differentiate between non-severe and severe CAP. Furthermore, levels of IL-6 in serum and BAL fluid were correlated. Finally, important inflammatory cytokines like TNFα and IL-17A were undetectable in BAL fluid or serum of CAP patients.

IL-6, IL-8 and IL-10 are three of the most studied cytokines in CAP. In line with previous studies, we found significantly increased IL-6 and IL-8 levels in BAL fluid in CAP patients [4, 6-7, 14, 18]. IL-10 was not detectable in BAL fluid of healthy individuals or non-severe CAP patients and only in five out of ten severe CAP patients. Two groups have previously reported low but detectable IL-10 levels in BAL fluid of CAP patients [4, 12]. Our inability to detect IL-10 in part of our patients could be due to differences in detection limits, study design or study population. Lee *et al.* studied only severe CAP patients on mechanical ventilation [12] and in Moret *et al*. there is a delay in sampling compared to our study,

because BAL samples were analyzed in patients with treatment failure at 72 hours after start of antibiotic treatment [4].

Whereas serum levels of IL-6 and IL-8, IL-10 and IL-22 were significantly higher in patients compared with healthy individuals, serum IFNγ levels were only significantly higher in severe CAP patients. In contrast to BAL fluid cytokine levels, serum levels of IL-6 and IL-10 and IFNγ, proved to be good tools to discriminate between non-severe and severe CAP. Hereby, IL-6 and IL-10 acted as acute phase responders, since at day 7 levels decreased to values similar to those found in healthy individuals, consistent with previous reports [5, 8, 10].

The observed strong local and systemic induction of IL-6 emphasizes the importance of this cytokine in the inflammatory response in pneumonia. Furthermore, the correlation between IL-6 in BAL fluid and serum suggests that IL-6 produced in the lung contributes at least in part to serum levels of this cytokine [7, 11]. Systemic IL-6 might therefore be a valuable biomarker to define severity of disease and act as a prognostic indicator in CAP patients [22-24]. Interestingly, in contrast to systemic IL-6, IL-10 and IFNγ concentrations, CRP levels could not differentiate between non-severe and severe CAP patients. Adding systemic IL-6 and/or IL-6 to IL-10 ratio measurements [25] to existing prognostic scales such as the PSI or CRB65, might therefore improve mortality prediction in CAP patients.

To our knowledge, no previous studies of CAP patients have included IFNγ measurements in BAL fluid. We found that (i) IFNγ levels in BAL fluid were significantly elevated in severe CAP patients compared with healthy individuals, (ii) systemic levels were significantly increased in severe CAP patients compared with non-severe CAP patients or healthy individuals, and (iii) systemic levels correlated with PSI. In contrast to IL-6 and IL-10, concentrations of IFNγ in serum did not show a significant correlation with the CRB65 disease severity scores. This may be related to the finding that our total group of CAP patients

exhibited significantly increased concentrations of IL-6 and IL-10 but not IFNγ in the serum. Although many cells have the capacity to produce IFNγ, it is the hallmark cytokine of Th1 cells. *In vitro* experiments with *S. pneumoniae* demonstrated the importance of Th1 cytokine production in early phases of disease [26]. The Th2 cytokine IL-4 and the Th17 cytokine IL-17A were not detectable in BAL fluid or in serum of CAP patients in early or late phases of disease. IL-22 is an IL-10 family cytokine member and can be produced by Th17 cells [27]. Importantly, in an experimental model of Gram-negative pneumonia, it has been shown that IL-22 can augment epithelial antimicrobial activity, thereby providing a crucial role in mucosal host defence in mice [2]. In BAL fluid, 60% of severe CAP patients had detectable but not significantly elevated levels of IL-22. Our finding that in serum of patients, both with non-severe and severe CAP, levels of IL-22 were significantly elevated supports the importance of this cytokine in the host response in human pneumonia.

Several factors can potentially influence inflammatory responses in patients with CAP. Previous studies showed that high doses of ICS may affect the immune system [28]. In our study population, we found lower concentrations of IFNγ in the BAL fluid of ICS users, compared with non-users. Because most ICS users were non-severe CAP patients (Table 1), it is possible that the lower levels of IFNγ in BAL fluid of non-severe CAP patients, is in fact due to ICS use. Similar to other studies [4, 13], we found a large scatter in cytokine concentrations in both BAL fluid and serum. One explanation could be that the type and magnitude of cytokine secretion varies between different causative pathogens [29-30], although in our cohort we did not detect a relation between bacterial species and cytokine levels.

The present study has several limitations that should be considered. First, the number of patients included was small, although comparable to other studies of local inflammatory responses in CAP [6, 14]. Nevertheless, we were able to classify the study cohort into both

non-severe and severe CAP patients and to determine both local and systemic cytokine concentrations in all patients. Another limitation relates to uncontrolled factors present before patients entered our study. Patients were admitted to the hospital at different disease stages and some of them had already started antibiotic treatment. Although we did not find a relationship between time of onset of symptoms or prior antibiotic use and cytokine levels, we cannot completely exclude the possibility of modulation of the inflammatory response and cytokine expression by these factors. Because COPD is associated with substantial chronic inflammation, COPD is an important potential confounder in CAP. Comparison of the five COPD with the 15 non-COPD CAP patients in our study did not reveal significant differences in local or systemic cytokine levels. However, the low number of CAP patients without comorbidity, ICS or antibiotic treatment (only 4 out of 20), precluded statistical analysis of confounding factors.

In conclusion, our study provides a comprehensive analysis of cytokine profiles in CAP. We show that systemic levels of IL6, IL-10 and IFNγ can discriminate between non-severe and severe CAP patients. Levels of IL-6, IL-8 and IFNγ in BAL fluid were significantly higher in patients than in healthy individuals, but did not correlate with disease severity. We also found a correlation between IL-6 levels in BAL fluid and serum of patients. These results show the importance of the systemic inflammatory response in CAP and further emphasize the importance of IL-6, but also of IFNγ in the local and systemic inflammatory response in patients with CAP. Future studies should show whether measurements of these cytokines are valuable to improve prognosis predictions.

Abbreviations

BAL	Bronchoalveolar lavages
CAP	Community-acquired pneumonia
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
ICS	Inhaled corticosteroids
IFN	Interferon
IL	Interleukin
PSI	Pneumonia Severity Index
Th	T helper
TNF	Tumor necrosis factor

Disclosure

All authors declare that they have no competing interests.

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Table 1. General characteristics of the study population

	CAP (total) n=20	Non-severe CAP (PSI 1-3) n=10	Severe CAP (PSI 4-5) n=10	Healthy controls n=10
Mean (SD) age in years	60.6±19	54.5±18.4	66.6±15.8	54.8±5.7
Sex, no. (%)				
Male	13 (65)	6 (60)	7 (70)	6 (60)
Female	7 (35)	4 (40)	3 (30)	4 (40)
Smoking, no. (%)	8 (40)	4 (40)	4 (40)	4 (40)
Comorbidity, no. (%)				n/a
COPD	5 (25)	4 (40)	1 (10)	
Heart disease	6 (30)	1 (10)	5 (50)	
Neurological disorder	4 (20)	1 (10)	3 (30)	
Chronic renal disease	1 (5)	-	1 (10)	
Diabetes mellitus	2 (10)	1 (10)	1 (10)	
PSI class, no. (%)				n/a
I	3 (15)	3 (30)	-	
II	5 (25)	5 (50)	-	
III	2 (10)	2 (20)	-	
IV	6 (30)	-	6 (60)	
V	4 (20)	-	4 (40)	
Mechanical ventilation, no. (%)	4 (20)	-	4 (40)	n/a
Mortality, no. (%)	2 (10)	-	2 (20)	n/a
Bacteriaemia, no. (%)	5 (25)	1 (10)	4 (40)	n/a
Prior antibiotic use, no. (%)	5 (25)	3 (30)	2 (20)	n/a
ICS	5 (25)	4 (40%)	1 (10)	n/a
Microbiological species, no. (%)	14 (70)	7 (70)	7 (70)	n/a
Streptococcus pneumoniae	7 (35)	3 (30)	4 (40)	
Stenotrophomonas maltophilia	1 (5)	1 (10)	-	
Pseudomonas aeruginosa	1 (5)	1 (10)	-	
Streptococcus pyogenes	1 (5)	-	1 (10)	
Staphylococcus aureus	1 (5)	1 (10)	-	
Haemophilus influenzae	1 (5)	-	1 (10)	
H1N1	1 (5)	-	1 (10)	
Adenovirus	1 (5)	1 (10)	-	
Unknown	6 (30)	3 (30)	3 (30)	

CAP: community-acquired pneumonia. SD: standard deviation. COPD: chronic obstructive pulmonary disease. PSI: pneumonia severity index. ICS: inhaled corticosteroids. n/a: not applicable.

Figure legends

Figure 1. BAL fluid cytokine levels upon admission in CAP patients and in healthy individuals. CAP patients were classified as non-severe CAP (NSCAP, PSI classes 1-3) or as severe CAP (SCAP, PSI classes 4 or 5). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between groups were first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001.

CAP: community-acquired pneumonia. SCAP: severe CAP. NSCAP: non-severe CAP. HC: healthy control. IL: interleukin. IFN: interferon. N.D.: not detectable.

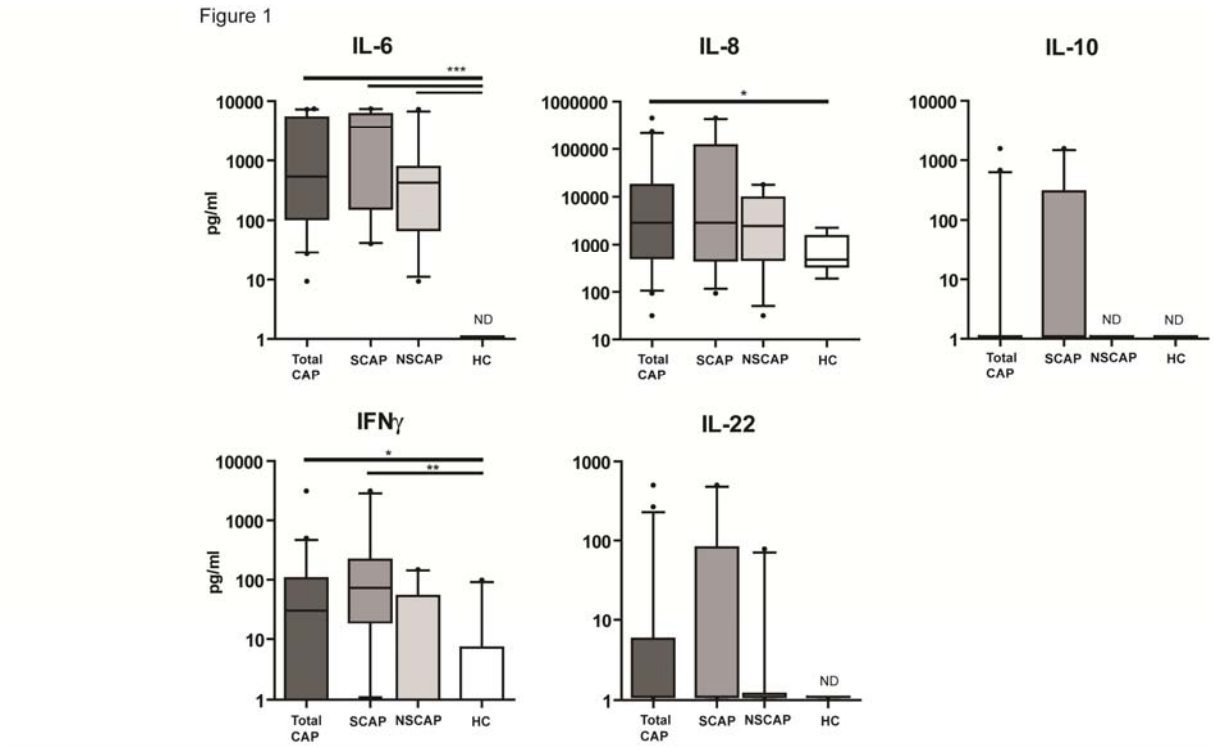

Figure 2. Serum cytokine levels upon admission in CAP patients and in healthy individuals. CAP patients were classified as non-severe CAP (NSCAP, PSI classes 1-3) or as severe CAP (SCAP, PSI classes 4 or 5). Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences between groups were

first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test.*: p<0.05; **: p<0.01; ***: p<0.001.

CAP: community-acquired pneumonia. SCAP: severe CAP. NSCAP: non-severe CAP. HC: healthy control. IL: interleukin. IFN: interferon. N.D.: not detectable.

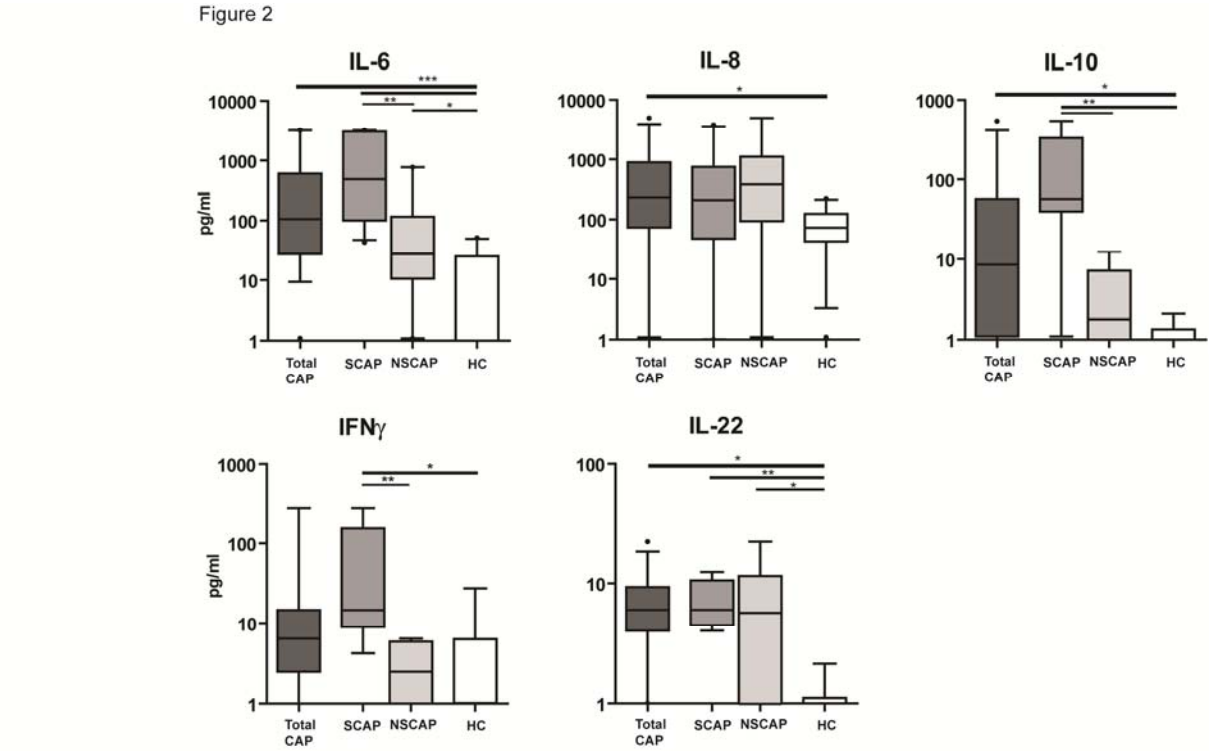

Figure 3. Serum cytokine levels upon admission, at day 7 and day 30 after admission in CAP patients and in healthy individuals. Data are shown as box and whisker plots with 10th and 90th percentiles. Bold lines represent median values. Differences in serum levels in CAP patients over time were tested as paired data with Wilcoxon signed rank test. Differences between different time points in CAP patients and healthy individuals were first tested with Kruskall Wallis tests and when significant, pair wise tested with the Mann Whitney U test. *: p<0.05; **: p<0.01; ***: p<0.001. CAP: community-acquired pneumonia. Adm: Admission. HC: healthy control. IL: interleukin. IFN: interferon.

Figure 3

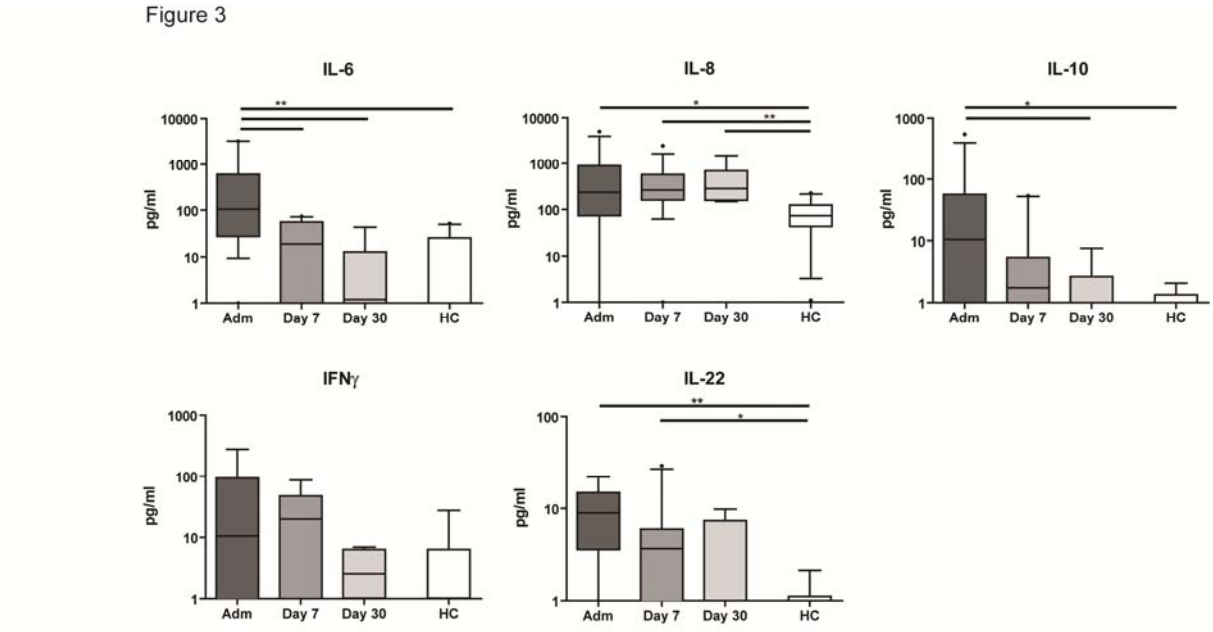

Figure 4. Correlations of serum cytokine levels with pneumonia severity index upon admission.

PSI: pneumonia severity index. IL: interleukin

Figure 4