Research article

Systemic interleukins levels in community-acquired pneumonia and their association with adverse outcomes

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Abstract

*Introduction: Community-acquired pneumonia (CAP) is still one of the major causes of morbidity and mortality worldwide. Pro-inflammatory and anti-inflammatory interleukins have been studied to elucidate the role that inflammation plays in its pathogenesis. The aim of this study is to investigate inflammation in CAP, by analyzing in dynamic, serum levels of six interleukins (IL) and their predictive value regarding adverse outcomes. **Materials and methods**: Forty adult patients with CAP, admitted in the Teaching Hospital of Infectious Diseases, Cluj-Napoca, Romania from December 2015 to February 2017, were enrolled in this study. Serum levels of pro-inflammatory: IL-1β, TNF-α, IL-6, anti-inflammatory: IL-10 and IL-4, along with IL-17A were analyzed in dynamic, on day 1 and day 4.The receiver – operator curves (ROC) were used to analyze the outcome prediction of IL. **Results**: Serum levels of IL-1β, IL-6, TNF-α and IL-10 have decreased significantly in dynamic, while IL-4 increases. IL-17A has acted like a pro-inflammatory cytokine. We have found a correlation between IL-6 and IL-10 (r=0.429, p=0.000), IL-6 and IL-17A (r=0.295, p=0.008) and IL-10 and IL-17A (r=0.475, p=0.000). Out of 40 patients, 9 had adverse outcomes, consisting in 9 relapses from which 1 died. IL-6 discriminates alone between adverse and favorable outcomes. With multivariate analysis and multiple regression of all combined IL, we have found that there is a predictive model regarding adverse outcomes. **Conclusion**: IL-10 and IL-17A behave like pro-inflammatory cytokines. IL-6 is a predictive marker for adverse outcomes alone. All IL studied together have an impact on adverse outcomes.*

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Introduction

Community-acquired pneumonia (CAP) remains one of the major causes of morbidity and mortality worldwide, despite the advances in diagnostic methods and treatment and it is the leading cause of severe sepsis [1,2]. Recent studies have been focused on the role that inflammation plays in the pathogenesis of this disease. Inflammation is part of the host's immune response against infection and if uncontrolled, it can have deleterious consequences for the host [3,4]. Several cytokines have been identified in immunopathogenesis of CAP, but most studies that analyzed them, have reported conflicting results and were limited to a single serum determination. IL-1β, IL-6, IL-8 and TNF-α as pro-inflammatory, and IL-10 as anti-inflammatory cytokines, have been the most studied in CAP [1,5]. IL-4 is another anti-inflammatory cytokine that has been postulated to have important role in allergic diseases, but also in the immune response against *Mycoplasma pneumoniae* [6,7]. IL-17A has recently been involved in the immune response during CAP, playing an important role in acute inflammation induced by infectious agents in the airway. [7,8].

This study aimed to investigate inflammation in CAP, by testing serum levels of IL-1β, TNF-α and IL-6 as pro-inflammatory cytokines, IL-10 and IL-4 as anti-inflammatory cytokines and the relationship between them and IL-17A. We studied the interleukin dynamics on admission and on day 4 of hospitalization and their predictive value regarding adverse outcomes.

Materials and methods

Patients and study design

A prospective study was carried out from December 2015 to February 2017 in the Teaching Hospital of Infectious Diseases Cluj-Napoca, Romania and it included adult patients with community-acquired pneumonia defined according to the IDSA [9]. The study protocol was approved by the medical ethics committee of both the Hospital and "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania. A written informed consent was obtained from the patients or closest relatives in each case. Exclusion criteria were represented by: age under 18 years old, pregnancy, HIV infection, active tuberculosis, known malignancies and autoimmune diseases requiring immunosuppressive drugs. Patients were followed-up 90 days after discharge.

Patients were enrolled in the study group within 24 hours of hospital admission. We recorded demographical data, clinical data, length of stay (LOS) in the hospital, various scores for pneumonia severity [10,11], usage of antibiotics prior to hospital admission, presence of complications such as sepsis, according to ACCP/SCCM criteria [12], acute respiratory failure and pleurisy, adverse outcomes during follow-up (relapses or death).

Venous blood samples for cytokine measurements were collected from each patient within the first 24 hours following admission, between 7 and 9 AM, and then again on day 4 of hospitalization. After centrifugation, the serum from patients was split into 2-4 cryotubes and stored at -70º C until it was analyzed.

In order to study the relationship between CAP and inflammation, we analyzed serum levels of cytokines using commercially available enzyme immunoassay technique (ELISA) (IL-1β, IL-4, IL-6, IL-10 and IL-17A using BioVendor®Research and Diagnostic products, USA; TNF-α using Hycult® biotech assay,USA). The detection limits were 0.3 pg/ml for IL-1β, 1.3 pg/ml

for IL-4, 0.92 pg/ml for IL-6, 1.0 pg/ml for IL-10 and 0.5 pg/ml for IL-17A.

Statistical analysis

For statistical analysis, to test for the normality of data, we used Shapiro-Wilk test, for nonparametric data, , the chi square, Wilcoxon, Friedmantests.The Spearman rank correlation test was used in order to assess the relationship between interleukin age and pneumonia severity index. The receiver – operator curves (ROC) with the level of significance set at $p< 0.05$, was used to analyze the accuracy of prediction by estimating the area under the curve. We performed ROC curves using Excell MedCalc 9.6 version and the multivariate analysis with StatMed. For logistic regression (Wald chi, ENTER method) we used SPSS 16.0 (SPSS Inc, Chicago, IL, USA) and MedCalc v12.

Results

Forty CAP patients were included in this study, with the mean age 63.45 ± 18.410 (range 23-87); 27 (67.5%) were men and 23 (57.5%) came from the urban areas. The average LOS in the hospital was 8.58 ± 3.137 days, with no significance between gender (p=0.628) or age (p=0.246). Prior to hospitalization, 8 patients had antibiotic treatment within the last 2 weeks. Etiology was established for 10 patients (25%): 2- *Strepto-coccus pneumoniae*, 3- *Haemophilus influenza*, 1- *Haemophilus parainfluenzae*, 1- *Chlamydia pneumoniae*, 1- *Serratia marcenscens (from community)*, 2- *Influenza virus* (1 A, 1 B).

Regarding complications, 13 patients had respiratory sepsis, 13 pleurisy and 39 had different degrees of acute respiratory failure. The Pneumonia Severity Score (PSI) and CURB-65 were calculated for each patient at admission. Regarding PSI, our patients were included as follows: 2 patients in risk class I, 4 in class II, 5 in class III, 20 in class IV, and 9 in class V.

For CURB-65: 6 patients were included in risk group 1, 21 in group 2 and 13 in group 3. We also used SMART-COP score for 38 of our patients (2 cases had incomplete data). The majority (24 patients) were included in low risk of needing intensive respiratory or vasopressor support (IRVS), 6 had moderate risk of IRVS, and 8 were considered to have severe CAP (7 with high risk of IRVS and 1 with very high risk).

The serum levels of six cytokines were determined at admission and on day 4. Their median values and differences between day 1 and day 4 are presented in table 1 [table 1 inserted here].

As shown above, the values of IL-1β, IL-6, IL-10 and TNF-α decreased significantly between day 1 and day 4. IL-4 values increased significantly on day 4 compared to day 1, while IL-17A seemed to act as a pro-inflammatory cytokine, but its values remained almost unchanged (figure 1)

There is a positive correlation between IL-1β and IL-6, TNF-α, IL-4; IL-6 and TNF-α, IL-17A, IL-10, IL-4; IL-4 and IL-17A, IL-10; and between IL-10 and IL-17A. (table2)

There is no correlation between patients' age and interleukin levels (IL-1β: p=0.983; IL-6: p=0.481; TNF-α: p=0.459; IL-17A: p=0.268; IL-4: p=0.656; IL-10: p=0.548), nor between gender and interleukin levels (IL-1β: p=0.062; IL-6: p=0.461; TNF-α: p=0.965; IL-17A: p=0.977; IL-4: p=0.355; IL-10: p=0.170).Likewise, we found no significant correlations between PSI risk score and cytokine levels (IL-1β: p=0.483; IL-6: p=0.730; TNF-α: p=0.775; IL-17A: p=0.744; IL-4: p=0.112; IL-10: p=0.152)Out of the 40 patients under study, 9 (22%) had adverse outcomes (9 relapses, from which 1 died). We questioned if any interleukin value on day 1 (prior to antibiotic treatment) was predictive for adverse outcomes and the results were that only IL-6 had significant contribution (p=0.043, cut-off= 28.46 pg/mL), discriminating between adverse outcomes and favourable outcomes.

Table 1. Differences between the values of the cytokines on day 1 and day 4

Interleukins		Median	IQR	Statistical results	
				p-value	Statistic test
IL1β	day 1	6.025	1.506	0.000	Z=-4.664
	day 4	5.092	0.796		
IL4	day 1	21.515	6.427	0.021	Z=-2.301
	day 4	24.789	10.709		
IL6	day 1	57.053	48.714	0.000	Z=-4.033
	day 4	36.026	12.980		
IL17A	day 1	8.342	10.850	0.925	U=-0.094
	day 4	7.845	6.951		
IL10	day 1	8.098	4.9412	0.000	U=-4.032
	day 4	6.098	1.979		
TNFα	day 1	12.872	3.502	0.006	U=-2.748
	day 4	10.234	2.968		
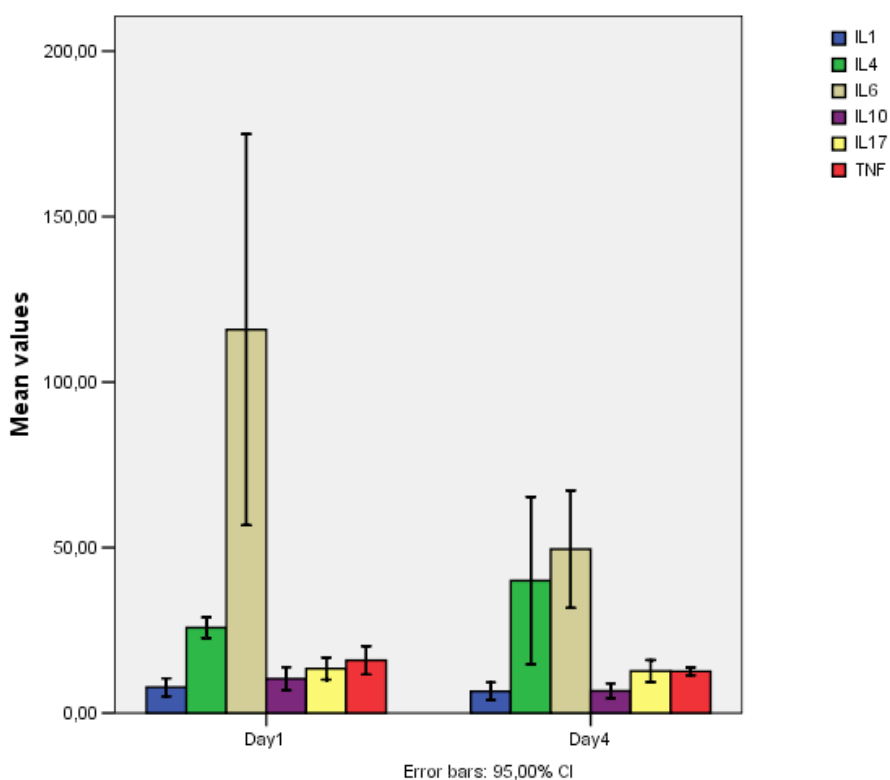
Fig.1. Serum concentration of interleukins on admission and on day 4 of hospitalization

IL-4 (p=0.056, cut-off=18.89 pg/mL) and IL-10 (p=0.07, cut-off=3.783 pg/mL) tend to reach statistical significance. The ROC curve analysis for adverse outcome prediction on day 1 is shown in figure 2.

The multivariate analysis of all combined interleukins on day 1 showed that there was a predictive model regarding adverse outcomes, AUC = 0.887 with CI95% [0.760, 0.913] (p =0.001). The model explains 87% of the variation of the outcomes.

We then studied if any of the interleukins were predictive for adverse outcomes on day 4. None reached statistical significance (figure 3).

Regarding the impact on adverse outcomes, the multivariate analysis of all combined interleukins has shown that the model explains roughly 48% of the variation in the outcomes (Wald chi2 test (p=0.025), Cox&Snell (R2=0.304), Nagelkerke (R2=0.481) and Hosmer and Lameshow Test (p=0.42) were used). In multiple regression, using ENTER method, for adverse outcomes as the dependent variable, only IL-1β and IL-6 had statistical significance on day 4 (table 3).

We questioned what happened between day 1 and day 4, so we studied the behaviour of each interleukin in every case. The behaviour of IL-1β differed from day 1 to day 4 in every patient (Marginal Homogeneity Test= 286.64, p=0.08). Therefore, we applied Wilcoxon Signed Ranks test (z=-4.685, p=0.001) which indicated that IL-1β values decreased from day 1 to day 4 in most patients. The exceptions were 2 patients, from which 1 died and the other was aggravated. IL-6 had similar behaviour (Marginal Homogeneity Test p= 0.042; Wilcoxon Signed Ranks Test p=
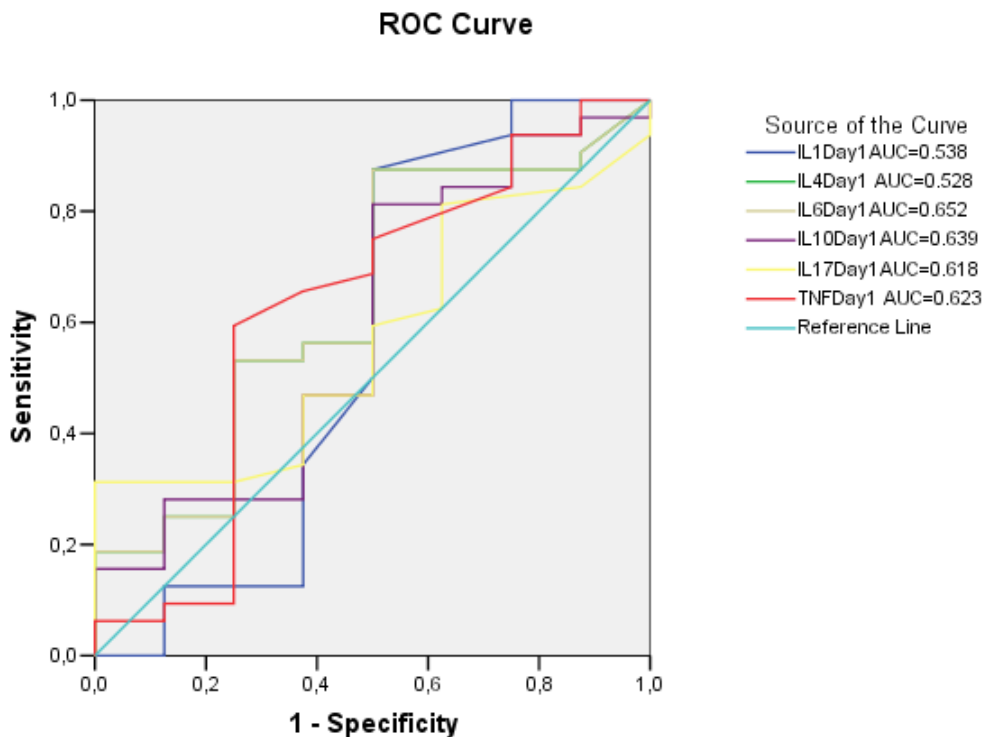
Fig.2. The ROC curve analysis for adverse outcome prediction on day 1
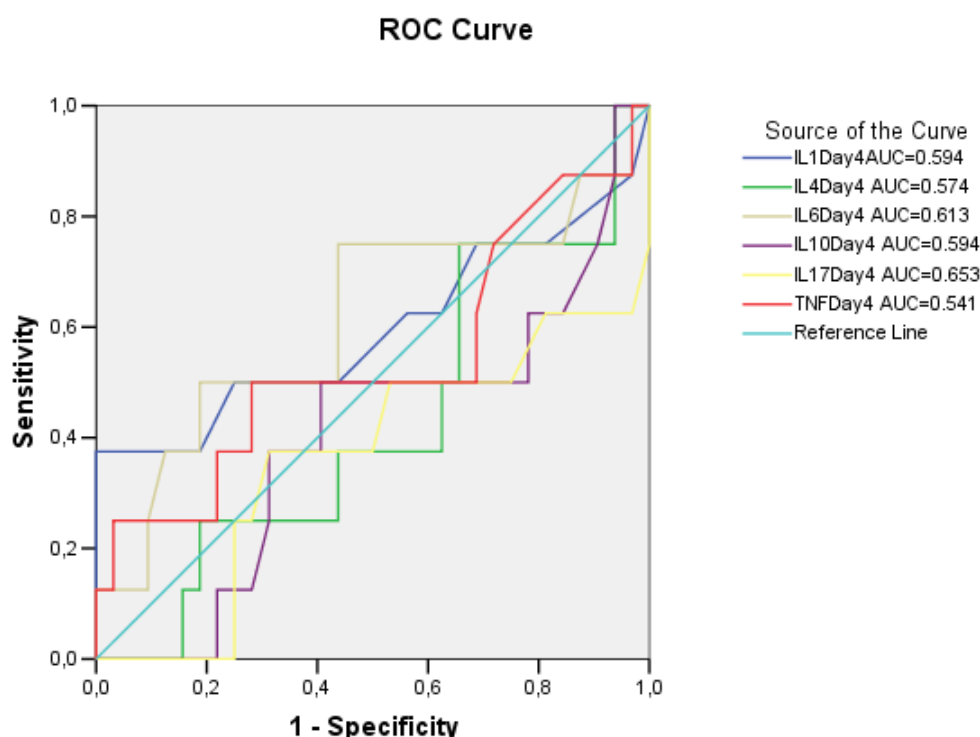
ROC Curve

Fig.3. The ROC curve analysis for adverse outcome prediction on day 4

0.003), except for 7 patients, from which 1 relapsed and only one had severe increase of the value on day 4 and he was the one who died. IL-10 had also a similar behaviour (Marginal Homogeneity Test p=0.049, Wilcoxon Signed Ranks Test p=0.001), with 6 exceptions, from which 1 died and 2 relapsed. IL-4 had opposite behaviour, meaning the values increased on day 4 (marginal Homogeneity Test p=0.03, Wilcoxon Signed Ranks Test p=0.001), but there were 13 exceptions, from which only 2 had relapses.

Discussion

Epidemiological studies tried to find the impact of age and gender on CAP caused by different

Table 3. The general multivariable logistic model for predicting mortality

Variable	Coefficient(B)	S. E.	P	Adjusted OR	95% CI for OR
IL1_D4	2.409	1.529	0.015	11.122	0.556 to 22.49
IL4_D4	-0.216	0.156	0.166	0.421	0.593 to 1.094
IL6_D4	0.016	0.013	0.027	0.978	0.991 to 1.043
IL10_D4	-0.056	0.279	0.840	1.304	0.547 to 1.634
IL17_D4	-0.040	0.148	0.787	1.362	0.719 to 1.284
TNF_D4	-0.851	0.546	0.119	0.975	0.147 to 1.245
Constant	0.701	4.994	0.888	2.016	0.801 to 3.786

pathogens. They found that incidence rates were higher in males and increased by age [13]. Our findings are similar to that. Studies reported significant variations in mean LOS for patients with CAP in Europe and United States of America [14,15,9]. Rozenbaum M. *et al* reported a mean LOS of 6.7 days, while Garau J. *et al* found a mean LOS of 11.5 days. Our study population had a mean LOS of 8.5 days, which was shorter than in most similar cohorts in Europe. The etiology was found in only 25 % of the cases, which was lower than in other reports. We used CURB-65, PSI, and SMART-COP scoring systems to predict mortality and the need for IRVS, at admission. The majority of our patients were included in PSI class risk 4 and 5 and CURB-65 group 2 and 3, but only 1 patient died. Regarding the need for IRVS calculated with SMART-COP, most of our cases had low risk.

This study aimed to analyze the relationship between pro and anti-inflammatory cytokines in the course of CAP, trying to bring a contribution to the knowledge of the disease, to a better understanding of its immunopathogenesis. Many studies have analyzed interleukin levels at a single time point, but dynamic changes in cytokines may have greater clinical value.

Similar to others, we have found no relationship between interleukin values and gender or age [1,7]. Bacci *et al* found an association between TNF-α and gender, and IL-1 was correlated with age, but the sample size was small, with only 26 subjects [16].

Previous studies found that systemic levels of IL-1β, IL-6 and TNF-α are usually elevated in patients with CAP, but they analyzed cytokines serum levels by only one determination [3,17]. G. Antunes *et al* showed a temporal pattern of these cytokines, with the serum levels declined rapidly over 5 days and postulated that pro-inflammatory cytokines were associated with poor prognosis in CAP [1]. M. Holub *et al* found that elevated levels of IL-6 and TNF-α decreased within 3 days after admission, to levels similar to that of healthy subjects [18]. Similar with these findings, we found a decrease in the serum levels of IL-1β, IL-6 and TNF-α within 4 days of antibiotherapy, acting as acute phase proteins. One explanation is the start of antibiotic treatment, and, in some cases, corticotherapy, which reduces the inflammatory response. In our study, similar to others [16], we found correlations between pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. The statistical model we used showed that high serum levels of IL-6 on day 1 associated with adverse outcomes consisted in relapses or death after 90 days of discharge. Similarly, Bacci M *et al* demonstrated that serum levels of IL-6 and TNF-α were correlated with worse outcomes [16]. There are several studies postulating that IL-6 is a marker of severity in CAP both in adults and children [7,19,20,21].

IL-4 has been best studied in the pathogenesis of asthma [22]. A recent study by M.J Giuffrida *et al* showed that there was no difference in serum levels of IL-4 and other cytokines studied, in asthmatic and non-asthmatic patients with bacterial, viral or mixed lung infection [23]. However, there are limited data regarding systemic levels of IL-4 in adults with CAP, most studies being concentrated on paediatric population and the role in the immune response against *Mycoplasma pneumonia* [6,24,25]. M.S Paats *et al* found that levels of IL-4 and IL-17A could not be detected in serum nor in bronchoalveolar lavage fluid (BAL) of the patients with CAP [7]. Unlike them, we found detectable serum levels of IL-4 on admission in almost all of our patients, with 2 exceptions, and in 27 cases their levels increased within 4 days of treatment. From the rest, 2 had relapses, and the others had only slightly decreased in their IL-4 values. Out of 38 patients, only 2 were known to have asthma, controlled at the time of hospital admission. Our results reinforce the idea that the immune response during bacterial infection is probably similar to the

early phases of the immune response in asthma [23]. We also found a positive correlation between IL-4, IL-6, IL-17A and IL-10 that can be explained by a common origin, meaning T-helper cells. [26]

Regarding IL-10, we found an opposite behaviour to IL-4, paradoxically, the decline of values from day 1 to day 4, acting as an acute phase biomarker, consistent with previous reports [1,3,5,7]. Similar to these studies, we found a correlation between IL-6 and IL-10. A possible explanation for the different behaviour of IL-10 compared to IL-4 could be that they have different dynamics. Also, at the time of admission, patients were in different stages of pneumonia, a possible explanation for their differences.

It has been proven that IL-17A has a protective role in host defense against airway pathogens in mice, but it is also involved in the local and systemic immune response in human pneumonia [27,28]. In a recent study, F. Higa *et al* postulated that this cytokine might play a role in legionnaire disease [29]. M.S. Paats *et al* studied the systemic and local involvement of IL-17A in CAP, among other cytokines, but its levels in both BAL fluid and serum of patients or healthy controls were not found [7]. In our study, we found detectable levels of IL-17A in 38 patients, behaving mostly as pro-inflammatory cytokines, with decreasing levels from day 1 to day 4, although it did not reach statistical significance. Also we found a correlation between IL-17A, IL-6 and surprisingly IL-10, reinforcing the pro-inflammatory effect.

In this study we found a relationship between IL-6, IL-1β, IL-4 and IL-10 and adverse outcomes (relapses and mortality). However, we have not found a correlation between cytokine levels and PSI risk class, partly because of the small number of subjects studied and also because we evaluated the severity scores only at admission. Endeman *et al* have also found no association between cytokine levels and this severity score

[5]. Kellum *et al* described the systemic cytokine response to infection and severe sepsis in a large cohort of patients with CAP, suggesting that individuals with high serum levels of both pro and anti-inflammatory cytokines had an increased risk of severe sepsis and death [30].

The present study has some limitations that should be considered. Firstly, the number of patients included was small, although we obtained relevant results for CAP. Secondly, we searched for systemic response only, not local immune response. Ideally, both responses should have been studied. However, many of the prognostic scores in CAP relate to the systemic rather than pulmonary effects of the biomarkers [31]. Another limitation is the prior prescription of antibiotics in 20% of our patients, which has certainly modulated the inflammatory response and cytokine expression. Also, the etiology was established in only 10 cases, which made it impossible to carry out statistical tests demonstrating the relationship between cytokines and etiology.

Conclusion

In conclusion, based on a comprehensive analysis of the systemic cytokine response in CAP, we have proved that IL-6 is a predictive marker for adverse outcomes and should be routinely analyzed at admission. IL-17A and IL-10 behave like pro-inflammatory cytokines, underlining the fact that the dynamic of these cytokines depends on several factors, such as the time of admission and prior usage of antibiotics. Although the number of the patients was small, we demonstrated a model in which all the studied combined interleukins had an impact on adverse outcomes.

Authors' contribution

RET (Conceptualization; Investigation; Project administration; Writing – original draft)
IBN (Data curation; Investigation; Validation)
LB (Data curation; Formal analysis; Investiga-

tion; Validation)

TLP (Conceptualization; Supervision; Writing – review & editing)

VC (Conceptualization; Project administration; Supervision; Writing – review & editing)

LMS (Conceptualization; Data curation; Formal analysis; Validation)

MSL (Conceptualization; Methodology; Supervision; Visualization; Writing – review & editing)

Disclosure of interest

The authors declare no conflict of interests.

Abbreviations

CAP= community-acquired pneumonia
IL= interleukin
TNF= tumor necrosis factor
IDSA= Infectious Diseases Society of America
LOS= length of stay
ACCP/SCCM= American College of Chest Physicians/Society of Critical Care Medicine
ELISA= Enzyme linked immunosorbent assay
ROC= receiver operator curve
BAL= bronchoalveolar lavage fluid

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