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Original Article

Diagnostic and clinical value of C-reactive protein and interleukin-6 serum levels in children with *Streptococcus pneumoniae*

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Abstract: Objectives: To determine the impact of C-reactive protein (CRP) and interleukin (IL)-6 on *Streptococcus pneumoniae* (*S. pneumonia*). Methods: Eight-eight children with community acquired pneumonia (CAP) were enrolled. Blood or pleural fluid cultures or polymerase chain reaction (PCR) assays was performed to identify the causes of CAP. Serum levels of CRP and IL-6 were analyzed. Pearson correlation analysis was performed to assess the relationship between levels of CRP and IL-6. Receiver operating characteristic (ROC) analysis was used to investigate the diagnostic tests. Results: Among the 88 patients, 75 cases were identified as *S. pneumonia* (34 cases), other types of bacterial pneumonia (12), *viral pneumonia* (22), and *mycoplasma pneumonia* (7). The levels of CRP and IL-6 were both significantly increased in *S. pneumonia* patients compared to *viral pneumonia* or *mycoplasma pneumonia* ($P < 0.05$). Additionally, there were strong relationships between CRP and IL-6 level in patients with *S. pneumonia* ($R^2 = 0.893$, $P = 0.003$). There were relatively weak relationships in other types of bacterial pneumonia ($R^2 = 0.604$, $P = 0.037$), but there were no relationships in patients with *viral pneumonia* ($R^2 = 0.232$, $P = 0.580$) or with *mycoplasma pneumonia* ($R^2 = -0.226$, $P = 0.591$). ROC showed that area under the curve (AUC = 0.841), with CRP threshold value of 250 mg/L and IL-6 of 800 pg/ml (sensitivity: 86.7%, specificity: 87.1%) for distinguishing between *S. pneumonia* from other pneumonia. Conclusion: Combined application of CRP and IL-6 has clinical usefulness in distinguishing between *S. pneumonia* from other pneumonia.

Keywords: *Streptococcus pneumoniae*, C-reactive protein, interleukin-6

Introduction

Streptococcus pneumoniae is a primary cause of invasive diseases such as bacterial pneumonia, meningitis, bacteremias, and acute otitis media in children with significant mortality worldwide [1]. It has been estimated that approximately 11% (8-12%) of all deaths in children aged 1-59 months can be attributed to pneumococcal disease, especially in developing countries [2, 3]. The widespread emergence of antimicrobial resistance resulted in increased burden of diseases caused by *S. pneumonia* [4]. In spite of great efforts to improve the diagnostic yield, rapid and accurate diagnosis of *S. pneumonia* is limited due to the inadequate available clinical, radiologic, and laboratory diagnostic methods. For example, polymerase chain reaction (PCR) tests in blood are less sensitive, time-consuming and expensive than the Binax NOW urinary antigen test [5]. The yield of cultures in patients with *S. pneumonia* is low, and isolation rates are even lower in patients who have received antibiotics prior to cultures [6]. Besides, clinically, it is difficult to differentiate between *S. pneumonia* and other bacterial pneumonia according to the clinical signs or radiographic findings. Therefore, it is essential to explore new markers for early differential diagnosis of *S. pneumonia*.

C-reactive protein (CRP) is an acute phase protein that increases 4-6 h triggered by an inflammatory response and peaks at 36-50 h [7], which plays significant roles in regulation of the inflammatory response, innate host defense, and clearance of damaged cells [8]. It is measured routinely in clinical care and functions as

a precipitin for the C-polysaccharide of the *S. pneumonia* cell wall [9]. The serum levels of CRP are increased in patients with community-acquired pneumonia (CAP), especially *S. pneumonia* or *L pneumonia*, and have been considered as a useful marker for establishing the diagnosis of CAP [10]. Persistently raised serum levels indicate the failure of treatment or accompany the complications [11, 12], while the declined levels suggest the recovery [13]. Recently, it has been reported that CRP enhances immunity and cytokines responses to *S. pneumonia* by interaction with Fcγ receptors [9, 14]. Cytokine interleukin-6 (IL-6) is a main regulator of induction of CRP in hepatocytes at the transcriptional level, and this effect can be enhanced by IL-1β [15]. We hypothesized that combined application of the biomarkers CRP and IL-6 have clinical usefulness in distinguishing between *S. pneumonia* from other pneumonia.

Therefore, the primary aim of this study was to determine the impact of inflammatory biomarkers CRP and IL-6 levels on distinguishing between *S. pneumonia* from other pneumonia. Our study might provide a clue as to early differential diagnosis of *S. pneumonia*.

Material and methods

Patients

This study was approved by the Hospital Medical Ethics Committees, and signed informed consent was obtained from all caregivers enrolled in the study. Between April 2014 and May 2015, a total of eighty-eight consecutive children (47 boys and 41 girls) aged 2-59 months with CAP were recruited. The inclusive criteria were listed as below: (1) Fast breathing [respiratory rate (RR)] ≥ 50/min if age 2-11 months or ≥ 40/min if age 12-59 months); (2) Any one of symptoms: oxygen saturation (SPO_2) < 90%, cyanosis, grunting, incapability to drink, or altered consciousness; (3) Shadows in bilateral lung tissues (X-ray examination). Patients were excluded if they were suspected or confirmed pulmonary tuberculosis, end-stage renal disease, congestive cardiac failure, human immunodeficiency virus (HIV), chronic cardiopulmonary symptoms (for > 2 weeks). Those who were subjected to injectable antibiotics treatment (within 2 weeks), immunosuppressive treatment (for > 3 months) were also excluded.

Radiology

Chest radiographs were screened at the time of enrollment. All chest radiographs were independently reviewed by two senior radiologists who were unaware of clinical and laboratory findings. Any disagreement was resolved by discussion and standardized and/or mutually exclusive diagnoses were assigned by the two radiologists.

Microbiology

Microbiological tests were routinely completed according to standard clinical laboratory procedures. Bacterial culture, viral culture, or direct fluorescent antibody (DFA) test performed to confirm organism. Blood cultures were performed with the BacT/Alert Blood Culture System® (BioMérieux). Pleural fluid Gram stain and cultures were performed if clinically indicated after diagnostic and therapeutic pleurocentesis. DFA staining test and cultures were performed to identify a panel of viruses such as adenovirus, parainfluenza 1, 2, and 3, respiratory syncytial virus (RSV), and influenza A and B.

Blood collection and laboratory analysis

Blood samples (five-milliliter) were collected within 24 h of enrollment before feeding and processed within 3 h of collection and frozen at -70°C until assayed. All samples were grouped equally for serological assays (serum levels of CRP and IL-6) and for DNA amplification (PCR tests). Serum levels of CRP and IL-6 were analyzed by chemiluminescent enzyme immunometric assay using an Immulite 1000 luminometer (Siemens 1, Germany). Each result was the average of duplicate analyses.

PCR tests

Blood or pleural fluid samples were lysed and DNA was extracted, amplified and detected according to a standard method [16]. DNA was extracted QIAGEN QIAamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA was amplified using the GenomiPhi V2 DNA amplification kit (GE Healthcare, Buckinghamshire, United Kingdom). The product of DNA amplification was detected by dot-blot hybridization [17]. A 10 colony-forming units/mL of amplified product was resolved on an electrophoresis in polyacrylamide gel and visualized under UV light.

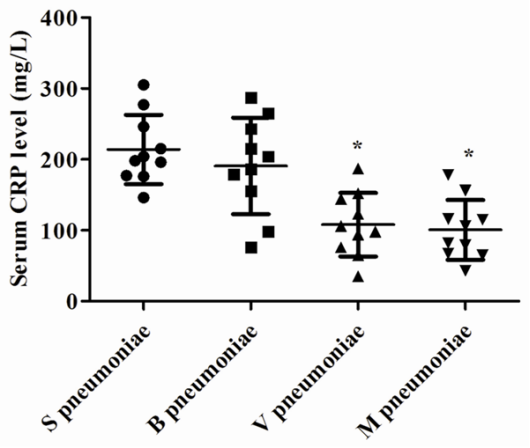

Figure 1. Serum levels of CRP among the four groups. CRP, C-reactive protein; *S. pneumonia*, Streptococcus pneumoniae; *B pneumonia*, bacterial pneumonia; *V pneumoniae*, viral pneumonia; *M pneumoniae*, *mycoplasma pneumonia*. $^{*}P < 0.05$ compared to *S. pneumonia.*

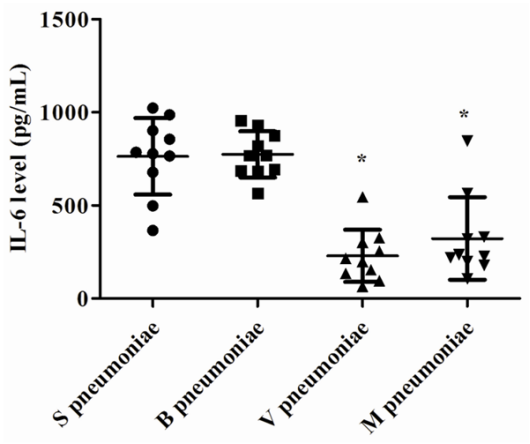

Figure 2. Serum levels of IL-6 among the four groups. IL, interleukin; *S. pneumonia*, Streptococcus pneumoniae; *B pneumonia*, bacterial pneumonia; *V pneumoniae*, viral pneumonia; *M pneumoniae*, *mycoplasma pneumonia.* $^{*}P < 0.05$ compared to *S. pneumonia.*

Statistical analysis

Continuous variables, expressed as the mean ± standard deviation (SD), were analyzed by statistical package for the social sciences (SPSS) software (version 19.0; SPSS Inc., Chicago, IL). Serum levels of CRP and IL-6 were compared using the Wilcoxon's signed-rank test. A Pearson correlation analysis was then used to assess the relationship between levels of CRP and IL-6. Receiver operating characteristic (ROC) analysis was used to investigate the diagnostic properties of the tests. A statistical significance was defined when $P < 0.05$.

Results

Analysis of the causes of pneumonia in children

In order to explore the expression levels of CRP and IL-6 and their clinical value in diagnosis of in *S. pneumonia*, 88 children with LRIs were enrolled. After blood or pleural fluid cultures or PCR assays, 75 cases were made with a definitive diagnosis. Among the 75 cases, 34 cases was *S. pneumonia*, 12 cases were other types of bacterial pneumonia (e.g. *Staphylococcal pneumonia* or *haemophilus influenzae pneumonia*), 22 cases were diagnosed with *viral pneumonia*, and 7 cases experienced *mycoplasma pneumonia*.

Serum levels of CRP

The serum levels of CRP were determined. As shown in **Figure 1**, the levels of CRP were significantly increased in patients with *S. pneumonia* compared to patients with *viral pneumonia* or *mycoplasma pneumonia* ($P < 0.05$). Although the levels of CRP were higher than those in the patients with other types of bacterial pneumonia, there were no statistical differences ($P > 0.05$).

Serum levels of IL-6

The serum levels of IL-6 were the highest in patients with *S. pneumonia* among the four groups, while were the lowest in patient with *viral pneumonia*. There were significant differences in levels of IL-6 between patients with *S. pneumonia* and *viral pneumonia*, and between patients with *S. pneumonia* and *mycoplasma pneumonia* (both $P < 0.05$). However, no significant differences were found between patients with *S. pneumonia* and other types of bacterial pneumonia ($P > 0.05$) (**Figure 2**).

Correlation analysis of CRP and IL-6

In order to confirm the clinical diagnostic value of CRP and IL-6 in *S. pneumonia*, we further performed the correlation analysis between the expression levels of CRP and IL-6 among the patients (**Figure 3A-D**). The results showed

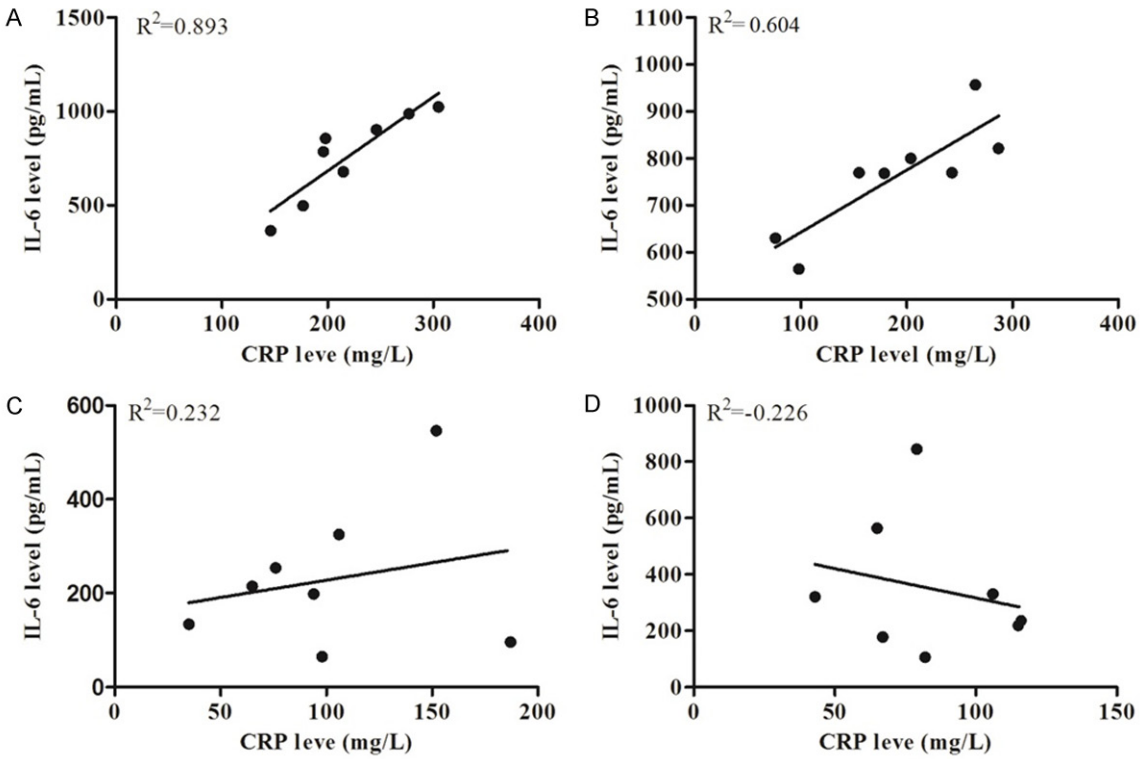

Figure 3. Correlation analysis of CRP and IL-6. A. Correlation analysis of CRP and IL-6 in patients with *S. pneumonia*; B. Correlation analysis of CRP and IL-6 in patients with other types of bacterial pneumonia; C. Correlation analysis of CRP and IL-6 in patients with *viral pneumonia*; D. Correlation analysis of CRP and IL-6 in patients with *mycoplasma pneumonia*. CRP, C-reactive protein; IL, interleukin; *S. pneumonia*, Streptococcus pneumoniae; *B pneumonia*, bacterial pneumonia; *V pneumoniae*, viral pneumonia; *M pneumoniae*, *mycoplasma pneumonia*.

that there were strong relationships between serum CRP level and serum IL-6 level in patients with *S. pneumonia* (R^2 = 0.893, *P* = 0.003), while there were relatively weak relationships between serum CRP level and serum IL-6 level in patients with other types of bacterial pneumonia (R^2 = 0.604, *P* = 0.037). However, there were no relationships between serum CRP level and serum IL-6 level in patients with *viral pneumonia* (R^2 = 0.232, *P* = 0.580), and patients with *mycoplasma pneumonia* (R^2 = -0.226, *P* = 0.591).

ROC curves results

To further distinguish between *S. pneumonia* and other types of bacterial pneumonia, we performed ROC curves to identify the threshold value. The results showed that when CRP was higher than 250 mg/L and meantime IL-6 level was higher than 800 pg/mL can be used as specific diagnostic criteria for the diagnosis of *S. pneumoniae*. The area under the curve (AUC) was 0.841 (*P* = 0.025), while the sensitivity and specificity were 86.7% and 87.1%, respectively (**Figure 4**). The results indicated that CRP (> 250 mg/L) and IL-6 (800 pg/mL) could distinguish between S. pneumonia from other types of bacterial pneumonia.

Discussion

In the present study, our results showed that the levels of CRP and IL-6 were significantly increased in patients with *S. pneumonia* and they showed a strong positive correlation. However, there were no relationships in patients with *V pneumonia* or *M pneumonia*. Additionally, combination of CRP (> 250 mg/L) and IL-6 (800 pg/mL) could distinguish between *S. pneumonia* from other types of bacterial pneumonia. Our results confirm that combination of CRP and IL-6 has clinical usefulness in distinguishing between *S. pneumonia* from other pneumonia.

Early differential diagnosis of *S. pneumonia* in children is of great significance for the early

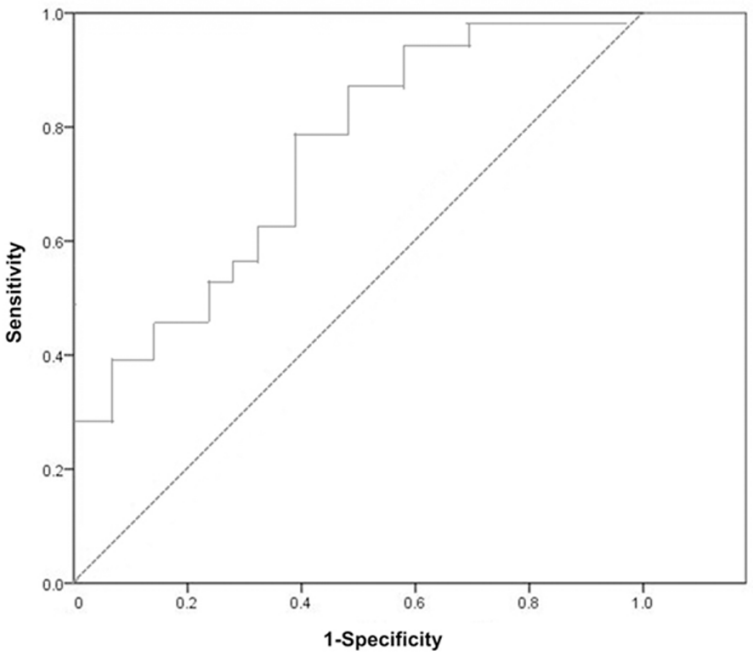

Figure 4. ROC curves for combination of CRP and IL-6 for discrimination between *S. pneumonia* and other types of pneumonia. ROC, receiver operating characteristic; CRP, C-reactive protein; IL, interleukin; *S. pneumonia*, Streptococcus pneumoniae.

diagnosis and rational drug use, possibly shortening the course of disease and reducing the mortality rate. However, it is a great challenge to make a specific diagnosis of pneumococcal infection, and it has heavily relied on the traditional culture methods until now. Although standard culture of blood or pleural fluid is a valuable technique for the diagnosis of bacterial pneumonia, it is time-consuming and frequently negative with low sensitivity and poor recurrence rate. Besides, sputum culture may represent colonization in adults with chronic obstructive pulmonary disease (COPD) [18]. Furthermore, the frequency of culture of pneumococci would be reduced if prior administration of antibiotic treatment before sampling. In addition, the ability of routine laboratory tests or chest radiographs examination to discriminate between bacterial and viral causes of CAP in children still poorly. Invasive investigations such as fiberoptic bronchoscopy may increase the diagnostic yield; however, it seems only regarded as a routine diagnostic procedure for patients with severe disease [19]. Recently, various nonculture tests such as PCR test using nucleic acid amplification or antigen detection have been developed and are being implemented in the diagnosis of pneumococcal infections. But this molecular diagnosis is time-consuming, expensive and not available in all hospitals. Binax NOW Streptococcus pneumoniae, a relatively new and rapid diagnosis of pneumococcal infections by detecting C polysaccharide antigen in urine, has represented good sensitivity in both adults and children even after antibiotic treatment has commenced [20-22], making complicated pneumonia treatment suitably and early. However, several studies have suggested that a high false positive rate were found in Binax NOW when compared to blood pneumococcal PCR in healthy children with and without pneumococcal carriage [23]. Additionally, although a generally high diagnostic specificity (> 90%), the diagnostic sensitivity is variable and frequently low. Half or more than half patients with identified pneumococcal disease by using culture method still remain negative for urine antigen [24]. Thus, so far no antigen detection tests have been demonstrated their sufficiently effectiveness and specificity for routine detection of respiratory infection [25, 26]. Hence, it is valuable to identify the markers of infection for discriminating between bacterial and viral causes of pneumonia for clinical treatment guidelines and follow up.

The role of Procalcitonin (PCT), CRP and IL-6 as early tools to diagnose infectious and inflammatory diseases has been extensively and widely studied [27-29]. Ahn *et al.* has suggested that pneumonia might be unlikely to be caused by mixed bacterial infection if the results representing combination of low CRP and PCT [28]. Bellmann *et al.* concluded that CRP in combination with PCT was of value for the differential diagnosis between *L. pneumophila* and *S. pneumoniae* infections [29]. A previous study suggested that PCT was a potential marker for children admitted to hospital with a threshold of 1 μg/L [30]. Although the

researcher showed that PCT was more sensitive and specific and had greater positive and negative predictive values than CRP and IL-6; our results indicated that CRP in combination with IL-6 had clinical usefulness in *S. pneumonia*. One main reason might be that we combined application of CRP and IL-6 but rather application of CRP or IL-6 alone. The levels of CRP and IL-6 in different pneumonia indicated that CRP and IL-6 levels were significantly increased in patients with *S. pneumonia* compared to patients with *viral pneumonia* or patients with *mycoplasma pneumonia*. Besides, there were strong relationships between CRP levels and IL-6 levels in patients with *S. pneumonia*; however, no relationships were found in patients with *viral pneumonia* or patients with *mycoplasma pneumonia*. Therefore, differential diagnosis between *S. pneumonia* and *viral pneumonia* or *mycoplasma pneumonia* was clear according to the levels of CRP and IL-6 and the correlations between CRP and IL-6. To further distinguish between *S. pneumonia* and other types of bacterial pneumonia, ROC curves were performed. We found that when CRP was higher than 250 mg/L and meanwhile IL-6 level was higher than 800 pg/mL could distinguish between *S. pneumonia* and other types of bacterial pneumonia with relatively higher sensitivity (86.7%) and specificity (87.1%).

In conclusion, combination of CRP and IL-6 has clinical usefulness in distinguishing between *S. pneumonia* from other pneumonia. These two classic inflammatory markers are relatively simple and easy to detect as serum indicators. Our study might provide a rapid and accurate identification of *S. pneumoniae* and contribute to the insight into appropriate antimicrobial therapy.

Disclosure of conflict of interest

None.

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References

[1] Lynch JP and Zhanel GG. Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention. Semin Respir Crit Care Med 2009; 30: 189-209.

[2] Pneumococcal conjugate vaccine for childhood immunization--WHO position paper. Wkly Epidemiol Rec 2007; 82: 93-104.

[3] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS and Cherian T. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 2009; 374: 893-902.

[4] Lynch JP and Zhanel GG. Streptococcus pneumoniae: does antimicrobial resistance matter? Semin Respir Crit Care Med 2009; 30: 210-238.

[5] Smith MD, Sheppard CL, Hogan A, Harrison TG, Dance DA, Derrington P, George RC; South West Pneumococcus Study Group. Diagnosis of Streptococcus pneumoniae infections in adults with bacteremia and community-acquired pneumonia: clinical comparison of pneumococcal PCR and urinary antigen detection. J Clin Microbiol 2009; 47: 1046-1049.

[6] Naheed A, Saha SK, Breiman RF, Khatun F, Brooks WA, El Arifeen S, Sack D, Luby SP; Pneumococcal Study Group. Multihospital surveillance of pneumonia burden among children aged < 5 years hospitalized for pneumonia in Bangladesh. Clin Infect Dis 2009; 48 Suppl 2: S82-89.

[7] McWilliam S and Riordan A. How to use: C-reactive protein. Archives of disease in childhood. Arch Dis Child Educ Pract Ed 2010; 95: 55-58.

[8] Black S, Kushner I and Samols D. C-reactive Protein. J Biol Chem 2004; 279: 48487-48490.

[9] Mold C and Du Clos TW. C-reactive protein increases cytokine responses to Streptococcus pneumoniae through interactions with Fc gamma receptors. J Immunol 2006; 176: 7598-7604.

[10] Almirall J, Bolibar I, Toran P, Pera G, Boquet X, Balanzo X and Sauca G. Contribution of C-reactive protein to the diagnosis and assessment of severity of community-acquired pneumonia. Chest 2004; 125: 1335-1342.

[11] Menendez R, Martinez R, Reyes S, Mensa J, Polverino E, Filella X, Esquinas C, Martinez A, Ramirez P and Torres A. Stability in community-acquired pneumonia: one step forward with markers? Thorax 2009; 64: 987-992.

[12] Coelho L, Povoa P, Almeida E, Fernandes A, Mealha R, Moreira P and Sabino H. Usefulness of C-reactive protein in monitoring the severe community-acquired pneumonia clinical course. Crit Care 2007; 11: R92.

[13] Hansson LO, Hedlund JU and Ortqvist AB. Sequential changes of inflammatory and nutri-

tional markers in patients with community-acquired pneumonia. Scand J Clin Lab Invest 1997; 57: 111-118.
[14] Thomas-Rudolph D, Du Clos TW, Snapper CM and Mold C. C-reactive protein enhances immunity to Streptococcus pneumoniae by targeting uptake to Fc gamma R on dendritic cells. J Immunol 2007; 178: 7283-7291.
[15] Kushner I, Jiang SL, Zhang D, Lozanski G and Samols D. Do post-transcriptional mechanisms participate in induction of C-reactive protein and serum amyloid A by IL-6 and IL-1? Ann N Y Acad Sci 1995; 762: 102-107.
[16] Michelow IC, Lozano J, Olsen K, Goto C, Rollins NK, Ghaffar F, Rodriguez-Cerrato V, Leinonen M and McCracken GH Jr. Diagnosis of Streptococcus pneumoniae lower respiratory infection in hospitalized children by culture, polymerase chain reaction, serological testing, and urinary antigen detection. Clin Infect Dis 2002; 34: E1-11.
[17] Salo P, Ortqvist A and Leinonen M. Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. J Infect Dis 1995; 171: 479-482.
[18] Korsgaard J, Moller JK and Kilian M. Antibiotic treatment and the diagnosis of Streptococcus pneumoniae in lower respiratory tract infections in adults. Int J Infect Dis 2005; 9: 274-279.
[19] Korsgaard J, Rasmussen TR, Sommer T, Moller JK, Jensen JS and Kilian M. Intensified microbiological investigations in adult patients admitted to hospital with lower respiratory tract infections. Respir Med 2002; 96: 344-351.
[20] Athlin S and Stralin K. The Binax NOW Streptococcus pneumoniae test applied on nasopharyngeal aspirates to support pneumococcal aetiology in community-acquired pneumonia. Scand J Infect Dis 2013; 45: 425-431.
[21] Casado Flores J, Nieto Moro M, Berron S, Jimenez R and Casal J. Usefulness of pneumococcal antigen detection in pleural effusion for the rapid diagnosis of infection by Streptococcus pneumoniae. Eur J Pediatr 2010; 169: 581-584.
[22] Adegbola RA, Obaro SK, Biney E and Greenwood BM. Evaluation of Binax now Streptococcus pneumoniae urinary antigen test in children in a community with a high carriage rate of pneumococcus. Pediatr Infect Dis J 2001; 20: 718-719.
[23] Lees EA, Ho DK, Guiver M, Mankhambo LA, French N and Carrol ED. Comparison of Binax NOW urine antigen test and pneumococcal DNA assay using qPCR, before and after nasopharyngeal swabbing in healthy Malawian children. New Microbes New Infect 2015; 8: 4-6.
[24] Skerrett SJ. Diagnostic testing for community-acquired pneumonia. Clin Chest Med 1999; 20: 531-548.
[25] Gillespie S. Molecular techniques for the diagnosis of respiratory bacterial infection. Curr Opin Pulm Med 1999; 5: 174-178.
[26] Smith PR. What diagnostic tests are needed for community-acquired pneumonia? Med Clin North Am 2001; 85: 1381-1396.
[27] Uusitalo-Seppala R, Koskinen P, Leino A, Peuravuori H, Vahlberg T and Rintala EM. Early detection of severe sepsis in the emergency room: diagnostic value of plasma C-reactive protein, procalcitonin, and interleukin-6. Scand J Infect Dis 2011; 43: 883-890.
[28] Ahn S, Kim WY, Kim SH, Hong S, Lim CM, Koh Y, Lim KS and Kim W. Role of procalcitonin and C-reactive protein in differentiation of mixed bacterial infection from 2009 H1N1 viral pneumonia. Influenza Other Respir Viruses 2011; 5: 398-403.
[29] Bellmann-Weiler R, Ausserwinkler M, Kurz K, Theurl I and Weiss G. Clinical potential of C-reactive protein and procalcitonin serum concentrations to guide differential diagnosis and clinical management of pneumococcal and Legionella pneumonia. J Clin Microbiol 2010; 48: 1915-1917.
[30] Moulin F, Raymond J, Lorrot M, Marc E, Coste J, Iniguez JL, Kalifa G, Bohuon C and Gendrel D. Procalcitonin in children admitted to hospital with community acquired pneumonia. Arch Dis Child 2001; 84: 332-336.