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Systemic cytokines and chemokines on admission of children hospitalized with community-acquired pneumonia

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ABSTRACT

Community-acquired pneumonia (CAP) is the main cause of death in children under-5 years worldwide and *Streptococcus pneumoniae* is the most common bacterial agent. However, it is difficult to identify pneumococcal infection among children with CAP. We aimed to assess association between any cytokine/chemokine and pneumococcal infection in childhood CAP. Furthermore, we evaluated the diagnostic value of cytokine/chemokine for pneumococcal infection. This prospective study was conducted at an Emergency Room, in Salvador, Brazil. Children < 5-years-old hospitalized with CAP in a 21-month period were evaluated. On admission, clinical and radiological data were collected along with biological samples to investigate 20 etiological agents and determine serum cytokines (interleukin (IL)-8, IL-6, IL-10, IL-1β, IL-12, TNF-α, IL-2, IL-4, IL-5, γ-interferon), and chemokines (CCL2, CCL5, CXCL9, CXCL10) concentration. From 166 patients with etiology detected, pneumococcal infection was detected in 38 (22.9%) cases among which the median IL-6(pg/ml) was 31.2 (IQR: 12.4–54.1). The other 128 cases had other causative agents detected (*Haemophilus influenzae*, *Moraxella catarrhalis*, atypical bacteria and viruses) with the median IL-6 concentration being 9.0 (IQR: 4.1–22.0; $p < 0.001$). The area under the ROC curve for IL-6 to predict pneumococcal CAP was 0.74 (95%CI: 0.65–0.83; $p < 0.001$). By multivariate analysis, with pneumococcal CAP as dependent variable, IL-6 was an independent predictor for pneumococcal infection (OR = 5.56; 95%CI: 2.42–12.75, cut-off point = 12.5 pg/ml; $p = 0.0001$). The negative predictive value of IL-6 under 12.5 pg/ml for pneumococcal infection was 90% (95%CI: 82–95%). Independently significant difference was not found for any other cytokines/chemokines. Serum IL-6 concentration on admission is independently associated with pneumococcal infection among children under-5 years hospitalized with CAP.

1. Introduction

Community-acquired pneumonia (CAP) remains the main single cause of death and a frequent cause of hospital admissions in children under-5 years worldwide, with 1 million estimated deaths in 2015 and approximately 15 million estimated admissions in 2010 [1,2].

It is currently very difficult to establish the etiological diagnosis of CAP. This is mostly due to lack of rapid tests with sensitivity and specificity high enough to be appropriately employed in emergency rooms or primary health-care settings, particularly among children [3]. Among the several putative etiological agents of childhood CAP, *Streptococcus pneumoniae* has been identified as the most common bacterial agent [3,4]. Nevertheless, clinical and radiological parameters, as well as presently available laboratory biomarkers have been found to be useless to distinguish pneumococcal cases from non-pneumococcal cases [3,5].

S. pneumoniae induces an intense inflammation in the lungs with the release of cytokines and chemokines from innate immunity. This process activates alveolar macrophages and systemic neutrophils to promote the clearance of pneumococcal strains [6,7]. Some cytokines are recognized to play a pivotal role in innate defense against *S. pneumoniae*, such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β, in the first moment of infection, being followed by IL-6, IL-8 and IL-10 [6–8].

Since the 1990's, several studies have been searching for association between inflammatory cytokines and bacterial CAP in adults [9,10].

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Few other studies have investigated this issue among children, but none has presented a practical and useful conclusion so far [11–13]. Thus, we aimed to evaluate further if there was association between serum cytokineś or chemokineś levels on admission and pneumococcal infection among children hospitalized with CAP.

2. Material and methods

2.1. Study design

This was a prospective study conducted at the Emergency Room of the Federal University of Bahia Hospital, in Salvador, Northeast, Brazil, from September 2003 to May 2005. Every child aged < 5 years hospitalized due to CAP was evaluated upon admission. The diagnosis was made by the pediatrician on duty. Diagnosis was based upon fulfillment of the following criteria: (1) respiratory complaints plus (2) fever or difficulty breathing plus (3) pulmonary infiltrates on the chest radiograph (CXR) taken at admission. Written informed consent was signed by parents/legal guardians before recruitment. The exclusion criteria were: (1) chronic lung disease, except asthma, (2) underlying co-morbidities, (3) other concurrent infections, (4) suspected or diagnosed immunodeficiency, or (5) transfer from other health-care units. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and it was approved by the Ethics Committee of the Federal University of Bahia.

2.2. Patients

Community-dwelling children were evaluated upon admission, when demographic and clinical data, blood samples and nasopharyngeal aspirates (NPA) were collected. At this moment, a chest radiograph was taken. Afterwards, an independent radiological evaluation was performed by a pediatric radiologist blinded to clinical and laboratory data. Two to four weeks later, the patients were invited to return for a follow-up visit, when a second blood sample was collected for serological assays and comparison of specific IgG titers.

2.3. Controls

We recruited a convenience sample of 30 asymptomatic healthy children. Eligibility requirements for controls included age < 5 years and performance of elective surgery. The blood sample was collected at the time of anesthesia induction after having received written informed consent from parents/legal guardians. Data on birth date, gender, surgery and collection date were registered.

2.4. Microbiological assays

Investigation of etiology comprised the performance of several tests to search for the same etiological agent; this procedure was carried out for every included case. Infections by the following pathogens were searched for: *S. pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Simkania negevensis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, rhinovirus, respiratory syncytial virus, influenza virus A and B, parainfluenza viruses types 1, 2 and 3, enterovirus, adenovirus, human metapneumovirus and human bocavirus. The investigation of these infections and the frequency of these etiological agents analyzed by age distribution have already been published [14–17].

In short, respiratory viruses investigation consisted of searching for viral antigens in NPA by time-resolved fluoroimmunoassay with monoclonal antibodies and comparison of virus-specific paired serum IgG titers determined by enzyme-immunoassay (ELISA) (influenza A and B viruses, respiratory syncytial virus, parainfluenza viruses type 1, 2, and 3, and adenovirus), when a ≥ 3-fold antibody titers increase in paired serum samples was considered diagnostic [18]. Reverse transcription-polymerase chain reaction (PCR) assays for the detection of rhinovirus, enterovirus, and human metapneumovirus were performed [16,19]. Human bocavirus was investigated by quantitative PCR of NPA and serum, IgG increase determination in paired serum samples, and searching for IgM and IgG avidity by ELISA [20]. Bacterial infections caused by *S. pneumoniae*, non-typable *H. influenzae*, *M. catarrhalis*, *S. aureus*, and *S. pyogenes* were investigated by blood culture (Automated Bact/Alert Organon) before the beginning of antimicrobial treatment. Bacterial infection by *S. pneumoniae* was also sought by an in-house ELISA which measured IgG antibodies to pneumococcal pneumolysin and pneumococcal C-polysaccharide in paired serum samples. A ≥ 2-fold or ≥3-fold increase, respectively, in antibody titres, was considered diagnostic [21]. For *H. influenzae* and *M. catarrhalis* infections, Ig (polyvalent) antibodies against whole bacterial cell antigens (a mixture of 10 different strains) were measured and a≥ 3-fold antibody increase between paired serum samples was considered diagnostic [21]. Pneumolysin-PCR was also used for the detection of *S. pneumoniae* DNA in blood buffy-coat collected upon admission [22]. An in-house microimmunofluorescence test was used to measure IgG, IgA and IgM antibodies to *C. pneumoniae* and *S. negevensis*, using purified, formalized elementary bodies of strains Kajaani 6 in *C. pneumoniae* [23] and ATCC strain Z (ATCC, Catalog No. VR-1471) in *S. negevensis* tests [24]. Diagnosis was based on a ≥ 4-fold increase in IgG or IgA antibodies between paired sera or on the presence of IgM antibodies (a titer of ≥10). *M. pneumoniae* infection was investigated by testing for specific IgM by using a commercial ELISA kit (Platelia, Bio-Rad, Marnes La Coquette, France) [25]. *C. trachomatis* IgG antibodies were measured by a commercial, solid-phase ELISA (Ani Labsystems Ltd., Vantaa, Finland). The laboratory diagnosis was based on signal to cut-off (S/CO) values, which were ≥1.4 S/CO [26].

2.5. Cytokines and chemokines assays

Serum concentrations of inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNFα and IL-12); Th1/Th2 cytokines (IL-2, IL-4, IL-5 and γ-interferon [IFNγ]); and chemokines (CCL2, CCL5, CXCL9, CXCL10) were measured in the residual serum from the etiology tests using the Cytometric Bead Array Human Inflammatory Cytokine Kit, Human Th1/Th2 Cytokine Kit and Human Chemokine Kit, respectively (BD Biosciences Pharmingen, San Diego, CA, USA). Flow cytometry (BD FACSArray) and the Software FCAP Array (BD Biosciences Pharmingen, San Diego, CA, USA) was used to perform the acquisition and the analysis, respectively. Lower limits of detection were: IL-8, 3.6 pg/ml; IL-1β, 7.2 pg/ml; IL-6, 2.5 pg/ml; IL-10, 3.3 pg/ml; TNFα, 3.7 pg/ml; IL-12, 1.9 pg/ml; IL-2, 2.6 pg/ml; IL-4, 2.6 pg/ml; IL-5, 2.4 pg/ml; IFNγ, 7.1 pg/ml; CCL5, 0.2 pg/ml; CXCL9, 2.5 pg/ml; CCL2, 2.7 pg/ml; CXCL10, 2.8 pg/ml. The maximum quantifiable level considered was supplied by the manufacturer: 10,000 pg/ml for all cytokines and chemokines. The assays were performed on two separate occasions by a technician blinded to etiological and clinical information. The residual serum samples from the etiology tests were kept frozen at −80 °C until cytokines and chemokines were measured. The same measurements and analyses were performed in the serum collected from healthy children (controls).

2.6. Statistical analysis

For the purpose of analysis, the cases were classified into two groups: cases with pneumococcal infection or with non-pneumococcal infection. The former group comprised cases with bacteremic pneumococcal infection (that is, *S. pneumoniae* was isolated from blood culture) and cases with non-bacteremic pneumococcal infection (*S. pneumoniae* was not isolated from blood culture but pneumolysin-PCR was positive and/or IgG increase against pneumococcal pneumolysin and/or pneumococcal C-polysaccharide was found, according to the used cut-offs). The group with non-pneumococcal infection comprised

cases with viral infection, or atypical bacterial infection or pyogenic bacterial non-pneumococcal infection. Viral infection was diagnosed when only viral infection was detected; atypical bacterial infection was diagnosed when infection by *M. pneumoniae*, *C. trachomatis*, *C. pneumonia*, or *S. negevensis* was detected irrespective of viral infection having also been detected, and pyogenic bacterial non-pneumococcal infection was diagnosed when infection by *H. influenzae*, *M. catarrhalis*, *S. aureus*, or *S. pyogenes* was found irrespective of other agents. The group of healthy children comprised the controls.

Tachypnea was defined as respiratory rate ≥50 breaths/min in children aged 2–11 months and respiratory rate ≥40 breaths/min in children aged 12–59 months [27]. Fever was defined as axillary temperature equal to or higher than 37.5 °C [28].

Continuous variables were presented as median (interquartile range [IQR]). Bivariate analysis was performed with Chi-Square test or Fisher's Exact test to compare proportions, as appropriate, Mann-Whitney *U* test to compare medians, and Spearmańs correlation test to compare 2 continuous variables. To analyze the diagnostic value of cytokines/chemokines, Receiver Operator Characteristic (ROC) curve was calculated. To define the predictive value of cytokines/chemokines (predictor variables) for pneumococcal pneumonia (outcome variable), a multivariable logistic regression model by enter method was constructed, and the 95% confidence interval (95% CI) of the Odds Ratio (OR) was calculated. Cytokines/chemokines which significantly differed in the bivariate analysis were selected for the multivariable analysis, which was performed in a model adjusted for age. All tests were 2-tailed with a significance level of 0.05. A Bonferroni correction was used when required to allow for multiple comparisons. Sensitivity, specificity, positive and negative predictive values along with the respective 95% CI were calculated for the cytokine or chemokine found to be independently associated with pneumococcal infection in the multivariable analysis. To address generalizability, a further analysis was done with the inclusion of the 50 cases with undetected etiology in the group of non-pneumococcal infection. SPSS software (version 9.0) was used for analysis. The main interest in IL-6 was not defined prior to the onset of the study. Exclusion criteria were chosen to address potential confounders. Blinding at cytokines and chemokines measurement was performed to address potential bias. Cases with any missing biological sample were excluded. The sample size provided the study with a power of 80% to detect as statistically significant a difference as low as 50% between proportions of exposure among cases with or without pneumococcal infection and, also, an odds ratio of 3.03 or more for a range of frequency of exposure among cases without pneumococcal infection from 20% to 60%.

Different data from the same research project have already been published. The novelty in this paper is the presentation of systemic cytokines and chemokines levels measured upon admission of the patients, along with the comparison of the cytokines and chemokines levels distribution between patients with or without pneumococcal infection.

3. Results

3.1. Study population

A total of 322 patients were evaluated, out of which 45 (14%) were excluded because they fulfilled one of the exclusion criteria. A further exclusion was due to serum sample unavailability for cytokine/chemokine measurement (n = 61) or undetected etiology (n = 50) (Fig. 1). Thus, the study group comprised 166 patients. Overall, the median (IQR) age was 17 (10–28) months. There were 98 (59.0%) boys. None had previously received either pneumococcal or influenza vaccines. On the contrary, 81% had received *H. influenza*e type b vaccine according to information retrieved from their vaccination cards. Among the 30 healthy controls, the median (IQR) age was 41 (26–59) months and 23 (76.7%) were boys. Surgeries comprised herniorrhaphy (56.7%), posthectomy (16.7%), exeresis of thyroglossal cyst (9.9%), orchidopexy (6.7%), correction of polydactyly (6.7%), and exeresis of hemangioma (3.3%).

Pneumococcal infection was detected in 38 (22.9%) cases, among which 9 (23.7%) were bacteremic and 29 (76.3%) were non-bacteremic cases (Fig. 1); among the non-bacteremic cases, 4 had positive pneumolysin-PCR and 25 had diagnostic pneumococcal antibody increase. Other causative agents were detected in 128 cases (77.1%), which were grouped into the non-pneumococcal infection subgroup. In this subgroup, there were 84 (65.6%) cases of viral infection, 26 (20.3%) cases of atypical bacterial infection and 18 (14.1%) cases of pyogenic bacterial non-pneumococcal infection (Fig. 1). The baseline characteristics of the study group and the comparison of the subgroups are presented in Table 1. The only significant difference was the higher frequency of alveolar infiltrate among patients with pneumococcal infection in comparison with patients with non-pneumococcal infection. Table 2 shows the frequency of detected etiological agents in each etiological subgroup. Infection by *C. pneumoniae*, *S. aureus*, and *S. pyogenes* was not found.

3.2. Cytokines and chemokines measurement

From all measured cytokines, we found detected levels of IL-8, IL-6, and IL-10 and their overall median (IQR) values were 78.0 (30.7–244.3) pg/ml, 10.6 (4.6–30.6) pg/ml, and 3.6 (3.1–4.4) pg/ml, respectively. Detected chemokines were CXCL10 and CCL2 and their respective median (IQR) values were 74.2 (36.1–164.8) pg/ml and 19.1 (10.8–22.8) pg/ml. Among healthy controls, the median (IQR) values of IL-8, IL-6, IL-10, CCL2, and CXCL10 were 11.2 (3.4–89.6) pg/ml, 4.5 (4.1–4.9) pg/ml, 13.5 (12.2–14.8) pg/ml, 106.4 (97.5–117.3) pg/ml, and 96.8 (82.4–99.4) pg/ml, respectively (Table 3). *P* Value was < 0.001 for comparisons of IL-8, IL-6, IL-10, and CCL2, whereas *P* Value was 0.2 for the comparison of CXCL10 between all cases and controls.

3.3. Bivariate analysis

By comparing the median concentrations of serum cytokines/chemokines between the two etiological subgroups of patients (pneumococcal *versus* non-pneumococcal pneumonia), IL-6 and CXCL10 showed significant statistical difference (Table 3). No correlation was found between IL-6 and axillary temperature upon admission (p = 0.2).

Table 4 depicts the median (IQR) concentrations of IL-6 in the serum of children with CAP caused by virus, atypical bacteria or pyogenic bacteria besides *S. pneumoniae*. By comparing IL-6 distribution in each of these subgroups, *P* value was < 0.001 when the subgroup with pneumococcal infection was compared with either the subgroup with viral infection or the subgroup with atypical bacterial infection; *P* value was 0.008 when the subgroup with pneumococcal infection was compared with the subgroup with pyogenic bacterial non-pneumococcal infection. According to Bonferroni correction, *P* value < 0.005 was considered significant.

The median (IQR) serum concentrations of IL-6 of children with bacteremic pneumococcal pneumonia and with non-bacteremic pneumococcal pneumonia were 31.7 (22.6–44.0) pg/ml and 30.6 (11.1–75.4) pg/ml, respectively. There was no difference in IL-6 distribution between these subgroups (p = 0.6) (Table 4).

By comparing the IL-6 distribution in the bacteremic pneumococcal subgroup with the IL-6 distribution in the subgroups with viral infection, atypical bacterial infection, or with the pyogenic bacterial non-pneumococcal infection, *P* value was 0.006, < 0.001, 0.01, respectively. Likewise, by comparing the IL-6 distribution in the non-bacteremic pneumococcal subgroup with each of these subgroups, *P* value was 0.002, < 0.001, 0.027, respectively (Table 4).

By comparing the median (IQR) concentrations of CXCL10 in the serum of children with CAP caused by virus, atypical bacteria or pyogenic bacteria besides *S. pneumoniae* with the median (IQR)

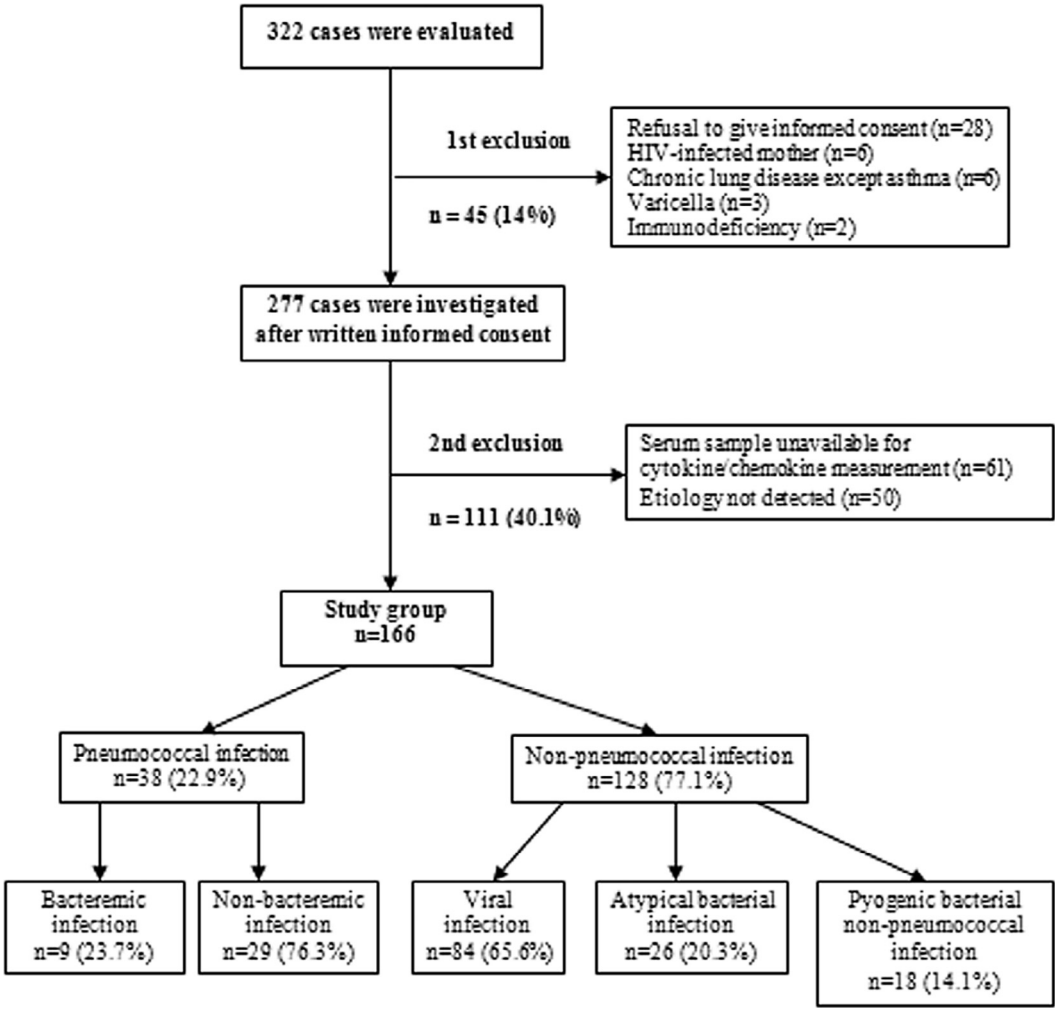

Fig. 1. Flow-chart of recruitment of children hospitalized with community-acquired pneumonia referred for etiology investigation and cytokine/chemokine measurement.

concentrations of CXCL10 in the serum of children with pneumococcal pneumonia, no difference was found (Table 4).

All comparisons were made with the cytokines/chemokines levels detected in two independent assays. The same statistical significances were found in both analyses.

3.4. Predictive value of IL-6

The diagnostic value of IL-6 as a linear variable to predict pneumococcal CAP was calculated with the ROC Curve and the area under the curve was 0.74 (95%CI: 0.65–0.83; $p < 0.001$) (Fig. 2). The optimal threshold value was 12.5 pg/ml, when sensitivity was 76.3% (95%CI: 60.0–88.0%) and specificity was 63.3% (95%CI: 54.0–71.0%).

By using 12.5 pg/ml as the cutoff point to dichotomize IL-6, we found that the negative predictive value of IL-6 under 12.5 pg/ml for pneumococcal infection was equal to 90% (95%CI: 82–95%) (Table 5). By considering IL-6 ≥ 12.5 pg/ml (as categorical variable), the multivariable logistic regression found OR equal to 5.559 (95%CI = 2.424–12.747; $p = 0.0001$) for pneumococcal infection in a model adjusted for age (OR 1.036 for age; 95%CI: 0.742–1.448; $p = 0.8$).

3.5. Predictive value of CXCL10

The chemokine CXCL10 achieved lower value in the area under the ROC curve (0.62; 95% CI: 0.52–0.72; $p = 0.02$) and the OR in the logistic regression model as continuous variable was borderline (1.0018; 95%CI: 1.0003–1.0033; $p = 0.02$). Therefore, IL-6 was identified as the cytokine/chemokine with significant result in bivariate analysis, in the ROC curve and in multivariable analysis.

3.6. Generalizability

To address generalizability, the analysis was repeated with the inclusion of the 50 cases with undetected etiology in the group of non-pneumococcal infection (Tables 3 and 5). In this case, the diagnostic value of IL-6 as a linear variable to predict pneumococcal CAP was calculated with the ROC Curve and the area under the curve was 0.74 (95%CI: 0.65–0.83; $p < 0.0001$); likewise, the diagnostic value of CXCL10 as a linear variable to predict pneumococcal CAP was calculated with the ROC Curve and the area under the curve was 0.65 (95%CI: 0.55–0.74; $p = 0.004$). In the multivariable logistic regression of IL-6 ≥ 12.5 pg/ml (predictor) as categorical variable for pneumococcal infection (outcome) OR was 3.957 (95%CI: 1.906–8.216; $p = 0.0002$) adjusted for age (OR = 0.954; 95% CI: 0.696–1.308; $p = 0.8$). In the multivariable logistic regression of CXCL10 (predictor) as continuous variable for pneumococcal infection (outcome) OR was 1.0023 (95%CI: 1.0008–1.0039; $p = 0.004$) adjusted for age (OR = 0.921; 95% CI: 0.670–1.267; $p = 0.6$).

4. Discussion

In this study, IL-6 increase was identified as independently associated with pneumococcal infection among children under 5 years of

Table 1
Baseline characteristics of children hospitalized with community-acquired pneumonia with or without pneumococcal infection.

Characteristics	Pneumococcal infection		*P* Value[c]	All children
	Yes (n = 38)	No (n = 128)		(n = 166)
Male gender[a]	24 (63.2)	74 (57.8)	0.6	98 (59.0)
Age (mo)[b]	19 (10–28)	16 (9–2 6)	0.5	17 (10–28)
Age strata[a]				
< 2 mo	0	4 (3.1)	0.6	4 (2.4)
2–11 mo	11 (28.9)	40 (31.3)	0.8	51 (30.7)
12–23 mo	15 (39.5)	42 (32.8)	0.4	57 (34.3)
24–59 mo	12 (31.6)	42 (32.8)	0.9	54 (32.5)
Symptoms				
Duration of disease (days)[b]	8 (5–1 4)	7 (4–1 4)	0.3	7 (4–1 4)
Cough[a]	38 (1 0 0)	126 (98.4)	1.0	164 (98.8)
Duration of cough (days)[b]	10 (5–1 5)	7 (4–1 5)	0.3	7 (4–1 5)
Fever[a]	37 (97.4)	122 (95.3)	1.0	159 (95.8)
Duration of fever (days)[b]	5 (4–8)	5 (3–7)	0.2	5 (3–8)
Difficulty breathing[a]	32 (84.2)	108 (84.4)	1.0	140 (84.3)
Duration of difficulty breathing (days)[b]	4 (2–7)	4 (3–7)	0.6	4 (3–7)
Signs upon admission[a]				
Tachypnea	35 (92.1)	106 (82.8)	0.2	141 (84.9)
Crackles	22 (57.9)	92 (72.4)	0.09	114 (69.1)
Fever	24 (63.2)	77/126 (61.1)	0.8	101/164 (61.6)
Axillary temperature (°C)[b]	37.9 (37.0–38.5)	37.7 (37.0–38.2)	0.6	37.8 (37.0–38.3)
Radiological findings[a]				
Alveolar infiltrate	32/37 (86.5)	81/121 (66.9)	0.02	113/158 (71.5)
Pleural effusion	5/37 (13.5)	12/121 (9.9)	0.6	17/158 (10.8)

[a] Results in n (%).
[b] Results in median (IQR).
[c] *P* Value according to Chi-Square or Fisher's Exact test, as appropriate (for categorical variables) or Mann Whitney U test (for continuous variables).

Table 2
Frequency of etiological agents found in each etiological subgroup among children hospitalized with community-acquired pneumonia, established etiology and serum cytokines/chemokines measured upon admission.

Etiological subgroup	Frequency n (%)
Pneumococcal infection (n = 38)	
Sole infection	12 (31.6)
Co-infection with respiratory virus	26 (68.4)
Co-infection with other pyogenic bacteria	3 (7.9)
Co-infection with atypical bacteria	2 (5.3)
Viral infection (n = 84)	
Sole infection	67 (79.8)
Respiratory virus co-infection	17 (20.2)
Identified viral infections	
Rhinovirus	27 (32.1)
Parainfluenza viruses types 1, 2, 3	22 (26.2)
Respiratory syncytial virus	21 (25.0)
Human bocavirus	11 (13.1)
Influenza viruses A and B	7 (8.3)
Human metapneumovirus	6 (7.1)
Adenovirus	6 (7.1)
Enterovirus	5 (6.0)
Atypical bacterial infection (n = 26)	
Sole infection	12 (46.2)
Co-infection with respiratory virus	13 (50.0)
Atypical bacterial co-infection	1 (3.8)
Identified atypical bacterial infections	
M. pneumoniae	13 (50.0)
C. trachomatis	11 (42.3)
S. negevensis	3 (11.5)
Pyogenic bacterial non-pneumococcal infection (n = 18)	
Sole infection	7 (38.9)
Co-infection with respiratory virus	9 (50.0)
Co-infection with respiratory virus and atypical bacteria	2 (11.1)
Identified pyogenic bacterial non-pneumococcal infections	
H. influenzae	15 (83.3)
M. catarrhalis	3 (16.7)

age hospitalized with CAP. Additionally, we demonstrated that IL-6 levels under 12.5 pg/ml have high negative predictive value for pneumococcal infection among these patients. To the best of our knowledge, this is the first time that this association is reported with such evidence among children and the threshold of 12.5 pg/ml for IL-6 is found. As a matter of fact, IL-6 distribution was compared between cases with and without pneumococcal infection when cases with pneumococcal infection had or did not have bacteremia; this comparison was repeated by considering only bacteremic cases in the group of pneumococcal infection, or by considering only non-bacteremic cases in the group of pneumococcal infection. The same results were found in these three distinct analyses. Curiously, IL-6 distribution did not differ within the pneumococcal infection subgroup, that is, when bacteremic and non-bacteremic patients were compared. Herein, no other causative agent elicited such IL-6 increase. It is important to recall that cytokines and chemokines levels were determined twice in each serum sample and the statistical analysis of both measurements performed in serum samples collected at admission pointed to the same results. Moreover, the results were the same when cases with undetected etiology were included in the subgroup with non-pneumococcal infection (Tables 3 and 5).

IL-6 has been reported to be an important pro-inflammatory cytokine in defense against pneumococcus [6,7], but its role remains not completely understood. IL-6 is within the pyrogenic response pathway but we did not find correlation between axillary temperature upon admission and serum IL-6 level. Herein, IL-6 showed a potential value as a biomarker for pneumococcal CAP among hospitalized children. A positive correlation between IL-6 and C reactive protein (another biomarker) levels has been reported in children with CAP, but not with a specific pathogen [11,13]. In the pediatric population, there is only one previous study in which etiology of CAP was investigated by an expanded panel of tests and 15 different cytokines/chemokines were measured, but no association was found between IL-6 and any specific etiology. Therein, cytokines/chemokines were measured using the Human Cytokine Multiplex Antibody Bead Kit. Indeed, the number of children studied was limited (n = 55) and, most important, the diagnosis of pneumococcal infection was based solely on positive or negative blood culture or pneumolysin-based PCR assays. In that study, the median (IQR) of IL-6 among 12 children with pneumococcal infection was 89 (43–203) pg/ml whereas it was 82 (50–381) pg/ml among 8 children with viral infection. Of note, all children had negative blood culture and 12 had positive pneumolysin-based PCR assays [13]. By comparing their results with ours, it is possible to infer that, therein, pneumococcal infection occurred in the subgroup labeled as viral infection and the pneumococcal infection was not identified. This finding may be due to the fact that those authors only searched for invasive pneumococcal infection. Therefore, they could not exclude cases with non-invasive pneumococcal infection among patients grouped in the viral infection category.

Another study compared the serum levels of IL-8 and IL-6 between children with CAP caused by *M. pneumoniae* (detected by serology) and children with CAP caused by pyogenic bacteria, including *S. pneumoniae* (detected only by blood culture). No difference in IL-6 levels between these two groups was found. Nevertheless, according to the techniques described to detect the etiological agents, pneumococcal infection was not ruled out in the group of *M. pneumoniae* infection. Again, non-invasive pneumococcal infection was not sought. Furthermore, one specific group of pneumococcal infection was not

Table 3
Comparison of median (interquartile range) concentrations (pg/ml) of detected cytokines and chemokines in serum from children hospitalized with community-acquired pneumonia with or without pneumococcal infection, without (n = 128) or with (n = 178) the inclusion of cases with undetected etiology in the sub-group with non-pneumococcal infection, and from healthy controls.

Cytokines and chemokines[a]	Pneumococcal infection					Healthy controls
	Yes (n = 38)	No (n = 128)	*P* Value[b]	No (n = 178)	*P* Value[b]	
IL-8 (pg/ml)	120.1 (27.9–408.8)	69.0 (29.7–206.7)	0.1	69.6 (25.8–204.6)	0.2	11.2 (3.4–89.6)
IL-6 (pg/ml)	31.2 (12.4–54.1)	9.0 (4.1–22.0)	< 0.001	0.0 (0.0–15.2)	< 0.001	4.5 (4.1–4.9)
IL-10 (pg/ml)	3.5 (3.0–4.3)	3.7 (3.1–4.4)	0.5	3.4 (3.0–4.6)	0.9	13.5 (12.2–14.8)
CXCL10 (pg/ml)	99.8 (50.3–306.8)	65.5 (33.6–135.3)	0.02	61.8 (32.1–133.2)	0.004	96.8 (82.4–99.4)
CCL2 (pg/ml)	19.8 (11.4–22.7)	19.0 (10.3–22.8)	0.5	18.9 (9.8–22.9)	0.5	106.4 (97.5–117.3)

[a] IL1β, IL-2, IL-4, IL-5, IL-12 TNFα, IFN-γ, CXCL9 and CCL5 did not show detectable levels.
[b] Determined by Mann-Whitney U test.

assessed for comparison with infection by other pathogens [12]. The main difference of our work is the etiological definition of CAP in the sample studied. Herein, various different microbiological techniques were applied with the objective to enhance the accuracy of the results. Notably, pyogenic bacterial infections, including pneumococcal infection, were investigated as invasive and non-invasive (non-bacteremic) presentations.

IL-6 was demonstrated to have limited value in distinguishing bacterial from viral infection among children, including pneumococcal infection *versus* infection by other pathogens [11]. However, therein, the blood samples to perform the cytokine analysis were collected mostly (86%) after the beginning of antimicrobial therapy, and the decrease of IL-6 has been documented to follow the antimicrobial treatment in adults hospitalized with CAP [29,30].

IL-6 increase was significantly associated with bacteremia in a case-control study that assessed children with fever without source and positive blood culture (58% for *S. pneumoniae*) and children with fever without source and negative blood culture [31]. In such a case, children with fever without source rarely have non-bacteremic pneumococcal infection. That means, these children most probably do not have pneumococcal infection when blood culture is negative. This is not the case among children with CAP, when the vast majority of pneumococcal infections are non-bacteremic [32].

In adults, levels of IL-6 measured in patients with bacteremic pneumococcal CAP were significantly higher than in patients with *M. pneumoniae* CAP [9]. In addition, adults with pneumococcal CAP presented significantly higher serum concentrations of IL-6, on admission, than those with CAP caused by *Legionella pneumophila*, *M. pneumoniae,*

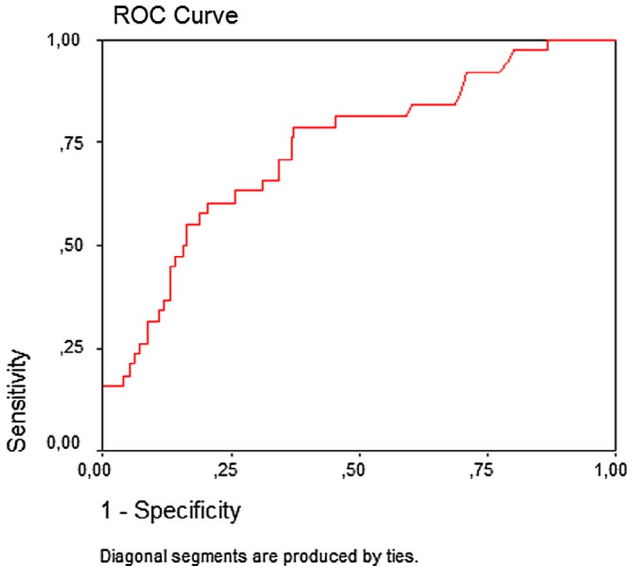

Fig. 2. Receiver Operating Characteristic (ROC) curve for interleukin-6 (predictor variable) to predict pneumococcal community-acquired pneumonia (outcome variable) among children.

C. pneumoniae, Coxiella burnetti or respiratory viruses (influenza viruses, parainfluenza viruses, adenovirus and respiratory syncytial virus) [30]. These results in adults are concordant with ours among children. The lower frequency of non-bacteremic pneumococcal infection among

Table 4
Median (interquartile range) concentrations (pg/ml) of IL-6 and CXCL10 in serum from children hospitalized with community-acquired pneumonia in distinct etiological subgroups.

Cytokines and chemokines	Pneumococcal infection			Non-pneumococcal infection		
	Overall n = 38	Bacteremic n = 9	Non-bacteremic n = 29	Virus	Atypical bacteria	Pyogenic bacteria besides *S. pneumoniae*
IL-6 (pg/ml)	31.2 (12.4–54.1)	31.7 (22.6–44.0)	30.6 (11.1–75.4)	9.5 (3.7–23.4)	7.3 (4.1–10.8)	9.5 (6.7–20.0)
CXCL10 (pg/ml)	99.8 (50.3–306.8)	150.5 (66.4–426.5))	97.2 (49.9–249.5)	69.9 (33.5–168.1)	68.8 (34.2–135.6)	44.2 (32.7–107.2)

Value by Mann-Whitney U test for:		
Compared etiological subgroups	IL-6	CXCL10
Overall pneumococcal infection X virus	< 0.001	0.05
Overall pneumococcal infection X atypical bacteria	< 0.001	0.2
Overall pneumococcal infection X pyogenic bacteria besides *S. pneumoniae*	0.008	0.009
Bacteremic pneumococcal infection X virus	0.006	0.1
Bacteremic pneumococcal infection X atypical bacteria	< 0.001	0.2
Bacteremic pneumococcal infection X pyogenic bacteria besides *S. pneumoniae*	0.01	0.04
Non-bacteremic pneumococcal infection X virus	0.002	0.1
Non-bacteremic pneumococcal infection X atypical bacteria	< 0.001	0.3
Non-bacteremic pneumococcal infection X pyogenic bacteria besides *S. pneumoniae*	0.027	0.02
Bacteremic X non-bacteremic pneumococcal infection	0.6	0.6

According to Bonferroni correction, *P* value < 0.005 was considered significant.

Table 5
Diagnostic value of serum IL-6 equal to or higher than 12.5 pg/ml for pneumococcal infection among children hospitalized with community-acquired pneumonia without (n = 128) or with (n = 178) the inclusion of cases with undetected etiology in the subgroup with non-pneumococcal infection.

IL-6 ≥ 12.5 pg/ml	Pneumococcal infection				
	Yes	No	Total	No	Total
Yes	29	47	76	50	79
No	9	81	90	128	137
Total	38	128[a]	166	178[b]	216

Sensitivity: 29/38 = 76.3% (95%CI: 60.0–88.0%)[a].
Specificity: 81/128 = 63.3% (95%CI: 54.0–71.0%)[a]; 128/178 = 71.9% (95%CI: 65.0–78.1%)[b].
Positive predictive value: 29/76 = 38.2% (95%CI: 28.0–49.0%)[a]; 29/79 = 36.7% (95%CI: 26.6–47.7%)[b].
Negative predictive value: 81/90 = 90.0% (95%CI: 82.0–95.0%)[a]; 128/137 = 93.4% (95%CI: 88.3–96.7%)[b].

these adult patients in comparison with pediatric patients may explain this concordance [33].

This study has some limitations. Because of ethical reason, direct analysis to search for the presence of microorganisms in the lungs was not performed. As a result, etiology was established on a presumptive basis. However, a panel with different diagnostic tests to search for the same pathogen was used. Most importantly, pneumococcal infection was sought by blood culture, PCR for the detection of pneumococcal DNA in blood buffy-coat and serum antibodies titers increase in paired samples. This procedure enabled us to investigate invasive and non-invasive pneumococcal infection. The serological tests to diagnose non-bacteremic pneumonia are not well validated yet, because there is no gold standard for this kind of infection. Nonetheless, less than 3% of the cases diagnosed by increased IgG titers in paired serum samples may be due to acquisition of a new pneumococcal strain in the nasopharynx instead of being a true pneumococcal infection [34].

In conclusion, IL-6 is independently associated with pneumococcal infection among children under-5 years hospitalized with CAP.

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Conflicts of interests

None for all authors.

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