CLINICAL SCIENCE

The behavior and diagnostic utility of procalcitonin and five other inflammatory molecules in critically ill patients with respiratory distress and suspected 2009 influenza a H1N1 infection

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OBJECTIVES: During the 2009 influenza A H1N1 pandemic, it became difficult to differentiate viral infections from other conditions in patients admitted to the intensive care unit. We sought to evaluate the behavior and diagnostic utility of procalcitonin, C-reactive protein and four other molecules in patients with suspected 2009 Influenza A H1N1 infection.

METHODS: The serum levels of procalcitonin, C-reactive protein, tumor necrosis factor α, interferon γ, interleukin 1β, and interleukin 10 were tested on admission and on days 3, 5, and 7 in 35 patients with suspected 2009 H1N1 infection who were admitted to two ICUs.

RESULTS: Twelve patients had confirmed 2009 influenza A H1N1 infections, 6 had seasonal influenza infections, and 17 patients had negative swabs. The procalcitonin levels at inclusion and on day 3, and the C-reactive protein levels on day 3 were higher among subjects with 2009 influenza A H1N1 infections. The baseline levels of interleukin 1β were higher among the 2009 influenza A H1N1 patients compared with the other groups. The C-reactive protein levels on days 3, 5, and 7 and procalcitonin on days 5 and 7 were greater in non-surviving patients.

CONCLUSION: Higher levels of procalcitonin, C-reactive protein and interleukin-1β might occur in critically ill patients who had a 2009 H1N1 infection. Neither procalcitonin nor CRP were useful in discriminating severe 2009 H1N1 pneumonia. Higher levels of CRP and procalcitonin appeared to identify patients with worse outcomes.

KEYWORDS: Severe respiratory distress syndrome; C-reactive protein; Biomarker; Sensitivity; Specificity.

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INTRODUCTION

The 2009 influenza A H1N1 (2009 H1N1) pandemic generated a significant burden to health care services around the world (1,2). This virus was first identified in Mexico and the United States (March-April 2009) and quickly spread worldwide (3,4). In Brazil, as in some other southern hemisphere countries, the outbreak started in June, lasted approximately 18 weeks, and was responsible for at least 1,600 deaths (2,5).

No potential conflict of interest was reported.

The 2009 H1N1 infection caused a broad spectrum of clinical syndromes, ranging from afebrile upper respiratory illness to fulminating viral pneumonia and acute respiratory distress syndrome (2,6-8). The real-time reverse transcriptase polymerase chain reaction (rRTPCR) analysis performed on respiratory secretions has a sensitivity of 98 to 100% and a specificity of 100% in identifying the 2009 H1N1 infection (9). However, with a turnaround time of 2-3 days, its results do not contribute to the initial therapeutic decisions. The use of biomarkers in patients with suspected or confirmed bacterial infections has been investigated primarily in the critical care setting. Procalcitonin (PCT) has been proven to be accurate in discriminating bacterial from viral infections (10,11). However, only a few studies have evaluated the behavior of PCT in patients with severe

2009 H1N1 pneumonia (12-15). It has been suggested that the immune response against the 2009 H1N1 virus might diverge from that observed against the seasonal influenza virus (16). Interestingly, elevated levels of PCT have been observed in patients with viral 2009 H1N1 infection even when the patients did not have a concomitant bacterial infection (13,14).

In this study, we sought to evaluate the behavior and the diagnostic utility of the circulating serum levels of PCT in patients with suspected 2009 H1N1 infection and severe acute respiratory illness. To further evaluate the immune response in those individuals, we investigated the levels of C-reactive protein (CRP), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin 1β (IL-1β) and interleukin 10 (IL-10) in those patients.

MATERIALS AND METHODS

Study setting and subjects

This was a prospective observational study conducted in two mixed medical and surgical intensive care units (ICUs) in two university hospitals in southeastern Brazil. From August to November 2009, all the patients aged ≥14 years old that were admitted to one of the two participant ICUs with suspected 2009 H1N1 infection were evaluated for potential eligibility. The following inclusion criteria were used: *(1)* severe acute respiratory illness defined as dyspnea plus bilateral infiltrates on the x-ray; *(2)* suspicion of 2009 H1N1 infection and lack of any other obvious etiology to explain the respiratory symptoms; and *(3)* a stay of at least 24 h in the ICU. The patients who fulfilled the inclusion criteria were included in the study on the first day of ICU admission. The study was approved by the Universidade Federal de Minas Gerais' Ethic Committee, and written informed consent was obtained from all the patients or the next of kin. To conduct this study and write this report, we observed the Standard for Reporting of Diagnostic Accuracy checklist and recommendations (17).

Study procedures

A dedicated fellow (MBSP) visited the participating ICUs daily to follow the included subjects and to identify new eligible patients. Demographic, clinical, and laboratory data were recorded at inclusion and then daily. The radiographic diagnostics and microbiological examinations (i.e., cultures of urine, blood, blind bronchoalveolar lavage, and tracheal aspirates) were performed at the discretion of the treating physicians. Severity and organ dysfunction at admission were defined based on the Acute Physiology and Chronic Health Evaluation II (APACHE II) (18) and the Sepsis-Related Organ Failure Assessment (SOFA) scores (19). All-cause hospital mortality, ICU length of stay and hospital length of stay were also recorded. No diagnostic or therapeutic intervention was performed as part of the study protocol.

Identification of the 2009 H1N1 virus

All the included patients were submitted to the collection of nasopharyngeal swabs or aspirates upon admission to the ICU. These samples were tested for 2009 H1N1 in a public reference laboratory according to the Centers for Disease Control (CDC) rRTPCR Protocol for the Detection and Characterization of Swine Influenza (version 2009) (20).

PCT and CRP measurement

Peripheral blood samples were collected in the morning using vacuum tubes (BD Vacutainer SST II Plus plastic tubes; Becton Dickinson Diagnostic Systems, São Paulo, Brazil). After centrifugation, the serum was stored at -80℃ until analyzed. The circulating plasma PCT and CRP levels were measured at inclusion (baseline) and on days 3, 5, and 7 following inclusion until the time of patient death or ICU discharge. The PCT levels were measured using an enzyme-linked fluorescent immunoassay (PCT Vidas Brahms, bioMérieux, France) with an assay sensitivity of 0.05 μg/L, which was approximately fourfold higher than the mean normal levels. The circulating CRP levels were measured using dry chemistry with the Ektachem 950ICR System (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY, USA). The detection limit for CRP was 7 mg/dL. Values above 10 mg/dL were considered abnormal.

Cytokine Measurements

The plasma levels of TNF-α, IFN-γ, IL-10, and IL-1β were measured using the enzyme-linked immunoassay (ELISA) sandwich (Duoset R & D Systems, Minneapolis, MN, USA) with pairs of marked antibodies. These cytokines were tested at inclusion and on days 3, 5, and 7 according to the ICU length of stay.

Statistical analysis

The categorical variables are expressed as numbers and percentages. The continuous variables are stated as the means ± SD for normally distributed variables and as medians and interquartile ranges (IQR) for non-normally distributed variables. The comparability among the groups was analyzed using the χ^2 tests (Yates' test or Fisher's exact test), the two-sample t test, the Mann-Whitney U test or the Kruskall-Wallis test. The ROC curves were built to establish the accuracy of the inflammatory molecules tested in the identification of patients infected by 2009 H1N1. The positive and negative predictive values were calculated once the best cutoffs for these molecules were defined. The Spearman test was used to establish correlations between parameters. The data collected were analyzed using the SPSS software (SPSS 17.0; SPSS Inc., Chicago, United States). To compare the three studied groups regarding the behavior of the CRP and PCT levels over time, i.e., for the four measurements (baseline, day 3, day 5, and day 7), we fitted a linear mixed-effect model using *R* software (*lme*4 and *nlme* packs). Therefore, the CRP and PCT levels were transformed to normally distributed variables with square root and natural logarithm calculations, respectively. Significance was reported as a *p*-value of 0.05 or less.

RESULTS

Characteristics of the study population

Forty-nine patients were assessed for eligibility. Five patients did not meet the inclusion criteria: two patients had an alternate diagnosis, and three patients stayed in the ICU for less than 24 hours. Among the 44 remaining patients, nine were excluded from the final analysis: for eight patients, we were unable to obtain the results for 2009 H1N1 rRTPCR from the reference laboratory probably due to inaccurate measurements, and in one patient, the results of the circulating levels of PCT upon admission were unavailable.

Table 1 - Patients' main characteristics.

	Influenza A H1N1 (n = 12)	Seasonal Influenza (n = 6)	Negative rRTPCR (n = 17)	*p*-value[a]
Age (mean ± SD)	37.5 (21.6)	39.2 (11.8)	39.7 (22.0)	0.81
Sex (male, %)	6 (50)	1 (17)	7 (41)	0.39
Underlying medical condition (n, %)	8 (67)	3 (50)	10 (59)	0.78
Asthma	0 (0)	1 (17)	3 (18)	
Chronic obstructive pulmonary disease	2 (17)	0 (0)	1 (6)	
Diabetes	1 (8)	0 (0)	2 (12)	
Chronic cardiovascular disease	2 (17)	1 (17)	2 (12)	
Chronic renal disease	1 (8)	0 (0)	0 (0)	
Immunosuppression	1 (8)	1 (17)	1 (6)	
Pregnancy	2 (17)	0 (0)	3 (18)	
Obesity	1 (8)	1 (17)	0 (0)	
Apache 2 (median, IQR)	14.5 (11.0)	11.0 (5.0)	10.0 (8.0)	0.24
SOFA at admission (median, IQR)	6.0 (6.0)	3.0 (2.0)	2.0 (2.0)	0.006
PaO_2/FiO_2 at admission (median, IQR)	160 (127)	200 (119)	172 (136)	0.31
Positive cultures at admission (n, %)[b]	3 (25)	0 (0)	3 (18)	0.32
Blind-BAL or tracheal aspirate	2 (17)	0 (0)	2 (12)	
Blood	2 (17)	0 (0)	1 (6)	
Vasopressors (n, %)	11 (91.7)	3 (50)	8 (47.1)	0.04
Hydrocortisone (n, %)	7 (58.3)	2 (33.3)	9 (52.9)	0.60
Endotracheal intubation (n,%)	11 (91.7)	3 (50)	12 (70.6)	0.14
Mechanical ventilation (median days, IQR)	10 (11)	12 (29)	4 (23)	0.91
Acute kidney injury (n, %)[c]	8 (66.7)	2 (33.3)	3 (17.6)	0.03
Hemodialysis (n, %)	6 (50)	2 (33.3)	3 (17.6)	0.18
ICU LOS (median days, IQR)	12 (14)	17.5 (25)	7 (21)	0.65
Hospital LOS (median days, IQR)	16.5 (21)	28.5 (55)	18 (21)	0.85
Mortality (n, %)	5 (41.7)	0 (0)	6 (35.3)	0.18

LOS- length of stay; IQR- interquartile range; SOFA – sequential organ failure assessment
[a]For comparison among the three groups, significant if < 0.05.
[b]One patient with H1N1 infection had both the respiratory secretion and the blood cultures positive for *Staphylococcus aureus*.
[c]According to the AKIN criteria[21]

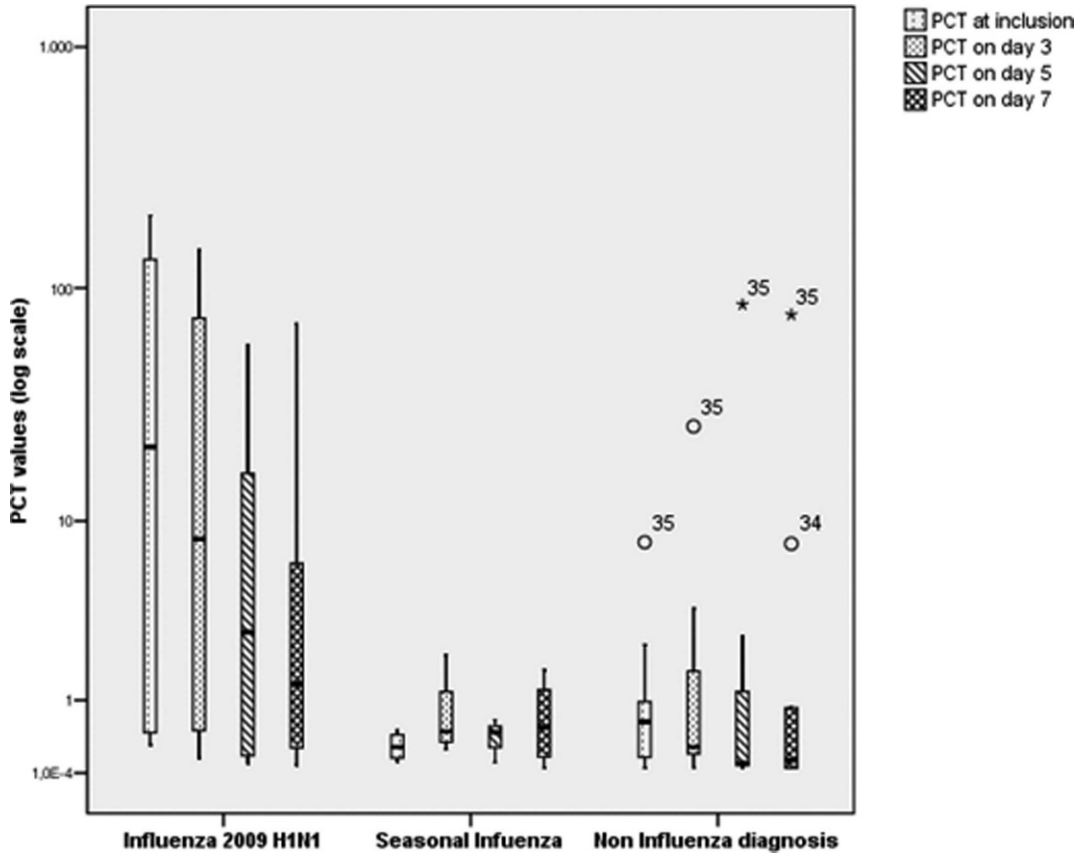

Figure 1 - The circulating levels of procalcitonin observed in the studied patients at the four points of measurement, according to the respective group.

The demographic (i.e., age and gender) and the primary baseline clinical characteristics (i.e., comorbidities, SOFA score and APACHE score) were similar between the included and excluded patients (data not shown). The rRTPCR analysis was performed in a median of 6 (*range*: 0-16) days after the onset of symptoms. Twelve (34.3%) patients had confirmed 2009 H1N1 infections, 6 (17.1%) patients had seasonal influenza infections and 17 (48.6%) patients yielded swabs that were negative for the influenza virus. The most frequent diagnoses among individuals with negative rRTPCR were undefined pulmonary disease with bilateral infiltrates (5 patients), microbiologically confirmed community-acquired pneumonia (3 patients), severe asthma and pulmonary embolism (2 patients each). The patients' clinical characteristics are displayed in table 1. The SOFA score measured at inclusion was higher among 2009 H1N1-infected individuals compared with the other groups; the use of vasopressors and the occurrence of acute renal failure were also higher among these patients (21).

Thirty-two (91.4%) patients had blood cultures obtained at admission, and 16 (61.5% of those who underwent tracheal intubation) provided respiratory samples that were tested for bacterial agents. Positive blood culture results were observed in two patients of the 2009 H1N1 group and in one patient with negative rRTPCR results. Concerning the respiratory samples, two patients in the 2009 H1N1 group (both with *S. aureus*) and two patients in the negative rRTPCR group (1 with *S. aureus* and 1 with *K. pneumoniae*) had positive results.

Plasma levels of PCT and CRP

The circulating levels of PCT at inclusion and on day 3 were significantly higher among subjects with confirmed H1N1 infections (median 7.22 μg/L, IQR: 95.92) compared with the patients in the seasonal influenza group (0.28 μg/L, IQR: 1.39) and the patients without influenza (0.85 μg/L, IQR: 3.29); $p = 0.005$ and $p = 0.015$, respectively. Similar results were observed when the circulating levels of CRP that were tested on day 3 were compared among the three groups: medians of 179 mg/dl (IQR: 274) in the 2009 H1N1 group, 110 mg/dl (IQR: 119) in the seasonal influenza group, and 123 mg/dl (IQR: 106) in the patients without an influenza infection ($p = 0.024$). A smaller, although still significant, difference was observed for both markers when a subgroup analysis excluding the three patients with bacteremia at inclusion was performed.

Regarding the changes in the levels of CRP and PCT during the first seven days of follow-up in all the studied patients, the levels of both markers decreased with time ($p = 0.003$ for PCT and $p = 0.079$ for CRP). The trends were independently compared among the groups for each marker. For both markers, the patients in the 2009 H1N1 group were primarily responsible for this trend. When the entire period of time was considered, the CRP and PCT values were significantly lower in the seasonal influenza ($p = 0.029$ for CRP and $p = 0.009$ for PCT, respectively) and noninfluenza groups ($p = 0.041$ for CRP and $p = 0.010$ for PCT, respectively) compared with the H1N1 patients.

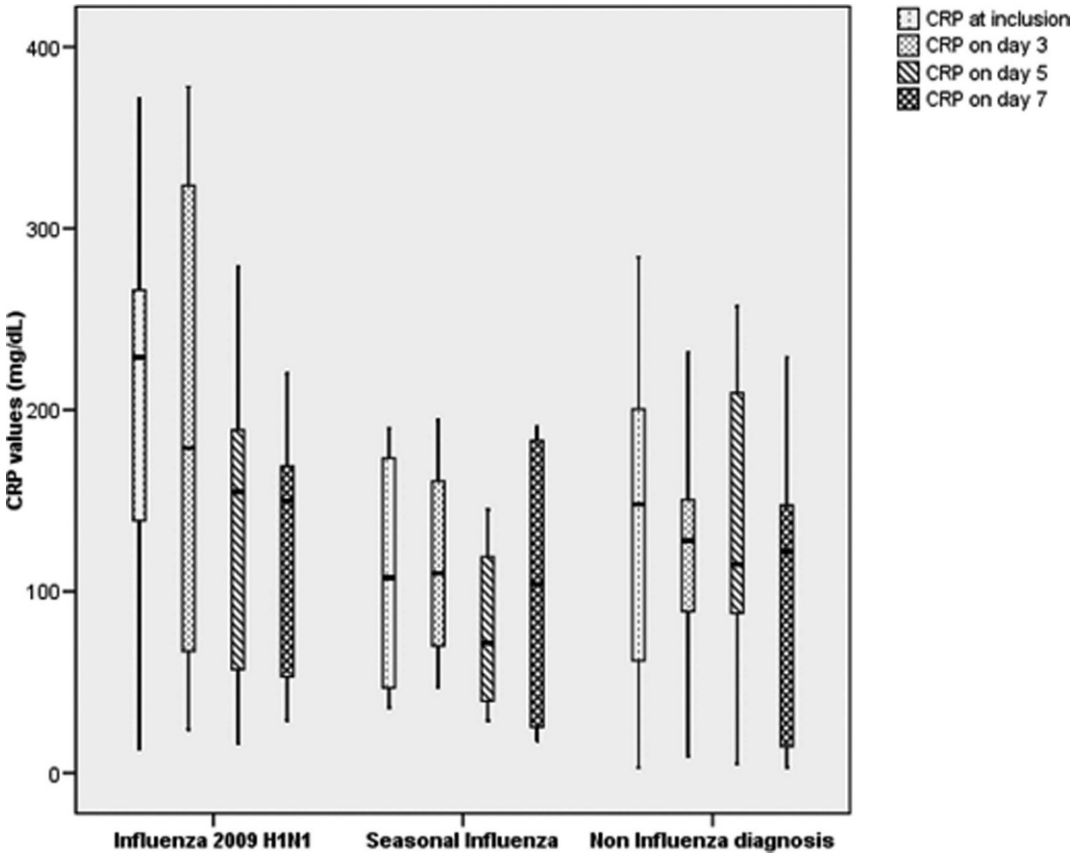

Figure 2 - The circulating levels of C-reactive protein observed in the studied patients at the four points of measurement, according to the respective group.

Plasma levels of cytokines

Except for the baseline levels of IL-1β, which were proven to be significantly higher among the patients in the 2009 H1N1 group ($p = 0.014$) compared with the other groups, no other difference was observed concerning the circulating levels of the tested cytokines. The AUC for IL-1β values indicating H1N1 infection was 0.79 (CI, 0.62 to 0.96); at a cutoff point set at 30 pg/ml, the sensitivity was 91%, and the specificity was 65%. The levels of IL-1β remained significantly higher among patients infected with 2009 H1N1 influenza when the subgroup with bacteremia was excluded from the analysis ($p = 0.04$).

Inflammatory molecules and outcome

We further evaluated the time course of the tested molecules in relation to in-hospital mortality. Eleven (31.4%) out of the 35 included patients died during hospitalization. In the analysis that compared the CRP and PCT levels with the outcome, the CRP levels on days 3, 5 and 7 ($p = 0.047$, 0.012 and 0.008, respectively) and the PCT levels on days 5 and 7 ($p = 0.019$ and 0.001, respectively) were significantly higher in non-surviving patients. No studied cytokine was associated with all-cause hospital mortality.

DISCUSSION

In this observational study on critically ill patients with suspected 2009 H1N1 infection, we observed higher circulating levels of PCT and CRP among 2009 H1N1-infected individuals compared with patients with seasonal influenza and non-influenza-related respiratory distress. The IL-1β levels were also higher among 2009 H1N1 subjects compared with the two other studied groups on admission. The increased levels of PCT and CRP throughout the course of the disease were associated with higher mortality.

Many studies have tested the role of PCT as a tool to differentiate infectious and noninfectious systemic inflammatory response syndrome (SIRS) (22-25) and to distinguish between bacterial and viral infections (10,11). Most of the studies observed better results with PCT than with CRP and other markers in discriminating these conditions. Moreover, the PCT levels during the first days of antibiotic therapy

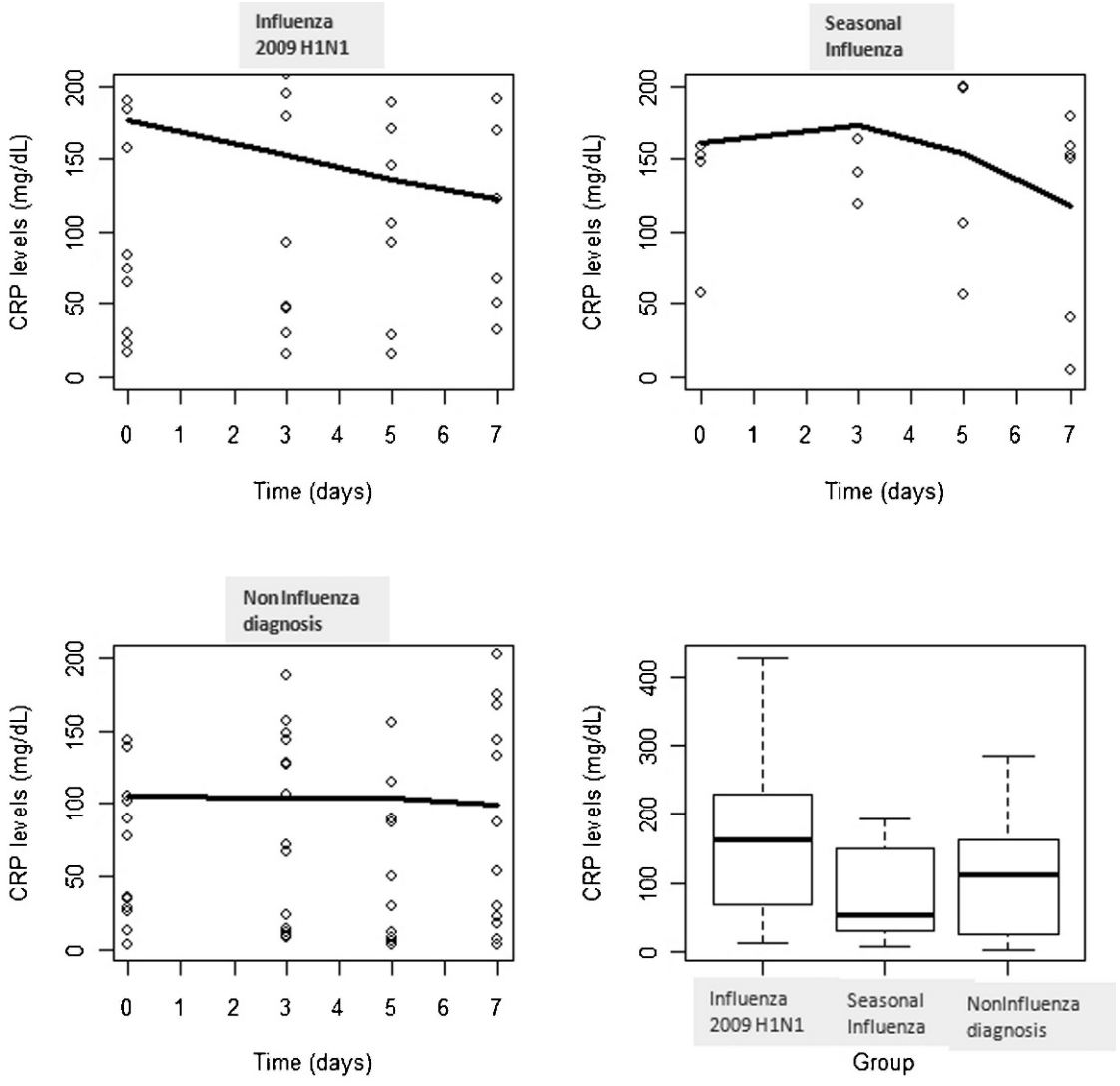

Figure 3 - The circulating levels of CRP during the first seven days of follow-up in the three studied groups.

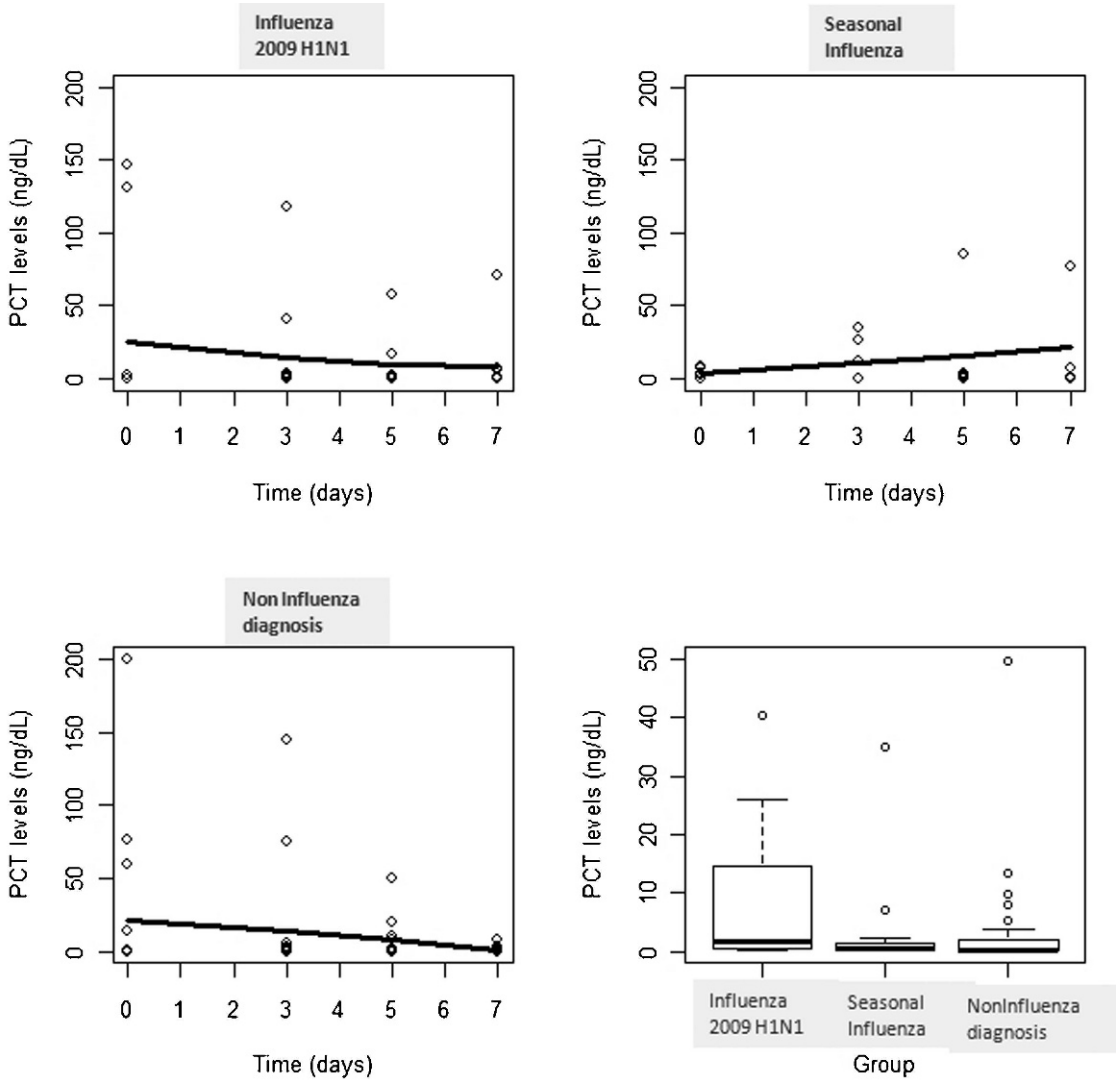

Figure 4 - The circulating levels of PCT during the first seven days of follow-up in the three studied groups.

seem to be an accurate marker for clinical response and outcome (26). The higher levels of PCT presented by the patients with 2009 H1N1 infection in the present study challenges the notion that the levels of this marker would remain normal or only slightly elevated among patients with viral conditions. As suggested by the SOFA scores, the most probable explanation of our results is the significantly greater severity of the 2009 H1N1 subjects. Increased PCT concentrations have already been shown to be associated with higher severity scores (*i.e.*, SOFA score) in critical care patients with sepsis (27). Both the PCT and CRP levels were useless in discriminating 2009 H1N1 infection from other causes of respiratory distress in our study.

Although this possibility was uniformly present among the 35 studied subjects, we cannot rule out unidentified bacterial coinfection among the 2009 H1N1 patients as a hypothetical reason to explain their elevated CRP and PCT serum levels. Guervilly *et al.* observed that patients infected with this virus might present high PCT levels despite the absence of bacterial coinfection (13). Similar results were reported by Cunha *et al.* in subjects with a definite or probable 2009 H1N1 diagnosis (14).

Conversely, several authors have observed lower levels of circulating PCT among patients with an isolated 2009 H1N1 infection compared with those with a bacterial or mixed (bacterial and viral) infection (12,28). In a multicenter, retrospective study conducted in 23 French ICUs, Cuquemelle *et al.* investigated the initial circulating levels of PCT presented by 52 patients admitted with confirmed 2009 H1N1 infection. They found that the PCT levels of 0.8 μg/L combined with alveolar pulmonary infiltrates are strongly suggestive of bacterial coinfection (OR12.9; 95% CI 3.2–51.5) (15).

Regarding the tested cytokines, only the baseline levels of IL-1β were higher among 2009 H1N1-infected subjects. The IL-1β levels also maintained their association with H1N1 infection in the analysis, excluding the patients with bacteremia. At a cutoff of 30 pg/ml, the IL-1 β levels accurately identified the patients infected with the virus. The levels of IL-1 β have been found to be elevated in patients with severe 2009 H1N1 infections (29). It has been shown that a proinflammatory response predominates in patients with severe 2009 H1N1 infections (30). This inflammatory state might at least partially explain the high levels of CRP and PCT observed.

In our study, the PCT levels tested on days 5 and 7 were significantly higher among non-survivors compared with survivors. It has been shown that the PCT trends, not the baseline values, are associated with the outcome in intensive care patients (22,31). Thus, the decrease in the PCT levels might have identified individuals with better outcomes. In other words, patients with persistently high PCT levels had higher mortality. Elevated CRP levels have also been associated with poor outcomes in intensive care patients (32) and in patients with severe community-acquired pneumonia (33). We found that the circulating CRP levels tested on days 3, 5, and 7 also increased in the non-survivors compared with the survivors.

This study has several limitations. First, our sample size was small, and the investigation was conducted in only two university hospitals. Second, we were not able to obtain lower respiratory samples from some of the patients that underwent invasive mechanical ventilation, which could have been responsible for a small number of negative RT-PCR results. The nasopharyngeal samples, however, were obtained in the acute phase of the disease and before the institution of antiviral therapy in most cases. Additionally, the sensitivity of rRTPCR for influenza in the nasopharyngeal swabs is between 97.8 and 100%, and the specificity is 100% (9). Third, we were unable to obtain culture results from respiratory samples in 38.5% of the subjects who underwent tracheal intubation. Finally, we did not investigate *Streptococcus pneumoniae* or *Legionella pneumophila* urinary antigens.

In conclusion, in this prospective observational study, we observed higher levels of PCT, CRP and IL-1β among critically ill 2009 H1N1-infected patients compared with subjects with seasonal influenza infection and noninfluenza diagnoses. Neither PCT nor CRP was useful in the discrimination of severe 2009 H1N1 infection from other causes of respiratory failure. The elevated levels of PCT and CRP might have been due to the greater severity of certain cases with a concomitant substantial inflammatory response, although bacterial coinfection could not be definitively ruled out. The IL-1β values had a higher discriminative value for 2009 H1N1 infection, and they were not correlated with positive blood or respiratory cultures. Overall, the PCT levels on days 5 and 7 and the CRP levels on days 3, 5, and 7 following admission were associated with all-cause hospital mortality.

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AUTHOR CONTRIBUTIONS

Paiva MB designed the study, supervised data collection, collected data, and drafted the manuscript. Botoni FA participated in the study design and data collection. Teixeira Jr AL and Miranda AS performed the immunoassays. Oliveira CRA and Abrahão JO helped to draft the manuscript. Faria GM helped with the data collection. Nobre V conceived the study, participated in its design and coordination and helped to draft the manuscript.

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