S100A9 as an inflammatory marker in hospitalized COVID-19 patients

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Abstract

Objective: To evaluate serum levels of S100A9 as an inflammatory marker of unfavorable outcomes in patients with COVID-19 in a cross-section study.

Material: Participants in this study were allocated from reference care units for the diagnosis of COVID-19 in the western region of Bahia, Brazil, from May 2020 to June 2021.

Methods: Blood samples were collected from control group, patients with RT-qPCR positive for COVID-19 with different clinical manifestations of the disease and cured COVID-19 patients. Cytokines and inflammatory mediators (S100A9, D-dimer IL-6, IL-10, TNF-a, IL-12p70) were analyzed by ELISA/CBA.

Results: Hospitalized COVID-19 patients presented increased production of S100A9, IL-6, IL-10, TNF-α, IL-12p70, and D-dimer when compared with not hospitalized patients, not infected or cured. Spearman analysis revealed a positive correlation between S100A9 and inflammatory cytokines/D-dimer. ROC curve analyses demonstrated accurate sensitivity and specificity for S100A9 and IL-6.

Conclusion: S100A9 serum levels were elevated in hospitalized patients associated with unfavorable outcomes and poor prognosis in COVID-19. Thus, using S100A9 in association with other standardized parameters in clinical protocols may be a useful tool to predict critical clinical evolution during SARS-COV-2 infection.

Introduction

COVID-19 (coronavirus disease 2019) is a multisystemic disease caused by SARS-CoV-2 coronavirus, first described in China in December 2019 [1]. Symptomatic patients present clinical variability, from mild flu-like symptoms to severe viral pneumonia [2].

Advanced age and the presence of comorbidities such as diabetes, cardiovascular disease and obesity have been identified as predictors of poor prognosis in the context of COVID-19 [3]. However, even younger and generally healthy individuals may have complications associated with respiratory failure and require invasive ventilatory support [1]. Both virus-specific virulence factors and host inflammatory response are implicated in determining disease severity and patient clinical outcome [4], [5].

Ineffective innate immunity followed by impaired adaptive immune response and hyperinflammation can lead to microthrombosis and tissue damage, resulting in Acute respiratory distress syndrome (ARDS), multiple organ failure, and death[6]. In addition, tissue damage induced by SARS-CoV-2 infection can lead to the release of damage-associated molecular patterns (DAMP) which, in turn, induce the activation and recruitment of immune cells that regulate the production of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and chemokines that will orchestrate the innate immune response [7].

Abnormal blood levels of pro or anti-inflammatory cytokines, chemokines and other mediators have been associated with unfavorable outcomes in COVID-19 [8], [9]. In particular, high levels of IL-6 correlated with

an increased risk of death [9], [10]. In addition, patients who require ICU (intensive care unit) admission have higher plasma levels of inflammatory mediators such as IL-2, IL-7, IL-10 and TNF-α [10], [11]. A better understanding of the immunopathogenesis of COVID-19 may allow the identification of prognostic markers, targeted and personalized therapeutic interventions.

The S100 family proeins are damage-associated molecular patterns (DAMPs) that are in the cytoplasm and/or nucleus of a wide range of myeloid lineage cells (neutrophils and monocytes) and are involved in the regulation of cellular processes, such as cell cycle progression and differentiation [12]. Calprotectin is a heterodimer formed by the union of S100A9/MRP14 with S100A8/MRP8 proteins, capable of regulating myeloid cell functions. They are Toll-like receptor 4 (TLR4) and receptor for advanced glycation end-products (RAGE) ligands and therefore are involved in the innate immune response [13]. Moreover, S100A9 has been shown to be an inflammatory mediator in the activity of vascular and inflammatory diseases that culminate in thrombotic events [12], a hallmark associated with COVID-19 [14].

Recent studies have shown that serum calprotectin is closely related to the negative progression of COVID-19, through the participation of myeloid lineage cell like neutrophils in the blood and lung [15], [16]. It is also known that during hyperinflammation occurs the onset of emergency myelopoiesis, which releases immature myeloid cells in the peripheral blood and lungs in response to infection[15]. Thus, S100A8/A9 complex released from neutrophils has been considered a biomarker of COVID-19 infection and it is also involved in the induction of cytokine storms [15], [17].

S100A9 as homodimer has calprotectin-independent properties and in the presence of zinc, it undergoes a conformational change and becomes a ligand for the pro-inflammatory receptors Receptor for Advanced Glycation End products (RAGE) and Toll like receptor 4 (TLR4) [18]. However, there are few studies that demonstrate the participation of S100A9 in the context of the innate immune response, especially in COVID-19. In this way, the present study intend to evaluate the potential of the S100A9 as a predictor associated poor prognosis in hospitalized patients in COVID-19.

Material And Method

Sample collection and study population

The samples used for this observational cross-sectional study were collected from individuals with suspected SARS-COV-2 infection who were part of the group of patients positive for COVID-19; frontline healthcare professionals in the fight against COVID-19 who made up the group of patients negative for COVID-19 and cured COVID-19 patients, in the western region of Bahia, from May 2020 to June 2021. Data collection was performed using an epidemiological form, applied during the samples collection to perform the diagnostic test, upon application of the informed consent form (TCLE) and COVID-19 notification epidemiological sheet, required by the Brazilian Ministry of Health. At this stage, demographic data, as well as the duration of symptoms, quality, and intensity of symptoms were collected.

Individuals enrolled in the study had their blood samples collected in tubes containing EDTA, as well as nasopharyngeal swab samples in L15 medium for investigation of SARS-COV-2 infection. Samples were collected until the 14th day of symptoms at the Leonídia Ayres Laboratory and Hospital do Oeste in Barreiras - Bahia and at the Coronavirus collection units in the municipalities of the western region of Bahia.

The groups were divided according to the following criteria: Not infected or negative (n = 11), composed of health professionals who had negative RTqPCR result; Positives not hospitalized (n = 21), composed of individuals with symptoms such as fever, dry cough and fatigue, and no indication for hospitalization; Positive hospitalized (n = 30), composed of individuals admitted to the Intensive Care Unit with symptoms of fever above 38°C, dyspnea and desaturation (below 90%), with or without mechanical ventilatory support; Cured (n = 25), composed of convalescent individuals from COVID-19 who did not have serious illness. The collection of the cured group was carried out in a period between 1 to 3 months after infection.

A total of 87 individuals of both sexes, of legal age, were selected for the study, whose samples were sent to the Laboratory of Infectious Agents and Vectors (LAIVE) of the Federal University of Oeste da Bahia for analysis. This work was approved by the National Ethics and Research Commission - CONEP, under the number CAAE 30629520.6000.0008.

Diagnosis of SARS COV 2 by RTqPCR and biomarker´s dosage: The nasopharyngeal swab samples were subjected to RNA extraction, following the protocol of the kit "RNA + DNA purification based on a spin column" CELLCO®, and then were subjected to the reaction in Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), following the CDC kit protocol for identification of the N gene. Dosage of inflammatory mediators: Blood samples were centrifuged at 1500 RPM for 5 minutes for plasma collection. The cytokines IL-6, IL-10, TNF-α and IL-12p70 were simultaneously quantified using the cytometric bead array assay (Human Inflammatory Cytokine Kit, BD®, Biosciences®), according to manufacturer's instructions. Beads were analyzed in a flow cytometer BD FACSCanto II (BD®, Biosciences®, San Diego, CA, USA) for cytokine quantitation. The dosage of S100A9 was performed following the protocol and guidelines of the kit manufacturer (Human S100A9 DuoSet ELISA, R&D Systems®). The dosage of D-dimer, following the protocol and guidelines of the manufacturer of the kit (The Human D-Dimer ELISA, THERMO®), that recognizes exclusively natural and recombinant human D-dimer. Only patients with mild, moderate, or severe symptoms and the group cured performed the dosage of D- dimer.

Secondary data

The neutrophil count was obtained only from hospitalized patients, who were admitted to the Hospital do Oeste in Barreiras-Bahia, a regional referral unit for the care of severe cases of COVID-19. From hematological reports, it was possible to identify the neutrophil population of the patients. The method used in the dosage was flow cytometry in blood samples with EDTA, using the "CELLTAC G ®" equipment.

Statistical analysis

After dividing the groups of patients and subsequently measuring the reported biomarkers, the results were plotted and analyzed using the “GraphPad PRISM” and “STATISTIC” software. The D'Agostino and Pearson, Shapiro Wilk and KS Normality tests were performed to analyze the data distribution, where it was found that the data are non-parametric.

The relations between genera versus sample group and age versus sample group were performed with Pearson´s Chi-square and Kruskal-Wallis Test, respectively. To analyze the association between the occurrence of signs of severity, co-morbidities with the analyzed groups, Pearson's Chi-square was performed.

To analyze the production of cytokines and calprotectin between the study groups, a non-parametric Kruskal-Wallis Test was performed. For the analysis of correlations, Spearman's correlation was performed. For IL-6 and calprotectin parameters a ROC curve was performed to determine sensitivity and specificity and area under the curve.

Results

Demographic data of patients.

In our study, no gender predominance was found among the analyzed patients, nor an association between gender and the sample groups ($p = 0.20$). Nonetheless, some aging differences were observed between medians of the groups: negatives x positive hospitalized ($p < 0.0001$); positive not hospitalized x positive hospitalized ($p < 0 .0001$) and positive hospitalized x cured ($p < 0.0001$).

Clinical data of patients

Among the symptoms presented by the patients, an association was found between the clinical profile of all groups and the occurrence of fever ($p < 0.005$). An association was also found between the occurrence of headache and the groups of positive not hospitalized patients ($p < 0.0001$) and positive hospitalized patients ($p = 0.0001$). Likewise, an association was found between the occurrence of myalgia between groups of patients, where the not hospitalized positive ($p < 0.00001$) and hospitalized positive ($p = 0.01$) groups differed from the others. (Table 1)

Table 1
Symptoms and comorbidities presented by patients according to the epidemiological notification form.

		NEGATIVE	POSITIVE NOT HOSP	POSITIVE HOSP	CURED
SYMPTOMS	Fever	9,1%	81%	63,3%	4%
	headache	18,2%	71,4%	10%	40%
	Sore throat	0	42,9%	6,7%	4%
	Dry coach	0	57,1%	83,3%	12%
	Myalgia	18,2%	52,4%	6,7%	12%
	Edema	0	4,8%	0	12%
	Dyspnea	9,1%	14,3%	93,3%	24%
	Diarrhea	18,2%	28,6%	6,7%	12%
	Anosmia	9,1%	38,1%	6,7%	20%
	Vomit	0	9,5%	0	4%
		NEGATIVE	**POSITIVE NOT HOSP**	**POSITIVE HOSP**	**CURED**
COMORBITIDIES	Hipertention	9%	0	36,7%	4%
	Diabetes Mellitus	0	0	30%	4%
	Asthma	9%	4,7%	0	0

The occurrence of sore throat also differs between groups, where only the group of positive patients not hospitalized differs from the others ($p < 0.00001$) and dry cough differs between all analyzed groups ($p < 0.002$), with the exception of positive patients not hospitalized ($p = 0.3$). The occurrence of dyspnea is associated among the analyzed patients, where all groups differ, with the highest frequency being for positive hospitalized patients ($p < 0.00001$). (Table 1)

Regarding comorbidities, an association was found between the occurrence of diabetes and hypertension in relation to groups of patients, where positives not hospitalized ($p = 0.04$) and positives hospitalized ($p < 0.0001$) differed from the others for diabetes and positives not hospitalized ($p = 0.02$) and hospitalized positives ($p < 0.00001$) differed from the others for hypertension. (Table 1)

Clinical data of hospitalized patients

In all hospitalized in ICU patients, it was possible to detect leukocytosis with neutrophilia and associated lymphocytopenia as a hematological finding. More than half of the patients required mechanical ventilatory support. About 25% of patients died. (Table 2).

Table 2
Clinical data of hospitalized patients.

Laboratory Findings (Hospitalized patients)		
Parameter	**Average ± SD**	**Reference values**
Hemoglobin (g/dl)	11,8 (± 2.21)	Famale: 11 to 16.4 g/dl
	12,3 (+/- 2.1)	Male: 13.5 to 18 g/dl
Leukocits (cels/ mm^3)	14292,6 (± 6308.3	4000 to 10000/mm^3
Neutrophils (cels/mm^3)	12558.3 (± 5616.3)	2800 to 5250 /mm^3
Lymphocits (cels/mm^3)	881.8 (± 482.4)	1400 to 3150 /mm^3
Platelets (un/mm^3)	281.812 (± 129.465)	150.000 to 450.000 /mm^3
Respiratory Status		
Mechanical ventilation	15	54%
No mechanical ventilation	13	46%
Clinical Outcome		
Death	7	25%
Discharged	21	75%

The production of inflammatory mediators and S100A9 is increased in hospitalized patients

For a better understanding of S100A9 role in exacerbating the inflammatory response in COVID-19, other known inflammatory markers related to poor prognosis in infection were used as a reference. Cytokines such as IL-6, TNF-α, IL-10 and IL-12p70, fibrinolysis product (D-dimer) were measured in plasma from not infected individuals, patients infected with SARS-Cov-2 or cured from the disease.

S100A9 production was increased in hospitalized patients, when compared to not hospitalized patients (Fig. 1A). IL-6 and IL-10 production was increased in the hospitalized patients, when compared to not infected control or cured. (Fig. 1B and 1C)

TNF-α production was increased in hospitalized patients for COVID-19 when compared to controls and not hospitalized patients (Fig. 1D). Following the same dynamics, the production of IL-12p70 was also higher in hospitalized patients compared to the negative group for SARS-CoV-2 and not hospitalized patients (Fig. 1E). Interesting, there was no difference in TNF-α and IL-12p70 production between positive hospitalized patients and cured individuals (Figs. 1D and 1E).

Correlation between inflammatory mediators in COVID-19.

To analyze the behavior of S100A9 against other markers of clinical importance in COVID-19, Spearman correlations were performed. Was found a positive correlation between the parameters IL-6 x S100A9 (Fig. 2A); D-dimer X S100A9 (Fig. 2B); IL-10 x S100A9 (Fig. 2C); TNF-α x S100A9 (Fig. 2D); IL-12p70 x S100A9 (Fig. 2E);

Furthermore, the main cytokine involved in hyperinflammation in COVID-19, IL-6, was also correlated with IL-10 and D-dimer, where a positive correlation was also found between the data. (Fig. 2F and 2G).

IL-6 and S100A9 as predictors of severity in COVID-19.

To assess the predictive ability of S100A9 and IL-6 parameters as markers of severity in COVID-19, a Receiving Operating Curve (ROC) analysis was performed with SARS-CoV-2 positive (hospitalized and not hospitalized) patients. The graphical representation of the curve showed a sensitivity rate of 100% and specificity of 90% for IL-6, while the sensitivity and specificity rate of S100A9 was 75% and 76.4%, respectively. Both, IL-6 and S100A9 presented a suitable performance as a predictor of severity in COVID-19, with area under curve (AUC) and confidence interval (CI) of AUC = 0.97 (CI: 87.6–100%) and AUC = 0.72 (CI: 53.2–90.2%), respectively. (Fig. 3A and B).

Discussion

The present study demonstrated the association of plasmatic S100A9 with other severity markers described in the literature, such as the cytokine IL-6 and D-dimer, both considered markers of poor prognosis in COVID-19. In addition, other cytokines of clinical importance such as IL-10, TNF-α and IL-12p70 were also measured and compared with the production of S100A9, where a positive association was found in the production of such markers.

In our study, increased S100A9 levels was found in COVID-19 hospitalized patients compared to positive not hospitalized patients, which suggests that S100A9 can be directly related to unfavorable outcomes during SARS-COV-2 infection. In a recent work, increased S100A9 gene expression were found in SARS-CoV-2 infected lung epithelial cells suggesting a potential therapeutic role for S100A9 blocking [19]. These same blocking perspective, the Paquinimod, a specific inhibitor of S100A8/A9, can rescue the pneumonia with substantial reduction of viral loads in SARS-CoV-2-infected mice [20].

Several studies have shown remarkably high levels of serum calprotectin (S100A8/S100A9) in patients admitted with COVID-19 [15], [16], [21]. In addition, elevated serum calprotectin levels on day 1 or 2 of hospitalization are associated with the requirement for mechanical ventilation during admission, a finding that should be evaluated in expanded prospective cohorts [16].

Moreover, due to its function as an inflammatory marker, S100 family proteins may also have a direct role in the auto-amplifying thrombo-inflammatory storm in COVID-19, through the mobilization and activation of innate immune sensors such as TLR4 [22]. In the same way, hyperactivity of the coagulation system is a common finding in severe COVID-19 [23]. Many patients exhibit a prothrombotic profile, including high

levels of fibrin degradation products (D-dimer), high levels of fibrinogen and low levels of antithrombin [24]. In our study, S100A9 production was correlated with plasma D-dimer. This data suggests an increased risk for the development of thrombotic events due to synergistic action between such markers since S100A9 homodimer has also been described as playing an important role in thrombus formation in animal models [12].

As a major cytokine in the “cytokine storm” and hyperinflammation during SARS-CoV-2 infection, IL-6 presented increased levels in plasma from severe patients as described in other studies [5], [25], [26]. In addition, cytokines such as IL-10, TNF- α, IL-12p70, also were augmented in hospitalized patients compared to patients with not hospitalized patients. Interestingly, patients cured of COVID-19 remained with high levels of IL-12p70 and TNF-α. This could be related to late effects of cytokine storm associated with post-infection [27].

Increased blood levels of IL-10 is also correlated with disease severity in patients with COVID-19 [11], [28]. The cellular source and specific effects of increased IL-10 in patients with severe COVID-19 remain undefined [21]. However, it is interesting to note that IL-10 inhibits the expression of HLA class II molecules and a similar event is described in patients with severe COVID-19 [29]. It is also possible that increased IL-10 may suppress antiviral immune responses dampening resistance to bacterial infections in patients with COVID-19 [28].

For range of clinical manifestations, we found a positive association between the occurrence of symptoms, such dyspnea, with comorbidities, like diabetes, hypertension and age over 60 years, data that corroborate previously published studies [3]. A recent work described that, both chronic and intermittent hyperglycemia, which leads to increased production of inflammatory monocytes and neutrophils through enhanced S100A8 and S100A9 signaling on RAGE (receptor for advanced glycation end-products), in an NF-κB dependent pathway [30]. In our study, more than 32% of positive hospitalized patients had diabetes as a co-morbidity, one of the main risk factors in COVID-19.

The pathogenesis of COVID-19 involves the production of inflammatory mediators that will determine the response pattern and consequently the patient's clinical evolution. The infection is characterized by a broad inflammatory signature, with increased levels of soluble biomarkers indicative of immune cell activation, including monocytes/macrophages, neutrophils, T and B lymphocytes and epithelial cells, with an important role in homeostasis imbalance [31]. Providing new insights into the immunological effectors that may contribute to the immunopathogenesis of COVID-19 suggest that certain immune-based biomarkers, identified at the beginning of patient admission or during hospitalization, may indicate an increased risk of mortality in infected patients. The identification of these biomarkers is particularly important for the decision of early interventions, such as treatment with new drugs that are in the clinical study phase, such as molnupiravir [32].

In our study, we demonstrated the potential role of the S100A9 protein as a candidate of severity biomarker in the evolution of SARS-CoV-2 infection. In the context of a pandemic, where quick decision-making in the clinical environment is a crucial factor to minimize risks and reduce mortality, the

availability of tools capable of predicting unfavorable outcomes is increasingly necessary. Thus, S100A9 can be a molecule to be explored within this perspective.

Abbreviations And Acronyms

COVID-19 (coronavirus disease 2019); DAMP (damage associated molecular patterns);; IL-6 (interleukin-6); IL-10 (interleukin- 10), IL-12p70 (interleukin – 12p70); RAGE (receptor for advanced glycation end products); SARS-COV-2 (severe acute respiratory syndrome coronavirus 2); TLR-4 (toll like receptor -4); TNF- α (tumor necrosis factor α);

Declarations

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Figures

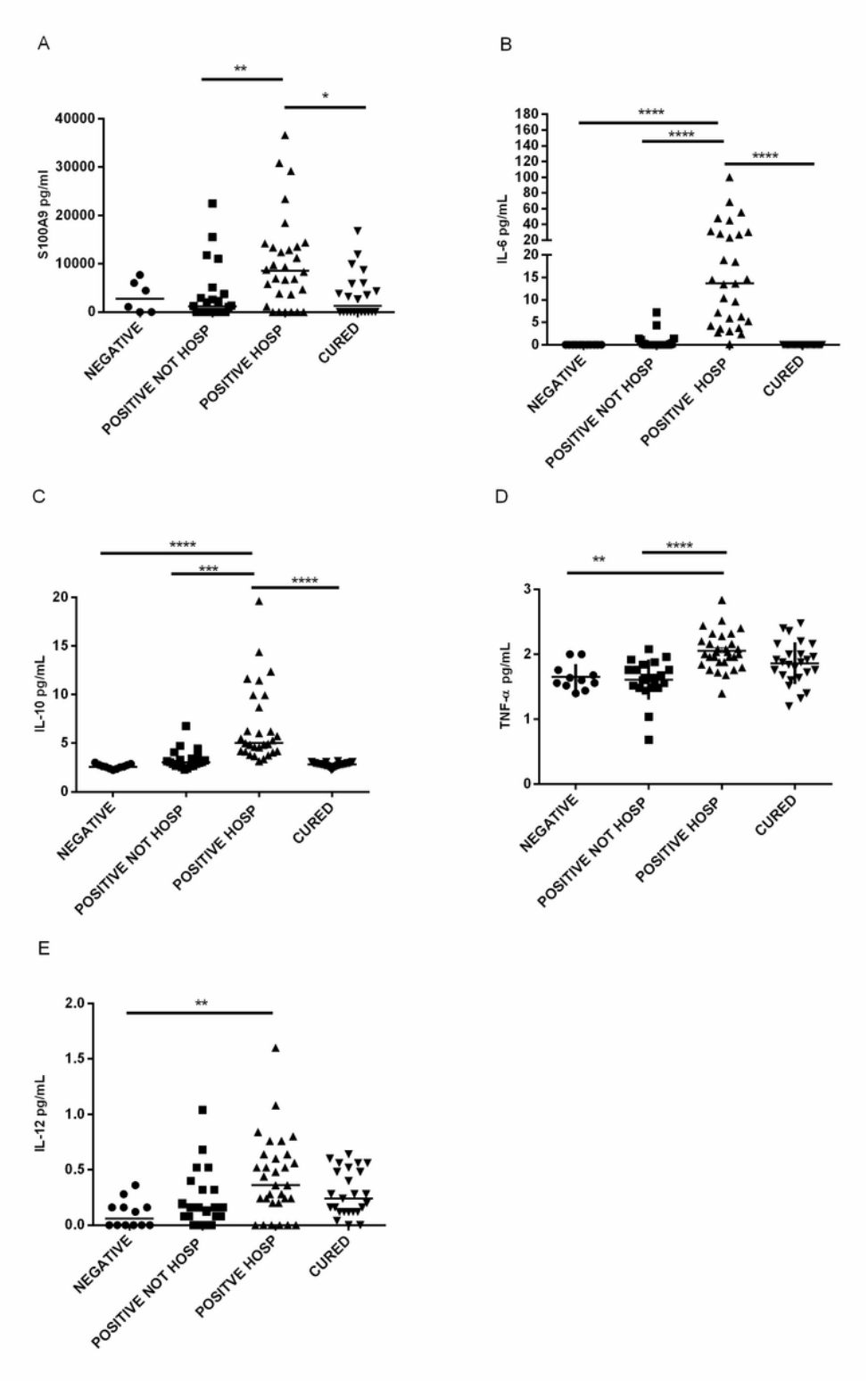

Figure 1

The production of inflammatory mediators and S100A9 is increased in positive hospitalized patients for COVID-19: Cytokine assay by CBA (cytometric beads array) and S100A9 by ELISA (Human S100A9 DuoSet ELISA) in plasma from individuals classified into groups: Negative for COVID-19, positive not hospitalized , positive hospitalized and cured of SARS-CoV-2 infection . Kruskal-Wallis statistical test. A:

S100A9 (*p=.002* **); B: IL-6 (*p<.0001* ****); C: IL-10 (*p<.0001* ****); D: TNF-α (*p< .0001* ****); E: IL-12p70 (*p=.003* **)

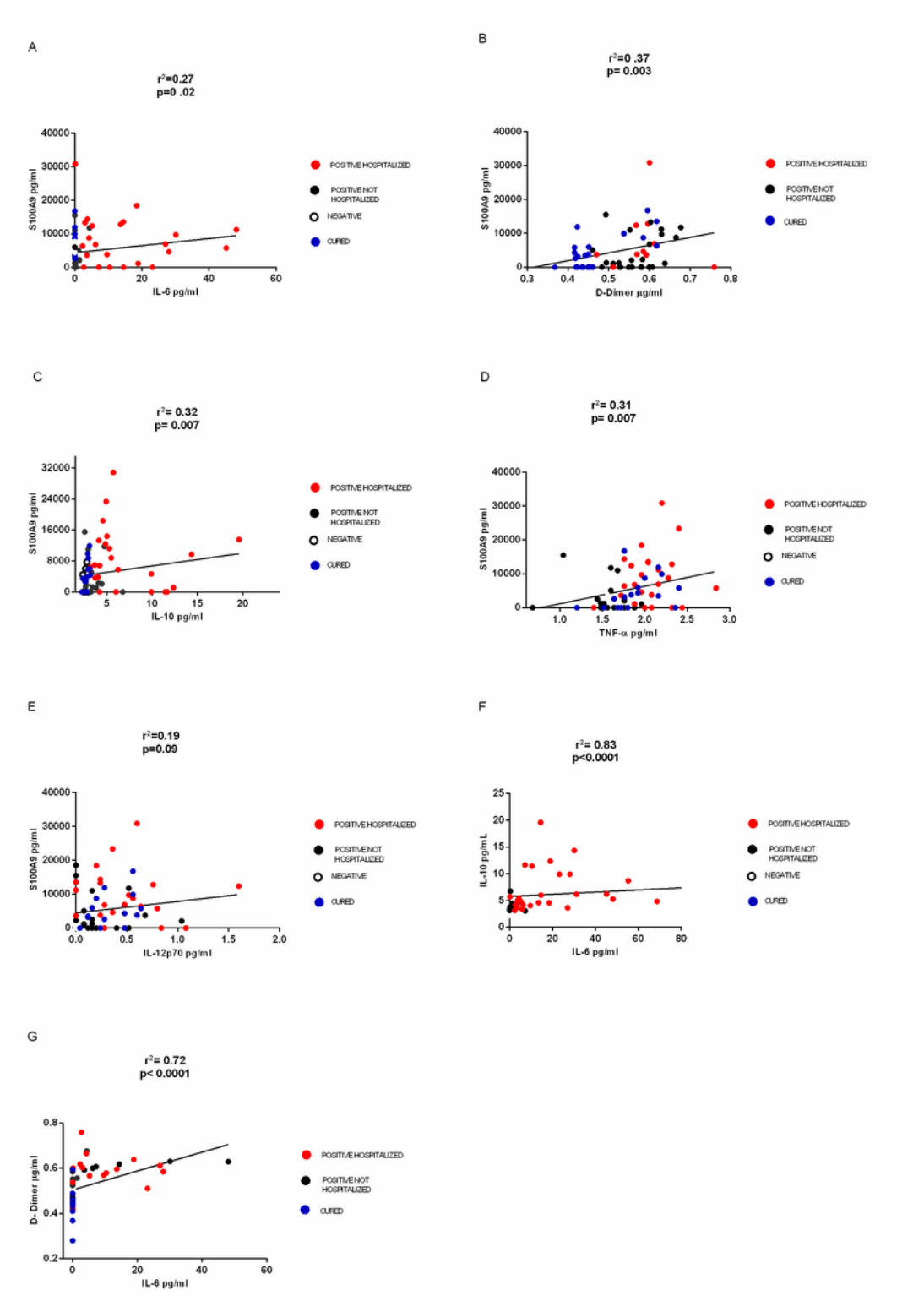

Figure 2

The production of S100A9 is positively correlated with severity markers associated with COVID-19: Spearman's correlation between mediators in COVID-19. A: Correlation between IL-6 and S100A9 (*r= .27; p=.02*;), B: Correlation between D-dimer and S100A9 (*r=.37; p< .0003*); C: Correlation between IL-10 and S100A9 (*r= .32; p= .007*), D: Correlation between TNF-α and S100A9 (*r=,31*; *p=.007*), E: Correlation between IL-12 and S100A9 (*r=.19; p=.09*), F: Correlation between IL-6 and IL-10 (*r= ,83*; *p=,0001*), G: Correlation between IL-6 and D- dimer (*r= ,72 ; p= ,0001*).

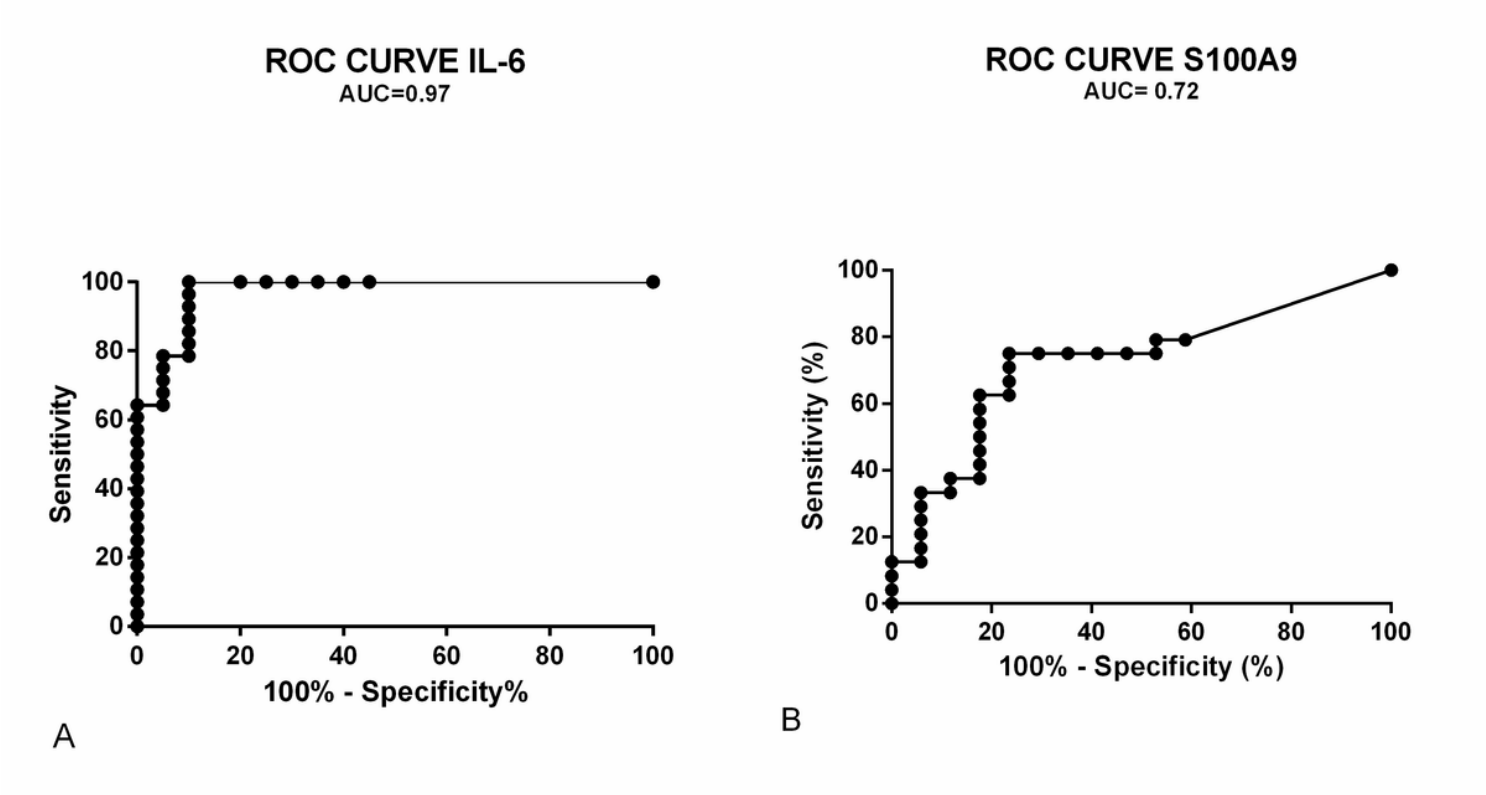

Figure 3

ROC curve analysis to assess sensitivity and specificity of the parameters IL-6 and S100A9 as predictors of severity in COVID-19. A: IL-6 (area under the curve = 0.97; cut off > 1,860 pg/ml; CI: 87.6-100%); B: S100A9 (area under the curve = 0.72; Cut off > 3190 pg/ml; CI: 53.2-90.2%).