Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Acute kidney injury associated to COVID-19 leads to a strong unbalance of circulant immune mediators

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ABSTRACT

Background: Severe cases of coronavirus disease 2019 (COVID-19) have increased risk for acute kidney injury (AKI). The exacerbation of the immune response seems to contribute to AKI development, but the immunopathological process is not completely understood.
Objectives: To analyze levels of circulant immune mediators in COVID-19 patients evolving with or without AKI. We have also investigated possible associations of these mediators with viral load and clinical outcomes.
Methods: This is a longitudinal study performed with hospitalized patients with moderate to severe COVID-19. Serum levels of 27 immune mediators were measured by a multiplex immunoassay. Data were analyzed at two timepoints during the follow-up: within the first 13 days of the disease onset (early sample) and from the 14th day to death or hospital discharge (follow-up sample).
Results: We studied 82 COVID-19 patients (59.5 ± 17.5 years, 54.9% male). Of these, 34 (41.5%) developed AKI. These patients presented higher SARS-CoV-2 viral load ($P = 0.03$), higher frequency of diabetes ($P = 0.01$) and death ($P = 0.0004$). Overall, AKI patients presented significantly higher and sustained levels ($P < 0.05$) of CCL-2, CCL-3, CCL-4, CXCL-8, CXCL-10, IFN-γ, IL-2, IL-6, TNF-α, IL-1Ra, IL-10 and VEGF. Importantly, higher levels of CCL-2, CXCL-10, IL-2, TNF-α, IL-10, FGFb, and VEGF were observed in AKI patients independently of death. ROC curves demonstrated that early alterations in CCL-2, CXCL-8, CXCL-10, IFN-γ, IL-6, IL-1Ra and IL-10 show a good predictive value regarding AKI development. Lastly, immune mediators were significantly associated with each other and with SARS-CoV-2 viral load in AKI patients.
Conclusions: COVID-19 associated AKI is accompanied by substantial alterations in circulant levels of immune mediators, which could significantly contribute to the establishment of kidney injury.

1. Introduction

Since December 2019, the new coronavirus (severe acute respiratory syndrome coronavirus 2 - SARS-CoV-2) has spread worldwide, causing the global outbreak of coronavirus disease 2019 (COVID-19) [1,2]. The clinical spectrum resulting from SARS-CoV-2 infection is broad, ranging from asymptomatic to critical cases [2]. In severe cases, systemic involvement and multi-organ dysfunction are highly prevalent, especially for older patients and those presenting pre-existing diseases [3].

In this context, acute kidney injury (AKI) has been associated with

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severe COVID-19, with incidence rates varying from 0.5 to 29%, particularly among patients admitted at intensive care units (ICU) [4-6]. Biopsy findings show that patients can develop different types of lesions. Glomerular injury can course with collapsing segmental and focal glomerulosclerosis, tubular damage is often diagnosed as acute tubular necrosis and vascular lesions (e.g., thrombotic microangiopathy) are also present [7,8]. Importantly, AKI associated with severe COVID-19 increases the risk for poor clinical outcomes, including the worsening of previous chronic kidney disease (CKD) and other comorbidities, in-hospital death, and post-COVID CKD development [9-11].

Although the pathophysiology of kidney injury in COVID-19 is diverse and multifactorial, comorbidities such as diabetes, hypertension, and coronary disease have been identified as risk factors for AKI development [4,5,10]. Nevertheless, several factors could be involved in the pathogenesis of kidney damage in SARS-CoV-2 infection, such as the hyper-activation of the angiotensin-converting enzyme 2 (ACE2) receptor, dysregulation of the renin-angiotensin-aldosterone system (RAAS), pro-coagulant stimuli, and the establishment of an exacerbated immune response, usually referred as "cytokine storm" [12,13].

Altogether, even though various mechanisms have been proposed to elucidate the pathophysiology of AKI secondary to COVID-19, the immunopathological process is not yet fully understood. Thus, we aimed to analyze circulant levels of mediators such as anti- and pro-inflammatory cytokines, chemokines, and growth factors during the hospitalization period of moderate-to-severe COVID-19 in patients evolving with and without AKI. Moreover, we investigated the potential of immune mediators as predictive biomarkers for disease worsening.

2. Methods

2.1. Study design and data collection

We conducted a longitudinal observational study, which was performed at the Hospital Universitário Antônio Pedro (HUAP - Niterói, Rio de Janeiro, Brazil) during the initial phase of the COVID-19 pandemic in Brazil (April to August 2020). This project was approved by the Research Ethics Committee of the Universidade Federal Fluminense (CAAE: 30623520.5.0000.5243). We included patients with a suspect diagnosis of SARS-CoV-2 infection admitted at HUAP (Fig. 1). Exclusion criteria was: negative RT-PCR result for SARS-CoV-2, CKD in advanced stages (4 or 5) or bladder cancer at the time of admission and unavailability of serum samples.

HUAP attends high complexity cases (e.g., cancer, autoimmune disease, heart surgeries, kidney transplants) in the Metropolitan Region II of Rio de Janeiro State. During the initial phase of COVID-19 pandemics, was reference treatment center for moderate to severe cases. These cases were usually characterized by the presence of persistent fever and cough, dyspnea, and hypoxia in association with pulmonary involvement.

Patient data (e.g., gender, skin color, age, presence of comorbidities, and routine laboratory tests) were obtained from the patient's charts. Also, from medical records we identified the main clinical outcomes, development of AKI, and/or death, in addition to other variables such as intensive care unit admission, mechanical ventilation, and therapeutic schemes.

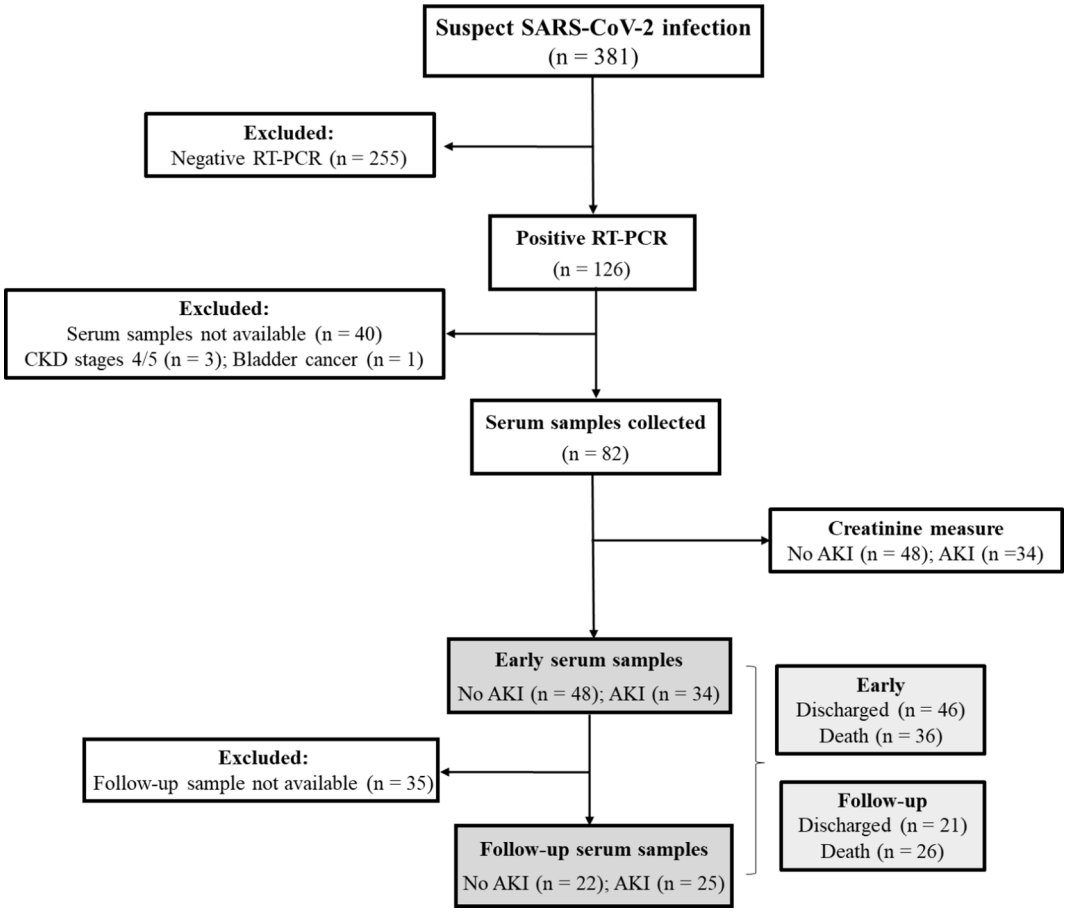

Fig. 1. Flowchart demonstrating the inclusion of patients with suspect SARS-CoV-2 infection during April to August 2020.

2.2. AKI definition

AKI cases were diagnosed using "The Kidney Disease: Improving Global Outcomes (KDIGO) 2012 Clinical Practice Guideline for Acute Kidney Injury", which defines AKI as an increase in serum creatinine (SCr) by 0.3 mg/dL within 48 h or increase in SCr to 1.5 times in comparison to baseline. AKI stages were categorized according to the fold-increase in SCr during the hospitalization, as follows: stage 1 = 1.5–1.9 times baseline; stage 2 = 2.0–2.9 times baseline; and stage 3 = >3 times baseline or increase in SCr to 4.0 mg/dL or the initiation of renal replacement therapy [14].

2.3. Diagnosis of SARS-CoV-2 infection

COVID-19 cases were confirmed within the first week from the onset of symptoms (~5 days) by RT-PCR, which was performed at the Multiuser Laboratory for Research Support in Nephrology and Medical Science (LAMAP/UFF). Briefly, nasopharyngeal or tracheal aspirate samples were collected by the medical team, and RNA extraction was performed using the QIAamp Viral RNA kit (Catalog no. 52906, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Subsequently, RT-PCR was performed in three separate reactions per specimen for each target (N1, N2, and the internal control RP) using the 2019-nCOV RUO Kit (catalog no. 10006770, Integrated DNA Technologies, Inc - IDT, Iowa, USA) and the GoTaq® Probe 1-Step RT-qPCR (Catalog no. A6121, Promega Corporation, Wisconsin, USA). Amplification was performed using the 7500 System (Applied Biosystems, ThermoFisher Scientific, California, USA), and cycle threshold (Ct) values for N1, N2, and RP genes were analyzed.

For viral load determination, a quantitative RT-PCR (RT-qPCR) was performed using the Bio GeneCOVID-19 PCR Kit (catalog no. G089-3, lot. 0001, Bioclin/Quibasa, Minas Gerais, Brazil) targeting the SARS-CoV-2 E, N, and RdRp gene regions, following the manufacturer's instructions. Data was reported as copies/mL, and the lower limit of detection was two copies/mL. Amplification was also performed using the 7500 System.

2.4. Blood sampling and laboratory tests

Peripheral blood samples were collected during the hospitalization period of COVID-19 patients. "Early samples" were collected during the first 13 days of the disease onset (8 ± 4 days) and "follow-up samples" were obtained from the 14th day of hospitalization to death or hospital discharge (15 ± 4 days). The decision to analyze the data at these timepoints was taken based on the mean length of hospitalization of our cohort, which was ~ 25 days (between 4 and 5 weeks).

Serum levels of immune mediators were assessed through a high-performance microbead 27-plex assay (Cat no. M500KCAF0Y, Bio-Rad, Hercules, CA, USA). We analyzed circulating levels of (i) chemokines [chemokine C-C ligand (CCL)-2/monocyte chemoattractant protein 1/MCP-1, CCL-3/macrophage inflammatory protein 1α, CCL-4/macrophage inflammatory protein 1β, CCL-5/RANTES, CCL-11/eotaxin, interferon C-X-C motif chemokine ligand (CXCL)-8 and CXCL-10/gamma-induced protein 10]; (ii) cytokines [tumor necrosis factor-α/TNF-α, interferon γ/IFN-γ, interleukin 1 receptor antagonist/IL-1Ra; and interleukins (IL)-1β, −2, −4, −5, −6, −7, −9, −10, −12p70, −13, −15, and −17]; and (iii) growth factors (granulocyte–macrophage colony-stimulation factor/GM-CSF; granulocyte colony-stimulating factor/G-CSF; basic fibroblast growth factor/FGFb; platelet-derived growth factor BB/PDGF-BB, and vascular endothelial growth factor/VEGF). Serum samples were filtered using a 0.22 μm syringe filter with a minimum volume of 50 μL of per sample. Results were expressed as pg/mL, and samples were tested on a Bio-Plex 200 instrument (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

2.5. Statistical analysis

GraphPad Prism (GraphPad Software 8.0, San Diego, CA, USA) was used for the statistical analyses. Data were expressed as mean ± standard deviation (SD) or n (%). Differences between two independent groups were assessed by *t*-test or Mann-Whitney test according to the variable's distribution. Paired analysis of longitudinal data was performed using Repeated Measures ANOVA or Friedman test with their respective post hoc tests. Fisher's exact test was used to analyze differences between proportions. The ability of circulant immune mediators to predict clinical outcomes was evaluated by the area under the curve (AUC) after performing the receiver operating characteristic curves (ROC) for these parameters. Spearman's coefficients were assessed to investigate correlations between variables. *P*-values were considered significant when < 0.05.

The J48 method was used for developing best-fit trees aiming to select the minimal set of phenotypic features that efficiently segregated groups. For this, we considered 0.25 for pruning confidence (-C parameter) and 2 to minimum number of instances (-M parameter). The Leave-one-out cross validation (LOOCV) was calculated to estimate the accuracy of the generated model. These analyses were performed using the Weka software (Waikato Environment for Knowledge Analysis, version 3.6.11, University of Waikato, New Zealand). The heat map analysis was carried out using the heatmap.2 function in the R (Project for Statistical Computing Version 3.0.1) and gplots package.

3. Results

3.1. Profile of COVID-19 patients

From April to August 2020, we attended 381 patients suspected of SARS-CoV-2 infection. Of these, 126 had positive RT-PCR results. From 86 patients with serum samples, four patients were excluded: three had CKD in stages 4 or 5, and one patient had bladder cancer. Thus, for the analysis of circulant immune mediators, we evaluated 82 patients. Fig. 1 illustrates the inclusion of patients from the original cohort. Overall, patients presented a mean (±SD) age of 59.5 ± 17.5 years, and 54.9% (n = 45) were male. Patients presented various comorbidities such as diabetes (36.6%), cardiovascular disease (64.6%), onco-hematological disease (37.8%) and obesity (23.2%). Clinical and demographic characteristics of patients are presented on Table 1.

Importantly, the demographic and clinical profile of COVID-19 patients was also evaluated according to the development of AKI. In our cohort, 34 (41.5%) patients developed AKI during COVID-19 progression. Of those, three developed AKI stage 1 (8.8%), six AKI stage 2 (17.6%), and 25 AKI stage 3 (73.5%) requiring renal replacement therapy. We identified that patients with AKI were more frequently diabetic ($P = 0.01$) and, as expected, they also presented a higher frequency of complications during hospitalization, such as intensive care admission, invasive mechanical ventilation support ($P < 0.0001$), and use of medications such as antibiotics and amines. When evaluating the parameters obtained in the RT-qPCR, we observed that SARS-CoV-2 viral load was significantly higher in patients who developed AKI ($P = 0.03$). Lastly, AKI was associated with increased mortality (70.6% vs. 25%, $P = 0.0004$).

3.2. COVID-19 associated AKI is accompanied by significant alterations in circulant immune biomarkers during disease progression

We evaluated serum levels of several immune mediators in both time points according to the development of AKI (Fig. 2). We identified that the early samples of AKI patients showed significantly higher levels of CCL-2 ($P = 0.002$), CCL-3 ($P = 0.02$), CXCL-8 ($P = 0.005$), CXCL-10 ($P = 0.0002$), IFN-γ ($P = 0.001$), IL-2 ($P = 0.03$), IL-6 ($P = 0.005$), TNF-α ($P = 0.003$), IL-1Ra ($P = 0.001$), IL-10 ($P = 0.001$), FGFb ($P = 0.03$). Except for CCL-2 and IL-2, these immune mediators were also significant higher

Table 1
General characteristics of COVID-19 hospitalized patients.

Characteristics	Total (n = 82)	No AKI (n = 48)	AKI (n = 34)	*P*-value
Age (years, mean ± SD)	59.5 ± 17.5	60.8 ± 18.5	59.5 ± 17.5	0.6
Male, n (%)	45 (54.9)	24 (50)	21 (61.8)	0.4
White (self-declared), n (%)	35 (42.7)	18 (35.3)	19 (51.3)	0.2
Comorbidities, n (%)				
Diabetes	30 (36.6)	11 (22.9)	19 (55.9)	0.01
CVD	53 (64.6)	29 (60.4)	24 (70.6)	0.4
Immunosuppression	19 (23.2)	11 (22.9)	8 (23.5)	0.9
Oncohematological disease	31 (37.8)	18 (37.5)	13 (38.2)	0.9
Obesity	19 (23.2)	10 (20.8)	9 (26.5)	1.0
Complications during hospitalization, n (%)				
Admission at the ICU	49 (59.7)	19 (39.6)	30 (88.2)	<0.0001
Mechanical ventilation	38 (46.3)	9 (18.7)	29 (85.3)	<0.0001
Renal replacement therapy, n (%)		–	26 (76.5)	–
Days on RRT (mean ± SD)		–	8.4 ± 6.1	–
AKI staging, n (%)				
1	–	–	3 (8.8)	–
2	–	–	6 (17.6)	–
3	–	–	25 (73.5)	–
Length of hospitalization (days), mean ± SD	25.4 ± 21.3	23.5 ± 21.5	27.6 ± 21.3	0.4
SARS-CoV-2 viral load (log_{10}copies/mL)	4.03 ± 1.4	2.74 ± 1.6	3.47 ± 1.6	0.03
Treatment during hospitalization, n (%)				
Oseltamivir	21 (25.6)	5 (10.4)	16 (47)	0.0003
Meropenem	21 (25.6)	5 (10.4)	16 (47)	0.0003
Azitromycin	25 (30.5)	6 (12.5)	19 (55.8)	<0.0001
Ceftriaxone	19 (23.2)	5 (10.4)	14 (41.2)	0.003
Polymyxin B	16 (19.5)	3 (6.2)	13 (38.2)	0.0005
Tazocin	15 (18.3)	1 (2.1)	14 (41.2)	<0.0001
Vancomycin	16 (19.5)	1 (2.1)	15 (44.1)	<0.0001
Hydroxycloroquine	6 (7.3)	–	6 (17.6)	0.004
Fluconazole	2 (2.4)	–	2 (5.9)	0.2
Amines	37 (45.1)	9 (18.7)	28 (82.3)	<0.0001
Death, n (%)	36 (43.9)	12 (25)	24 (70.6)	0.0004

Data is expressed as mean ± SD or n (%). *P*-values were calculated using Mann-Whitney test or qui-square test and were considered statistically significant when < 0.05. CKD = chronic kidney disease, Ct = cycle threshold, CVD = cardiovascular disease, ICU = intensive care unit, RRT = renal replacement therapy (dialysis).

in the follow-up samples of AKI patients when compared to "No AKI". Moreover, the analysis of heatmaps also show the significant unbalance between circulating immune mediators in AKI patients in both timepoints (Fig. 3**A**). Importantly, considering all mediators, the decision trees derived from the J48 method demonstrated that CCL-2 and TNF-α were the best mediators to discriminate AKI in the early timepoint and CCL-3 and IL-1Ra showed the best performance in the follow-up timepoint (Fig. 3**B**)

Subsequently, the paired analysis revealed that patients who developed AKI presented a significant increase of CCL-3 ($P = 0.03$), CCL-4 ($P = 0.03$), CXCL-8 ($P = 0.0004$), IL-2 ($P = 0.02$), TNF-α ($P = 0.008$) and IL-1Ra ($P = 0.008$) during COVID-19 progression; in addition to decreased CXCL-10 levels ($P = 0.03$). In the group that did not develop renal dysfunction, levels of CXCL-10 ($P = 0.006$) and IL-10 ($P = 0.03$) were significantly lower in the follow-up samples. These results are also demonstrated on Fig. 2.

Of note, other inflammatory parameters routinely assessed during hospitalization were also analyzed (Supplementary Table 1). We identified that C-reactive protein (CRP, $P = 0.009$) levels were significantly higher in AKI patients, but no differences were observed for ferritin ($P = 0.5$) and lactate dehydrogenase (LDH, $P = 0.3$). Differences were also not identified for hematological parameters, such as the absolute number of lymphocytes ($P = 0.9$).

3.3. Alterations in circulant immune mediators in COVID-19 associated AKI occur independently of death

Fig. 3**C** shows that COVID-19 patients who develop AKI and evolved to death present stronger alterations on serum levels of various immune mediators in comparison to AKI patients who survived. To discriminate AKI in association with death, decision trees highlights that CXCL-10 and the combination of CXCL-10 and IL-6 were the best mediators for early and follow-up timepoints, respectively (Fig. 3**D**).

Despite that, we decided to investigate whether the changes in inflammatory mediators would be associated with AKI regardless of death. For this, serum levels of immune mediators were compared among all patients who died according to AKI development. Overall, we observed that AKI patients who died still present significantly higher concentrations of CCL-2 ($P = 0.02$), CXCL-10 ($P = 0.04$), IL-2 ($P = 0.03$), TNF-α ($P = 0.02$), IL-10 ($P = 0.002$), VEGF ($P = 0.02$), and FGFb ($P = 0.02$) when compared to COVID-19 patients who died with no significant alterations on kidney function. Except for FGFb, these differences were significant at both time points. These results are illustrated on Fig. 4.

3.4. Circulant immune biomarkers could be used as predictors of AKI in COVID-19 patients

In order to recognize possible biomarkers that could be useful as predictors of AKI in hospitalized COVID-19 patients, ROC curves were plotted for the levels of immune mediators obtained in early samples, considering "AKI" and "AKI + death" as outcomes (Table 2). Overall, we observed that CCL-2, CXCL-8, CXCL-10, IFN-γ, IL-6, IL-10, and IL-1Ra showed a predictive potential for AKI ($P < 0.01$); and, except for IL-6 and CXCL-8, serum levels of these immune mediators presented AUC values > 0.7. When analyzing "AKI + death" as the final outcome, CCL-2 and CXCL-10 showed the highest AUC values (AUC 0.879, $P = 0.0006$ and AUC 0.841, $P = 0.002$, respectively).

3.5. Immune mediators are associated with each other and with SARS-CoV-2 viral in AKI patients

We developed matrix correlations for all circulating levels of immune mediators in COVID-19 patients according to the development of AKI (Fig. 5). Overall, we identified that, in AKI patients at early timepoint, chemokines were significantly correlated with each other and with other anti- and pro-inflammatory cytokines, especially CCL-2, CCL-3, CCL-4 and CXCL-8. Moreover, IL-6 and IFN-γ also show different correlations with various immune mediators. Among growth factors, we highlight the associations between G-CSF and FGFb with other mediators; and IL-1Ra showed more associations when we evaluated anti-inflammatory cytokines. Importantly, when compared to patients who did not developed AKI, the strength of these associations were reduced or absent. At the same time, AKI patients who died showed slightly increased coefficients for some associations. Of note, similar patterns of matrix correlations for the follow-up timepoint are demonstrated on **Supplementary** Fig. 1.

In COVID-19, as well as for other viral infections, it is suggested that

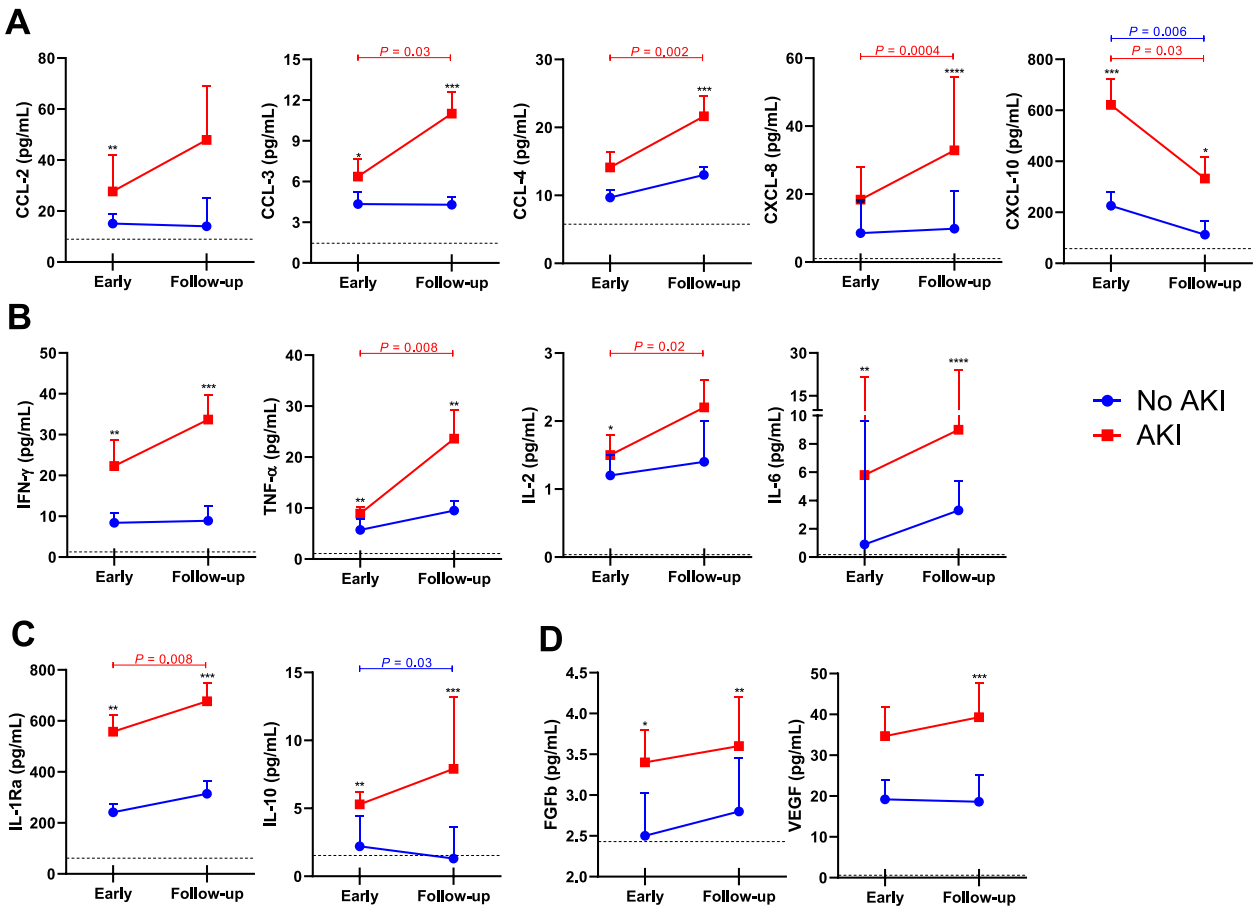

Fig. 2. Analysis of immune mediators of COVID-19 hospitalized patients evolving with and without AKI. Circulating levels of (A) chemokines, (B) pro-inflammatory cytokines, (c) anti-inflammatory cytokines and (D) growth factors were assessed by a multiplex assay. Data is shown as median ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate comparison between groups (No AKI vs. AKI) in each time point (*t* test/Mann-Whiney test). P-values on horizontal bars indicate the comparison of time points (early vs. follow-up samples) for each group - blue lines for "No AKI" and red lines for "AKI" - (paired *t* test/Wilcoxon).

higher viral load is associated with poor outcomes [16-18]. Thus, we investigated if SARS-CoV-2 viral load could be associated with serum levels of immune biomarkers in patients who developed AKI. We observed that SARS-CoV-2 viral load was significantly correlated with CCL-2, ($P = 0.01$) IFN-γ ($P = 0.001$), CXCL-8 ($P = 0.03$), IL-9 ($P = 0.01$) and IL-5 ($P = 0.01$). The best correlation coefficient was observed for IFN-γ (Spearman's r = 0.786). These results are shown on Table 3.

4. Discussion

SARS-CoV-2 is known not only to cause severe pulmonary impairment but also to affect multiple tissues and organs, such as the gastrointestinal tract, liver, and kidneys [19]. In this context, studies have been performed to investigate the prevalence, risk factors, and mechanisms of kidney dysfunction during COVID-19. Considering that an exacerbated immune response is regarded as a determinant factor for disease severity [20,21], we aimed to assess serum levels of several immune mediators in hospitalized COVID-19 patients with moderate to severe disease evolving with and without AKI.

Overall, our results show that COVID-19 patients who develop AKI presented substantial alterations in serum levels of inflammatory mediators. Thus, higher concentrations of chemokines (CCL-2, CCL-3, CCL-4, CXCL-8 and CXCL-10), growth factors (FGFb and VEGF), and cytokines either pro-inflammatory (IFN-γ, IL-2, IL-6 and TNF-α) or anti-inflammatory (IL-1Ra and IL-10) were observed in the first weeks after the onset of symptoms, which significantly increased throughout the hospitalization.

Several studies have demonstrated that SARS-CoV-2 infection induces hypercytokinemia, which leads to a state of hyperinflammation in association with uncontrolled and sustained activation of T cells and macrophages, especially in patients with the severe forms of the disease [15,20,22,23]. Thus, our results suggest that these dramatic changes in inflammatory mediators indicate that an unbalanced immune response can also be involved in the establishment of kidney injury associated with COVID-19. Moreover, one may suggest that alterations in anti-inflammatory mediators accompanies the excess of pro-inflammatory cytokines and chemokines in a probable attempt to suppress the inflammatory process. In this context, other studies reported that IL-10 is a cytokine that "should be watched" in COVID-19 [24-26]. Furthermore, Henry and colleagues (2020) demonstrated that IL-10 is a strong predictor of disease severity and AKI [26].

Anderberg and colleagues (2021) recently assessed the profile of circulant immune mediators in critically ill COVID-19 patients in a cross-sectional study. They observed that high serum levels of IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-7, CXCL-8, IL-10, IL-13, IL-17a, G-CSF, IFN-γ, CXCL-10, CCL-2, and TNF-α were associated with AKI; however, they did not find correlations with CCL-3 and CCL-4 [27]. In fact, the dysregulation of the immune response significantly contributes to kidney disease. Besides the autoimmunity pathways, the loss of immunological homeostasis results in complement activation and glomerular injury with sustained activation of B and T cells leading to tissue damage and immune complex deposition on the glomerular basal membrane and blood vessels [28]. During AKI, damaged kidney cells induce the activation of resident macrophages and dendritic cells, which leads to the secretion of cytokines and chemokines, resulting in tissue inflammation [29].

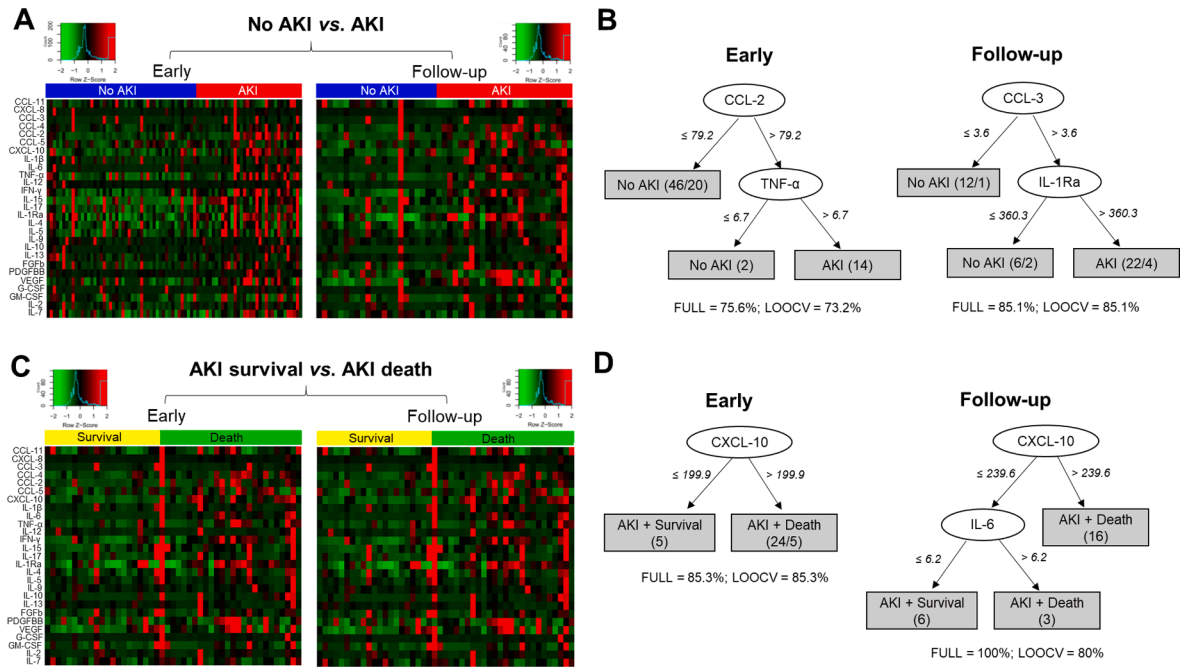

Fig. 3. Exploring the profile of immune mediators in COVID-19 patients according to AKI development. Heatmaps show the landscape of immune mediators between "No AKI" and "AKI" (A) and "AKI + survival" and "AKI + death" (C). Best-fit decision trees identified the mediators that efficiently segregated "No AKI" from "AKI" (B) and "AKI + survival" from "AKI + death" (D). Levels were placed in the root of the tree according to the cytokine/chemokine value (pg/mL) that best divided groups. The total of classified registers (correct and incorrect) for each class are given in parentheses for each terminal node with the Full training (FULL) and Leave-one-out cross-validation (LOOCV) accuracies. If incorrectly classified registers exist, they will appear after slash "/".

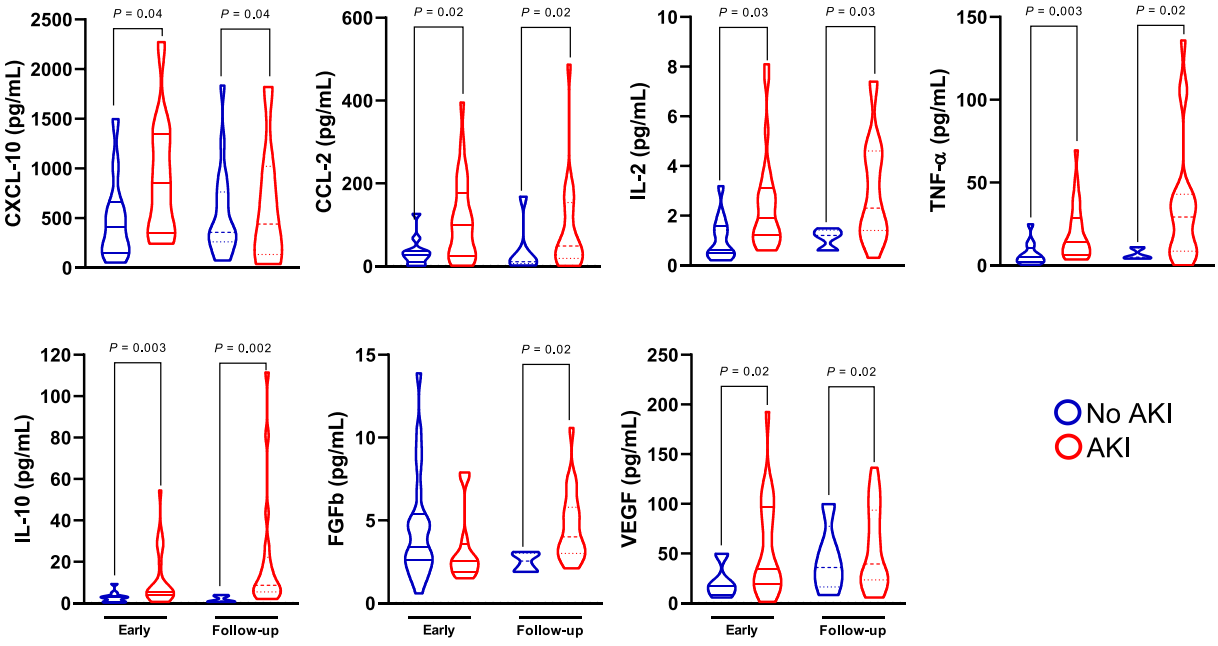

Fig. 4. Analysis of immune mediators of COVID-19 hospitalized patients who died according to the development of AKI. Data is shown as violin plots. *P*-values indicates comparison between groups (No AKI vs. AKI; *t* test/Mann-Whitney).

In this study, we also observed that, when compared to COVID-19 patients who died with normal kidney function, AKI patients still showed higher levels CCL-2, CXCL-10, IL-2, TNF-α, IL-10, FGFb, and VEGF. As the frequency of death by COVID-19 was high in the group that developed AKI (70.6%), our objective was to evaluate whether changes in inflammatory mediators would be associated with AKI, regardless of death as the final event. Since the cytokine storm has been closely associated with severe COVID-19 and death [20], these results strengthen the hypothesis that a hyperinflammatory state also contributes to renal dysfunction in COVID-19. This could be a reflection of direct kidney injury promoted by SARS-CoV-2 infiltration [30,31], with activation of the innate immune response [29] or reduction of plasma clearance of cytokines due to kidney function impairment [32].

We have also investigated the potential of circulating immune biomarkers in predicting AKI during COVID-19 progression. ROC curves demonstrated that alterations in CCL-2, CXCL-8, CXCL-10, IFN-γ, IL-6,

Table 2
Parameters from ROC curves of circulant immune mediators as predictors of AKI or AKI + death in COVID-19 hospitalized patients at "Early" timepoint.

Parameters	AUC	95% CI	Cut-off	Sens (%)	Spec (%)	*P*-value
A) No AKI *vs.* AKI						
CXCL-10	0.739	0.627–0.853	>314.2	72.7	63.8	0.0003
IFN-γ	0.722	0.602–0.842	>14.1	70.9	63.4	0.001
IL-10	0.717	0.599–0.835	>3.15	71.9	65.9	0.001
IL-1Ra	0.713	0.594–0.831	>349	69.7	63.8	0.001
CCL-2	0.703	0.584–0.822	>22.0	63.6	61.7	0.002
CXCL-8	0.682	0.563–0.802	>17.9	54.5	80.5	0.006
IL-6	0.681	0.560–0.802	>1.75	63.6	65.9	0.006
B) AKI + survival *vs.* AKI + death						
CCL-2	0.879	0.764–0.994	>21.5	83.3	80.0	0.0006
CXCL-10	0.841	0.694–0.988	>341.6	79.2	70.0	0.002
IL-6	0.752	0.591–0.913	>2.7	75.0	70.0	0.02
CXCL-8	0.725	0.532–0.917	>15.4	66.7	70.0	0.04

AUC = area under the curve; CI = confidence interval; sens = sensitivity; spec = specificity.

IL-1Ra, and IL-10 during the early acute phase of the disease might have diagnostic value. Importantly, we observed that when using "AKI + death" as outcome, the predictive power was increased, especially CCL-2 and CXCL-10, which presented AUC values > 0.8. Previous studies have reported that cytokines can be used as predictive biomarkers for COVID-19 severity, especially IL-6 and IL-10, as demonstrated by a recent *meta*-analysis [24]. Interestingly, a combined analysis of different cytokines can help to predict COVID-19 prognosis [25]; however, a multivariate analysis was not performed in the present study. Considering the mediators which showed the best values, our results were, once again, similar to Anderberg and colleagues (2021), which reported that IL-1Ra, IL-6, CXCL-8, IL-17a, CXCL-10, and CCL-2 presented the strongest associations with AKI (Spearman coefficients > 0.7) [27].

In order to explore the hypothesis that high viral load is directly associated with exacerbation of the immune system, leading to increased disease severity and AKI, we sought to determine the associations between circulant levels of immune mediators, and SARS-CoV-2 viral load. Studies show that increased viral load is associated with COVID-19 severity [16-18]. Moreover, a recent study demonstrated that SARS-CoV-2 urinary viral load was significantly associated with AKI development [33]. Interestingly, Bermejo-Martin and colleagues (2020) reported that SARS-CoV-2 viral load is correlated with several inflammatory mediators, in addition to coagulation factors and markers of endothelial dysfunction in critically ill patients; however, the frequency of AKI was not reported [34]. Here, we observed that serum levels of CCL-2, CXCL-8, IFN-γ, IL-9 and IL-5 are associated with higher SARS-CoV-2 viral load only in COVID-19 patients who developed AKI. The strongest correlation coefficient was observed for IFN-γ, which is a potent antiviral cytokine and a potential candidate for therapeutic strategies [35].

Besides the cytokine storm, other immunological events are pivotal for the establishment of the host's response against the virus [22]. A significant increase in acute phase proteins is observed by different studies [36,37]. In our study, patients who developed AKI also showed a significant increase in CRP levels, but differences were not observed for serum ferritin and LDH. Moreover, SARS-CoV-2-induced lymphocyte apoptosis also have a impact on COVID-19 immunopathogenesis [38,39]. However, lymphopenia was not associated with renal dysfunction in the present study. Finally, an impaired type I IFN-response [40,41], NLR3P inflammasome hyper-activation [42], decreased cytotoxic capacity of $CD8^+$ lymphocytes, and natural killer cells [43-45] has also been associated with COVID-19 poor outcomes. In this regard, it would be interesting to investigate these parameters in COVID-19 patients who develop AKI.

Other mechanisms have been suggested as determinants for the establishment of kidney injury in COVID-19. Some factors such as hypoxia, sepsis, and septic shock (which implicates in hemodynamic alterations), in addition to baseline diseases such as hypertension and diabetes, were demonstrated to be closely associated with AKI development in COVID-19 patients [4,5,10]. In our study, diabetes was also associated with an increased risk for AKI. Moreover, as previously mentioned, kidney cells are rich in ACE2 receptors and the auxiliary protein trans membrane serine proteases, especially podocytes and proximal tubule cells, which are essential for viral cell entry [46]. Thus, SARS-CoV-2 is capable of infecting these cells and promoting a direct cytopathic effect [30,31]. Interestingly, the recent study from Lite and colleagues (2021) showed that, by an interactome analysis, that ACE2 could mediate immunological interactions in different tissues [47].

Our study has some limitations. Besides the small number of patients, the analysis of serum levels of immune mediators could not be performed at daily or weekly basis to better understand the kinetics of these biomarkers. Studies have shown that early urinary alterations in dipstick tests can be detected in COVID-19 patients [48]; however, the majority of patients did not have results from analysis of urinary sediments in their charts. Importantly, the intrinsic conditions associated with severe COVID-19, such as the hemodynamic changes associated with the development

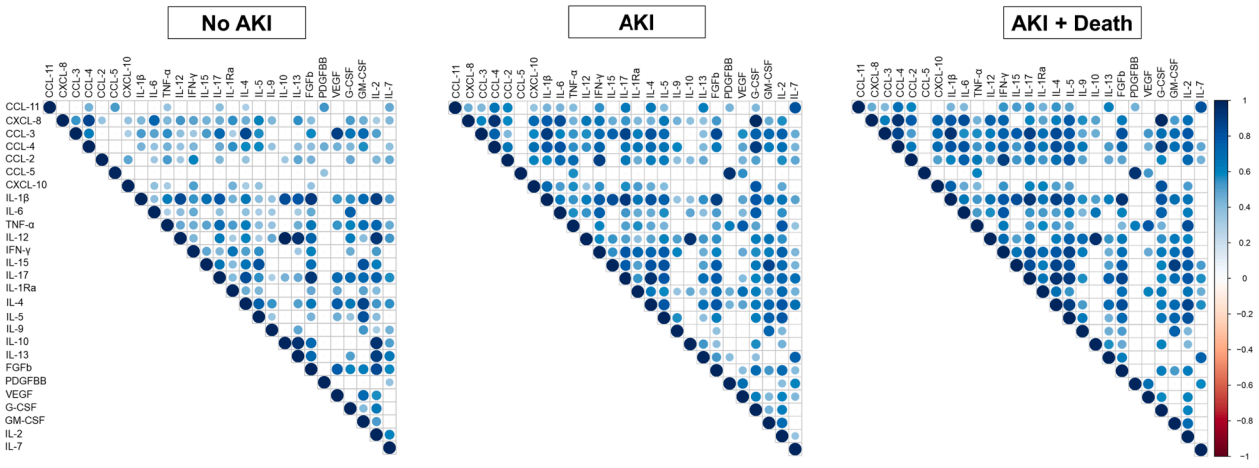

Fig. 5. Correlation matrix for comparing associations between immune mediators at early timepoint in COVID-19 patients according to AKI development. Spearman's coefficients are represented in different colors, as demonstrated by the vertical bar on the right.

Table 3
Correlation between immune mediators and SARS-CoV-2 viral load in COVID-19 patients with AKI.

Parameters	SARS-CoV-2 Viral load
Chemokines	
CCL-2	**0.637***
CXCL-8	**0.588***
CXCL-10	0.379
CCL-11	0.341
CCL-5	−0.324
CCL-3	0.269
CCL-4	0.011
Pro-inflammatory cytokines	
IFN-γ	**0.786****
IL-9	**0.663***
IL-6	0.495
TNF-α	0.352
IL-7	0.297
IL-12p70	0.278
IL-15	0.201
IL-2	0.198
IL-1β	0.135
IL-17	0.110
Anti-inflammatory cytokines	
IL-5	**0.673***
IL-10	0.341
IL-4	0.326
IL-1Ra	0.313
IL-13	−0.204
Growth factors	
G-CSF	0.544
GM-CSF	0.428
FGFb	0.171
PDGFbb	0.126
VEGF	0.118

Data is shown as Spearman's coefficients. Significant correlations are shown in bold (*$P < 0.05$, **$P < 0.01$).

administration of several medications (e.g., vasoactive amines and antibiotics), can also affect renal function [8,13]. In this study, as well as in other "real-life" reports focusing on the analysis of kidney injury in COVID-19, it was not possible to investigate AKI dissociated of secondary factors related to COVID-19 disease severity itself. Intensive care scores such as the Sequential Organ Failure Assessment (SOFA) could not be assessed. Finally, we could not compare levels of inflammatory mediators according to the degree of AKI since the majority of our patients were classified as KDIGO stage 3. Further studies should also consider to evaluate the profile of circulating immune mediators in AKI associated with COVID-19 in comparison to other ethiologies of kidney injury.

5. Conclusion

Altogether, our findings suggest that AKI associated with moderate-to-severe SARS-CoV-2 infection is accompanied by dramatic alterations in circulant levels of cytokines, chemokines, and growth factors. Moreover, this unbalance was associated with AKI independently of death and is significantly correlated with high viral load. This suggests that the loss of immunological homeostasis significantly contributes to the establishment of kidney injury in COVID-19. Lastly, early changes in these biomarkers could be considered as potential predictors of AKI and death in COVID-19 patients.

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CRediT authorship contribution statement

Thalia Medeiros: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Gabriel Macedo Costa Guimarães:** Data curation, Investigation, Writing – review & editing. **Fabiana Rabe Carvalho:** Data curation, Investigation, Writing – review & editing. **Lilian Santos Alves:** Data curation, Investigation, Writing – review & editing. **Ana Carolina Campi-Azevedo:** Data curation, Investigation, Writing – review & editing. **Andréa Teixeira-Carvalho:** Data curation, Investigation, Writing – review & editing. **Laurence Rodrigues do Amaral:** Formal analysis, Software, Writing – review & editing. **Olindo Assis Martins-Filho:** Conceptualization, Funding acquisition, Formal analysis, Writing – review & editing. **Jocemir Ronaldo Lugon:** Conceptualization, Formal analysis, Writing - review & editing. **Jorge Reis Almeida:** Conceptualization, Funding acquisition, Formal analysis, Writing – review & editing. **Andrea Alice Silva:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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