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First and Second Waves of Coronavirus Disease 2019 in Madrid, Spain: Clinical Characteristics and Hematological Risk Factors Associated With Critical/Fatal Illness

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OBJECTIVES: This study aims to determine similarities and differences in clinical characteristics between the patients from two waves of severe acute respiratory syndrome coronavirus-2 infection at the time of hospital admission, as well as to identify risk biomarkers of coronavirus disease 2019 severity.

DESIGN: Retrospective observational study.

SETTING: A single tertiary-care center in Madrid.

PATIENTS: Coronavirus disease 2019 adult patients admitted to hospital from March 4, 2020, to March 25, 2020 (first infection wave), and during July 18, 2020, and August 20, 2020 (second infection wave).

INTERVENTIONS: Treatment with a hospital-approved drug cocktail during hospitalization.

MEASUREMENTS AND MAIN RESULTS: Demographic, clinical, and laboratory data were compared between the patients with moderate and critical/fatal illness across both infection waves. The median age of patients with critical/fatal coronavirus disease 2019 was 67.5 years (interquartile range, 56.75–78.25 yr; 64.5% male) in the first wave and 59.0 years (interquartile range, 48.25–80.50 yr; 70.8% male) in the second wave. Hypertension and dyslipidemia were major comorbidities in both waves. Body mass index over 25 and presence of bilateral pneumonia were common findings. Univariate logistic regression analyses revealed an association of a number of blood parameters with the subsequent illness progression and severity in both waves. However, some remarkable differences were detected between both waves that prevented an accurate extrapolation of prediction models from the first wave into the second wave. Interleukin-6 and D-dimer concentrations at the time of hospital admission were remarkably higher in patients who developed a critical/fatal condition only during the first wave ($p < 0.001$), although both parameters significantly increased with disease worsening in follow-up studies from both waves. Multivariate analyses from wave 1 rendered a predictive signature for critical/fatal illness upon hospital admission that comprised six blood biomarkers: neutrophil-to-lymphocyte ratio (≥ 5; odds ratio, 2.684 [95% CI, 1.143–6.308]), C-reactive protein (≥ 15.2 mg/dL; odds ratio, 2.412 [95% CI, 1.006–5.786]), lactate dehydrogenase (≥ 411.96 U/L; odds ratio, 2.875 [95% CI, 1.229–6.726]), interleukin-6 (≥ 78.8 pg/mL; odds ratio, 5.737 [95% CI, 2.432–13.535]), urea (≥ 40 mg/dL; odds ratio, 1.701 [95% CI, 0.737–3.928]), and D-dimer (≥ 713 ng/mL; odds ratio, 1.903

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[95% CI, 0.832–4.356]). The predictive accuracy of the signature was 84% and the area under the receiver operating characteristic curve was 0.886. When the signature was validated with data from wave 2, the accuracy was 81% and the area under the receiver operating characteristic curve value was 0.874, albeit most biomarkers lost their independent significance. Follow-up studies reassured the importance of monitoring the biomarkers included in the signature, since dramatic increases in the levels of such biomarkers occurred in critical/fatal patients over disease progression.

CONCLUSIONS: Most parameters analyzed behaved similarly in the two waves of coronavirus disease 2019. However, univariate logistic regression conducted in both waves revealed differences in some parameters associated with poor prognosis in wave 1 that were not found in wave 2, which may reflect a different disease stage of patients on arrival to hospital. The six-biomarker predictive signature reported here constitutes a helpful tool to classify patient's prognosis on arrival to hospital.

KEY WORDS: blood biomarkers; coronavirus disease 2019; critical/fatal illness; first and second wave of infection; prognosis; risk factors; severe acute respiratory syndrome coronavirus 2

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global health threat, with more than 83.3 million people infected worldwide, leaving over 1.8 million deaths as of January 3, 2021 (1). Although the coronavirus outbreak first emerged in Wuhan (Hubei province, China) in December 2019 (2), it spread quickly throughout the world, endangering the health and well-being of all people, but especially vulnerable populations. Spain ranks among the top nine countries with the highest COVID-19 prevalence and death toll across the globe, with nearly 1.9 million reported cases and over 50,000 confirmed deaths, as of January 3, 2021 (1). After a first wave of infection in March–April 2020, Spain experienced one of the most draconian lockdowns in the world to control the spread of the virus. However, with the ease of restriction measures, a second wave of SARS-CoV-2 infection surged across the country since mid-July, and Madrid was hit hard again (3).

The clinical spectrum of SARS-CoV-2 infection is broad, ranging from asymptomatic infection or mild symptoms of mainly an upper respiratory tract infection in over 80% of the cases to a more severe condition and death, usually in older adults and people with certain preexisting medical conditions (4). Some common clinical manifestations in patients suffering critical COVID-19 disease include pneumonia, respiratory failure, acute respiratory distress syndrome (ARDS), venous thrombosis, lung thromboembolism, lung fibrosis, septic shock, systemic inflammatory response, kidney damage, cardiovascular damage, blood-vessel damage, and multiple organ failure (5–7). Early detection of patients likely to develop critical illness is an urgent need to provide proper care and optimize the use of hospital resources. The aim of this study was to analyze the clinical characteristics of patients admitted to hospital during the first and second waves of SARS-CoV-2 infection and to identify a blood biomarker COVID-19 prognostic signature of critical/fatal illness. This signature could help clinicians to triage patients with a foreseeable poor prognosis at admission to hospital.

MATERIALS AND METHODS

Study Design and Data Collection

This retrospective observational study analyzed data from a total of 193 patients with COVID-19 admitted to the Fundación Jiménez Díaz University Hospital (Madrid, Spain) from March 4, 2020, to March 25, 2020 (first wave), and 83 COVID-19 patients from July 18, 2020, to August 20, 2020 (second wave), with available data on outcome (i.e., discharge or mortality). All patients were confirmed to have SARS-CoV-2 infection by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Inclusion criteria included: admission to hospital, positivity for SARS-CoV-2 infection as assessed by RT-PCR, clinical symptoms and radiologic evidence suggestive of the disease, aged greater than or equal to 18 years, not pregnant, or breastfeeding. Patients with missing hematological data, lack of complete electronic clinical data, or incomplete follow-up resolution at the cutoff date were excluded (**Supplementary Fig. S1**, http://links.lww.com/CCX/A508). This study was performed in accordance with the Fundación Jiménez Díaz University Hospital Ethics Committee and to the tenets of the Declaration of Helsinki. Clinical, laboratory, and outcome data were

obtained from electronic medical records. This study was approved by the Research Ethics Committee of the Jiménez Díaz Foundation (EO085-20-IIS-FJD). Blood laboratory tests were done at hospital admission, and then every 4–6 days in inpatients with stable vital signs or daily in critical patients. Thirty-three parameters were examined from blood samples at the Fundación Jiménez Díaz University Hospital central laboratory. Neutrophil-to-lymphocyte ratio (NLR) was determined by dividing neutrophil count by lymphocyte count. Follow-up studies were conducted with data from both waves over 45 days from hospital admission, and herein represented by three time frames: at hospital admission, after 4–6 days of hospitalization, and the day before resolution (discharge or mortality). The degree of disease severity was categorized as moderate (patients who did not require ICU admission), critical (ICU admission), and fatal (nonsurvivor) conditions. ICU admission criteria included ARDS (chest imaging by chest radiograph or CT scan, with bilateral opacities not fully explained by effusions, lobar/lung collapse or nodules, respiratory failure not fully explained by cardiac failure or fluid overload, Pao_2/Fio_2 < 150 mm Hg with positive end-expiratory pressure ≥ 5 cm H_2O, within 1 wk of a known clinical insult or new/worsening respiratory symptoms), or septic shock (quick Sequential Organ Failure Assessment score ≥ 2 points, vasopressor therapy needed to elevate mean arterial pressure ≥ 65 mm Hg, and lactate > 2 mmol/L [18 mg/dL] despite adequate fluid resuscitation).

Patient Treatment

The standard treatment for SARS-CoV-2 pneumonia, approved by the Pharmacy Commission of the Fundación Jiménez Díaz University Hospital included, in the first wave, a drug cocktail comprising different combinations and doses of: lopinavir/ritonavir (200/50 mg, two tablets/12 hr, 7 d), hydroxychloroquine (first day 400 mg/12 hr, followed by 200 mg/12 hr, 4 d), or chloroquine (500 mg/12 hr, 5 d), doxycycline (100 mg/12 hr, 5 d) or azithromycin (500 mg/24 hr, 5 d), levofloxacin (500 mg/24 hr, 5 d), low-molecular-weight heparin (bemiparin [therapeutic dose: 115 international units (IU)/kg/24 hr, intermediate dose: 80 IU/kg/24 hr, prophylactic dose: 2,500–3,500 IU/24 hr] or enoxaparin [therapeutic dose: 1.5 mg/kg/24 hr– 1 mg/kg/12 hr, intermediate dose: 1 mg/kg/24 hr, prophylactic dose: 20–40 mg/24 hr]), cyclosporine (starting at 100 mg/d [< 60 kg weight], 150 mg/d [60–80 kg weight], and 200 mg/d [> 80 kg weight], and then considered for scaling doses to 150, 200, and 300 mg/d, respectively, after 48 hr, with subsequent individualized scaling thereafter), and corticosteroids (methylprednisolone [125–250 mg daily pulses, 1–3 d, as induction therapy, followed by 40–80 mg/d]). Tocilizumab (400 mg, single dose) was recommended in severe interstitial pneumonia, rapid progression requiring ventilatory support, extrapulmonary organ failure, and mostly in the case of a severe systemic inflammatory status (as a reference, a threshold of 40 pg/mL for serum interleukin [IL]-6 levels and of 400 ng/mL for D-dimer were suggested). A second dose was considered if partial response in individualized cases.

In the second wave, the drug cocktail included corticosteroids (dexamethasone [40–60 mg daily pulses, 1–3 d, followed by 8–16 mg/d] or methylprednisolone as above), antibiotics, low-molecular-weight heparin (bemiparin or enoxaparin as above), remdesivir (200-mg IV followed by 100-mg IV, 10 d), and tocilizumab (< 75 kg: 400 mg; ≥ 75 kg: 600 mg, single dose) in patients with Fio_2 ≤ 0.6 and at least two of the following parameters: ferritin greater than 1,000 ng/mL or in progression, D-dimer greater than 1,000 ng/mL or in progression, C-reactive protein greater than 10 ng/mL or in progression, or IL-6 greater than 40 pg/mL upon admission.

Statistical Analysis and Development of the Prognostic Signature

Continuous variables were expressed as median and interquartile range (IQR) values, and compared using independent group *t* test for normally distributed data, or Mann-Whitney *U* and Kruskal-Wallis tests for non-normally distributed data. Categorical variables were described as frequency rates and percentages, and compared using the chi-square test or Fisher exact test as appropriate. Cutoff values for each biomarker were established using the Youden index (8, 9) on a receiver operating characteristic (ROC) curve with statistically significant area under the curve. The same value was used for both waves unless otherwise specified. For the given cutoff values, continuous variables were dichotomized to conduct logistic regression analyses. Associations between the biochemical parameters and critical/fatal COVID-19 condition were first assessed with univariate binary

logistic regression analyses. Different combinations of statistically significant biomarkers on univariate analyses were used to fit a multivariate binary logistic regression model and screened for the most powerful determiners to identify a signature capable of reliably predicting disease prognosis in wave 1. Our dataset was randomly split into two subsets to train (77% of data) and test (23% of data) the model. Such model was also tested in wave 2. The predictive performance of the signature was evaluated and optimized to obtain the highest accuracy, precision, recall, and F1-score. The model was validated with the area under the ROC curve (AUROC). Odds ratio (OR), 95% CI, and *p* values were calculated for all biomarkers included in logistic regression models. Statistical analyses and regression models were conducted using IBM SPSS Statistics Version 27 (IBM Corporation, Armonk, NY) and Python 3.6 (Python Software Foundation, Fredericksburg, VA) with scikit-learn library. A two-sided *p* value of less than 0.05 was considered statistically significant.

RESULTS

Demographic Characteristics and Comorbidities in COVID-19 Inpatients

Data from 276 patients admitted to hospital between March 4, 2020, and March 25, 2020 (first wave), and between July 18, 2020, and August 20, 2020 (second wave), with confirmed COVID-19 and meeting the inclusion criteria, were analyzed in our study. Demographic and comorbidity features of the first and second COVID-19 waves are shown in **Table 1**. The median age of critical/fatal patients was 67.5 (IQR, 56.75–78.25) and 59.0 years (IQR, 48.25–80.50 yr) for the first and second COVID-19 waves, respectively, whereof 64.5% (first wave) and 70.8% (second wave) were men. Disease severity increased with age, and hardly any patient (<1%) was less than 30 years old. The two most prevalent comorbidities observed in COVID-19 patients from both waves were hypertension and dyslipidemia, with diabetes ranking behind (Table 1). However, less than 11% of the patients presented chronic obstructive pulmonary disease and asthma, or were under immunosuppressive therapy (Table 1). A comorbidity burden equal or greater than 2 was associated with poor prognosis in wave 1 (Table 1). The median body mass index of COVID-19 patients was over 25 in both waves (Table 1), indicating a prevalence of overweight in COVID-19 patients. Bilateral pneumonia was a common feature in COVID-19 patients, and respiratory failure at the time of hospital admission was observed in 75.0–83.9% of patients who suffered a critical/fatal condition (Table 1). Furthermore, O_2 saturation at hospital admission was associated with poor prognosis in wave 1, but not in wave 2 (Table 1).

Blood Laboratory Analyses at Hospital Admission and Their Relationship With Disease Prognosis in Waves 1 and 2 of COVID-19

As shown in **Table 2**, significant differences in several parameters determined at hospital admission were closely associated with the subsequent disease severity in both COVID-19 waves. NLR dramatically increased in patients from both waves who eventually suffered critical/fatal condition, as compared with moderate patients ($p < 0.001$). Likewise, high levels of several inflammatory markers, including C-reactive protein and ferritin, were detected in all COVID-19 patients at the time of hospital admission, especially in the critical/fatal cohort in both waves (C-reactive protein: $p < 0.001$ [wave 1] and 0.025 [wave 2]; ferritin: $p < 0.001$) (Table 2). Additional common changes in the critical/fatal group of both waves included rises in lactate dehydrogenase (LDH) ($p < 0.001$), urea ($p < 0.001$ [wave 1] and 0.033) [wave 2]), and blood urea nitrogen ($p < 0.001$ [wave 1] and 0.043 [wave 2]) (Table 2).

Interestingly, 25-hydroxyvitamin D levels were rather low in all COVID-19 patients, but no significant differences were found between the moderate and critical/fatal conditions (Table 2). Despite dyslipidemia was the second most common comorbidity in COVID-19 patients (Table 1), a rather low level of cholesterol was detected in most patients (Table 2).

Most of the blood parameters analyzed behaved similarly in the two waves of COVID-19, except for two remarkable differences, namely, IL-6 and D-dimer (Table 2). Both parameters were already highly increased in moderate patients from both waves at hospital admission. However, such values were dramatically increased in the critical/fatal cohort from the first wave, whereas no change was observed in the critical/fatal cohort from the second wave when compared with moderate patients (Table 2).

TABLE 1.

Demographic Characteristics, Preexisting Comorbidities, and Respiratory Conditions in Coronavirus Disease 2019 Inpatients From the Two Infection Waves

Demographics and Comorbidities								
	First COVID-19 Wave				Second COVID-19 Wave			
Characteristics	Moderate (*n* = 131)	Critical/ Fatal (*n* = 62)	*p*	Total (*n* = 193)	Moderate (*n* = 59)	Critical/ Fatal (*n* = 24)	*p*	Total (*n* = 83)
Sex, *n* (%)								
Male	69 (52.7)	40 (64.5)	0.121	109 (56.48)	31 (52.5)	17 (70.8)	0.126	48 (57.83)
Age, yr, median (IQR)	64.00 (52.00–72.00)	67.50 (56.75–78.25)	0.038	65.0 (54.00–75.00)	71.0 (51.00–88.00)	59.0 (48.25–80.50)	0.223	65.00 (50.00–86.00)
Distribution, yr, *n* (%)								
18–29	1 (0.76)	0 (0)	1.000	1 (0.52)	0 (0)	0 (0)	–	0 (0)
30–39	11 (8.4)	2 (3.23)	0.230	13 (6.74)	5 (8.5)	1 (4.2)	0.663	6 (7.23)
40–49	14 (10.7)	5 (8.1)	0.796	19 (9.84)	8 (16.6)	6 (25.0)	0.215	14 (16.87)
50–59	22 (16.8)	12 (19.4)	0.663	34 (17.62)	10 (16.9)	5 (20.8)	0.756	15 (18.07)
60–69	45 (34.4)	16 (32.1)	0.233	61 (31.61)	6 (10.2)	4 (16.7)	0.465	10 (12.05)
70–79	27 (20.6)	14 (22.6)	0.755	41 (21.24)	4 (6.8)	2 (8.3)	1.000	6 (7.23)
≥ 80	13 (9.9)	13 (21.0)	0.036	26 (13.47)	26 (44.1)	6 (25.0)	0.106	32 (38.55)
Prevalent comorbidities, *n* (%)								
Hypertension	56 (42.7)	30 (48.4)	0.462	86 (44.56)	26 (44.1)	14 (58.3)	0.238	40 (48.19)
Dyslipidemia	39 (29.8)	23 (37.1)	0.309	62 (32.12)	20 (24.5)	13 (56.5)	0.238	33 (39.76)
Diabetes	16 (12.2)	13 (21.0)	0.112	29 (15.03)	14 (23.7)	2 (8.3)	0.134	16 (19.28)
Chronic obstructive pulmonary disease	3 (2.3)	3 (4.8)	0.388	6 (3.11)	1 (1.7)	2 (8.3)	0.199	3 (3.61)
Asthma	7 (5.3)	2 (3.2)	0.721	9 (4.66)	4 (6.8)	1 (4.2)	1.000	5 (6.02)
Immunosuppressive therapy	5 (3.8)	3 (4.8)	0.713	8 (4.14)	2 (3.4)	1 (4.2)	1.000	3 (3.61)
Comorbidity burden, *n* (%)								
0	56 (43.4)	23 (35.9)	0.320	79 (40.9)	26 (44.1)	7 (29.2)	0.209	33 (29.8)
1	39 (30.2)	14 (21.9)	0.221	53 (27.5)	12 (20.3)	5 (20.8)	1.000	17 (20.5)
≥ 2	34 (26.4)	26 (40.6)	0.044	60 (31.1)	21 (35.6)	12 (50.0)	0.224	33 (39.8)
Body mass index, median (IQR)	27.17 (24.74–31.67)	28.28 (25.30–31.53)	0.416	27.44 (25.00–31.53)	25.91 (23.44–30.83)	32.62 (25.94–38.62)	0.042	26.42 (24.09–32.19)
Respiratory condition, *n* (%)								
Unilateral pneumonia	18 (13.7)	8 (12.9)	0.874	26 (13.47)	4 (6.8)	0 (0)	1.000	4 (4.82)
Bilateral pneumonia	106 (80.9)	54 (87.1)	0.287	160 (82.90)	32 (54.2)	22 (91.7)	0.005	54 (65.06)
Respiratory failure	72 (55.0)	52 (83.9)	< 0.001	124 (64.25)	14 (23.7)	18 (75)	< 0.001	32 (38.55)
O_2 saturation, median (IQR)	94.00 (90.00–96.00)	92.00 (88.00–95.00)	0.048	93.00 (89.00–96.00)	95.00 (93.00–97.00)	95.00 (92.85–96.80)	0.925	95.00 (93.00–97.00)

COVID-19 = coronavirus disease 2019, IQR = interquartile range.

Sex, age group distribution, preexisting comorbidities, body mass index, and respiratory condition of COVID-19 patients at hospital admission. Patients were classified according to the subsequent development of moderate or critical/fatal conditions. Respiratory failure was defined by a $Pao_2 < 60$ mm Hg, a $Paco_2 > 45$ mm Hg, or both.

TABLE 2.

Laboratory Findings of Inpatients Infected With Severe Acute Respiratory Syndrome Coronavirus-2 at Admission to Hospital From the Two Infection Waves

Laboratory Parameters							
		First COVID-19 Wave, Median (IQR)			Second COVID-19 Wave, Median (IQR)		
Laboratory Results	Normal Range	Moderate ($n = 131$)	Critical/Fatal ($n = 62$)	p	Moderate ($n = 59$)	Critical/Fatal ($n = 24$)	p
Blood cells and related parameters							
Red cells							
Red cells ($\times 10^6/\mu L$)	3.5–5.8	4.60 (4.30–4.90)	4.65 (4.10–4.90)	0.869	4.50 (4.10–4.90)	4.70 (4.12–5.05)	0.584
Hemoglobin (g/dL)	12–15	13.75 (12.50–14.50)	13.75 (12.17–14.62)	0.973	13.85 (12.15–15.00)	13.85 (12.70–14.97)	0.529
Platelets and coagulation							
Platelets ($\times 10^3/\mu L$)	150–450	203.00 (155.50–280.25)	197.00 (152.25–264.25)	0.616	213.00 (162.50–279.00)	218.00 (163.50–287.00)	0.678
Prothrombin time (s)	10–14	13.10 (12.25–13.80)	13.20 (12.20–15.00)	0.236	12.95 (12.10–14.40)	12.60 (12.30–13.57)	0.499
WBCs							
WBCs ($\times 10^3/\mu L$)	3.5–10.0	5.80 (4.610–8.00)	7.20 (5.31–11.67)	0.003	6.26 (4.46–10.69)	6.57 (4.41–8.78)	0.996
Lymphocytes (%)	20–45	18.85 (10.62–25.57)	12.30 (7.57–17.95)	< 0.001	19.90 (12.72–25.62)	10.40 (7.30–14.17)	< 0.001
Monocytes (%)	2–10	5.65 (3.97–8.45)	4.35 (3.10–6.62)	0.003	5.45 (3.82–7.87)	3.80 (2.05–5.00)	0.003
Neutrophils (%)	50–70	74.15 (61.25–83.12)	81.05 (76.15–87.17)	< 0.001	72.70 (66.77–81.60)	86.45 (79.30–89.60)	< 0.001
Eosinophils (%)	0–4	0.20 (0.00–0.60)	0.10 (0.00–0.225)	0.009	0.50 (0.10–1.20)	0.00 (0.00–0.27)	< 0.001
Basophils (%)	0.2–1.2	0.20 (0.10–0.40)	0.32 (0.17–0.40)	0.424	0.40 (0.12–0.50)	0.20 (0.10–0.40)	0.173
Lymphocyte count ($\times 10^3/\mu L$)	1.2–4.5	1.00 (0.72–1.50)	0.80 (0.60–1.00)	< 0.001	1.10 (0.72–1.40)	0.75 (0.52–1.00)	0.001
Monocyte count ($\times 10^3/\mu L$)	0.1–1.0	0.35 (0.2–0.575)	0.30 (0.20–0.50)	0.478	0.30 (0.30–0.50)	0.25 (0.10–0.47)	0.057
Neutrophil count ($\times 10^3/\mu L$)	1.7–7.0	4.20 (2.80–6.25)	6.70 (4.37–9.92)	< 0.001	4.60 (2.80–7.10)	5.45 (3.80–7.90)	0.156
Neutrophil-to-lymphocyte ratio	1–3	3.00 (2.00–7.00)	7.50 (5.00–12.25)	< 0.001	3.80 (2.71–6.54)	8.37 (5.94–12.92)	< 0.001
Lymphocyte populations							
CD3 (cells/μL)	880–2,600	742.50 (512.75–987.00)	652.50 (362.75–738.00)	0.030	449.00 (334.50–852.75)	336.00 (173.00–530.00)	0.074
CD4 (cells/μL)	500–1,600	480.00 (313.50–673.50)	364.00 (176.25–543.25)	0.108	240.00 (206.25–440.25)	162.00 (116.50–292.50)	0.042
CD8 (cells/μL)	150–1,000	228.00 (144.00–308.00)	180.00 (122.50–243.00)	0.136	161.50 (110.00–287.25)	138.00 (58.50–168.50)	0.161
CD4/CD8	1–4	2.32 (1.74–3.14)	2.67 (1.66–3.33)	0.726	1.59 (0.99–2.58)	1.75 (1.16–2.06)	0.924

(*Continued*)

TABLE 2. (*Continued*).

Laboratory Findings of Inpatients Infected With Severe Acute Respiratory Syndrome Coronavirus-2 at Admission to Hospital From the Two Infection Waves

Laboratory Parameters							
		First COVID-19 Wave, Median (IQR)			Second COVID-19 Wave, Median (IQR)		
Laboratory Results	Normal Range	Moderate (*n* = 131)	Critical/Fatal (*n* = 62)	*p*	Moderate (*n* = 59)	Critical/Fatal (*n* = 24)	*p*
Biochemical parameters							
Glucose (mg/dL)	74–109	95.00 (87.00–107.25)	108.00 (93.00–160.25)	< 0.001	108.50 (97.00–146.00)	125.50 (109.25–178.00)	0.087
Fibrinogen (mg/dL)	200–400	683.50 (546.75–758.00)	702.00 (601.00–792.00)	0.209	612.00 (513.25–709.25)	640.00 (550.25–803.00)	0.210
D-dimer (ng/mL)	68–494	573.50 (365.50–1,005.00)	786.00 (567.75–1,170.00)	0.005	520.50 (302.75–937.75)	466.00 (267.00–1,217.00)	0.973
Urea (mg/dL)	10–40	33.00 (24.25–42.00)	41.50 (30.75–63.50)	< 0.001	32.00 (24.00–51.00)	41.00 (34.00–58.75)	0.033
Blood urea nitrogen (mg/dL)	5–20	15.00 (11.25–20.00)	20.00 (14.75–31.00)	< 0.001	15.50 (11.25–24.75)	19.50 (16.00–27.75)	0.043
Bilirubin, total (mg/dL)	0.3–1.2	0.65 (0.40–0.80)	0.80 (0.50–1.05)	0.026	0.40 (0.30–0.60)	0.45 (0.30–0.60)	0.465
25-Hydroxyvitamin D (ng/mL)	> 30	14.80 (9.65–21.70)	15.80 (10.70–22.00)	0.676	16.10 (11.70–21.80)	13.50 (9.54–34.00)	0.663
Krebs von den Lungen-6 (U/mL)	< 650	287.50 (206.75–399.75)	525.50 (300.00–840.50)	0.005	302.50 (217.00–406.50)	328.00 (269.00–379.00)	0.496
Interleukin-6 (pg/mL)	< 7	36.90 (18.90–68.70)	85.85 (57.42–147.25)	< 0.001	12.95 (2.39–44.15)	12.50 (4.79–15.00)	1.000
Lactate dehydrogenase (U/L)	< 250	298.00 (245.75–387.25)	445.00 (317.00–521.00)	< 0.001	238.00 (193.00–293.00)	324.00 (246.50–397.50)	< 0.001
Albumin (g/dL)	3.5–5.2	3.50 (3.20–3.90)	3.30 (3.07–3.60)	< 0.001	3.80 (3.35–4.00)	3.60 (3.40–3.85)	0.119
C-reactive protein (mg/dL)	< 0.5	6.80 (3.49–14.00)	16.00 (5.90–24.91)	< 0.001	4.15 (1.92–9.55)	8.87 (3.05–16.10)	0.025
Transferrin (mg/dL)	200–360	165.00 (134.00–188.75)	149.50 (124.25–176.25)	0.018	171.00 (152.00–213.00)	170.50 (143.00–203.00)	0.522
Ferritin (ng/mL)	13–150	787.00 (364.00–1,618.00)	1,326 (702.50–2,034.00)	< 0.001	412.00 (236.00–772.00)	1,413.00 (630.00–1,699.00)	< 0.001
Cholesterol, total (mg/dL)	150–200	129.00 (113.00–151.75)	115.00 (100.50–136.75)	0.015	131.00 (114.00–180.00)	158.00 (107.00–180.00)	0.926

CD = cluster of differentiation, COVID-19 = coronavirus disease 2019, IQR = interquartile range.
Data are median (IQR) from the indicated number (*n*) of patients with available data.

COVID-19 Signature for Critical/Fatal Illness in the First Wave of Infection: Development and Validation

Considering only the first wave of infection, we determined optimal cutoff values for 11 of the most robustly altered hematological parameters in critical/fatal COVID-19 patients: neutrophil count (≥ 5.1 × 10^3/μL), lymphocyte count (≤ 0.9 × 10^3/μL), NLR (≥ 5), C-reactive protein (≥ 15.2 mg/dL), LDH (≥ 411.96 U/L), IL-6 (≥ 78.8 pg/mL), urea (≥ 40 mg/dL), D-dimer (≥ 713 ng/mL), transferrin (≤ 164 mg/dL), ferritin (≥ 1,152 ng/mL), and glucose (≥ 106.5 mg/dL). On univariate binary logistic regression, the above parameters, together with comorbidity burden (≥ 2) and age (≥ 67 yr), showed predictive ability for critical/fatal condition in wave 1 (**Fig. 1*A***). Multivariate binary logistic regression analyses using different combinations of the above parameters revealed that NLR, C-reactive protein, LDH, IL-6, urea, and D-dimer was the most powerful combination of risk factors to predict, at admission to hospital, a later critical/fatal illness. NLR was used to encompass changes in neutrophils and lymphocytes. The six-biomarker signature holds a predictive accuracy of 84% and an AUROC value of 0.886 (**Fig. 2*A***), which surpassed the use of each of the six indicators separately, and was not further improved by incorporating comorbidity burden, age, or any other biomaker. Both training and test groups displayed AUROCs of 0.88 (**Supplementary Fig. S2**, http://links.lww.com/CCX/A508), demonstrating the reliability of the model as a predictor of critical/fatal illness during the first wave of infection. NLR, C-reactive protein, LDH, and IL-6 persisted as independent risk factors for critical/fatal COVID-19, with statistically significant ORs in multivariate analysis (**Fig. 2*B***). When breaking down the comorbidity burden into the three most prevalent comorbidities (hypertension, dyslipidemia, and diabetes), NLR was the only parameter within the six-biomarker signature that resulted increased in all the three most prevalent comorbidities (no comorbidity-NLR: OR, 2.652 [95% CI, 1.002–7.0182], p = 0.050; hypertension-NLR: OR, 11.012 [95% CI, 3.595–33.726], $p < 0.001$; dyslipidemia-NLR: OR, 6.750 [95% CI, 1.318–34.565], $p = 0.022$; diabetes-NLR: OR, 16.100 [95% CI, 3.285–78.917], $p = 0.001$).

Validation of the COVID-19 Signature for Critical/Fatal Illness From Wave 1 in Wave 2: Differences Between Both Waves

The prediction model built with data from wave 1 led to an AUROC value of 0.874 when tested in wave 2 (**Supplementary Fig. S3A**, http://links.lww.com/CCX/A508), supporting the prediction value of the above COVID-19 signature for critical/fatal illness at hospital admission. However, only NLR and LDH were independently associated with poor prognosis in wave 2 in multivariate analysis (**Supplementary Fig. S3B**, http://links.lww.com/CCX/A508). IL-6 and D-dimer represented two major risk factors for critical/fatal illness on univariate (Fig. 1*A*) and multivariate (Fig. 2) analyses

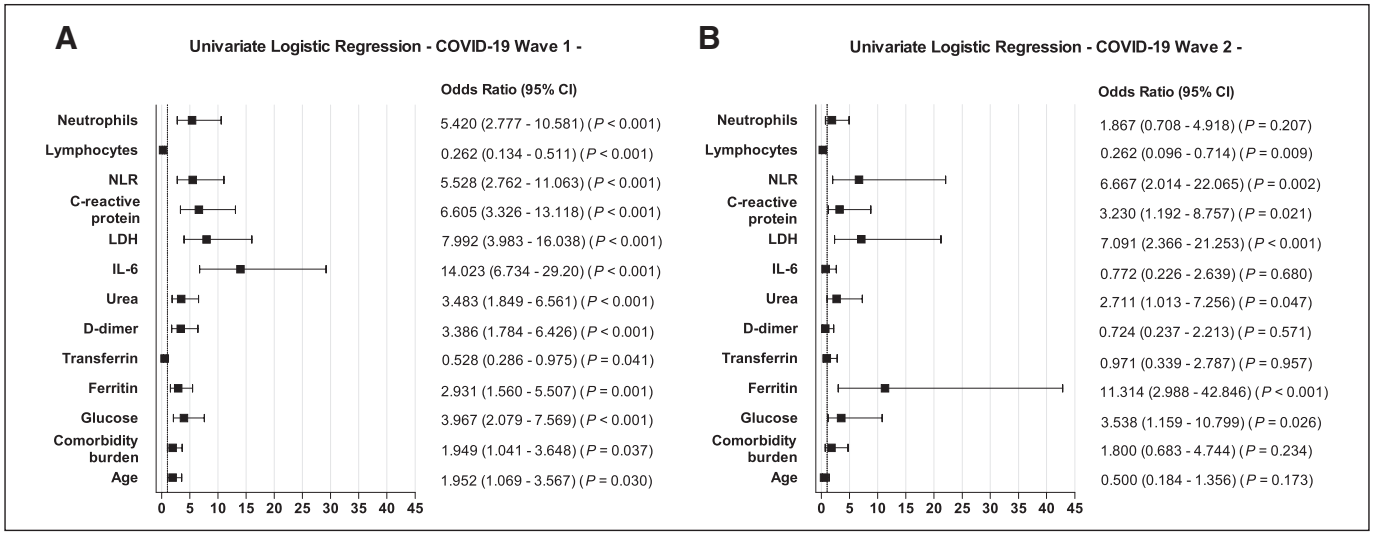

Figure 1. Univariate logistic regression analyses of risk factors associated with critical/fatal coronavirus disease 2019. Odds ratio plots, 95% CI, and p of biomarkers in wave 1 (**A**) and wave 2 (**B**). IL = interleukin, LDH = lactate dehydrogenase, NLR = neutrophil-to-lymphocyte ratio.

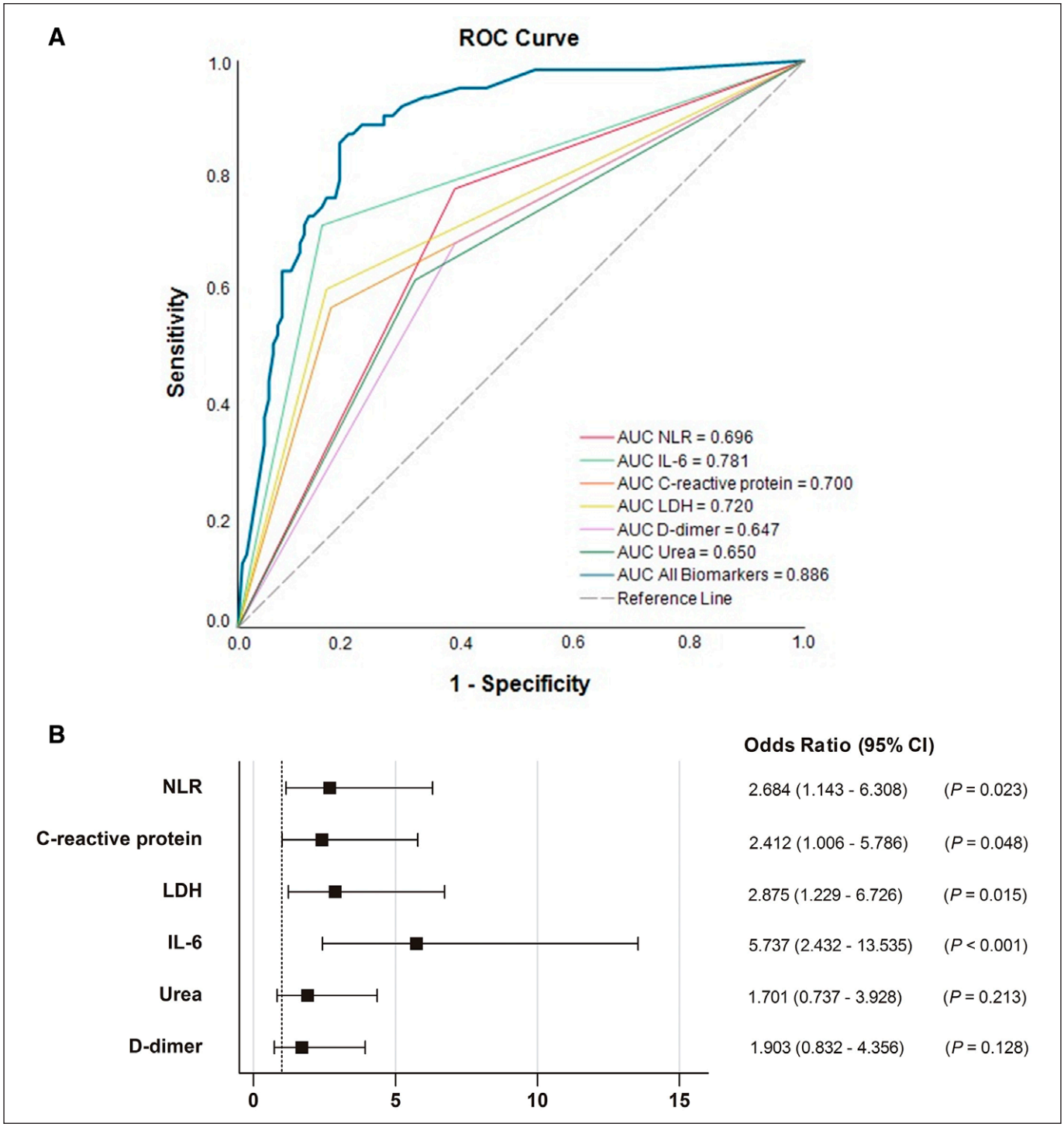

Figure 2. Identification of the six-parameter critical/fatal coronavirus disease 2019 prognosis signature during the first wave of infection. (**A**) Receiver operating characteristic (ROC) curves and area under the curve (AUC) analyses and (**B**) odds ratio plots, 95% CI, and *p* of the biomarkers included in the signature, alone or in combination, from multivariate logistic regression analysis. IL = interleukin, LDH = lactate dehydrogenase, NLR = neutrophil-to-lymphocyte ratio.

during the first wave of infection. However, no differences were detected in the levels of IL-6 and D-dimer, at hospital admission, between patients who developed a moderate or critical/fatal disease in wave 2 (Table 2 and **Fig. 1*B***). A comparison of univariate analyses of parameters associated with critical/fatal illness between both waves determined at hospital admission (Fig. 1), using the cutoff values described above for wave 1 (except for LDH ≥ 323 U/L, C-reactive protein ≥ 8.70 mg/dL, D-dimer ≥ 500.50 ng/mL,

IL-6 ≥ 12.60 pg/mL, glucose ≥ 108.5 mg/dL cutoff values in wave 2), showed similar associations and some discrepancies. Reduced lymphocyte count and increased NLR, C-reactive protein, LDH, urea, ferritin, and glucose were similarly associated with disease severity in both waves (Fig. 1). However, neutrophil count, IL-6, D-dimer, transferrin, comorbidity burden (≥ 2), and age were not associated with poor prognosis at hospital admission in wave 2, opposite to the effect observed in wave 1 (Fig. 1). These results, together with the similar values of O_2 saturation in moderate and critical/fatal patients at hospital admission in wave 2 (Table 1), suggest that patients arrived to hospital earlier in the disease course and in better health condition than in wave 1. Taken together, these data support that the six-biomarker signature identified in wave 1 is a reliable predictor of critical/fatal illness in COVID-19 at hospital admission, but some parameters are highly dependent on patient condition when admitted to hospital.

Follow-Up Studies With the COVID-19 Signature Biomarkers to Monitor Fatal Disease Development

We next monitored the COVID-19 parameters included in the signature in follow-up studies in patients from both waves with moderate disease who were discharged from hospital without ICU admission (n = 190) and nonsurvivors (n = 30). Remarkable differences between the moderate and fatal conditions over hospital stay were found in neutrophils ($p = 0.006$ to $p < 0.001$), lymphocytes ($p < 0.001$), NLR ($p < 0.001$), LDH ($p = 0.001$ to $p < 0.001$), ferritin ($p = 0.032$ to $p < 0.001$), C-reactive protein ($p = 0.048$ to $p < 0.001$), urea ($p < 0.001$), IL-6 ($p = 0.001$ to $p < 0.001$), and D-dimer ($p = 0.001$ to $p < 0.001$) (**Fig. 3**). Some parameters gradually increased over time in the fatal cohort (neutrophil count, H [2] = 9.772, $p = 0.008$; NLR, H [2] = 13.476, $p = 0.001$; urea, H [2] = 9.207, $p = 0.01$; IL-6, H [2] = 9.804, $p = 0.007$; D-dimer, H [2] = 6.77, $p = 0.034$, Kruskal-Wallis test), whereas lymphocyte count decreased over time (H [2] = 8.551, $p = 0.014$, Kruskal-Wallis test). Other parameters (LDH, ferritin, and C-reactive protein) were persistently elevated in the nonsurvivor cohort in comparison to discharged patients (from $p < 0.05$ to $p < 0.001$). These analyses further suggest that the above parameters behave as poor prognosis indicators during disease progression.

DISCUSSION

This study found that most hematological parameters behaved similarly across both waves of infection with regard to disease severity, but remarkable differences were found in IL-6 and D-dimer concentrations. Using data from patients who underwent a moderate or critical/fatal condition during the first wave of SARS-CoV-2 infection in Madrid (Spain), we identified and validated a six-blood biomarker signature (NLR, C-reactive protein, LDH, IL-6, urea, and D-dimer) that predicted critical/fatal COVID-19 prognosis at hospital admission in both waves. High levels of the above blood parameters were associated with poor prognosis. Multivariate logistic regression analyses led to an AUROC value of 0.886, indicating that this COVID-19 signature is an accurate tool to assess poor prognosis upon hospital admission. Follow-up analysis further indicated that the COVID-19 signature reported here can be used, together with an increase in neutrophil count and ferritin level, and a decrease in lymphocyte count, to monitor disease progression.

IL-6 and D-dimer, measured at hospital admission, were predictive factors of critical/fatal illness only in the first wave. However, both parameters were highly increased during illness progression to a fatal stage in both waves, and therefore, they behave as important markers of disease monitoring. Our results are compatible with a hyperinflammation condition in COVID-19 patients that, if not timely identified and treated, could lead to a cascade of irreversible inflammation-mediated processes resulting in a fatal outcome (10). The difference between the ability of IL-6 and D-dimer to act as risk factors of critical/fatal illness condition at hospital admission between the first and second waves strongly suggests that patients were admitted to hospital earlier in the disease course during the second wave, likely due to higher hospital bed availability. In support of this notion, moderate patients in the second wave showed lower levels of C-reactive protein, ferritin, IL-6, and D-dimer at admission to hospital when compared with the first wave, as well as a lower prevalence of pneumonia and respiratory failure, and a higher O_2 saturation value. On these grounds, it could be envisaged that patients in wave 2 arrived to hospital with a less inflammatory condition. An analysis of the similarities and discrepancies between the distinct biomarkers and parameters in both waves of infection clearly indicates that caution should be taken in interpreting

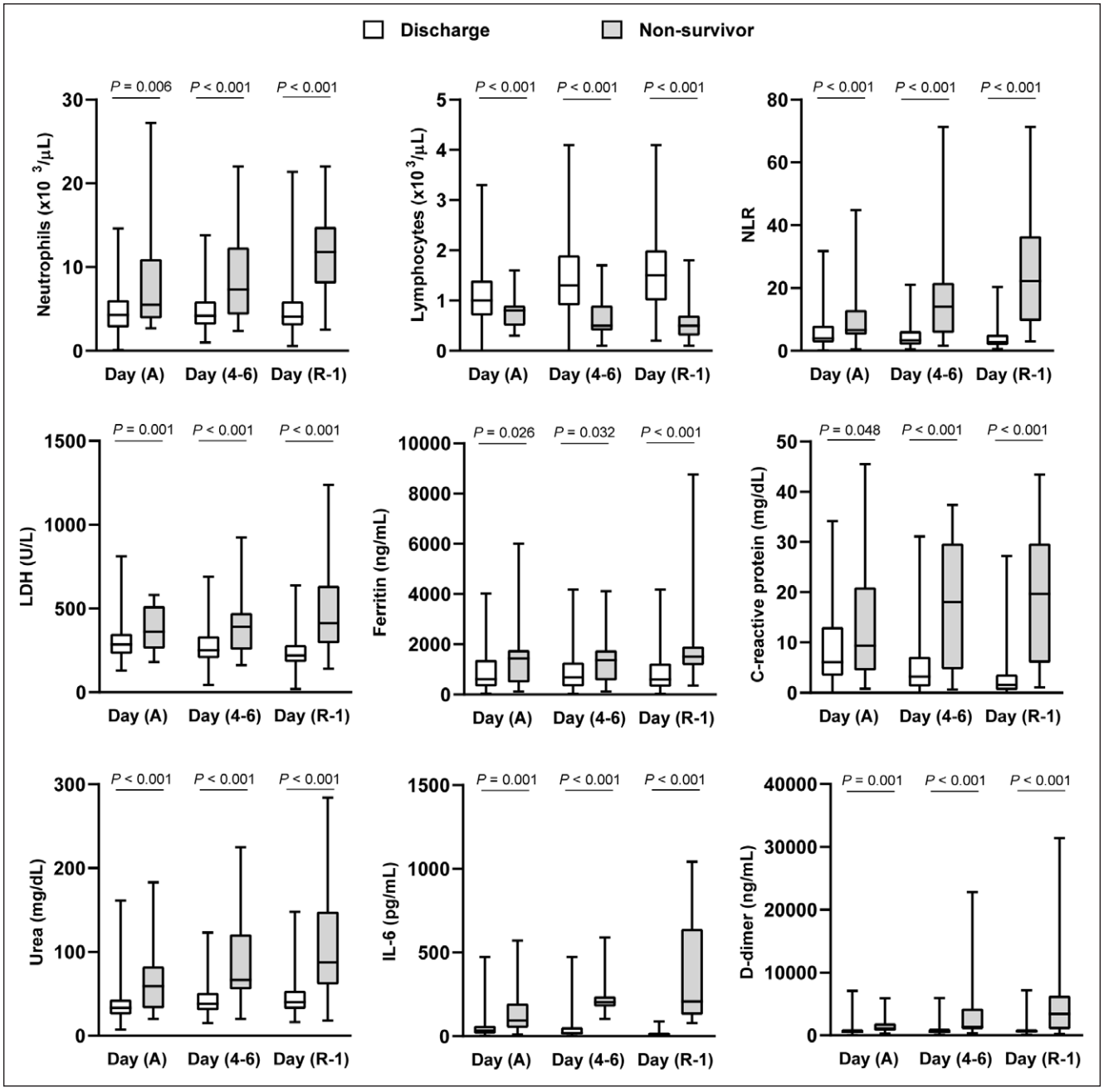

Figure 3. Follow-up studies with the biomarkers included in the coronavirus disease 2019 signature demonstrate its predictive ability for fatal disease progression. Follow-up data of moderate discharged patients and nonsurvivors collected at three time frames: upon hospital admission (day [A]), after 4–6 d of hospitalization (days [4–6]), and the day before resolution (day [R-1]). Box plots show the distribution of all observations of the indicated blood parameters in moderate discharged patients and nonsurvivors: median (*center line*), interquartile range (interquartile range, *box limits*), highest and lowest observations (*whiskers*). IL = interleukin, LDH = lactate dehydrogenase, NLR = neutrophil-to-lymphocyte ratio.

the meaning of the herein-reported COVID-19 signature for prognosis. The use of multivariable modeling to draw inference about the coefficients of individual biomarkers is known to be subject to bias, leading to a situation known as "Table 2 Fallacy" (11). In addition, by analyzing only hospitalized samples and finding that patients were admitted to hospital at different stages of their disease during both waves of infection, we tried to minimize collider bias (12) by calculating both waves separately, and testing how the model created with data from the first wave performed in the second wave.

A number of increasing prediction models and clinical risk prediction scores have been recently developed,

but they have been found to have a high risk of bias, are poorly reported, lack external validation, and are considered of limited clinical utility (13). A low validity was observed when prediction models, based on Chinese hospitals, were applied to U.K. patients (14), which could be explained by significant differences between the respective populations affected by SARS-CoV-2 in China compared with Europe, as well as between the respective healthcare systems. Here, by using a single-center study and, therefore, keeping several variables identical, we found that a predictive model developed with data from the first wave of infection could be validated in a second wave, but some risk biomarkers lost their independent significance. Our model, from a multivariate analysis, could be affected by a series of confounding, mediation, and collider biases (15), and therefore, it should be considered as a descriptive analysis that cannot be interpreted in causal terms, but that suggests candidate predictors of poor prognosis at hospital admission. Several biomarkers are highly dependent on the patient condition as well as on the level of other biomarkers at hospital admission. Despite some variability in certain biomarkers, inflammatory, cell death, and thrombotic and macrophage activating signatures remained associated with poor prognosis in both waves. Our prediction model includes a series of biomarkers that cover a wide spectrum of situations and highlight two biomarkers, NLR and LDH, related to inflammatory state and cell death, as the most prevalent ones in COVID-19 patients at the early stages of the disease.

A recent study from Wuhan (China) reported a five-parameter signature for COVID-19 progression that included neutrophil count, lymphocyte count, procalcitonin, older age, and C-reactive protein (16), but these parameters are common to any inflammatory process. Our COVID-19 signature, together with those biomarkers monitoring illness progression, seems to be more specific for the disease, as it includes parameters related to systemic inflammation, tissue damage, kidney dysfunction, and altered coagulation, four major hallmarks in critical/fatal COVID-19 (5, 17–19).

In line with the present study, additional reports have shown increased NLR and neutrophil count as risk factors for severe COVID-19 (20–22). This fact underscores that the damage wrought by SARS-Cov-2 is likely due to an exacerbated inflammatory response, affecting several tissues and organs. In addition, lymphopenia, contributing to high NLR levels, has been also associated with severe stages of the disease (23). The most frequent comorbidities present in critical/fatal COVID-19 patients, such as obesity, dyslipidemia, hypertension, and diabetes, have been related to increased NLR (24–26). Our follow-up studies indicate that NLR and neutrophils are increased during progression of COVID-19 toward a deadly stage and behave as risk factors for poor prognosis. Neutrophils constitute the majority of infiltrating cells in inflamed tissues (27). The amazing ability of neutrophils to infiltrate different organs (28) might explain the systemic inflammation in COVID-19 patients (5, 29). The LDH and C-reactive protein parameters, present in our COVID-19 signature, represent two established markers for tissue damage and inflammatory status, respectively, two major clinical features of COVID-19 (30). The presence of ferritin in our COVID-19 signature is in agreement with previous reports that associated an elevated ferritin level with poor outcome in COVID-19 (31). Serum ferritin is a well-known inflammatory marker as well as a leakage product from damaged cells (32). The presence of urea in our signature might be related to the association of kidney disease with inhospital mortality in COVID-19 patients (33).

IL-6 was found to be a risk factor of disease severity in our studies of the first infection wave, and it is highly increased during illness progression to fatal outcome in both waves. In this regard, tocilizumab, a recombinant humanized monoclonal antibody directed against IL-6 receptor, appears to be useful in severe COVID-19 patients (34–36). The herein-reported high increase of D-dimer concentration in COVID-19 progression is in agreement with the high number of venous thromboembolism events in critically ill inpatients, and the beneficial action of the anticoagulant therapy (7). Elevated D-dimer levels were found in nonsurvivors and could be associated with disseminated intravascular coagulation, venous thromboembolism, and pulmonary embolism observed in severe COVID-19 patients (37, 38). Our results indicate that IL-6 and D-dimer do not act as predictors at the time of hospital admission in the second wave, but they remain as strong indicators of illness progression to a critical condition. Recent evidence shows some contradictory results regarding the use of D-dimer concentration at hospital admission as a predictor for fatal illness (39, 40). A plausible

explanation is that patients, as stated above, could be at different stages of disease course at the time of hospital admission in distinct studies. In our case, it could be envisaged that by adhering the national guidance of "stay at home" to prevent hospitals being overwhelmed and avoid hospital collapse, patients arrived to hospital in significantly worse health condition in wave 1. In support of this, it is noteworthy that critical patients showed lower O_2 saturation levels than moderate patients ($p = 0.048$) in wave 1, whereas both moderate and critical patients in wave 2 showed similar O_2 saturation values in both moderate and critical patients and were even higher than in moderate patients in wave 1 (Table 1) at hospital admission. Furthermore, as stated above, patients at hospital admission showed lower levels of C-reactive protein, IL-6, and D-dimer in wave 2 when compared to wave 1. Thus, an earlier hospitalization in the second wave could explain many of the observed differences between the laboratory findings in both waves.

COVID-19 has no specific treatment and appears in a wide range of clinical forms with varying degrees of severity. Timely determination of patients who are likely to develop a critical condition is of pivotal importance to choose timely the correct treatment. This novel six-biomarker COVID-19 signature minimizes the number of biomarkers to measure upon hospital admission while maintaining specificity for COVID-19, and can be easily implemented in any hospital worldwide for the early identification of patients who require more intensive supportive therapies and timely treatments. The use of dichotomized data to generate our model, based on optimal threshold values, can help clinicians classify hazardous levels of biomarkers and thus anticipate patient's prognosis and guide a rapid decision-making in clinical practice.

Some limitations of this study should be noted. It was a single-center study and external validity is required to support widespread use of the herein-reported COVID-19 signature for triage and prognosis. In addition, a larger sample size might improve the statistical power of the study. Additional limitations include several multivariate biases discussed above, differences in the time elapsed from the onset of symptoms to hospital admission, and patient heterogeneity. Despite the above limitations, our results are expected to help formulate causal hypotheses in this disease.

CONCLUSIONS

We have identified a six-biomarker COVID-19 signature that predicted critical/fatal illness at hospital admission in a first wave on infection that was validated in a second infection wave. However, differences in some risk factors at the time of hospital admission with regard to disease severity were found between the first and second waves of infection, likely due differences in patient's health conditions on arrival to hospital, which may hamper the accurate extrapolation of all biomarkers from a first wave-derived risk prediction model to a second wave. The herein-reported COVID-19 signature constitutes a novel prognostic tool that could also be used to monitor disease progression. This COVID-19 signature constitutes an accessible, cost-effective, and easy protocol to be implemented in hospitals, allowing rapid identification and timely treatment of patients who may later develop a critical/fatal condition.

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