Poor Survival in COVID-19 Associated with Lymphopenia and Higher Neutrophile-Lymphocyte Ratio

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ABSTRACT **Background:** Immune cell counts in blood in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection may be useful prognostic biomarkers of disease severity, mortality, and response to treatment.

Objectives: To analyze sub-populations of lymphocytes at hospital admission in survivors and deceased from severe pneumonia due to coronavirus disease-2019 (COVID-19).

Methods: We conducted a cross-sectional study of healthcare workers confirmed with SARS-CoV-2 in convalescents (control group) and healthy controls (HC) diagnosed with severe COVID-19. Serum samples were taken at hospital admission and after recovery. Serum samples ≥ 25 days after onset of symptoms were analyzed for lymphocyte subpopulations through flow cytometry. Descriptive statistics, Kruskall-Wallis test, receiver operating characteristic curve, calculation of sensitivity, specificity, predictive values, and Kaplan-Meier analysis were performed.

Results: We included 337 patients: 120 HC, 127 convalescents, and 90 severe COVID-19 disease patients (50 survivors, 40 deceased). For T cells, total lymphocytes ≥ 800/μL, CD3+ ≥ 400/μL, CD4+ ≥ 180/μL, CD8+ ≥ 150/μL, B cells CD19+ ≥ 80/μL, and NK ≥ 34/μL subsets were associated with survival in severe COVID-19 disease patients. All subtypes of lymphocytes had higher concentrations in survivors than deceased, but similar between HC and convalescents. Leukocytes ≥ 10.150/μL or neutrophils ≥ 10,000/μL were associated with increased mortality. The neutrophil-to-lymphocyte ratio (NLR) ≥ 8.5 increased the probability of death in severe COVID-19 (odds ratio 11.68).

Conclusions: Total lymphocytes; NLR; and levels of CD3+, CD4+, CD8+, and NK cells are useful as biomarkers of survival or mortality in severe COVID-19 disease and commonly reach normal levels in convalescents.

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KEY WORDS: coronavirus disease-2019 (COVID-19), lymphocytes, mortality, neutrophil-to-lymphocyte ratio (NLR), survival

Coronavirus disease-2019 (COVID-19) has spread rapidly throughout the world [1]. The first case of COVID-19 detected in Mexico was on 27 February 2020. Since then, the number of patients has increased exponentially with a high mortality rate [2]. Severe COVID-19 is characterized by pneumonia, immune system dysregulation leading to lymphopenia, exhausted lymphocytes, and cytokine storm [3-5]. Decrease of T lymphocyte subsets has correlated with in-hospital death and severity of the illness [5,6]. It has been suggested that the reduction of immune cell counts in peripheral blood during viral infection may be caused by transfer of immune cells to sites of infection, such as the lungs and possibly by virus-induced destruction of T cells [6]. Therefore, lymphocyte subsets analysis may be possible prognostic factors of disease severity, mortality, and response to treatment [6-7]. Another useful prognostic factor is the neutrophil-to-lymphocyte ratio (NLR), which has been associated with an increased risk of death during hospitalization [8].

However, production of neutralizing antibodies (nAbs) can inhibit the viral replication and its detection may provide information on time course of virus infection [9,10]. A study reported that anti-severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) antibody levels differ significantly among COVID-19 patients according to different illness severities and outcome [10]. Therefore, our objective was to analyze the subpopulations of lymphocytes as well as NLR at hospital admission in survivors and deceased from severe pneumonia due to COVID-19 in comparison with convalescents and healthy controls.

PATIENTS AND METHODS

We included patients from the Hospital de Especialidades Centro Medico La Raza, IMSS in Mexico City, Mexico, who had been diagnosed with severe COVID-19 disease. The diagnosis of COVID-19 was performed according to the Guidelines of the Diagnosis and Treatment of New Coronavirus Pneumonia pub-

lished by the National Health Commission of China [11]. Patients were divided into four groups: survived and deceased patients who were confirmed by means of RT-qPCR nasopharyngeal swab test for SARS-CoV-2 infection classified with severe pneumonia at hospital admission [12,13]; outpatients who were healthcare workers confirmed with SARS-CoV-2 in convalescence, and a healthy control group without exposure to SARS-CoV-2 defined as those with negative nasopharyngeal swab test to RT-qPCR and negative antibodies againstSARS-CoV-2 by ELISA. Convalescence serum samples were collected 25 or more days after the onset of COVID-19 symptoms. The research was approved by the ethics and research committee of the hospital with registration number R-2020-3501-108. All the samples were collected after we received written informed consent and registered according to the international standards for the protection of privacy and personal information and to the Declaration of Helsinki and the current General Health Law in our country.

EQUIPMENT AND REAGENTS

The immunophenotyping was performed using the 8-color Multitest™ according to the instructions of the manufacturer (Becton, Dickinson and Company, USA). TBNK reagents were obtained from the manufacturer.

LABORATORY PROCEDURES

All blood samples were collected in fasting state early in the morning and were collected in ethylenediaminetetraacetic acid tubes. Samples were taken in hospitalized patients at the time of hospital admission, while samples in outpatients with COVID-19 were taken 15 days after they recovered. CD3+, CD4+, CD8+, NK, NKT, and CD19+T cells were analyzed by a flow cytometer.

The optimal concentration of already conjugated monoclonal antibody was added (V500, FITC, PE, PerCP-Cy5.5, APC, or APC-H7) to 100 µL of peripheral blood containing 106 cells. The mixture was incubated for 20 minutes at 4°C in darkness. The lysis of erythrocytes was conducted using a lysing FACS solution (Becton Dickinson San José, CA, USA) following manufacturer instructions. Stained cells were washed and re-suspended in a buffer of saline phosphates (PBS). The following monoclonal antibodies were used: CD45-v500, CD19-FITC, CD16+CD56-PE, CD3-PerCP, CD8-APC, and CD4-APC-H7. Analysis of flow cytometry was performed using the FACSCanto-II™ device (Becton Dickinson, USA). Data were obtained by FACSDiva' software.

STATISTICAL ANALYSIS

We employed descriptive statistics, with frequencies, percentages, medium and standard deviation, media and interquartile range (percentile 25–75th). Comparison of quantitative values among groups was made using the Kruskall-Wallis test due to data distribution. Receiver operating characteristic (ROC) curve was used for choosing the threshold cutoffs for each lymphocyte subtype, neutrophils count, and NLR as well as sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV). Area under the curve (AUC) was also reported. We performed a Kaplan-Meier curve according to the lymphocyte cut-off value and test of equality of survival distributions log rank (Mantel-Cox). Statistical analyses were performed using IBM Statistical Package for the Social Sciences statistics software, version 25 (SPSS, IBM Corp, Armonk, NY, USA).

RESULTS

We included 337 patients: 169 men and 168 women. Of the entire group of patients, 120 were controls, 50 survivors (27 men, 23 women), 40 deceased (26 men, 14 women), and 127 convalescents recently recovered of COVID-19 disease. The group of hospitalized patients included 53 men and 37 women who arrived at the hospital with severe COVID-19 disease in June and July 2020. They had a mean age of 55 ± 12 years, while controls and COVID-19 convalescent individuals had a mean age of 40 ± 10 years. Mechanical ventilation was required in 49 of 90 patients (54%) during their hospital stay. Deceased patients had a mean age of 60 ± 12 years old, compared to those who survived, with a mean age of 52 ± 10 years ($P = 0.037$). An example of flow cytometry in this study is shown in Figure 1.

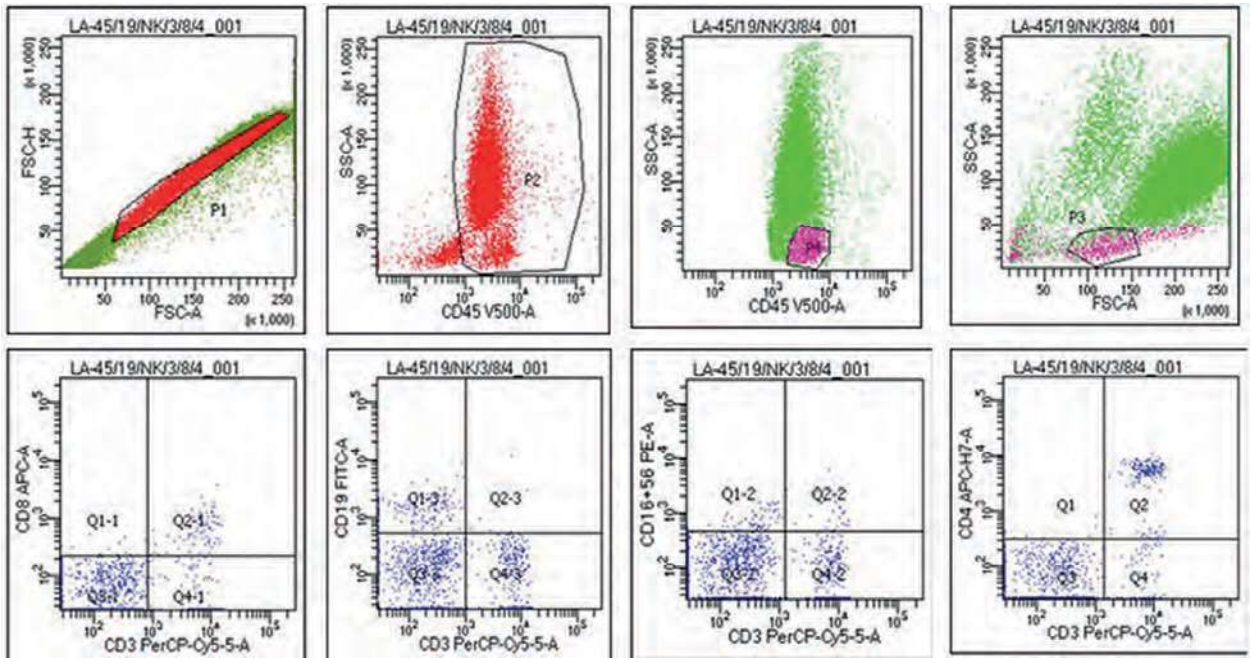
The included convalescents had a median of 39 days (11 to 96 days) after the first symptom of COVID-19 disease. In this group, the number of days after the first symptom was in direct correlation with the number of NK cells ($r = 0.300$, $P = 0.001$), NKT ($r = 0.213$, $P = 0.021$), and CD19 ($r = 0.287$, $P = 0.002$), but without correlation with total lymphocytes, CD3, CD4, and CD8 T cells.

Notably, there was a significant decrease in all T cell subpopulations at hospital admission; mainly in patients who died, as shown in Table 1. All lymphocyte subtypes were similar between COVID-19 convalescents and controls without history of SARS-CoV-2 infection. Total lymphocytes and all subpopulations, except NKT cells showed significant differences between survivors and deceased patients [Figure 2]. In addition, distribution evaluated in percentage of lymphocyte sub-populations was lower in deceased than survivors for CD3 (49 ± 11% vs. 57 ± 11%, $P = 0.001$), CD8 (22 ± 8% vs. 26 ± 8%, $P = 0.018$), and CD19 (11 ± 8% vs. 15 ± 8%, $P = 0.47$) but not for CD4 (25 ± 8% vs. 28 ± 8%, $P = 0.06$), NKT (2% vs. 2%), and NK (5% vs. 6%, $P = 0.06$).

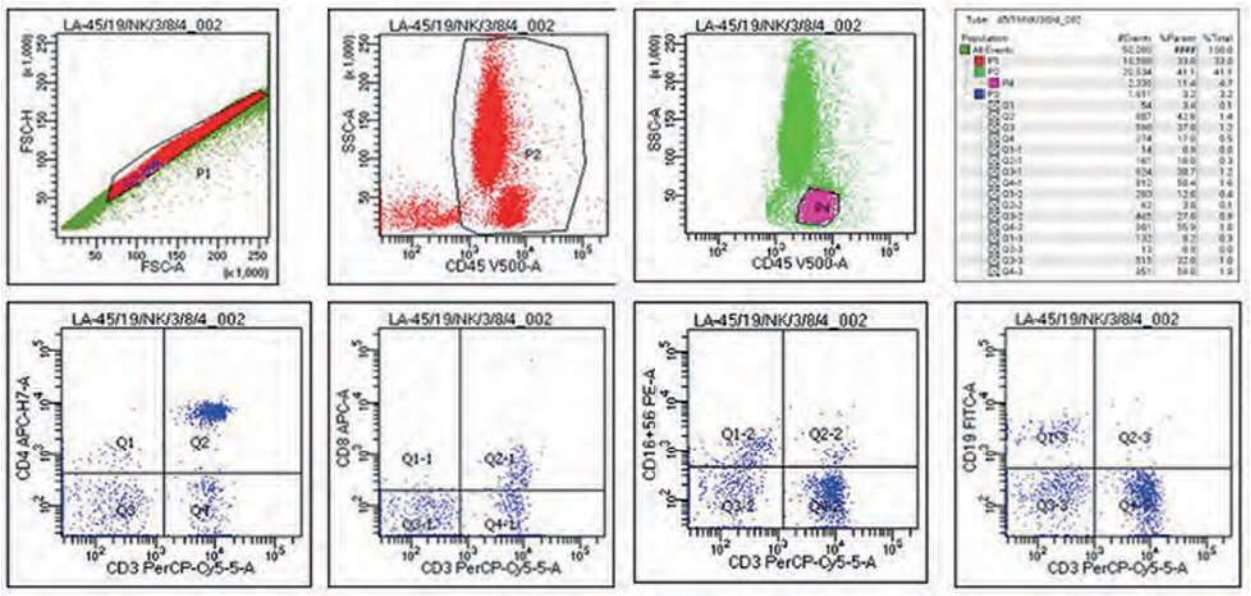
For survival prediction, the best cut-off point in total lymphocytes was for T cells, total lymphocytes ≥ 800/µL (AUC 0.735, 95%CI 0.634–0.841, $P < 0.001$), CD3+ ≥ 400/µL (AUC 0.745, 95%CI 0.649–0.851, $P < 0.001$), CD4+ ≥ 180/µL (AUC 0.700, 95%CI 0.606–0.821, $P = 0.001$), CD8+ 150/µL (AUC 0.742, 95%CI 0.645–0.848, $P < 0.001$), B cells CD19+ ≥ 80/µL (AUC 0.726, 95%CI 0.593–0.811, $P = 0.001$), and NK 34/µL (AUC 0.688, 95%CI 0.557–0.781, $P < 0.001$), NKT (AUC 0.638, $P = 0.130$) subsets were associated with survival in severe COVID-19 disease patients [Figure 3].

[Figure 1. Lymphocytes subpopulations in healthcare workers and patients with COVID-19
Phenotyping flow cytometry, from the total lymphocyte population. We selected the CD45 bright and considered the graph of granularity size. The lymphoid population was selected to define CD4 + (CD3 + / CD4 +), CD8 + (CD3 + / CD8 +), NK (CD16 + CD56 + / CD3-), NKT (CD16 + CD56 + / CD3 +), and B lymphocytes (CD19 +)

A] Lymphocyte subpopulation in a patient with critical lymphopenia



[B] Lymphocyte subpopulation in a patient with moderate lymphopenia



Regarding sensitivity, specificity, and predictive values for survival, we obtained the following data: total lymphocytes Se 73%, Sp 67%, PPV 70%, NPV 66%; CD3 Se 71%,Sp 56%,PPV 65%, NPV62%; for CD4Se71%, Sp56%,PPV 65%, NPV62%; for CD8Se75%, Sp61%, PPV69%,NPV 68%. Concerning toCD19, Se 75%, and Sp 54%,PPV 65%, NPV 65% and for NK, Se63%, Sp59%, PPV 64%,NPV 57%.

In-hospital death due to severe COVID-19, occurred 16 days

Table 1. Differences in the total amount of lymphocytes and subpopulations in the four groups

Lymphocyte subsets cells/μL mean (IQR)	Healthy controls, n=120**	Survivors, n=50**	Deceased, n=40**	Convalescents, n=127**
Total lymphocytes	1900 (1700–2300)	1104 (806–1200)*	635 (403–800)*	1800 (1500–2200)
CD3 cells/μL	1214(983–1531)	601 (465–706)*	314 (198–457)*	1122 (887.6–1441)
CD4 cells/μL	701.4 (520–845.5)	270 (209–345)*	155 (94–241)*	585.9 (455–790)
CD8 cells/μL	465.5 (342–589)	267 (190–322)*	126 (95–162)*	409.6 (321–589)
CD19 cells/μL	293.4 (203–367)	130(101–159)*	68 (21–102)*	263 (171.4–331.3)
NK cells/μL	222.3 (147–310)	51(30–84)*	20 (10–39)*	179.2 (109–280)
NKT cells/μL	52.8 (28.8–100.8)	8 (6–14)*	6 (3–11)*	68 (28.58–111.2)
Leukocytes × 10^3/μL	6.35 (5.8–6.9)	8.4 (7.4–9.9)*	12.75 (10.7–13.6)*	5.4 (5.1–6.1)
Neutrophiles × 10^3/μL	3.45 (2.8–3.96)	6.81 (5.5–7.9)*	10.96 (9.3–12.1)*	3.27 (2.8–4.0)
Neutrophil-to-lymphocyte ratio	1.56 (1.3–1.9)	6.6 (5.8–7.96)*	15.3 (11.6–20.2)*	1.87 (1.6–2.2)

Data are expressed as median (interquartile range)
**Total number of patients with available data
P values were calculated for survivors, deceased, and recovered cases compared to healthy controls
**P* = 0.001 from Kruskal-Wallis test and pos-hoc Bonferroni corrections

after admission in average (range 1–49) and hospital discharge due to improvement happened 11 days after admission (range 3–36), *P* = 0.017. Likewise, the length of hospital stay in patients with < 800/μL lymphocytes at admission were 11 days (1–49), while in those with ≥ 800/μL, it was 13 days (2–48 days), *P* = NS. At the end of the follow-up in hospitalized patients with severe COVID-19 disease, 29/43 (67.5%) with lymphocytes ≥ 800/μL and 14/38 (29%) with lymphocytes ≥800/μL, died [Figure 3]. In the survival analysis, medium survival time when lymphocytes were ≥ 800/μL was 36 days compared to 19 days with < 800 lymphocytes/μL (*P* = 0.002).

For mortality prediction the best cut-off point for NLR was ≥ 8.5 (AUC 0.789, 95%CI 0.685–0.892, *P* = 0.0001), Se 83%, Sp 73%, PPV 68%, NPV 84%; leucocyte count ≥ 10,150/μL (AUC 0.748, 95%CI 0.633–0.862, *P* < 0.0001, Se 72%, Sp72%, PPV 67%, NPV 77%), and neutrophiles ≥ 10,000/μL (AUC 0.749 95%CI 0.639–0.858, *P* < 0.0001, Se 75%, Sp75%, PPV 74%, NPV 71%) was associated to increased mortality.

In the Kaplan-Meier analysis, medium survival time occurred on day 18 (IQR 12–26) when NLR was ≥ 8.5 compared to day 35 (IQR 28–41) with NLR < 8.5, (log rank, Mantel-Cox, *P* = 0.001) [Figure3]. For patients with NLR ≥ 8.5, the relative risk of death increases 11.68 times (95%CI 4.0–33.5, *P* = 0.0001).

In addition, ratios (R) for neutrophiles (N) with each of the lymphocyte subpopulations (CD3+, CD4+, CD8+, CD19+, and NK) associated with mortality were calculated as follows: N/CD3R ≥ 20 (AUC 0.837, Se 80%, Sp 77%, PPV 75%, NPV 82%), N/CD4R ≥ 40 (AUC 0.823, Se 78%, Sp 79%, PPV 76%, NPV 80%), N/CD8R ≥ 50 (AUC 0.822, Se 73%, Sp 75%, PPV 71%, NPV 76%), N/CD19R ≥ 85 (AUC 0.810, Se 78%, Sp 72%, PPV 71%, NPV 79%), and N/NKR ≥ 200 (AUC 0.732, Se 68%, Sp 60%, PPV 59%, NPV 69%).

DISCUSSION

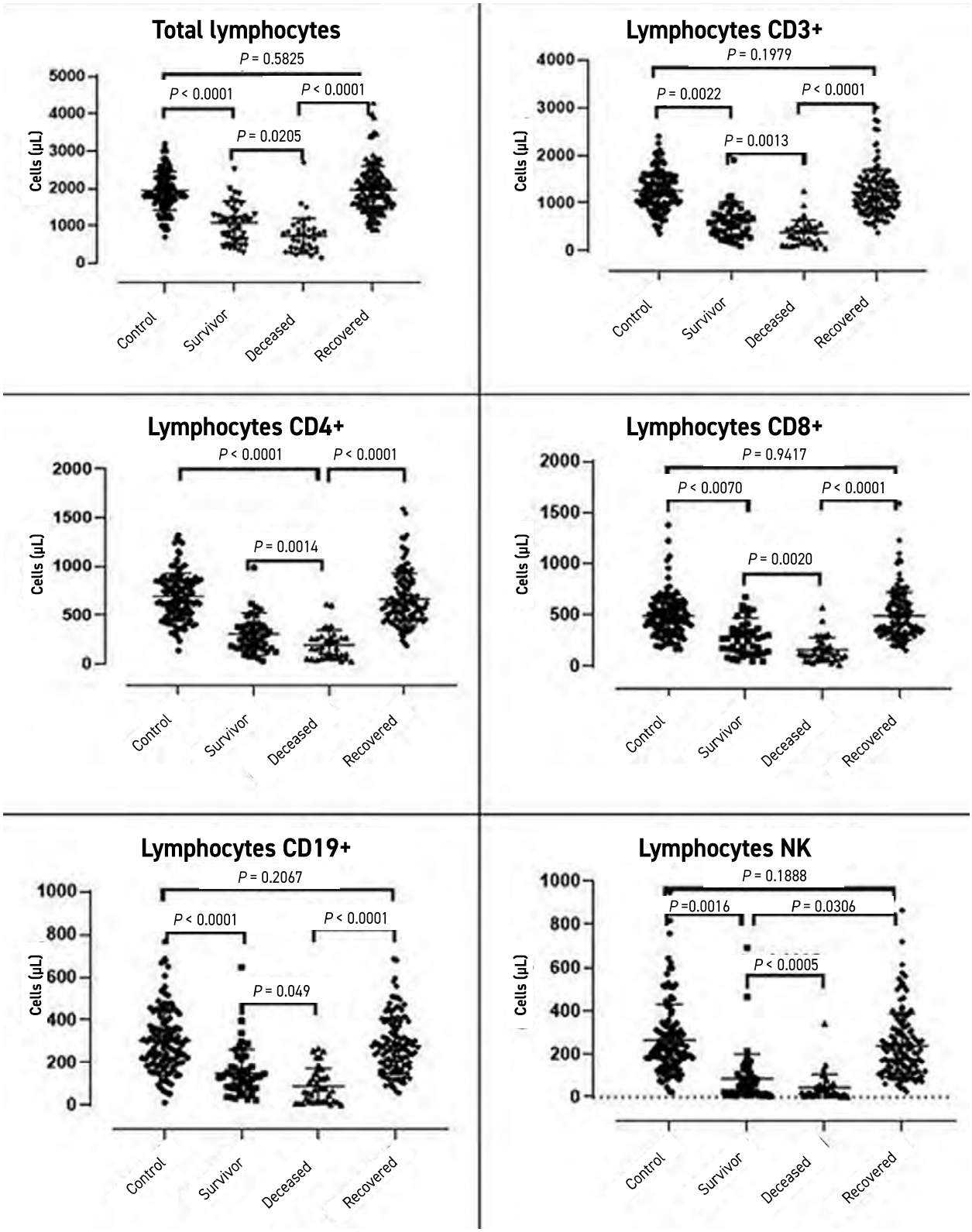
In this study we found that having at least 800/μL total lymphocytes predicted survival, while having neutrophiles > 10,000/μL or NLR ≥ 8.5 at hospital admission predicted mortality in patients with severe COVID-19 disease. Also, having at least CD3+ 400/μL, CD4+ 180/μL, CD8+150/μL, NK 34/μL, or CD19+ 80/μL lymphocyte subpopulations, predicted survival but did not predict hospital stay in those who arrived seriously ill. However, lymphocyte subpopulations in convalescents of COVID-19 disease recovered until they reach the level of healthy controls.

During the peak of the pandemic, COVID-19 patients often arrived at the hospital with severe lung disease and exceptionally low oxygen levels [14]. Some required immediate invasive ventilatory support. Early identification of prognostic factors in severe COVID-19 disease could help decide appropriate interventions. We experienced a mortality of 62.5%. The mortality in patients with severe disease had already been 62% among critically ill patients with COVID-19 and 81% among those requiring mechanical ventilation [12], similar to what we found in our patients.

Previously, the reduction of total lymphocytes to 600–800 × 109/L was identified as a biomarker of poor prognosis in 13 severe cases, compared to 800–1400 × 109/L in mild cases that survived or gradually recovered. Also, CD8+ T cells showed significant decrease in severe cases [15].

In Wuhan, China, in 138 patients hospitalized in the inten-

Figure 2. Total lymphocytes and subpopulations are represented in scatter and median ± IQR. *P* values after comparison with Kruskal-Wallis and Bonferroni corrections post-hoc analysis



sive care unit (ICU), lymphopenia of 800 (500–900 ×109/L) was found compared those who were not admitted to the ICU, with 900 (600–1200 ×109/L). This was the unique biochemical difference found on admission that was associated with mortality [16].

In another study with 27 patients, lymphopenia was commonly associated with severe COVID-19, and commonly after antiviral treatment increased from 900 to 1500 × 109/L. Lymphocyte subsets increased in CD4+ from 472 to 678, while inCD8+, they increased from 219 to 355 per microliter. However, B lymphocytes and natural killer cells did not change significantly [17].

We found that having at least 800 lymphocytes/µL at the time of hospitalization with severe COVID-19 illness was associated with a higher probability of survival. In our patients, the best prognostic indicator was blood levels of CD8+, although lower serum levels in all subpopulations were evidently associated with significant mortality. Nevertheless, the outcome at hospital discharge was not evaluated in this study.

Cytotoxic T lymphocytes and NK cells have an essential role for the control of SARS-CoV-2 infection. On the one hand, peripheral NK and CD8+ T cells are not only markedly decreased in severe COVID-19 disease, but also appear to be exhausted, leading to doubly insufficient antiviral action [18]. On the other hand, lymphocytes showed migration to infected tissues where inflammatory response is associated to increased cytokines production [19]. Although our patients presented a significant decrease in all the circulating lymphocytes studied, the recovered ones showed normal values quickly, unrelated to the time in which they were measured.

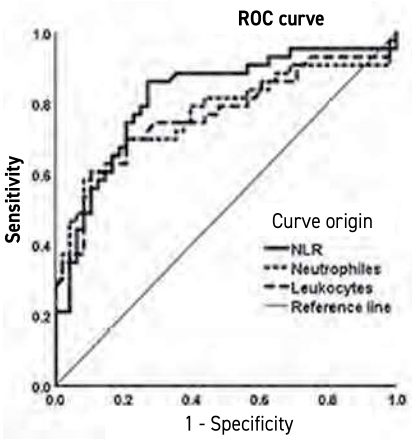
However, in convalescents a greater number of NK cells and B lymphocytes related positively to the number of days since the first symptom. The explanation for this requires further study.

The levels of total lymphocytes reported for mortality are similar to those that we found for predicting survival of patients infected with SARS-CoV-2 pneumonia. A peripheral blood lymphocyte count under 800/µL in a multivariate analysis predicted 4.5 times more risk of mortality [20]. In a cohort of 162 COVID-19 patients in Israel a similar value of 0.8K/ml lymphocytes was found in patients with severe disease [21]. We did not perform any multivariate analysis for mortality risk since the patients included were very homogeneous, with a prognosis of death greater than 50%.

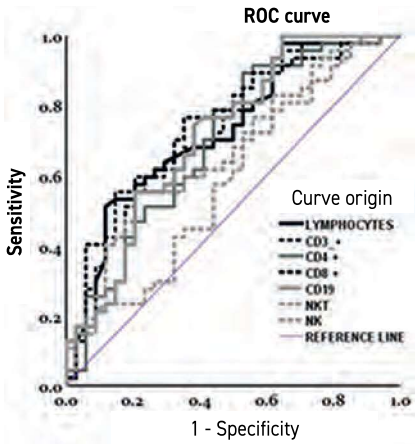
In addition to prognosis, hospital stay is a piece of information that can be predicted in serious diseases such as COVID-19. In a prospective cohort of 1840 patients admitted to a hospital in California and Washington, the ICU stay ranged from 1–30 days, averaging 10.6 days [22]. Hospital stay in severe diseases could be prolonged depending on risk factors such as lymphopenia associated to COVID-19. In our study, the average in length of stay was 16 for deceased and 11 days for survivors. In spite of having mild lymphopenia and better survival, our patients had a hospital stay of approximately 13 days compared to 11 days for patients with severe lymphopenia.

Figure 3. Receiver operating characteristic (ROC) curves for in-hospital mortality by COVID-19 disease, according to lymphocyte subpopulations and Kaplan-Meier, survival probability according to NLR on admission

[A] ROC curve for mortality



[B] ROC curve for survival



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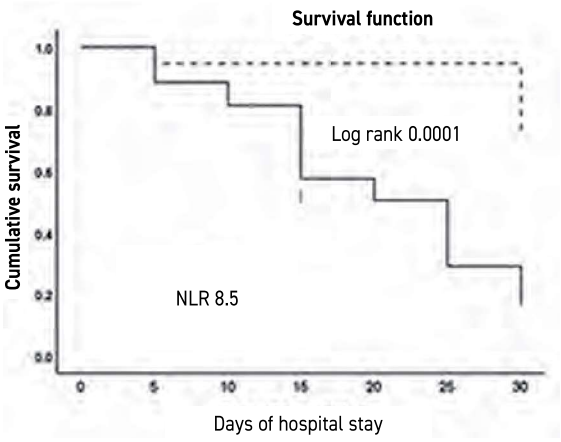

ROC curves used for the selection of the cut-off value in each subpopulation of lymphocytes.
[A] Neutrophiles (AUC 0.749, *P* = 0.0001), NLR (AUC 0.789, *P* = 0.0001)
[B] Total Lymphocytes (AUC 0.726, *P* = 0.001), CD3+ (AUC 0.737, *P* = 0.001), CD4+ (AUC 0.698, *P* = 0.002), CD8+ (AUC 0.742, *P* = 0.001), CD19+ (AUC 0.718, *P* = 0.001), NK (AUC 0.661, *P* = 0.014), NKT (AUC 0.594, *P* = 0.151). Leukocytes (AUC 0.748, *P* = 0.0001)
[C] Kaplan-Meier survival curves, Log rank (Mantel-Cox), *P* = 0.0001
ROC = receiver operating characteristic curve

	Follow-up days						
Number at risk	0	5	10	15	20	25	30
NLR › 8.5	50	45	35	21	16	10	5
NLR ‹ 8.5	40	36	24	14	12	10	4

Regarding NLR, like other studies we found that this baseline ratio predicts mortality. A recent study demonstrated that there was 8% higher risk of in-hospital mortality for each unit increase in this ratio [8]. In another study a value of NLR (8.7) was associated to severity but they did not analyze mortality in COVID-19 [23]. We found that the Neutrophil/lymphocyte subpopulation ratio is equally useful as the NLR in predicting mortality. The cut-off value of the NLR has been variable [8,23]. In an Israeli study in patients with SARS-CoV-2,lymphopenia was also found in 81%, as well as an increased NLR in 68%with values of NLR =5.78 and lymphocytes = 886 K/µl in patients with invasive ventilation [24].

In our study, we found that a NLR > 8.5 showed a high predictive value for mortality; therefore, we propose its use in routine clinical evaluation from admission to take early therapeutic decisions.

CONCLUSIONS

Both innate and acquired immunity are of great relevance, especially for the resolution of severe COVID-19 clinical presentation. Total lymphocytes in blood have the same value in survival prediction as the levels of CD3, CD4, CD8T cell, and NK cells subpopulations. Severe lymphopenia and high NLR are useful, available, and low cost biomarkers of in-hospital mortality. In a convalescent state after COVID-19 disease, the level of lymphocytes and their subpopulations fully recovered.

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A man is known by the company he keeps. A company is known by the men it keeps.
Thomas J. Watson (1874–1956), American businessman

The sun, with all those planets revolving around it and dependent upon it, can still ripen a bunch of grapes as if it had nothing else in the universe to do.
Galileo Galilei (1564–1642), Italian physicist and astronomer