

Severity-related Changes in Laboratory Results During Early Follow-up in COVID-19 Patients Treated with a Novel Cocktail of Stem Cells

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Abstract: *Background and aims:* Laboratory tests may play an important role in the follow-up of COVID-19 patients acting as indicators of risk for severity and death. This study aims to explore the significance of certain laboratory tests in the management of COVID-19 patients treated by an autologous novel stem cells cocktail plus standard care. *Methods:* The 69 hospitalized COVID-19 patients recruited in the experimental arm of a clinical trial [NCT04473170] were divided into moderate or severe groups as recommended by WHO. Initial and after 21 days of treatment, laboratory data were analyzed and compared for both groups. The variable association was analyzed using the symmetric Spearman correlation matrix. Multiple linear regression was used for biomarkers most described in COVID-19 by a multivariate study and disease severity association using relative risk (RR) to laboratory variables. *Results:* Positive and strong associations were evidenced between parameters related to coagulation and inflammation markers. We found the strongest positive relationship between the LDH enzyme and IL-6 ($r=0.81$), followed by D-dimer ($r=0.70$). The multivariate study showed a strong influence of D-dimer, IL-6, IgG, and ceruloplasmin on the increased LDH level, with a greater influence of the last ($R=0.71$, $p<0.0001$). RR showed a statistically significant and positive association with COVID-19 severity for WBC ($RR=45.2$); neutrophil/lymphocyte ratio (NLR) ($RR=3.8$); IL-6 ($RR=1.6$); lymphocyte/monocyte ratio (LMR) ($RR=1.5$); and $RR=1.3$ for platelets/lymphocyte ratio (PLR), ferritin, and LDH. *Conclusions:* Risk assessment of severity using this laboratory variable is important.

Keywords: COVID-19, SARS-CoV-2, Clinical Decision-Making, Risk Assessment, Risk Factors, Prognosis, IL-6, LDH

1. Introduction

Coronavirus disease 2019 (COVID-19) is a pandemic infectious disease caused by the novel Severe Acute

Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) [1]. Since its outbreak in Wuhan in December 2019, COVID-19 has affected more than 191 million patients all over the World and caused more than 4 million deaths, according to the World

Health Organization (WHO) [2].

The 2020 COVID-19 outbreak in Abu Dhabi was a *leitmotiv* for our group to assess a clinical intervention using a cellular therapy product containing autologous peripheral blood non-hematopoietic enriched stem cells cocktail (PB-NHESC-C) delivered by jet-nebulization plus the standard care provided by the UAE Ministry of Health and Prevention (MOHAP) COVID-19 guidelines [3].

The clinical spectrum of COVID-19 varies widely, going from asymptomatic to severe clinical conditions characterized by dyspnea and lethal complications, such as acute respiratory distress syndrome (ARDS), multi-organ failure, and septic shock [4] WHO recommended an ordinal 8-scores scale to monitor patient's management mainly based on oxygen saturation levels and other symptoms [5], but without including any laboratory variables. While mild or moderate disease can be exhibited by 80% of patients, severe and critical conditions were diagnosed in the remaining 20% [6].

Accumulating laboratory data revealed various abnormalities such as coagulopathy, myocardial injury, liver damage, kidney injury, and immune dysfunction in patients with severe COVID-19 [7–9], particularly in those fatal cases [10–13], but also in some patients with moderate disease. However, few studies assessed the role of laboratory markers in COVID-19 severity, progression, and outcome [8, 14, 15].

Therefore, a COVID-19 patient follow-up was performed by laboratory tests due to the increasing scientific interest in the searching for serological markers to shape the severity of COVID-19 and attribute their clinical importance, focusing on inflammatory markers as suggested by several authors [16–22]. All patients were clinically classified into two groups, moderate or severe disease, and recruited into the investigational arm of the clinical trial [ClinicalTrials.gov identifier (NCT number): NCT04473170].

2. Methods

2.1. Study Participants

A total of 69 patients with confirmed positivity to SARS-CoV-2 by a molecular test of nasopharyngeal swabs, a method based on World Health Organization (WHO) interim guidance for laboratory diagnosis of COVID-19 [23] were recruited, during hospitalization between April-July 2020 at Abu Dhabi SEHA hospitals, as part of the investigational treatment arm of the clinical trial. Therefore, patients were treated with a novel cocktail of autologous peripheral blood non-hematopoietic enriched stem cells (PB-NHESC-C) plus the conventional standard care approved in UAE and monitored for 28 days [3].

WHO classified disease severity by the guidelines of diagnosis and management for COVID-19 [5]. Thus, subjects were categorized into six scores numbers, from those eight ordinal numbers scale established by the guidance, and related to their disease severity and clinical manifestations on the day before treatment: Scores 5 to 7 represent patients hospitalized with severe disease (S-COVID): score seven associated to those

under ventilation plus additional support such as pressure, renal replacement therapy, and extracorporeal membrane oxygenation, score 6 to those with intubation and mechanical ventilation, and score 5 to patients with no invasive ventilation or high flow oxygen. While scores 4 - 3 were designed for hospitalized patients with moderate disease (M-COVID): score 4 associated with those using oxygen by mass or nasal prongs, and score 3 for hospitalized patients without oxygen therapy. Scores 2 -1 represent patients hospitalized and with ambulatory conditions; score 2 is associated with some limitation of activities, while score 1 is to those completely asymptomatic (non-hospitalized or released already from Hospitals). Dead patients are receiving a score of 8.

This work has been carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, as part of the clinical trial approved by a special conditional letter received from the Ministry of Health and Prevention of the UAE, via the Emirates Institutional Review Board (IRB) for COVID-19 Research Committee, on June, 25th 2020.

2.2. Treatment Intervention

The autologous stem cell cocktail was prepared from the peripheral blood of the selected patients by a patented procedure developed in the ADSCC Stem Cell Laboratory [24]. Then, it was administrated to the correspondent patient using a compressor (jet) nebulization at a flow rate of 5-6 L/min, as a total of two doses, 24 hours apart. The PB-NHESC-C product was characterized by Flow Cytometry and Immunofluorescence Microscopy techniques. In addition, other biochemicals test procedures were assessed for their complete characterization, like COVID-19 antibody titration and platelets-derived growth factors (PDGF) quantification [25].

2.3. Patients Clinical Variables Data Collection

The attending physicians collected and independently reviewed clinical data, including demography, medical history, clinical manifestations, laboratory blood test results, and clinical outcomes. We focused on the comprehensive laboratory results, including the following seven categories: the complete blood cell count {total white cells and platelets counts}, counted by Hematology Analyzer DHX900 (Beckman Coulter, USA), coagulative state {D-dimer, fibrinogen} using a Cobas t-511 (Roche, Switzerland), kidney function markers (Creatinine), humoral immune profile (C3, C4, IgG, IgA, IgM), cell damage (lactate dehydrogenase enzyme: LDH), and inflammatory factors (ferritin, IL-6, ceruloplasmin, and C-reactive protein: CRP), using a Chemistry Analyzer AU480 (Beckman Coulter, USA), as well as erythrocyte sedimentation rate (ESR), analysis of each patient was done on admission and about 21 days after initiation of treatment, according to the manufacturer's instructions and in compliance with ADSCC Standard Operation Procedures. In addition, SARS-Cov-2 RNA was assessed by real-time reverse transcription-PCR for diagnosis before and after treatment [23].

2.4. Statistical Analysis

No imputation was made for variables with missing data. Quantitative data with non-normal distribution were expressed as median values and interquartile range and statistically compared by Mann-Whitney U non-parametric test. Spearman correlation matrix was applied to analyze the possible association between laboratory parameters. A multiple linear regression model was further used to evaluate the contribution of other parameters in these variables. The relative risk (RR), with 95% confidence intervals, was calculated for the severe clinical condition regarding the rest of the laboratory parameters evaluated. Validation was carried out using a residual distribution graph. All statistical analyses were performed using Graph Pad software v.8 (La Jolla, USA) [26] and Med-Calc (<https://www.scistat.com/index.php>) [27]. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patient Characteristic

3.1.1. Gender and Nationalities

Data of the 69 patients with COVID-19 were analyzed. From them, 65 (94.20%) masculine and 5 (5.79%) feminine with 13 different nationalities (Afghanistan, Bangladesh, Egypt, India, Indonesia, Nepal, Pakistan, Palestine, Philippines, Somalia, Sudan, Syria, and the United Arab Emirates).

3.1.2. Patient's Clinical Scores Attribution

Of 49 (71.01%) patients classified as the moderate COVID-19 group (M-COVID), 37 (53.62%) were scoring 3, and 12 (17.39%) to score 4. Whereas in the severe group (S-COVID) of 20 (28.98%) patients, 3 (4.34%) represent the score 5, 2 (2.89%) the score 6, and 15 (21.74%) the critical subtype score 7.

3.1.3. Comorbidities

The comorbidities frequency by groups was 65% in S-COVID and 38.77% in M-COVID, respectively, with a moderate statistical significance ($p=0.0491$). Furthermore, only a unique type of comorbidity among the patients was reported in 38.46% and 24.48% in S-COVID and M-COVID groups, respectively ($p=1.00$). The pattern of various types of comorbidity was distributed differently between both study groups. The most frequently reported in M-COVID was hypertension: 13/49 (26.53%); followed by Diabetes mellitus: 8/49 (16.32%); obesity as well as dyslipidemia: 3/49 (6.12%). Anemia, asthma, and chronic renal disease represented 1/49 (2.04% of affected cases). In the S-COVID group, common comorbidities were: Diabetes mellitus in 9/20 patients (45.00%), followed by arterial blood hypertension 5/20 (25.00%), asthma 4/20 (20.00%), and dyslipidemia 3/20 (15.00%).

3.2. Laboratory Findings

3.2.1. Correlations Between Serum Inflammatory and Coagulation Markers Among Laboratory Variables

Using the non-parametric Spearman's correlation coefficient, relations between laboratory data with statically significant and positive correlations were observed between 8 clinical parameters related mainly to coagulation and inflammation; Indeed, fibrinogen level was positively associated with C3 ($r=0.51$) and ceruloplasmin ($r=0.51$). Moreover, a positive correlation was also determined between ESR and Ceruloplasmin ($r=0.63$), and ferritin level was positively associated with markers of inflammation, LDH ($r=0.58$), and ESR ($r=0.59$). Finally, a statistically significant and positive correlation was observed between LDH and IL-6 ($r=0.81$) as well as LDH and D-dimer ($r=0.70$). In an inverse relationship, no associations were found statistically significant (Shown in Figure 1).

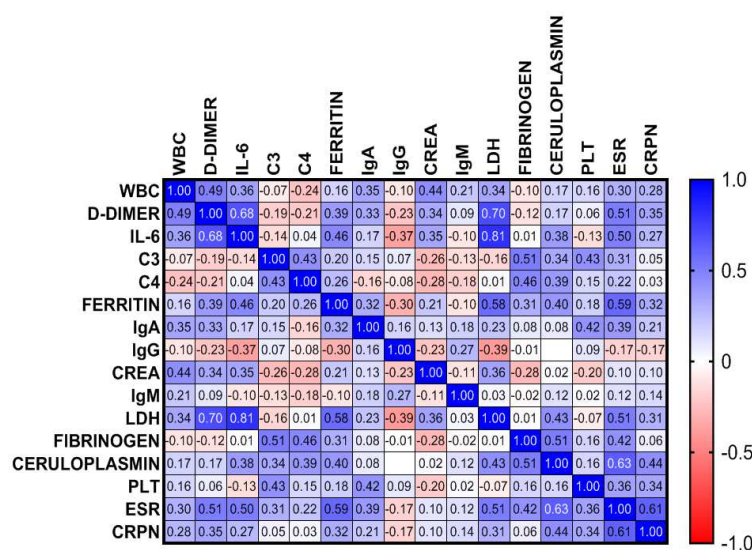


Figure 1. Symmetric Spearman correlation matrix between relevant clinical laboratory variables.

Legend: CREA: Creatinine; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; PLT: Platelets; LDH: Lactate dehydrogenase enzyme; IL-6: Interleukin-6; WBC: White Blood Cells.

3.2.2 Multivariate Linear Regression Analysis to Identify the Association Between Possible Predictive Biomarkers in COVID-19

A strong influence of D-dimer, IL-6, IgG, and ceruloplasmin on the increased level of LDH was found, with the more significant impact of the latter ($R=0.71$, $p<0.0001$, F-statistic test). The validation of the model was carried out with a residual distribution graph, the model that best fits is with the incorporation of all the variables of the study as explanatory, and nevertheless, a certain tendency to an open distribution pattern of the residuals was visualized, suggesting that the variance is not constant.

Furthermore, a comparison between blood and biochemical parameters, at the starting time of treatment, revealed that S-COVID compared to M-COVID group, has a significant

increase in IL-6 (450.06 vs. 10.05 pg/mL; $p=0.0001$), CRP (207.1 vs. 22.4 mg/L; $p=0.0001$), LDH (421 vs. 203 U/L; $p=0.0001$), D-dimer (1.78 vs. 0.3 $\mu\text{g/mL}$; $p=0.0001$), ESR (97.5 vs. 51.0; $p=0.0007$), ferritin (796.6 vs. 495.9 ng/mL; $p=0.0209$), creatinine (1.165 vs. 0.89 mg/dL; $p=0.0243$) and both neutrophil/lymphocyte ratio (NLR) (9.76 vs. 2.23 U; $p=0.0001$), and platelets/lymphocyte ratio (PLR) (293 vs. 167.8 U; ($p=0.0001$), due to a significant decrease in the lymphocytes. A significant increase tendency was observed in fibrinogen (545.5 vs. 790.0 mg/dL; $p=0.0086$), C3 (134.1 vs. 158.2 mg/dL; $p=0.0285$), C4 (37.04 vs. 48.68 mg/dL; $p=0.0413$), Hemoglobin (12.65 vs. 14.5 g/dL; $p=0.0001$), and lymphocyte/monocyte ratio (LMR) (1.50 vs. 2.67 U; $p=0.0001$) (Shown in Figure 2).

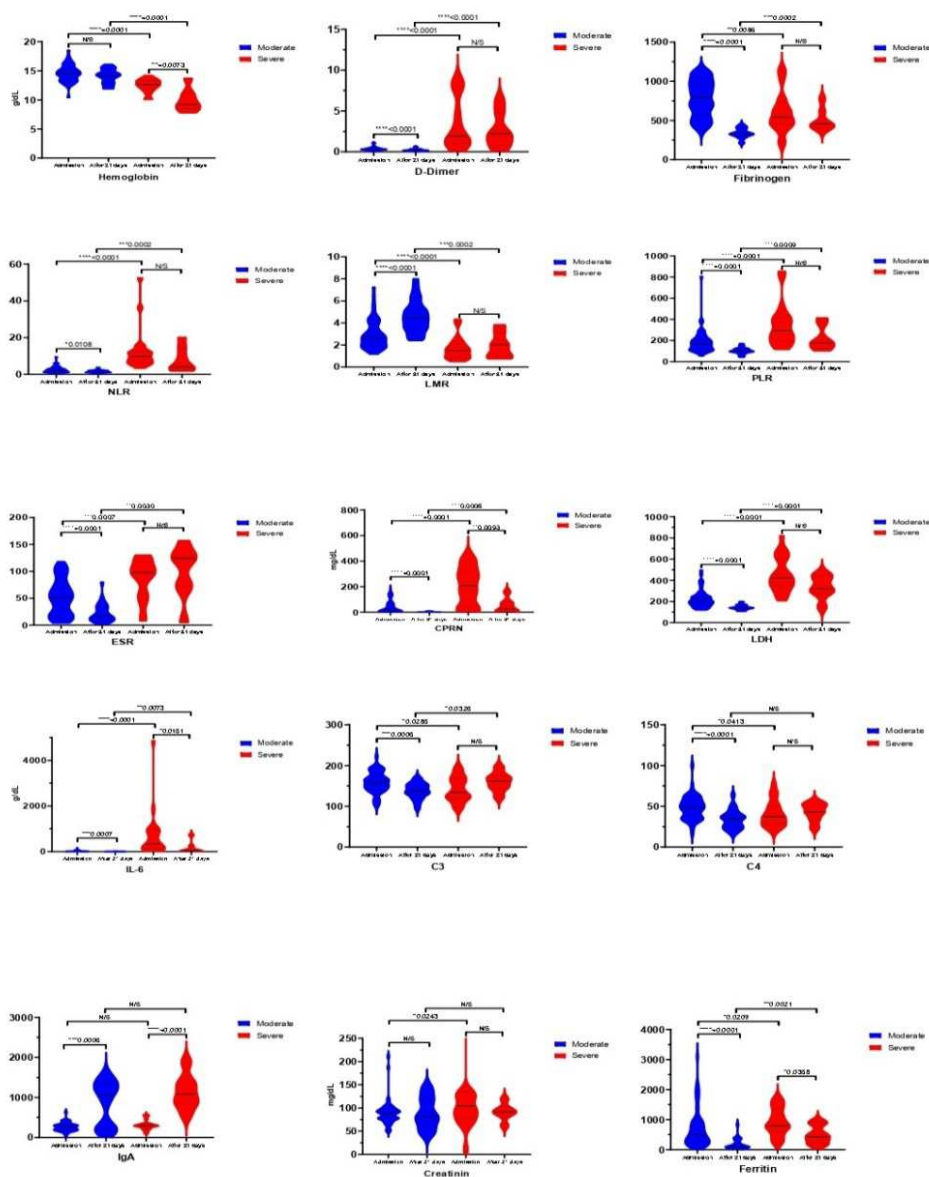


Figure 2. Median comparison of quantitative laboratory main variables using univariate analyzes by the Wilcoxon-Mann Whitney test.

Legend: CPRN: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; IL-6: Interleukine-6; LMR: Lymphocyte/Monocyte Ratio; NLR: Neutrophil/Lymphocyte Ratio; N/S: non-significant statistical difference; PLR: Platelets/Lymphocyte Ratio; *: $p<0.005$; **: $p<0.005$; *** $p<0.0005$.

Besides, when comparing the level of each parameter mentioned above, after 21 days of treatment, no relevant changes in the variation pattern were found between groups. Regarding the median IgA serum level, there was no statistical difference between both subgroups of patients, neither at the starting nor at the follow-up evaluations. Interestingly, comparing the treatment effect in each group, we found statistically significant differences among the laboratory parameters in M-COVID. Indeed, a substantial decrease in D-dimer, fibrinogen, NLR, LMR, PLR, ESR, CRP, LDH, IL-6, C3, C4 and ferritin was observed. Perhaps due to the efficacy of the novel treatment in alleviating the severity of the disease. Unfortunately, this decreasing tendency of laboratory markers was not observed in the same way in the S-COVID group.

3.2.3. Relative Risk Analysis for Severity

The clinical COVID-19 severity was related to alterations in blood test laboratory results; therefore, a further investigation was made taking the severe COVID-19 condition to evaluate the risk factor (RR) of some selected laboratory variables.

It was found that on the day before starting the stem cells cocktail treatment, some acute phase reactants showing alterations related to severity, which was accentuated after 21 days of treatment. At the starting evaluation time, RR showed a positive and statistically significant association for results like WBC (RR=45.2); NLR (RR=3.8); IL-6 (RR=1.6); LMR (RR=1.5); PLR (RR=1.3); ferritin (RR=1.3); and LDH (RR=1.3). Twenty-one days later, the magnitude of these RRs values increased considerably, except for WBC (RR=40.6); for the rest, NLR changed to RR=17.0, IL-6 to RR=5.7, MRL to RR=4.2, PLR to RR=3.4, ferritin to RR=2.9, and LDH to RR=2.6. Thus, anemia was associated 8.9 times more in severe patients than in moderate ones, and after 21 days, this association rose to 30.9 times. The same tendency was observed with the D-dimer (RR=3.5 moved to 6.8, respectively). Nevertheless, non-statistically significant associations were found for ESR, fibrinogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), C3, and C4 complement components (as seen in Figure 3).

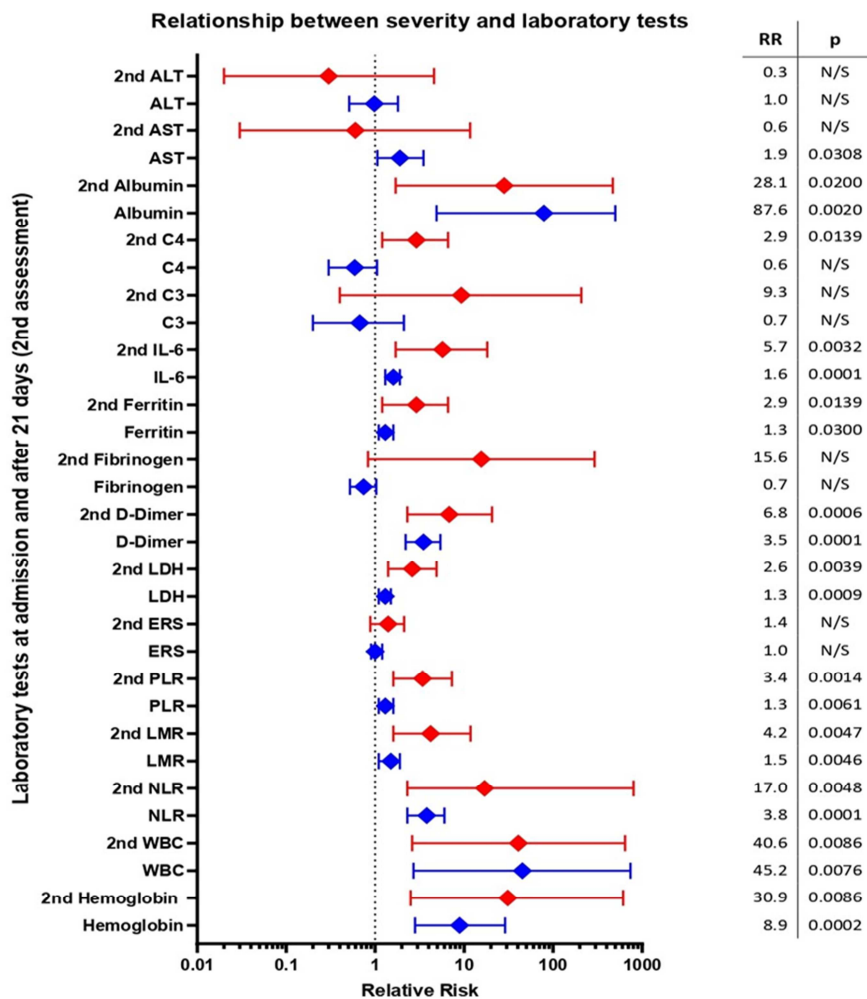


Figure 3. Relative Risk for COVID-19 severity of Laboratory test performed.

Legend: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ESR: Erythrocyte Sedimentation Rate; IL-6: Interleukine-6.; LDH: Lactate dehydrogenase enzyme; LMR: Lymphocyte/Monocyte Ratio; NLR: Neutrophil/Lymphocyte Ratio; N/S: Non-significant; p: statistical probability value; PLR: Platelets/Lymphocyte Ratio; PLT: Platelets; RR: Relative Risk; WBC: White Blood Cells.

4. Discussion

Comparing M-COVID and S-COVID groups in their hematological and biochemical profiles at the time of admission showed highly significant differences in all the parameters evaluated, indicating a positive association to the severity of the disease. Indeed, the findings showed a positive and robust association between parameters related to coagulation and acute phase reactants (APR), like D-dimer, fibrinogen versus IL-6, C3, C4, ESR, and ceruloplasmin. In addition, ferritin was also associated with markers of inflammation, and the most robust positive relationship was found between the LDH and IL-6, followed by D-dimer.

It has been postulated that SARS-CoV-2 may target alveolar macrophages via the angiotensin-converting enzyme-2 (ACE2) receptor, leading to physio-pathological mechanisms that mobilize a wide variety of biomolecules, mainly immunological. In the most severe cases, the prognosis can be markedly worsened by the hyperproduction of mainly proinflammatory cytokines, such as IL-1, IL-6, IL-12, IFN- γ , and TNF- α , preferentially targeting lung tissue [28].

The clinical characteristics of 140 patients and WBC levels were evaluated in a previous study done in China by Zhang et al. [8]. They found normal absolute total WBC but with decreased lymphocyte and eosinophil counts in most patients at admission. Their serial follow-up testing demonstrated further decreases in lymphocyte and eosinophil counts. These changes were confirmed by us when we found a positive correlation between decreased lymphocytes and disease severity.

Our study showed a significant difference between S-COVID and M-COVID groups at the starting time of treatment in the serum level of IL-6, CRP, LDH, D-dimer, hemoglobin, NLR, LMR, and PLR, followed by ESR, fibrinogen, ferritin, creatinine, C3, and C4. In this regard, our findings agree with a previous retrospective study reporting a significantly severe lymphopenia and a higher NLR, D-dimer, and fibrinogen levels [17]. The serial measurements of NLR and D-dimer levels also differed significantly between the two groups. Another study suggested that elevated NLR was significantly associated with disease severity, and it was an independent biomarker showing bad clinical outcomes [18]. Leukocytosis due to neutrophilia and lymphopenia with low CD3, CD4, CD8 subset absolute counts and or percentages and increased NLR were also associated with S-COVID. At the same time, thrombocytopenia was more observed in severe or critically ill and fatal cases [19, 20].

In the second evaluation 21 days later, similar differences between both groups were found in our series, among the same markers, like LDH, hemoglobin, and D-dimer. However, IL-6, NLR, LMR, PLR, ESR, fibrinogen, ferritin, and C3 slightly varied, but still significantly. No significant differences persisted when comparing creatinine and C4 in both groups.

At the initial evaluation and 21 days after the treatment, using the more commonly applied laboratory tests to evaluate

COVID-19 patients, we found some alterations of APR related to the severity with a further accentuation at 21 days. This result indicates the relevance of these variables as prognostic severity markers for COVID-19, independently of the treatment applied. At the starting time, we found a positive and statistically significant association for variables like WBC, NLR, IL-6, MRL, PLR, ferritin, and LDH. After 21 days, most of these statistic associations increased considerably, except for the WBC. It is essential to point out that about 21 days of the follow-up, NLR presented a 4.47 –times increased association with the severity, followed by IL-6 that showed 3.5, MRL 2.8, PLR 2.61, ferritin 2.2, LDH 2- times increased association. All of them are related to the severity.

On the other hand, anemia was associated 8.9 times more in S-COVID than in the M-COVID group at the initial evaluation. This association rose to 30.9 times after 21 days, and almost the same happened with the D-dimer. Other authors also found neutrophil counts, CRP, LDH, and ALT had an excellent predictive value for severity in follow-up studies [18]. Our data also revealed the importance of MRL, PLR, and ferritin.

Different studies analyzed and tried to identify correlation with disease progression of inflammatory markers. As in our series, statistically significant differences were found in the serum levels of CRP, ferritin, and IL-6, comparing M-COVID to S-COVID and critically ill groups. Supported by other studies, this study concluded that CRP, ferritin, and IL-6 levels were associated with disease progression [18–20].

Concerning coagulation markers, one large retrospective study also demonstrated that prothrombin time (PT), D-dimer, and fibrin degradation products (FDP) were significantly higher in critically ill patients compared with those with mild and severe disease. In addition, marker levels correlated positively with disease severity, sepsis-induced coagulopathy, and disseminated intravascular coagulation (DIC) scores are increased over time during COVID-19 progression [21]. Another study suggested coagulation parameters have good predictive value and discriminate between mild, severe, and critical disease states. They found significant coagulation abnormalities in patients with SARS-CoV-2 infection compared with healthy controls. Indeed, monitoring coagulation parameters may prove helpful for the early identification of severe cases [20], as in our series, the D-dimer marker showed.

Due to the short number of cases in this study, we could not perform a statistical analysis of the relevance of comorbidities. Nevertheless, other studies have demonstrated an association of the severity, disease progression, and adverse outcome for older male individuals (from 60 years old) and hypertension, Diabetes mellitus, cardiovascular disease, and a high body mass index [29-31]. However, only Diabetes mellitus and asthma showed a significative different frequency in severe compared with moderate cases. However, another group found inconsistent results for chronic obstructive pulmonary disease and asthma as risk factors [29].

Several studies compared the hematologic, biochemical,

inflammatory, and coagulation parameters in moderate and severe COVID-19 disease and reported comparable findings to our research [9, 32]. Han H *et al.* in China informed that CRP, ferritin, LDH, and ALT were significantly higher in severe cases than mild cases [33]. As the disease progressed from mild to severe or critical, a downward trend for lymphocytes and pre-albumin and albumin was observed. In contrast, the opposite was seen for total WBC count, neutrophil count, CRP, and LDH [34, 35]. A progressive worsening of specific laboratory parameters—including the already mentioned decreased lymphocyte count and increased NLR, CRP, ferritin, IL-6, IL-10, some coagulation parameters, and serum viral load were observed as the disease progressed. However, lymphocytopenia and age appeared to be the most critical determinant of disease severity [36].

Changes in coagulation parameters, including prolonged PT, elevated D-dimer, and elevated fibrinogen or FDP, were common findings in severe disease and no survivors [9, 37]. Prolonged PT and higher serum D-dimer levels are related to a hypercoagulable state rather than consumptive coagulopathy, and hyperfibrinogenemia leads to fibrin polymerization, thrombus formation, and eventually, complications or adverse outcomes [38].

Other biomarkers, such as LDH, creatinine kinase (CK), brain natriuretic peptide (BNP), AST, and ALT, have been associated in several studies with severe and critically ill diseases, and their levels likely indicate the adverse outcome [39, 40]. For example, the leading cause of death in SARS-CoV-2 infection was acute respiratory distress and respiratory failure. Still, systemic involvement with end-organ damage, including sepsis, thrombotic or hemorrhagic events, cardiac failure, liver failure, and renal failure, also contributed to the death [41].

Limitations for interpreting our results and the results of several other studies mentioned in this work are various. First, retrospective study designs predominate in many author's reports. Other causes are small sample sizes, multiple sampling biases, and lack of exact timeline of laboratory sample collection. Nevertheless, the information provided shows the importance of a defined timeline of studies, and serial sampling performance may help in clinical decision-making during the acute phase of the disease.

5. Conclusions

The severity of COVID-19 patients treated with the novel non-hematopoietic enriched stem cells was related to specific laboratory parameter alterations and significant heterogeneity in the range of results non-seen in the moderate COVID-19 patients treated with the same investigational product. Fundamentally, they were associated with anemia, inflammation markers, and coagulation disorders.

The results suggest a significant trend towards improvement and normalization of laboratory results in moderate patients. The severity of the alteration due to inflammation and cell damage markers was more significant in severe cases even after 21 days of follow-up.

As with other reports, our study suggests using serial measurements of these markers to predict disease course, severity, and even mortality. The relative risks calculated showed that the severity is associated with increased inflammation markers and coagulation alterations, especially for those parameters whose association strength increases at 21 days of follow-up.

Therefore, our study concludes that the dynamic changes in biomarker levels like WBC, NLR, IL-6, MRL, PLR, ferritin, LDH, hemoglobin, and D-dimer, may assist in predicting disease course, prognosis, and outcome, as another good way to monitor COVID-19 patients. However, we do not exclude the use of the WHO proposed severity score based on respiratory oxygen needs and other clinical complications found in critically ill patients. Consequently, prospective studies with larger cohorts and serial measurements with defined sampling collection timelines are imperative to confirm the current findings further.

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