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ORIGINAL INVESTIGATION / ÖZGÜN ARAŞTIRMA

Potential Biomarkers in the Diagnosis of Hemophagocytic Syndrome in COVID-19 Patients

COVID-19 hastalarında Hemofagositik Sendrom Tanısında Potansiyel Biyobelirteçler

Barış Boral[1], Erdinç Gülümsek[2], Hilmi Erdem Sümbül[2], Bilge Sümbül[3], Akkan Avcı[4]

[1]Department of Immunology, University of Health Sciences, Adana Health Practice and Research Center, Adana, Turkey
[2]Department of Internal Medicine, University of Health Sciences, Adana Health Practice and Research Center, Adana, Turkey
[3]Department of Medical Microbiology, Bezmialem Vakif University Faculty of Medicine, Istanbul, Turkey
[4]Department of Emergency Medicine, University of Health Sciences, Adana Health Practice and Research Center, Adana, Turkey

ABSTRACT

Objective: Hemophagocytic syndrome (HPS) is one of the very severe immunologic complications a COVID-19 patient may exhibit. HPS can cause cytokine storm syndrome and acute respiratory distress syndrome both of which can be fatal. The aim of this study was to determine the immunologic differences caused by HPS in COVID-19 patients early on so that appropriate treatment of immunologic complications can be provided.

Method: This study was a planned prospective study. Thirty patients who initially did not have HPS hospitalized for COVID-19 were included in the study. These patients were later separated into two groups based on having HPS or not. Both groups were examined according to CD subgroups, NK, and HLA parameters at the time of admission.

Results: The mean age and gender distribution of the HFS– and HFS+ patient groups were not statistically significant between them. Statistically significant results were obtained in the markers AST, LDH, albumin, CD3 (%), CD19 (%), and CD21 MFI (mean fluorescence intensity). In the univariate statistical analysis performed with CD21, correlations were found with age, lymphocyte count, CD3, and CD19 values. In linear regression analysis performed with these four parameters, which were found to be correlated, a negative relationship with age was confirmed. When the NK cells and NK cell subgroups (CD56bright/CD16–, CD56dim/CD16+) were examined, no statistically significant difference was found between the groups.

Conclusion: More frequently used laboratory markers such as CD3, CD19, and CD21 may be beneficial in the early diagnosis and management of treatment for HPS patients with COVID-19.

Keywords: CD3, CD19, CD21, COVID-19, hemophagocytic syndrome, peripheral lymphocyte subsets

ÖZ

Amaç: Hemofagositik Sendrom (HPS), bir COVID-19 hastasının sergileyebileceği çok ciddi immünolojik komplikasyonlardan biridir. HPS, her ikisi de ölümcül olabilen sitokin fırtınası sendromuna ve akut solunum sıkıntısı sendromuna neden olabilir. Bu çalışmanın amacı, COVID-19 hastalarında HPS'nin neden olduğu immünolojik farklılıkları erken dönemde belirleyerek immünolojik komplikasyonların uygun tedavisinin sağlanabilmesidir.

Yöntem: Bu çalışma prospektif araştırma olarak planlandı. Başlangıçta COVID-19 nedeniyle hastaneye yatırılan HPS'si olmayan 30 hasta çalışmaya dahil edildi. Bu hastalar daha sonra HPS olup olmamasına göre iki gruba ayrıldı. Her iki grup, başvuru sırasındaki CD alt grupları, NK ve HLA parametreler açısından incelendi.

Bulgular: HFS- ve HFS+ hasta gruplarının yaş ortalaması ve cinsiyet dağılımları açısından aralarında istatistiksel olarak anlamlı değildi. AST, LDH, albümin, CD3 (%), CD19 (%) ve CD21 (MFI - ortalama floresan yoğunluğu) belirteçlerinde istatistiksel olarak anlamlı sonuçlar elde edildi. CD21 ile yapılan tek değişkenli istatistiksel analizde yaş, lenfosit sayısı, CD3 ve CD19 değerleri ile korelasyonlar bulundu. İlişkili olduğu tespit edilen bu 4 parametre ile yapılan lineer regres-

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Address for Correspondence/Yazışma Adresi: Akkan Avcı, Department of Emergency Medicine, University of Health Sciences, Adana Health Practice and Research Center, Adana, Turkey
E-mail: drakkanavci@gmail.com **ORCID ID:** 0000-0002-4627-0909

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yon analizinde yaş ile negatif bir ilişki doğrulandı. NK hücreleri ve NK hücre alt grupları (CD56bright/CD16-, CD56dim/CD16+) incelendiğinde gruplar arasında istatistiksel olarak anlamlı bir fark bulunmadı.

Sonuç: CD3, CD19 ve CD21 gibi rutin laboratuvarlarda daha sık kullanılan belirteçler, COVID-19'lu HPS hastalarının erken tanısında ve tedavisinin yönetiminde faydalı olabilir.

Anahtar kelimeler: CD3, CD19, CD21, COVID-19, hemofagositik sendrom , periferik lenfosit alt grupları

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has rapidly affected the whole world after the first case appeared in Wuhan, China, in December 2019. It is an infectious disease, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It transmits among people via droplets and physical contact.[1,2]

COVID-19 infection exhibits different symptoms among patients. The infection may be asymptomatic among some patients or it may cause only mild symptoms. The asymptomatic patients or the patients who have mild symptoms cover the majority of the cases.[3] However, other patients may show severe immunologic complications such as hemophagocytic syndrome (HPS). HPS can cause cytokine storm syndrome and acute respiratory distress syndrome both of which can be fatal.[4]

For patients who show severe immunologic complications, effective treatment strategies are needed. Toward this goal, understanding the dynamic progression of SARS-CoV-2 infection is crucial. One aspect of the progression of the infection can be monitored via lymphocyte subsets as these cells play an important role in the continuation of immune system function.[5] Therefore, it is important to study lymphocyte subsets, which can enable us to develop novel biomarkers and therapeutic strategies for the early detection of patients who experience serious immunologic complications such as HPS. After virus infection, the total lymphocyte numbers and the subsets show different behaviors with different virus types. This indicates a potential correlation between lymphocyte subset alteration and viral pathogenic mechanism.[6] Recent studies showed that there is a clear decrease in the number of peripheral lymphocytes in COVID-19 patients, and this decrease precedes even the abnormal changes on the chest X-ray.[7–9] Even though it is reported that the number of peripheral lymphocytes decreases in COVID-19 patients, changes in lymphocyte subsets are still unknown, particularly in COVID-19 patients with HPS.

In this work, we study the peripheral lymphocyte subset alteration in COVID-19 patients. Among these patients, a subset of them has HPS. We explore a potential biomarker for the early detection of HPS. This study can help us to clarify pathogenesis and develop novel therapeutic strategies for COVID-19. Early recognition and appropriate treatment of immunologic complications can decrease the morbidity and mortality in COVID-19 infection.

METHOD

Study Population

This study was conducted with the necessary permission from Adana City Research and Training Hospital Ethics Committee (Adana City Research and Training Hospital, Clinical Research Ethics Committee, Date: April 8, 2020, Meeting Number: 54, Decision Number: 788). We recruited 30 inpatients with active COVID-19 with none of them initially having HPS. Nine of them later had HPS+, whereas 21 of them did not have HPS–.

We measured various immunologic and biochemical parameters at the time of their admission while still none of them were HPS+. Later, 9 patients were moved to HPS+ state based on the diagnostic guidelines by Henter et al.[10] and Janka,[11] which provided revised guidelines of the HLH Study Group of the Histiocyte Society.[12] Specifically, patients were categorized as HPS+ if five of the following eight criteria were positive: having at least two cell lines (hemoglobin<9 g/dL, platelet<100 000 µL, neutrophils<1000 µL) with cytopenia, fever greater than 38.5°C for more than 7 days, splenomegaly (ferritin>500 µg/L, fasting triglyceride>3 mmol/L and/or>60mg/dL, fibrinogen<150 mg/dL, sCD25=>2400 U/mL), decreased or absent NK cell activity, and hemophagocytosis in bone marrow, CSF, or lymph node. Among these criteria, we did not check the hemophagocytosis in bone marrow, sCD25, and NK cell activity, which leaves us with five criteria, and all of them should be positive for HPS+ diagnosis.

Real-Time Reverse Transcription PCR

We enrolled 30 patients with COVID-19, which was confirmed by detecting SARS-CoV-2 RNA in nasopharyngeal swab samples using a SARS-CoV-2 nucleic acid detection kit according to the manufacturer's protocol (Bioeksen, İstanbul, Turkey). All of these patients were initially admitted to Adana City Hospital from April 7 to July 14, 2020.

Data Collection

The following information on each patient was extracted from electronic medical records: age, sex, medical history, and laboratory findings.

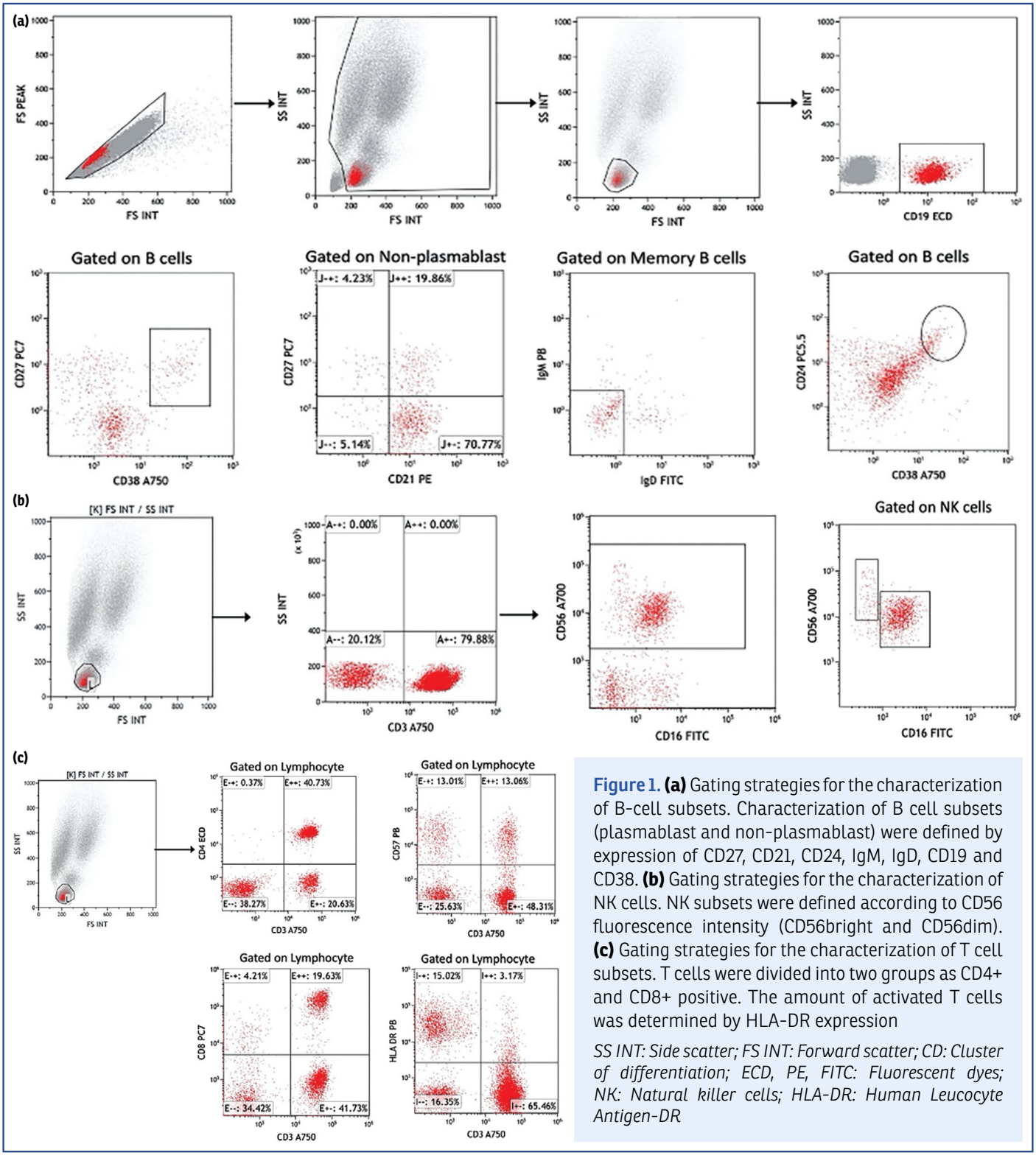

Figure 1. (a) Gating strategies for the characterization of B-cell subsets. Characterization of B cell subsets (plasmablast and non-plasmablast) were defined by expression of CD27, CD21, CD24, IgM, IgD, CD19 and CD38. **(b)** Gating strategies for the characterization of NK cells. NK subsets were defined according to CD56 fluorescence intensity (CD56bright and CD56dim). **(c)** Gating strategies for the characterization of T cell subsets. T cells were divided into two groups as CD4+ and CD8+ positive. The amount of activated T cells was determined by HLA-DR expression

SS INT: Side scatter; FS INT: Forward scatter; CD: Cluster of differentiation; ECD, PE, FITC: Fluorescent dyes; NK: Natural killer cells; HLA-DR: Human Leucocyte Antigen-DR

Flow Cytometric Immunophenotyping

Whole blood samples were analyzed by flow cytometry to determine the T cell, NK cell, and B cell subgroups phenotypically.[13] The gating strategy for T cell, NK cell, and B lymphocyte subgroups is provided in Figure 1a–c. Briefly, CD3+CD4+/CD8+/CD57+ T cell, CD16+CD56+/CD57+/CD3– NK cell, CD19+CD27+CD21+ memory B cell, CD19+CD27– naive B cell, CD19+CD38++CD24++ transitional B cell,

Table 1. Demographics and clinical characteristics

Variable	HFS–(n=21)	HFS+(n=9)	p
Age (years)	43.7±16.0	53.1±9.51	0.113
Sex (male/female)	11/10	7/2	0.186
Smoking, n (%)	5 (23.8%)	5 (55.6%)	0.097
Hypertension, n (%)	2 (9.5%)	3 (33.3%)	0.212
Diabetes, n (%)	3 (14.3%)	1 (11.1%)	0.822
Chronic obstructive pulmonary disease, n (%)	1 (4.8%)	1 (11.1%)	0.564
Alanine aminotransferase (U/L)	22.9±10.3	41.6±32.2	0.125
Glucose (mg/dL)	117.7±62.4	124.7±36.4	0.759
Aspartate aminotransferase (U/L)	28.6±7.46	49.4±21.7	**0.021**
Albumin (g/dL)	39.4±3.80	34.6±4.96	**0.009**
Direct bilirubin (mg/dL)	0.11±0.03	0.18±0.09	0.072
Total bilirubin (mg/dL)	0.45±0.14	0.73±0.33	0.053
Triglycerides (mg/dL)	118.5±74.4	58.3±11.1	0.210
Lactate dehydrogenase (U/L)	234.4±70.7	348.8±55.3	**<0.001**
High density lipoprotein	36.7±13.8	45.7±9.60	0.352
Gamma-glutamyl transpeptidase (U/L)	28.5±19.8	106.9±132.5	0.139
Alkaline phosphatase (U/L)	62.1±16.6	135.3±180.9	0.291
Sodium (mmol/L)	136.9±3.37	135.2±3.11	0.216
Potassium (mmol/L)	4.32±0.33	4.35±0.74	0.899
Blood urea nitrogen (mg/dL)	35.7±23.6	31.3±5.80	0.592
Creatinine (mg/dL)	0.88±0.42	0.77±0.19	0.479
Total protein (mg/dL)	70.9±4.34	66.7±4.18	0.242
Ferritin (µg/L)	222.2±379.8	269.5±270.1	0.820
White blood cell (10^3 μL^{-1})	5.66±1.92	6.53±3.59	0.392
Red blood cell (10^3 μL^{-1})	4.58±0.53	4.47±0.51	0.603
Hemoglobin (g/dL)	12.9±2.03	13.2±1.67	0.703
Platelets (10^3 μL^{-1})	209.8±73.3	225.9±131.2	0.670
Neutrophil (10^3 μL^{-1})	3.52±1.81	4.70±3.04	0.198
Lymphocyte (10^3 μL^{-1})	1.43±0.47	1.07±0.42	0.060
Monocytes (10^3 μL^{-1})	0.59±0.21	0.67±0.42	0.519
Mean platelet volume (fL)	8.85±0.79	8.78±1.04	0.843
Fibrinogen (mg/dL)	388.1±134.8	424.3±232.1	0.670
C reactive protein (mg/L)	83.6±73.8	132.3±28.9	0.259
Pro-brain natriuretic peptide (µg/L)	302.7±419.3	130.0±69.8	0.232
D-Dimer (µg/L)	744.4±1246.7	1347.4±1172.6	0.227
International ratio	1.07±0.09	1.05±0.10	0.817
High sensitivity troponin I (ng/L)	14.3±39.7	58.1±145.5	0.399
CD3+ (%)	77.2±6.35	71.7±6.44	**0.037**
CD3+/CD4+ (%)	44.1±8.70	45.2±7.01	0.745
CD3+/CD8+ (%)	29.3±6.90	25.4±6.46	0.161
CD57+/CD3+ (%)	11.7±4.67	12.4±4.63	0.708
CD57+/CD3– (%)	7.20±3.82	6.67±3.96	0.730
NK (%)	10.9±4.41	12.3±5.61	0.460

Table 1. Cont.

Variable	HFS–(n=21)	HFS+(n=9)	p
NKT (%)	2.71±1.48	3.67±1.50	0.120
CD19+ (%)	10.4±3.91	13.8±3.38	**0.034**
CD27+/CD19+/IgD– (%)	15.6±8.28	17.4±10.8	0.623
CD27–/CD19+ (%)	77.4±10.4	77.9±12.2	0.917
CD24+/CD38+ (%)	4.53±2.04	3.57±2.94	0.311
$CD56^{bright}$/CD16– (%)	0.21±0.23	0.28±0.18	0.458
$CD56^{dim}$/CD16+ (%)	9.81±4.77	9.07±4.86	0.702
HLA-DR+/CD3+ (%)	6.39±4.70	6.47±3.59	0.963
CD27+/CD38+ (%)	2.88±2.25	5.23±8.02	0.411
CD27+/CD21+ (%)	18.6±9.60	17.4±10.1	0.764
CD21 MFI (%)	16.3±4.90	11.1±2.91	**0.006**

HPS: Hemophagocytic syndrome; NK: Natural killer cells; NKT: Natural killer T cells; HLA-DR: Human Leucocyte Antigen-DR; MFI: Mean fluorescence intensity; CD: Cluster of differentiation

CD19+CD27+IgD– switched memory B cell, CD27+CD19+IgD+ non-switched memory B cell, CD27+CD38++ plasmablast, CD21 MFI (mean fluorescence intensity) were measured by multiple-color flow cytometry with human monoclonal anti-CD3-APC-A750 (Beckman Coulter, US), anti-CD4-ECD (Beckman Coulter, US), anti-CD8-PC7 (Beckman Coulter, US), anti-CD56-APC-A700 (Beckman Coulter, US), anti-CD16-FITC (Beckman Coulter, US), anti-CD19-ECD (Beckman Coulter, US), anti-CD27-PC7 (Beckman Coulter, US), anti-CD21-PE (Beckman Coulter, US), anti-CD24 PC5.5 (Beckman Coulter, US), anti-CD38-APC-A750 (Beckman Coulter, US), anti-IgD-FITC (Beckman Coulter, US) according to the manufacturer's instructions. Results were analyzed using Kaluza software (Beckman Coulter).

Statistical Analysis

Results were analyzed by SPSS 22.0 (Chicago, IL, USA), a statistical software package. The variables were grouped as categorical and continuous ones. The distribution of continuous variables was compared with normal distribution via Kolmogorov–Smirnov tests. Continuous variables were reported with their means and standard deviations (M±SD). Categorical variables were reported by the total number of patients who have that variable positive and the percentage of them over their category (HFS+ and HFS–). To compare the clinical, demographic, and laboratory results of the patients who were HFS+ and HFS–, Student's t-test was used. Statistical analysis among the groups is reported in Table 1 and Figure 2a, b. As discussed in depth in sections "Results" and "Discussion," our analysis found a strong correlation between CD21 MFI and HFS. Because we found the strongest correlation between HFS and CD21 MFI among the parameters we studied, we examined this potential biomarker further. To detect the correlated parameters with CD21 MFI, a univariate Pearson's correlation method was used. Statistically meaningful variables were added to the linear regression analysis. The variables were accepted as statistically meaningful when $p<0.05$.

RESULTS

General demographics and clinical characteristics of the patients are shown in Table 1. The median ages in the HPS– and HPS+ groups were 43 and 53 years, respectively, and were not significantly different (p=0.113). In accordance with a recent report,[14] individuals with COVID-19 had the underlying pulmonary disease (2/30 including moderate and severe, 6.6%) and were current or former smokers (10/30 including moderate and severe, 33.3%). Hypertension and diabetes were the most frequent comorbidities among the patients.

We measured various immunologic and biochemical parameters at the time of their admission, while none of the patients were HPS+ to find an early biomarker. We analyzed the level of alanine aminotransferase, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin, direct and total bilirubin, triglycerides, and gamma-glutamyl transpeptidase in HPS– and HPS+ COVID-19 patients. In our study, we found that the median levels of LDH at the time of blood draw were 234.4 µg/mL in HPS– and 348.8 µg/mL in HPS+ COVID-19+ individuals ($p<0.001$). We measured a higher level of AST in the HPS+ group compared with the

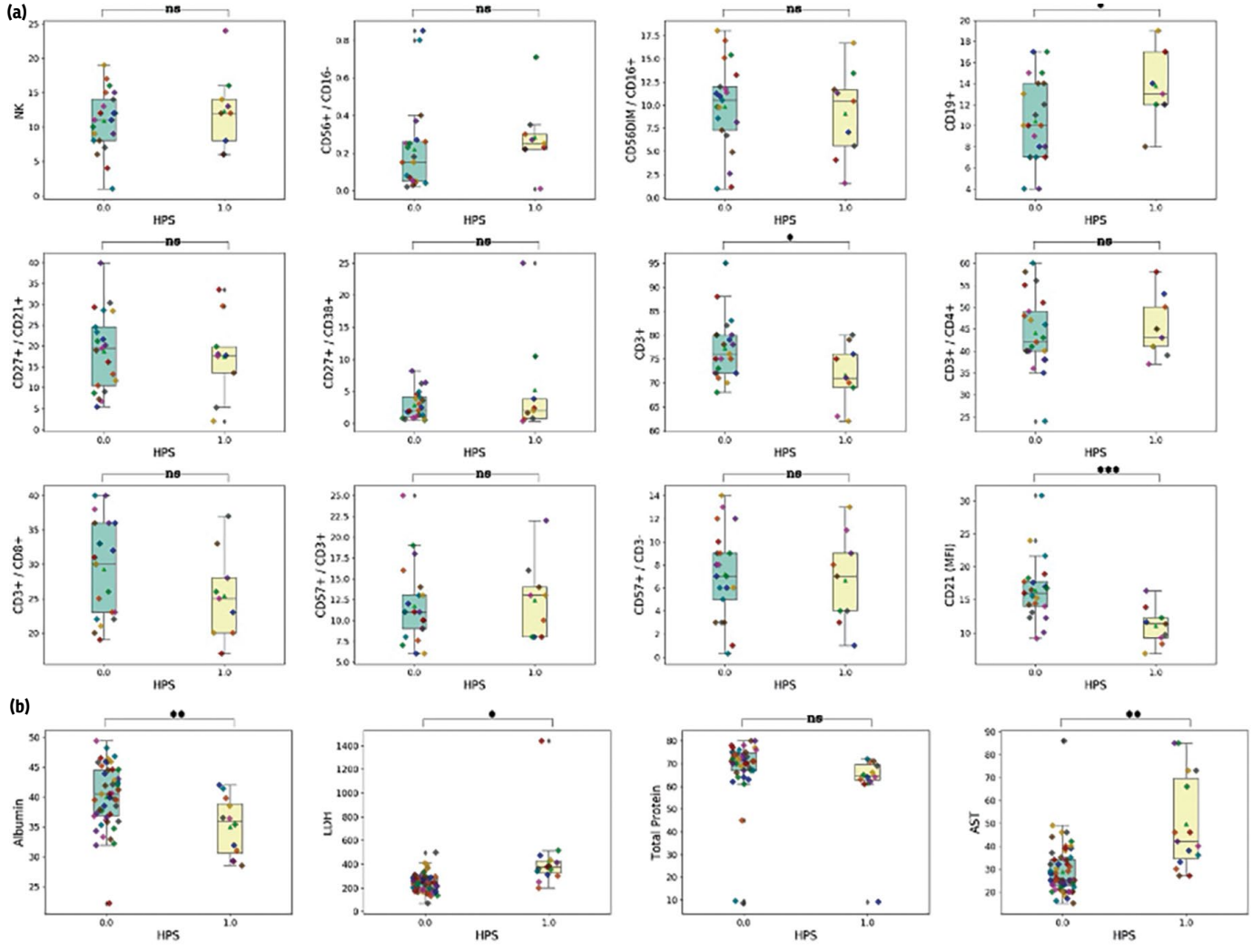

Figure 2. (a) Immunophenotyping of circulating lymphocyte subtypes and laboratory parameters in the HPS+ and HPS- groups during COVID-19 infection. Percentages of CD56bright and CD56dim NK cell, B cell and T cell subsets within the total lymphocyte. ***p<0.001, **p<0.01, *p<0.05, ns denotes not significant. **(b)** Immunophenotyping of circulating lymphocyte subtypes and laboratory parameters in the HPS+ and HPS- groups during COVID-19 infection. Albumin, LDH, Total Protein and AST values in patient groups. ***p<0.001, **p<0.01, *p<0.05, ns denotes not significant

HPS: Hemophagocytic syndrome; CD: Cluster of differentiation; NK: Natural killer cells; LDH: Lactate dehydrogenase; AST: Aspartate aminotransferase

HPS– group (p=0.021). Additionally, albumin levels were significantly lower in the HPS+ patients (p=0.009). A decrease in lymphocyte counts was observed in the HPS+ group, but we did not find significant differences between HPS+ and HPS– patient groups (p=0.06). There was no statistical difference between the groups for other laboratory parameters.

To assess the general landscape of immune responses, we carried out extensive immunophenotyping to identify the frequencies of circulating immune subsets in HPS– and HPS+ patient groups (Fig. 2a, b). Consistent with previous reports[5,15] in severe COVID-19 patients, CD3 T cell was found to be significantly lower in the group with HPS+ compared with HPS– (p=0.037). Although there was a significant decrease in CD3 rates, no statistical difference was found between the groups in terms of CD3 subsets (CD3+/CD4+, CD3+/CD8+) and active T cell (CD3+/HLA-DR+) (p=0.745, p=0.161, and p=0.963, respectively). Donors with HPS– had lower frequencies of CD19+ B cell compared with HPS+ group (p=0.037). However, the proportion of memory B cell, switch memo-

Table 2. Univariate and multivariate analysis between CD21 MFI and various variables

Variable	Univariate analysis		Multivariate analysis	
	p	r	p	r
Age (years)	**0.004**	–0.490	**0.038**	–0.385
Lymphocyte (10^3 μL^{-1})	**0.010**	0.436	0.302	0.177
CD3 (%)	**0.001**	0.542	0.274	0.242
CD19 (%)	**0.029**	–0.362	0.721	0.071

MFI: Mean fluorescence intensity; CD: Cluster of differentiation

ry B cell, and native B cell was not statistically significant between HPS+ and HPS– groups. B cell plasmablasts expanded in HPS+ donors compared with HPS–, but no statistically significant difference was found (p=0.365). The overall expression of CD21 in non-plasmablasts was decreased in HPS+ groups (p=0.006). In the univariate statistical analysis performed with CD21, correlations were found with age, lymphocyte count, CD3, and CD19 values. In linear regression analysis performed with these four parameters, which were found to be correlated, a negative relationship with age was confirmed (Table 2 and Fig. 3). When the NK cells and NK cell subgroups (CD56bright/CD16–, CD56dim/CD16+) were examined, no statistically significant difference was found between the groups.

DISCUSSION

HPS, also known as hemophagocytic lymphohistiocytosis, is an immune-mediated life-threatening disease. It results from dysfunctional natural killer cells and cytotoxic T cells. Overactivated NK and T cells cause an exaggerated inflammatory response coming from hypersecretion of proinflammatory cytokines and various chemokines. This cytokine storm could be pathogenically related to the development of the main clinical and laboratory features of HPS and contributes to tissue damage and progressive systemic organ failure.[16–19]

HPS is categorized as primary and secondary HPS. Primary HPS is caused by genetic factors whereas secondary HPS is acquired. One of the most common causes of secondary HPS is viral infections. The underlying mechanism associated with infection is related to the toll-like receptors (TLR). Enhanced antigen presentation and repetitive interferon gamma-dependent stimulation of Toll-like receptors are considered the fundamental mechanisms of uncontrolled activation of anti-

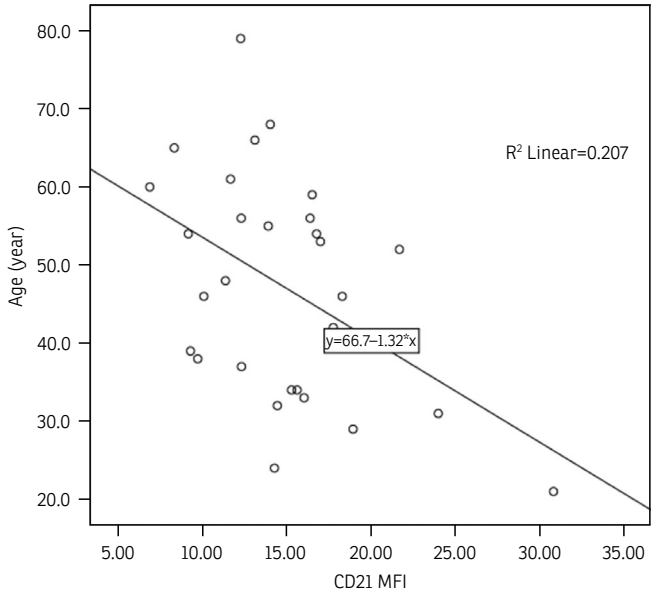

Figure 3. Linear correlation between CD21 MFI and age

CD: Cluster of differentiation; MFI: Mean fluorescence intensity

gen-presenting cells and T cells.[20,21]

In HPS patients with viral infections, the elevation of AST, LDH, and low levels of albumin and total protein have been reported in previous studies,[21–23] but the number of studies with lymphocyte subgroups, especially CD21 (complement receptor type 2, CR2), is limited. McCall et al.[24] reported an increase in CD8+ cells in almost half of their HPS+ patients with EBV infection. In the study conducted by Dalal et al.,[25] consistent with our findings, an increase in CD19+ cells, CD8+ cells, a decrease in CD4/CD8 ratios, and a decrease in CD3+ cells were reported in adult secondary HPS+ patients. The decrease in CD3+ cells has been shown to be inversely related to survival in secondary HPS+ individuals. An increase in $CD21^{low}$ B cells has been described in a variety of diseases associated with persistent immune stimulation as in chronic infection, immunodeficiency, or autoimmunity.[26,27] In our study, we observed downregulation of CD21 in our patients with HPS. This can be explained by the fact that viral infections and HPS result in TLR activation. TLR stimulation is known to decrease cell surface expression of CD21.[28–30] Decreased CD21 expression may be a valuable marker for predicting HPS in COVID-19 patients. Another finding of our study shows that there is an inverse correlation between CD21 marker and age in COVID-19 patients (Fig. 3 and Table 2) even though no correlation is reported between $CD21^{low}$ B cells and age in reference studies among healthy individu-

als.[13] This is consistent with the reports showing that older patients suffer from more severe symptoms of COVID-19.

In this work, we provide a valid data point from our 30 patients. There have been various studies on immunologic parameters where patients are grouped by the severity of their symptoms: severe and moderate COVID-19 patients.[5,15] Different from previous studies, we categorized our patients into two groups: the COVID-19 patients who later show HPS or not. We followed this grouping regime so that we can explore new parameters that can enable early prediction of HPS among COVID-19 patients. Statistically significant results were obtained in CD3, CD19, and CD21 markers in COVID-19 patients between the groups who will later have or not have HPS. It reinforces the idea that HPS is a common pathway formed as a result of the activation of various immunologic responses. Flow cytometry can identify HPS subtypes by detecting a deficiency of perforin expression or decrease in CD107a expression. However, these markers are not widely used in the routine laboratory and therefore are available only for a small number of patients.[31,32]

CONCLUSION

Therefore, we suggest that markers more frequently used in routine laboratories such as CD3, CD19, and CD21 may be useful in managing the treatment of HPS patients with COVID-19. Upcoming studies can extend our results with additional HPS+ patients. This can enable us to further understand the potential contribution of these phenotypic markers and subset alterations to COVID-19 pathogenesis.

Disclosures

Ethics Committee Approval: The study was approved by the Adana City Training and Research Hospital Clinical Research Ethics Committee (No: 788, Date: 08/04/2020).

Informed Consent: Written informed consent was obtained from all patients.

Peer-review: Externally peer reviewed.

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