Original Article

Analysis of lymphocyte subsets in COVID-19 patients: a retrospective observational study

Analisi delle sottopopolazioni linfocitarie in pazienti affetti da COVID-19: uno studio osservazionale retrospettivo (LINFO-COVID)

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Key words: COVID-19, lymphocyte subset, flow cytometry.

ABSTRACT

In this retrospective study, we analyzed the possible predictive changes in lymphocyte subsets of Coronavirus Disease 19 (COVID-19) hospitalized patients. We enrolled 107 COVID-19 patients older than 18 years of age, admitted to Alessandria Hospital with a confirmed diagnosis of SARS-CoV-2 infection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), who performed the analysis of lymphocyte subsets, between 2020 March 01 and 2021 May 31. Patients have been split up into two groups, based on clinical manifestations: group 1 non-severe disease (n=44) and group 2 severe disease (n=63), according to the World Health Organization (WHO) interim guidance. For the comparison between the two groups of patients, statistical significance was tested with the Mann-Whitney and the Chi-Square test. In the group of patients with severe disease, the blood cell analysis showed a significant reduction in the counts of total lymphocytes, absolute CD3 lymphocytes, and CD8 lymphocytes, as well as a significant reduction in monocyte percent and a very significant increase in neutrophil counts. From the results obtained, it can be stated that lymphocytopenia is associated with the progression of the disease and increased mortality.

In questo studio retrospettivo, sono stati analizzati possibili cambiamenti nelle sottopopolazioni linfocitarie in pazienti ospedalizzati affetti da malattia da Coronavirus19 (COVID-19). Sono stati arruolati 107 pazienti COVID-19 di età superiore ai 18 anni, ricoverati presso l'Azienda Ospedaliera di Alessandria con diagnosi confermata di infezione da SARS-CoV-2 mediante Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), che hanno eseguito l'analisi delle sottopopolazioni linfocitarie, tra il 01 marzo 2020 e il 31 maggio 2021. I pazienti sono stati suddivisi in due gruppi, in base alle manifestazioni cliniche: Gruppo 1 (malattia non grave (n=44)) e Gruppo 2 (malattia grave (n=63)), secondo le indicazioni dell'Organizzazione Mondiale della Sanità (OMS). Per il confronto tra i due gruppi di pazienti, la significatività statistica è stata testata con il test di Mann-Whitney e il test del Chi-quadro. Nel gruppo di pazienti con malattia grave, l'analisi delle cellule ematiche ha mostrato una riduzione significativa della conta di linfociti totali, dei linfociti CD3 assoluti e dei linfociti CD8, nonché una riduzione significativa della percentuale di monociti e un aumento molto significativo della conta dei neutrofili. Dai risultati ottenuti, si può affermare che la linfocitopenia è associata alla progressione della malattia e ad un aumento della mortalità.

INTRODUCTION

The year 2020 has been strongly marked by the pandemic of Coronavirus Disease 19 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The World Health Organization, on March 11, 2020, has declared the novel coronavirus outbreak a global pandemic. COVID-19 soon spread to more than 200 countries and regions around the world.[1-3]

Clinical manifestations of COVID-19 vary from patient to patient and may be asymptomatic, mild, moderate, or severe.[4]

Patients with mild disease have symptoms such as colds, fever, sore throat, anosmia, ageusia, cough, muscle pain, diarrhea, headache, fatigue, and abnormal CT scan, but in most cases the prognosis is good. In contrast, the condition of some patients progresses rapidly to Acute Respiratory Distress Syndrome (ARDS) or even Multi-Organ Failure (MOF) in a very short time and could

lead to a fatal outcome. Moreover, the new Coronavirus can not only cause interstitial pneumonia but also pulmonary thromboembolism, and other organs, such as brain, heart, and kidneys, can also be affected.[5]

Although age is the strongest risk factor for severe COVID-19 outcomes, patients with certain underlying medical conditions (diabetes, obesity, heart disease, cancer, and immunosuppression) are also at higher risk.[6,7]

Another important factor in the rapid progression of this disease is the immune response.[8]

Immune responses play an important role in the host's defense against viruses and pathological damage to the host. Therefore, a better understanding of the dynamic changes in lymphocyte populations in COVID-19 patients is a prerequisite for the development of efficient outcome-prediction tools.

A growing number of investigations have reported that at hospital admission, laboratory tests of severe patients showed leucopenia, lymphopenia and thrombocytopenia.[9-12]

In particular, several studies have shown that the progressive decrease in total T-lymphocyte counts, CD4+ T-lymphocytes, CD8+ T-lymphocytes, NK-lymphocytes, and B-lymphocytes were predictors of a worsening of the patient's conditions.[9-12]

Given these assumptions, this work aimed at collecting and analyzing data on peripheral blood lymphocyte subpopulations performed in 107 COVID-19 patients admitted to the Hospital of Alessandria in the period between 01 March 2020 and 31 May 2021. The objective was to assess the presence of any significant differences between patients with severe disease and patients with non-severe disease, in order to investigate the possible use of lymphocyte counts as prognostic predictive markers.

MATERIALS AND METHODS

Study design

This study is a single-center, retrospective observational study carried out at "SS. Antonio e Biagio e Cesare Arrigo" Hospital, approved by the Institutional Review Board (IRB) (protocol number ASO.LabAn.21.02) and conducted by the principles of the Declaration of Helsinki and the Good Clinical Practices guidelines.

For our study, we selected all consecutive patients older than 18 years of age, admitted to Alessandria Hospital with a confirmed diagnosis of SARS-CoV-2 infection by Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR), who performed the analysis of lymphocyte subsets, between the 1st of January 2020 and the 31st of May 2021.

A total of 107 hospitalized patients, who met the inclusion criteria, were selected. Patients discharged from the Emergency Department were excluded.

Data collection

Data from hospitalized patients were collected from the electronic medical records system (TrackCare) and paper-based medical records and recorded in a specific database created using the freely available Research Electronic Data Capture (*REDCap*) platform.[13]

Some variables of interest were also collected from the "COVID-19 Registry" database, an observational study carried out at the Hospital of Alessandria and approved by the IRB (protocol number ASO.IRFI.20.03).

The collected variables were: demographics, admission data, past and proximal medical history, onset symptoms, laboratory data (including lymphocyte subsets), chest X-ray or CT scan results, complications, performed treatments and outcome. For each patient, we calculated Charlson Comorbidity Index and Glasgow Coma Score if possible.

A pseudonymized code was assigned to each hospitalized patient included in the study.

Patient's classification

Based on clinical characteristics and according to the World Health Organizaition (WHO) interim guidance, patients were divided into two groups: Group 1, patients with mild or moderate disease (n=44), and Group 2, patients with severe or critical disease (n=63) (Table 1).

Laboratory procedures: flow cytometry

All cytofluorimetric tests for the lymphocyte subsets were performed on peripheral blood samples and collected in EDTA anticoagulant tubes at hospital admission, by standard procedures of the hospital laboratory.

Lymphocyte subsets were quantified with multi-parametric flow cytometry through AquiosCL (Beckman Coulter, Brea, USA), an automated flow cytometer that integrates the sample preparation and analysis phases, ensuring traceability of the entire analytical process. For each group of patients, we collected the following

Table 1. Classification of COVID-19 disease severity.

Group 1		Group 2	
Mild disease	Moderate disease	Severe disease	Critical disease
Symptoms as fever, cough, fatigue, dyspnea, myalgia, headache, diarrhea, nausea/vomiting, loss of smell/taste	X-ray findings of pneumonia; blood oxygen saturation levels (SpO_2)≥90% on room air	Mild or moderate clinical features plus at least one of the following manifestations:	Severe manifestations plus any other features that suggest the rapid progression of the disease
No pneumonia	No complications related to sever conditions	Respiratory rate >30 breaths/minute or severe respiratory distress	Respiratory failure with the need of mechanical ventilation
		SpO_2<90 on room air	Presence of Acute Respiratory Distress Syndrome (ARDS), sepsis or septic shock, acute pulmonary embolism, acute coronary syndrome, acute stroke and delirium

data: total lymphocytes (percent and absolute counts) and lymphocyte subset percent and absolute counts: total CD3 positive, CD3+CD4 positive, CD3+CD8 positive, CD3-CD16+CD56 positive and CD19 positive. Lymphocyte subsets were assessed using Beckman Coulter AquiosCL Tetra 1 and Tetra 2+ panel (PC5 labeled CD3, clone UCHT1, isotype IgG1; RD1 labeled CD4, clone SFCI12T4D11, isotype IgG1; ECD labeled CD8, clone SFCI21Thy2D3; RD1 labeled CD16, clone 3G8 and CD56, cloneN9O1, isotype IgG1; ECD labeled CD19, cloneJ3-119, isotype IgG1; FITC labeled CD45, clone B3821F4A, isotype IgG2b).

All tests were performed according to the instrument operative manual.

Statistical analysis

Patient's data were analysed anonymously and in aggregate form. A summary descriptive analysis was performed both on the whole population and on each of the two groups, separately.

Statistical significance for the comparison between the two groups was tested with the Mann-Whitney test for continuous data, Chi-Square test was used for categorical data. Qualitative data were expressed as absolute frequencies and percentages, quantitative data were represented as median and interquartile range.

Significant differences were established at a value of $p<0.05$. All statistical analyses were performed using SPSS software version 25 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA).

RESULTS

Demographics and baseline characteristics

The present study includes 107 patients admitted to the Alessandria Hospital between January 2020 and May 2022 with a diagnosis of COVID-19 confirmed by RT-PCR.

For the analysis, patients were divided into 2 groups: "mild/moderate disease" (n=44) and "severe/critical disease" (n=63).

Fifty-three point three percent of the total population were male (52.3% of the "mild/moderate disease" group, 54% of the "severe/critical disease" group). The average age of the total sample, expressed as a median and interquartile range, was 66 years (56-80), 64.5 for males (55.5-77.5), and 69.5 (55.75-80.25) for females. The ages of the patients ranged from 30 to 91 years.

Patients with "mild/moderate disease" had an average age of 64.5 years (54.25-81.75), males 61 (49-82), females 71 (55.5-81); the average age of patients with "severe/critical disease" was 70 years (56-78), 71 for males (58.25-77), 66 (55-80.5) for females.

No statistical differences were found in the age and sex composition of the two groups. Demographics and baseline characteristics are shown in Table 2.

Statistical significance for comparisons between the two groups was tested using the Mann-Whitney test for age and the chi-squared test for sex.

Clinical characteristics

As mentioned above, patients were divided into 2 groups based on clinical complications, symptoms, and need for mechanical ventilation, according to WHO interim guidance.

Patients with non-severe disease (n=44) showed typical symptoms of mild COVID-19 disease: Ageusia (n=2), Anosmia (n=2), Chills (n=1), Diarrhoea (n=8), Dyspnoea (n=16), Abdominal pain (n=3), Thoracic pain (n=2), Muscle pain (n=5), Fever (n=28), Headaches (n=2), Nausea (n=3), Rhinorrhoea (n=1), Fatigue (n=6), Cough (n=15), Vomiting (n=2).

During hospitalization, patients of Group 1 never developed serious respiratory complications: Access to Intensive Care Unit (ICU) (n=0), ARDS (n=0), deep vein thrombosis (n=1), pulmonary embolism (n=0), acute kidney failure (n=1), septic shock (n=0), sepsis (n=0), respiratory failure (n=5), heart failure (n=1), pneumonia (n=4) (short-term condition that resolved quickly). Of the 44 patients of Group 1, only 18 required High-Flow Nasal Canula (HFNC) oxygen.

Patients with severe/critical disease (n=63) at hospital admission showed the following symptoms: ageusia (n=2), anosmia (n=2), chills (n=1), diarrhoea (n=13), dyspnoea (n=39), abdominal pain (n=2), thoracic pain (n=2), muscle pain (n=3), fever (n=45), headaches (n=3), nausea (n=2), rhinorrhoea (n=0), fatigue (n=6), cough (n=24), vomiting (n=2).

During hospitalization, Group 2 developed more serious complications: Access to ICU (n=9), ARDS (n=5), deep vein thrombosis (n=2), pulmonary embolism (n=4), acute kidney failure (n=2), septic shock (n=2), sepsis (n=3), respiratory failure (n=42), heart failure (n=2), pneumonia (n=10). During the hospital stay, 51 patients needed at least HFNC oxygen. A total of 52 patients required Non-Invasive Ventilation (NIV), and for 7 of them became necessary mechanical ventilation.

Laboratory and flow cytometry findings

Lymphocyte subsets were quantified with multi-parametric flow cytometry.

Table 2. Sex and age distribution across the whole population, Group 1 and Group 2.

		Total patients n=107		Group 1 (Mild disease) n=44		Group 2 (Severe disease) n=63		p
		N	%	N	%	N	%	
Sex	Male	57	53,3	23	52,3	34	54,0	>0,999
	Female	50	46,7	21	47,7	29	46,0	
		Median (IQR)		Median (IQR)		Median (IQR)		
Age (years)	Total	66,00	(56,00-80,00)	64,50	(54,25-81,75)	70,00	(56,00-78,00)	0,761
Sex	Male	64,00	(55,50-77,50)	61,00	(49,00-82,00)	71,00	(58,25-77,00)	
	Female	69,5	(55,75-80,25)	71,00	(55,50-81,00)	66,00	(55,00-80,50)	

Statistical significance for comparisons between the two groups was tested using the Mann-Whitney test for age and the chi-squared test for sex.

All the results are presented in Table 3.

Group 2 showed a general reduction in the percentage of leucocytes, and in particular, a statistically significant decrease in the percentage of lymphocytes (p=0.004), monocytes (p=0.01) and neutrophils (p=0.001) was observed. The total neutrophil count was also found to be statistically higher in patients who developed the severe disease (p=0.02), while the absolute lymphocyte count was at the limit of significance (p=0.055).

With regards to the analysis of lymphocyte subpopulations, it can be stated that the group of patients who developed the most severe form of the disease showed a significant reduction in CD3 (p=0.012) and CD8 (p=0.019) lymphocyte counts compared to the patients who experienced the non-severe disease. Also, a reduction in CD4 lymphocyte count can be observed, although this result was at the limit of significance (p=0.052). Finally, the CD4/CD8 T cell ratio was analysed, but no significant differences were observed between the two groups of patients.

A graphic representation of the most representative data is presented in Figure 1.

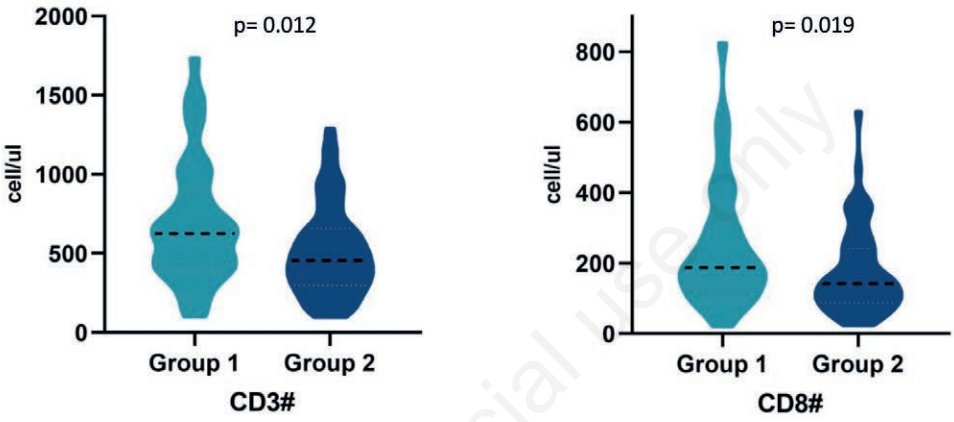

Figure 1. Absolute CD3 T cell counts (cells/ul) and absolute CD8 T cell counts (cells/ul) among patients enrolled in the study (Group 1 *vs* Group 2, Mann-Whitney test).

Table 3. Lymphocytes and lymphocytes subsets absolute counts and percentages and comparison between the two groups (Mann-Whitney test).

	Total patients n=107		Group 1 (Mild disease) n=44		Group 2 (Severe disease) n=63		p
	N	%	N	%	N	%	
CD3%	67	(57-73)	68,1	(62,25-75,75)	64	(54-73)	0,072
CD3# (cells/ul)	513	(359-714)	627	(429-869)	455	(297-657)	0,012*
CD4%	42	(33-49)	42	(35,3-48,8)	40	(32-50)	0,617
CD4# (cells/ul)	326	(210-477)	400	(223-546)	308	(184-431)	0,052
CD8%	22	(16-30)	23,5	(16-32)	21	(16-27)	0,294
CD8# (cells/ul)	162	(100-264)	188	(119-318)	142	(87-242)	0,019*
CD16+CD56%	14	(11-23)	14	(11-18,8)	14	(11-28)	0,408
CD16+CD56# (cells/ul)	117	(71-185)	121	(84-196)	110	(63-185)	0,357
CD19%	14	(9-20)	11,5	(7,5-16,7)	15	(10-20)	0,088
CD19# (cells/ul)	99	(60-163)	99	(49-186)	99	(62-142)	0,812
CD4/CD8	1,85	(1,2-3,2)	1,87	(1,1-3,1)	1,85	(1,3-3,2)	0,593
WBC (cells/ul)	7980	(5630-10440)	6540	(4590-10370)	8300	(6430-10720)	0,093
Neutrofili%	82,4	(75,2-88,2)	77	(69,7-87)	84,3	(79,1-89,5)	0,001*
Neutrofili# (cells/ul)	6830	(4290-9000)	4970	(3090-8910)	7310	(5110-9000)	0,020*
Monociti%	4,4	(3,1-6,3)	5,1	(3,4-7)	3,8	(2,8-5,2)	0,010*
Monociti#(cells/ul)	340	(230-510)	350	(220-630)	340	(230-480)	0,531
Linfociti%	10,3	(6,1-16,2)	13,8	(8,1-21,7)	9,3	(5,3-13,6)	0,004*
Linfociti#(cells/ul)	850	(590-1140)	930	(660-1190)	800	(480-1080)	0,055

*Statistically significant.

DISCUSSION

This study assessed the potential association of different lymphocyte subpopulations with disease severity criteria in patients hospitalized for COVID-19. Several studies have already evaluated the potential role of lymphocyte subpopulation as predictors of disease severity in COVID-19 patients.

In the population under investigation, 41.1% had mild/moderate disease and 58.9% had severe/critical disease. The most common clinical manifestations at disease onset in the mild/moderate group included fever, cough, and dyspnoea. The severe/critical cases were more likely to develop complications such as pneumonia, ARDS, and respiratory failure and to require oxygen therapy.

As in other viral infections, in COVID-19 T-cell cytotoxicity plays a key role in the elimination of infected cells. However, in our study, lymphocyte counts and especially CD8+ and CD3+ T-cells are significantly reduced in patients with severe disease compared to those with the non-severe disease.[10,14,15] Several studies have also identified a significant reduction in CD4+ T lymphocytes in patients with severe disease compared to patients with non-severe disease;[16] our work showed overlapping results, although at the limit of statistical significance, probably due to the small number of patients enrolled in the study.

Common viral infections typically lead to T-cell expansion and subsequent lymphocytosis. Conversely in COVID-19 patients there is simultaneous T-cell hyper-activation and depletion, a condition that results in damage to the organism. The excessive activation of the immune system leads to the production of pro-inflammatory cytokines causing multi-organ failure. Accordingly, literature shows that severe patients present an increase in IL-6.

IL-6 has been shown to be the cytokine associated with more severe forms of COVID-19,[17,] probably due to the development of the cytokine storm.[18] In a large meta-analysis of 7865 patients, a decrease in lymphocytes and an increase in IL-6 levels was found in the group of more severe cases of the disease compared to the milder ones.[19]

Unfortunately, in our study, we did not have this data available for a sufficient number of patients, so it was not possible to complete the analyses.

The study had several limitations. Since it was a retrospective observational study, some important clinical data such as procalcitonin, C-reactive protein, fibrinogen, and LDH were not available for all patients, so analyses of these parameters could not be included in this article.

Analyses on lymphocyte subpopulations in COVID-19 patients were only requested by some hospital departments, so for many in-patients, these data were not available.

As a result, the selected population was not large enough for statistical significance, but most of the results we obtained are consistent with those reported in the literature.

Therefore, further prospective studies with larger samples will be necessary to overcome these limitations.

CONCLUSIONS

In conclusion, the most relevant result in the study showed that a reduction in lymphocyte and lymphocyte subpopulation counts in COVID-19 patients admitted to the hospital is associated with disease progression and severity.

A statistically significant reduction in CD3+ and CD8+ lymphocyte subpopulation was observed. The reduction in CD4+, on the other hand, was at the limit of statistical significance, probably due to the small number of patients enrolled for the study.

The immune response to SARS-COV-2 infection represents one of the most important prognostic indicators for predicting disease severity, therefore it is important to identify early therapeutic targets to modulate the immunological status of these patients to reduce mortality.

In a pandemic scenario, the ability to stratify patients according to disease severity is a key prerequisite to identifying appropriate therapeutic approaches.

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Availability of data and materials: all data generated or analyzed during this study are included in this published article.

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