The immunological response of Egyptians to coronavirus disease-19 infection: a cohort study of lymphocyte populations and peripheral blood counts

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Background
The worldwide pandemic of COVID-19 infection that started in 2019 still lays its shadows over all populations of the world. COVID-19 infection presented with a spectrum of symptoms that varied from wave to wave, and also led to a wide number of long-term sequelae. Many immune system cells and cytokines were implicated in COVID-19 pathophysiology. Thus, many immuno-modulator and immuno-suppressive drugs were used in the management of severe cases. Lymphocytes are the key players of immune system, the change in their count and different subsets is expected to vary with COVID-19 infection.
Objective
The current study aimed to evaluate the role of peripheral blood lymphocyte subsets in predicting the outcome of COVID-19 patients and to investigate their correlation with different clinical and laboratory variables.
Materials and methods
The study included 64 patients hospitalized with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). They were stratified according to in-hospital mortality into survivors and nonsurvivors. Demographic, clinical and laboratory data were collected. Flowcytometric evaluation of lymphocyte subsets was done on admission.
Results and conclusion
Nonsurvivors showed lower relative lymphocyte count, higher absolute neutrophil count, and higher neutrophil to lymphocyte ratio (NLR) compared with survivors ($P = 0.034$, 0.006, 0.011; respectively). NLR at a cut off 15.3 had a sensitivity of 70.59% and specificity of 61.29% for predicting mortality in COVID-19 patients. The relative and absolute counts of lymphocyte subsets did not show a statistically significant difference between the two groups. Platelet count showed statistically significant positive correlation with absolute counts of total T lymphocytes, T helper, T cytotoxic, and B lymphocytes. The platelet to lymphocyte ratio (PLR), NLR and D-dimer results were negatively correlated with the total T lymphocytes, T helper, T cytotoxic, naïve T cytotoxic and B lymphocyte absolute counts.
The NLR, absolute neutrophil count and platelet count may serve as adjuvant predictors of survival in COVID-19 disease. Although lymphocyte subsets did not differ statistically across survival groups, their correlation with other possible prognostic markers may justify further investigation on their role in COVID-19 pathophysiology.

Keywords:
COVID-19, PLR, NLR, platelets, d-dimer, T-cell subset, flowcytometry, lymphocyte subset

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Introduction

A global pandemic has occurred since 2019 due to a novel coronavirus which resulted in severe acute respiratory syndrome (SARS-CoV-2). The disease was named COVID-19 as an abbreviation for Coronavirus Disease 2019. Immune system has a crucial role in the response to SARS-CoV-2 [1] with significant changes in different hematological and immune parameters among survivors and nonsurvivors [2]. Understanding the dynamics of immune system activation would help to predict prognosis through anticipating disease outcomes and probable complications. It would also help to determine the most relevant prognostic markers [3].

Starting with the basic laboratory tests, complete blood count (CBC) parameters were investigated to assess

their utility in exploring the immune status, diagnosis and prognosis of the COVID-19 patients. Many studies reported lymphopenia as a significant finding in patients with confirmed COVID-19 infection [4–6]. Bermejo-Martin and colleagues considered lymphopenia as a signature for severe COVID-19 infection and for associated pneumonia [7]. Significantly lower lymphocyte count was found in the elderly group in comparison to the younger age groups [8]. Shahid and colleagues suggested using absolute lymphocyte count and neutrophil to lymphocyte ratio (NLR) for ruling out a diagnosis of COVID-19 in clinically suspected patients, proposing that they could be used as a guide prior to referring patients for COVID-19 real time reverse transcription polymerase chain reaction (RT-PCR) testing [3,9].

It is noteworthy that NLR and platelet to lymphocyte ratio (PLR) have been investigated as independent predictors for prognosis of inflammation and COVID-19 severity and have been found to be significantly higher in severe cases in many studies [10,11]. Hence, it was suggested that they can be used as independent prognostic markers of disease severity [12] and to demarcate patients who might need intensive care unit (ICU) care [13].

Current knowledge indicated that T lymphocytes play an important role in the protection against coronaviruses. Therefore, it is of crucial importance to analyze the possible relationship of lymphocytes and their subpopulations in relation to the clinical outcome and complications of COVID-19. Evaluation of lymphocyte subsets could be helpful in predicting potential critical course of COVID-19 disease [14].

Cytotoxic CD8+ T cells produce perforins, granzymes and interferons which contribute to the elimination of COVID-19 virus [5]. A lower absolute number of both CD4+ and CD8+ T cells in COVID-19 infection were noted [14,15]. A more reduced fraction of CD8+ T cells was found to attribute to the development of acute respiratory distress syndrome (ARDS) in patients with severe COVID-19 infections [14]. This is indicative of the negative impact of COVID-19 induced disease on CD8+ T-cell fraction and functionality [14].

Also regarding severe cases of COVID-19, higher percentage of naïve helper T cells (CD3+CD4 +CD45RA+) and lower percentage of memory helper T cells (CD3+CD4+CD45RO+) was reported [14]. Other studies report increased CD45RO expression on CD4+ and CD8+ T cells in severe compared with mild cases [16]. Studies also reported the decline in B cells numbers with increased severity of illness [16,17].

There were no FDA approved antiviral for COVID-19, and most were symptomatic medications. Several studies proposed some candidate antiviral drugs. However, further experimental, and clinical trials were needed. Hence, the necessity of understanding the pathophysiology of this disease and its impact on the immune system [18].

This study aimed to evaluate the peripheral blood lymphocyte subsets by flowcytometry in hospitalized COVID-19 patients, and to investigate their association with clinical and laboratory variables. The patients were followed-up to record disease outcome, and to correlate it to different variables. These findings may help extend our understanding of the risk factors associated with increased mortality in the SARS-CoV-2 infection.

Materials and methods

Study design

A prospective single center study that was conducted in the period from June 2021 to January 2022, included 64 patients with confirmed COVID-19 infection by RT-PCR positive nasopharyngeal swabs. All patients were symptomatic adult patients (>18 years) who were admitted to the ICU in Ain Shams University Specialized Hospital El Obour, Cairo, Egypt, The Ethics Committee of Ain Shams University approved the protocol of this study. Informed consent was obtained from all the participants.

Data collection and clinical assessment

Demographic data (age and sex), comorbidities, date of onset of symptoms and hospital admission, vaccination history against COVID-19, respiratory rate, O2 saturation, treatment modality, respiratory support, and clinical outcome were assessed and recorded.

Patient classification

The patients' COVID-19 disease severity was assessed based on WHO COVID-19 clinical management guidelines. Accordingly, severe cases were defined as having severe pneumonia plus one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO_2 less than 90% on room air. Critical patients had either acute respiratory distress syndrome (ARDS), sepsis or septic shock. Patients with clinical signs of pneumonia but no signs of severe pneumonia, including SpO_2 greater than or equal to 90% on room air were identified as moderate cases, and those without

signs of pneumonia or hypoxia were identified as mild cases [19].

Patients were categorized according to their clinical outcome into non survivors when death occurred during hospitalization, and survivors who were discharged after being medically stable with no further need to be admitted to ICU.

Lymphocyte subsets evaluation by flowcytometry

Flow cytometric evaluation of the lymphocyte subsets was performed in the Immunogenetics Research Laboratory in the National Research Centre, Cairo, Egypt. It was done on whole blood collected on EDTA tubes using flow cytometry (BD Accuri C6 Cytometer, USA) to measure relative counts representing percentages of CD3 T lymphocytes, CD3CD4 T helper subset, CD3CD8 T cytotoxic subset, CD3CD4CD45RA naïve T helper cells, CD3CD4CD45RO memory T helper cells, CD8 CD45RA naïve T cytotoxic cells, CD8CD69 activated T cytotoxic cells, CD19 B lymphocytes and CD16 natural killer cells (NK cells). For this purpose, identification of different lymphocyte subsets was done on lysed whole blood by BD FACS^{TM} lysing solution using the monoclonal antibodies against following lymphocyte surface markers: CD3-PerCP, CD4-FITC, CD45RA-PE, CD4-PE, CD45RO-FITC, CD8-FITC, CD69-PE, CD19-PE, CD16-FITC (BD Biosciences, USA). The absolute counts of different lymphocyte subsets were calculated by multiplying the subset percentage by the absolute lymphocyte count. BD Accuri C6 software was used for the final analysis.

Laboratory assessment

The following routine laboratory tests were done for all patients at admission on the mentioned devices in the admitting hospital's central laboratory following standard procedures. CBC was done on ADVIA 560 (Siemens Healthcare diagnostics Inc., Germany) and Mindray BC_5130 (Jeevika Health care, China). The NLR and PLR were calculated. The D-dimer on VIDAS (Biomerieux, France), PT and aPTT (Acl elit pro, Instrumentation laboratory), serum ferritin and procalcitonin (Architect 100, ABBOT, USA) were also done. Serum C-reactive protein (CRP), Lactate dehydrogenase (LDH), Bilirubin, AST, ALT, total CK, CK-MB, Albumin, creatinine, BUN were done on Cobas C311 (Roche Diagnostics, Switzerland).

Statistical analysis

Data analysis packages used was SPSS version 21. Qualitative data was presented by number and percentage, quantitative data was presented by mean, standard deviation, median and interquartile range. χ^2 test and Fischer exact test were used for nonparametric qualitative data. For quantitative data student *t* test was used for parametric data and Mann Whitney test for nonparametric data. Test validity was calculated using ROC curve analysis, Youden index test of significance was used in validation with MedCalc. Pearson and spearman correlation tests were used between quantitative variables. Level of significance was set at *P* equal to or below 0.05.

Results

Demographic and baseline characteristics

From June 2021 to January 2022, 64 confirmed COVID cases were recruited in the current study. Cases were confirmed by positive RT-PCR for COVID-19. The age of patients ranged from 23 to 80 years with a mean of 61.5±14.3 years and male to female ratio 1.1/1. Comorbidities were found in 87.5% (56/64) of patients. The most common were diabetes 53.1% (34/64) and hypertension 53.1% (34/64). The mean time from onset of symptoms till sampling was 17.19±8.62 days and that from onset of symptoms till admission was 9.73±5.12 days. Fifty eight (90.6%) of patients were categorized as severe COVID-19 cases on admission, six (9.4%) were mild to moderate cases. The in-hospital mortality rate (nonsurvivors) was 48.4% (31/64). Nonsurvivors showed more comorbidities ($P=0.033$) especially diabetes ($P=0.004$), higher respiratory rate on admission ($P < 0.001$) and longer time from onset of symptoms to sampling ($P=0.045$) (Table 1).

Laboratory findings

The baseline laboratory parameters of the patients were analyzed to assess their contribution to in-hospital mortality. Relative lymphocyte count and platelet count (Plt) were significantly lower in nonsurvivors ($P=0.034$, 0.023, respectively). Conversely, total leucocyte count, neutrophil absolute count, neutrophil-to-lymphocyte ratio, Procalcitonin, Urea, conjugated bilirubin, CK-MB, lactate dehydrogenase, ferritin, D-dimers, troponin were significantly higher in nonsurvivors ($P=0.011$, 0.006, 0.011, 0.019, 0.007, 0.01, 0.009, <0.001, 0.038, 0.02, 0.009, respectively) (Table 2). Lymphopenia was a consistent finding in 46 (71.8%) of our patients with a median (IQR) lymphocyte count 0.6 (0.4-1.07)×10^9/l in all patients.

Flowcytometry of lymphocyte subsets

The relative and absolute counts of peripheral T-lymphocyte subsets, B and NK lymphocytes were

Table 1 Demographics and clinical characteristics of study population

Variable	Survivors *N* (%) 33 (51.6%)	Non survivors *N* (%) 31 (48.4%)	*P*- value	All patients (*N*=64)
Age (Mean±SD) (years)	58.4±14.9	65±13.1	0.063	61.5±14.3
Sex				
Male N (%)	18 (54.5%)	16 (51.6%)	0.915	34 (53.1%)
Female N (%)	15 (45.5%)	15 (48.4%)		30 (46.9%)
Comorbidities *N* (%)	26 (78.7%)	30 (96.7%)	**0.033**	56 (87.5%)
Hypertension *N* (%)	18 (54.5%)	16 (47.1%)	0.878	34 (53.1%)
Diabetes *N* (%)	13 (39.4%)	21 (61.8%)	**0.004**	34 (53.1%)
Heart disease (ISHD &AF) *N* (%)	9 (27.2%)	11 (35.4%)	0.432	20 (31.2%)
Chronic lung disease N (%)				
COPD/smoker	2 (6.1%)	1 (3.2%)	0.867	8 (12.5%)
BA	2 (6.1%)	3 (5.9%)		
Obesity (BMI >30) *N* (%)	0	3 (9.7%)	0.103	3 (4.6%)
CKD *N* (%)	2 (6.1%)	1 (3.2%)	1.000	3 (4.6%)
Time from onset of symptoms to admission (Mean±SD (days))	9.2±5.3	10.3±4.9	0.381	9.73±5.12
Time from onset of symptoms till sampling (Mean±SD (days))	15.1±7.5	19.4±9.3	**0.045**	17.19±8.62
Severity at admission *N* (%)				
Mild	3 (9.1%)	0	0.058	3 (4.6%)
Moderate	3 (9.1%)	0		3 (4.6%)
severe	27 (81.8%)	31 (100%)		58 (90.6%)
Clinical picture on admission				
RR/ min (Mean±SD)	25.6±4.5	32.5±6.2	**0.000**	28.86±6.35
O_2 saturation (%) (Mean±SD)	90.4±17.3	89.7±4.7	0.813	90.07±12.82
Treatment modality N (%)				
Antiviral and corticosteroid	8 (24.2%)	11 (35.5%	0.143	19 (29.6%)
Antiviral, corticosteroid and immunomodulators	21 (63.6%)	20 (64.5%)		41 (64.1%)
Antiviral only	4 (12.1%)	0		4 (6.2%)
Vaccination history (Sinopharm) *N* (%)	0 (0.0%)	2 (6.4%)	0.224	2 (3.1%)

Statistically significant *P* values are highlighted in bold. χ^2Chi square test with Fischer exact test was used for qualitative data. Student *t* test was used for quantitative data. AF, atrial fibrillation; BA, bronchial asthma; BMI, body mass index; CKD, chronic kidney disease; COPD, chronic obstructive lung disease; ISHD, ischemic heart disease; RR, respiratory rate.

determined. On comparing the results of the patients according to survival, no statistical difference was noted between survivors and non-survivors. However, median values of both relative and absolute counts of total T lymphocytes (CD3+ cells), T helper (CD3+CD4+), T cytotoxic (CD3+CD8+), naïve T helper (CD3+CD4+CD45RA+), memory T helper (CD3+CD4+CD45RO+), naïve T cytotoxic (CD8+CD45RA+) and B lymphocytes (CD19+) were higher in survivor's group. The median values of absolute counts of activated T cytotoxic cells (CD3+CD8+CD69+) and NK cells (CD16+) were also higher in the survivor's group but showed no statistical significance (Table 3).

Correlations with lymphocyte subsets

The CBC (Plt, PLR and NLR) and inflammatory parameters (D-dimer, ferritin and CRP) correlations with the lymphocyte subsets were assessed using the Spearman's test. Platelet count was positively correlated with the absolute counts of total T lymphocytes (CD3+ cells, $P=0.007$), T helper (CD3+CD4+, $P=0.015$), T cytotoxic (CD3+CD8+, $P=0.034$) and B lymphocytes (CD19+, $P=0.0096$), while no correlation was found with NK-cells (CD16+). The PLR and NLR were negatively correlated with the total T lymphocytes (CD3+ cells, $P < 0.001$), T helper (CD3+CD4+, $P=0.0013$, < 0.001), naïve T helper (CD3+CD4+CD45RA+, $P=0.04$, 0.01), memory T helper (CD3+CD4+CD45RO+, $P=0.001$, < 0.001), T cytotoxic (CD3+CD8+, $P=0.0015$, < 0.001), naïve T cytotoxic (CD8+CD45RA, $P=0.012$, < 0.001), B lymphocyte (CD19+, $P=0.0052$, 0.0058), and NK (CD16+, $P < 0.001$, 0.0078) absolute counts (Table 4 and Figs. 1–3).

Ferritin level did not show any correlation with the examined lymphocyte subsets. D-dimer was negatively correlated with the absolute counts of total T lymphocytes (CD3+ cells, $P=0.001$), T helper (CD3+CD4+, $P = 0.001$), T cytotoxic (CD3+CD8+, $P = 0.001$), naïve T cytotoxic (CD8+CD45RA, $P < 0.001$), B- Lymphocyte (CD19+, $P = 0.05$). CRP

Table 2 Baseline laboratory findings in the study population

Parameter	Survivors *N* (%) 33 (51.6%) Median [IQR]	Non-survivors *N* (%) 31 (48.4%) Median [IQR]	*P* value	All patients (*N*=64) Median [IQR]
TLC (10^9/l)	8.75 [7.27–12.24]	12.00 [8.6–21]	**0.011**	10.45 [7.6–15.22]
Neut (%)	89.30 [79.75–91.23]	92.00 [88.6–94.6]	**0.007**	90.3 [86.8–93.1]
Neut (10^9/l)	7.84 [5.53–11.03]	11.01 [7.75–18.88]	**0.006**	8.87 [6.8–13.84]
Ly (%)	6.30 [4.65–11.05]	5.60 [2.7–8]	**0.034**	5.95 [3.9–8.7]
Ly (10^9/l)	0.58 [0.41–1.05]	0.64 [0.39–1.11]	0.706	0.63 [0.41–1.07]
Hb (g/dL)	13.1 [11.1–13.8]	11.8 [10.4–13.4]	0.085	12.5 [10.6–13.7]
Plt (10^9/l)	251 [165–318]	167 [126–230]	**0.023**	200 [140–306]
NLR	14.16 [6.91–19.15]	15.87[15.88–35]	**0.011**	15.24[10.34–3.53]
PLR	399.2 [168.9–653.8]	254.3[171.7–403.6]	0.207	289.7 [185.6–502]
Procalcitonin (μg/l)	0.07 [0.03–0.17]	0.3 [0.05–1.34]	**0.019**	0.085 [0.04–0.31]
CRP (mg/l)	22.5 [5.8–47.2]	37.3 [8–116.6]	0.100	26.2 [7.5–82.6]
Urea (mg/dL)	65.5 [45.2–90.5]	82 [65–165]	**0.007**	71 [56–114]
Creatinine (mg/dL)	1.0 [0.88–1.43]	1.2 [1–2.2]	0.066	1.1 [0.9–1.9]
Conj bil (mg/dL)	0.2 [0.1–0.4]	0.4 [0.3–0.8]	**0.010**	0.3 [0.2–0.4]
Total bil (mg/dL)	0.3 [0.2–0.5]	0.3 [0.2–0.5]	0.623	0.3 [0.2–0.5]
AST (IU/l)	28 [18–50]	32 [20–58]	0.599	30 [19–55]
ALT (IU/l)	27 [22–49]	30 [17–42]	0.668	28 [20–42]
CK–MB (IU/l)	16 [13–22]	23 [18–32]	**0.009**	20 [14–25]
LDH (IU/l)	376.5 [289–478]	627 [450–947]	**0.000**	455 [342–663]
Ferritin (μg/l)	587 [372–1160]	925 [560–1473]	**0.038**	778 [447–1339]
D–Dimer (μg/l)	1.34 [0.46–2.72]	1.75 [0.93–7.31]	**0.020**	1.5 [0.6–3.6]
PT (Secs)	13.3 [12–15]	13.4 [12–17.5]	0.460	13.3 [12–16.3]
INR	1.13 [1–1.29]	1.17 [1.04–1.54]	0.221	1.15 [1.01–1.4]
aPTT (Secs)	29.9 [26.5–32.1]	31.4 [27.2–34.4]	0.286	30.3 [26.9–34]
Troponin (μg/l**)**	6.3 [1.9–18.75]	21.3 [7.9–62.9]	**0.009**	11.5 [3–50.45]

Statistically significant p values are highlighted in bold. Mann Whitney test was used. Reference values: TLC (10^9/l): 4.0–10.0; Neut (10^9/l): 2.0-7.0; Ly (10^9/l): 1.0-3.0; Hb (g/l): 12–16; Plt (10^9/l): 150–410; Procalcitonin (μg/l): 0-0.5; CRP (mg/l): 0–6; Urea (mmol/l): 2.14-7.14; Creatinine (μmol/l): 62–115; Conj Bil (μmol/l): <3.4; Total Bil (μmol/l): <20.5; AST (IU/l): 13-39; ALT (IU/l): 7–52; CK-MB (IU/l): 5-25; LDH (IU/l): 140–271; Ferritin (ug/l): male 30-400/ female 13-150; D-Dimers (μg/l): 0–500.00; PT (secs): 11.7–15.3; INR: 0.80–1.20; aPTT (secs): 26-40; Troponin (μg/l): 0-40. *ALT:* alanine transaminase; *aPTT*: activated partial thromboplastin time; *AST* : aspartate transaminase; *CK-MB:* creatine kinase mb; *Conj Bil* : Conjugated bilirubin; *CRP:* C-reactive protein; *Hb*, haemoglobin; *INR*: international normalized ratio; *LDH* : lactate dehydrogenase; *Ly*: lymphocyte; *Neut:* neutrophils; *NLR:* neutrophil-to-lymphocyte ratio; *PLR*: platelet lymphocyte ratio; *Plt*: platelets; *PT:* prothrombin time; *TLC:* total leucocyte count; *Total Bil*: total bilirubin.

Table 3 Lymphocyte subpopulations in the study population

Parameter	Survivors *N* (%) 33 (51.6%) Median [IQR]	Non-survivors *N* (%) 31 (48.4%) Median [IQR]	*P* value	All patients (N=64) Median [IQR]
CD3 (%)	31.8 [21.1–39.5]	23.4 [14.6–33.9]	0.133	29.1 [18.9–36.9]
CD3 (10^6/l)	181.8 [104.9–284.9]	169.1 [84.6–362.6]	0.41	176.8 [89.7–293.5]
CD3/CD4 (%)	15.7 [11.3–22]	11.3 [5–18.3]	0.086	12.6 [8.5–20.2]
CD3/CD4 (10^6/l)	113 [67.1–139.1]	70.6 [32.4–134]	0.215	95.5 [44.8–135]
CD3/CD4/CD45RA(%)	5.2 [2.1–9.6]	2.8 [1.2–7.9]	0.165	5 [1.8–7.7]
CD3/CD4/CD45RA (10^6/l)	35.2 [10.4–86.5]	24.2 [8–45.3]	0.28	34.4 [9.6–51]
CD3/CD4/CD45RO (%)	7.1 [1.7–8.8]	5.3 [2.4–8.4]	0.674	6.5 [2.9–8.5]
CD3/CD4/CD45RO (10^6/l)	43.5 [12.8–100.8]	23.2 [11.6–60.7]	0.49	39.2 [14.1: 61.5]
CD3/CD8 (%)	11 [4.2–18]	8.3 [4.9–15.8]	0.694	10.5 [4.7–15.9]
CD3/CD8 (10^6/l)	71.8 [24.8–112]	50.6 [28.2–142.3]	0.783	57.6 [26.6–112]
CD8/CD45RA (%)	7 [3.3–14.1]	6.7 [2.9–12.8]	0.422	6.7 [3.3–13.6]
CD8/CD45RA (10^6/l)	53.2 [14.2–87.6]	33.8 [15–108.2]	0.569	45.2 [15–86.9]
CD3/CD8/CD69 (%)	0.6 [0.3–1.25]	0.6 [0.2–1.5]	0.788	0.6 [0.3–0.8]
CD3/CD8/CD69 (10^6/l)	4.5 [1.4–9.2]	2.7 [1.2–8.6]	0.549	3.3 [2.4–6.4]
CD16 (%)	15.1 [9.7–23]	17.3 [8.2–25]	0.667	15.5 [9–24]
CD 16 (10^6/l)	99.1 [42.2–206.5]	86.9 [49.1–251.7]	0.672	95.5 [48.4–232.2]
CD19 (%)	9.6 [4.4–16.9]	7.6 [3.1–11.5]	0.147	9 [4–13.7]
CD19 (10^6/l)	60 [23.9–107.3]	55.2 [15.7–98.3]	0.528	60 [18.6–96]

Mann Whitney test was used.

Table 4 Correlation between lymphocyte subpopulation absolute counts and laboratory parameters

	Plt count	PLR	NLR	D–dimer
CD3 (10^6/l)				
Rho	0.3457	–0.44658	–0.62379	–0.434
P value	**0.007**	<**0.001**	<**0.001**	**0.001**
CD3/CD4 (10^6/l)				
Rho	0.31355	–0.40877	–0.6021	–0.412
P value	**0.015**	**0.0013**	<**0.001**	**0.001**
CD3/CD4/CD45RA (10^6/l)				
Rho	0.27946	–0.34459	–0.43917	–0.316
P value	0.11525	**0.04**	**0.01**	0.073
CD3/CD4/CD45RO (10^6/l)				
Rho	0.02826	–0.5693	–0.61958	–0.355
P value	0.88215	**0.001**	<**0.001**	0.054
CD3/CD8 (10^6/l)				
Rho	0.27515	–0.40339	–0.48784	–0.411
P value	**0.034**	**0.0015**	<**0.001**	**0.001**
CD8/CD45RA (10^6/l)				
Rho	0.24192	–0.3305	–0.4499	–0.467
P value	0.06983	**0.012**	<**0.001**	<**0.001**
CD3/CD8/CD69 (10^6/l)				
Rho	0.17033	–0.52747	–0.36264	–0.262
P value	0.57797	0.06395	0.22332	0.366
CD 16 (10^6/l)				
Rho	0.19493	–0.48872	–0.34296	–0.141
P value	0.13901	<**0.001**	**0.0078**	0.282
CD19 (10^6/l)				
Rho	0.33439	–0.35897	–0.35453	–0.254
P value	**0.0096**	**0.0052**	**0.0058**	**0.050**

Statistically significant *P* values are highlighted in bold. Rho: Spearman's rank correlation coefficient.

was negatively correlated with T cytotoxic (CD3+CD8+, $P=0.21$) and naïve T cytotoxic (CD8+CD45RA, $P=0.009$) absolute counts (Table 4).

ROC analysis of NLR

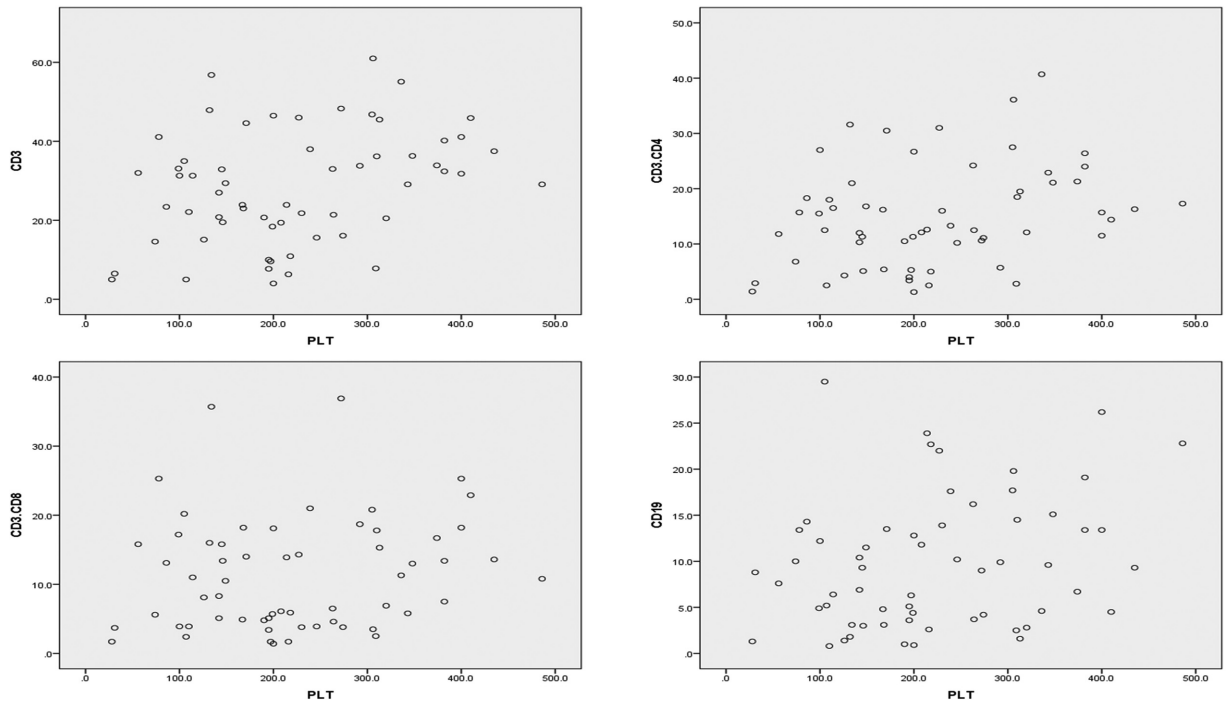
The ROC analysis for NLR revealed a cutoff value of 15.33 was associated with in-hospital mortality with sensitivity of 70.6% and specificity of 61.29% (AUC = 0.685, $P=0.005$, CI 0.558-.795) (Fig. 4).

Discussion

This study was conducted on 64 hospitalized COVID-19 patients. Most of the patients were severe cases 58 (90.6%), mostly unvaccinated for COVID-19, with an in-hospital mortality 31 (48.4%) for all patients. As for clinical risk factors among nonsurvivor's group and in concordance with peer studies, diabetes was a statistically significant risk factor for mortality Li and colleagues, Lima-Martinez and colleagues [20,21]. This can be explained by diabetic patient's higher susceptibility to cytokine storm and hyperinflammation [22]. Other factors including age, gender and other comorbidities did not show any statistical difference between the survivors and nonsurvivors' groups.

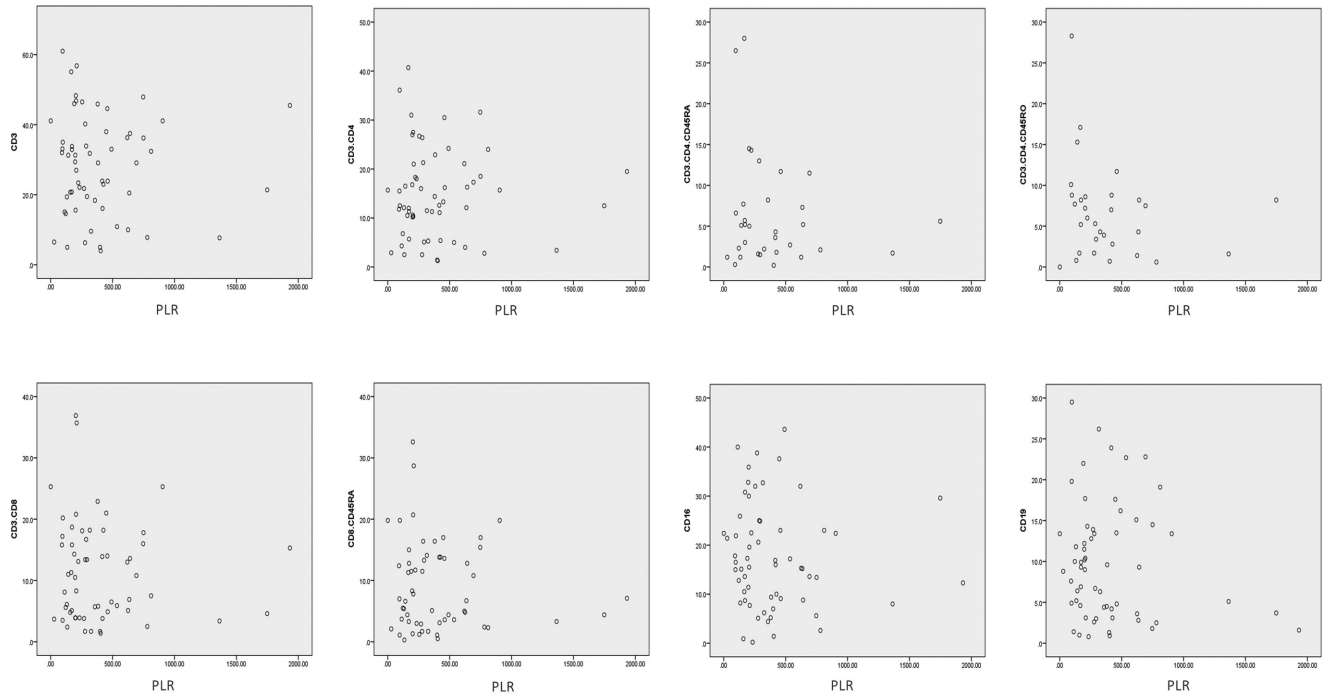
Absolute lymphopenia was a consistent finding in 46 (71.8%) of the patients. Nonsurvivors showed lower

Figure 1



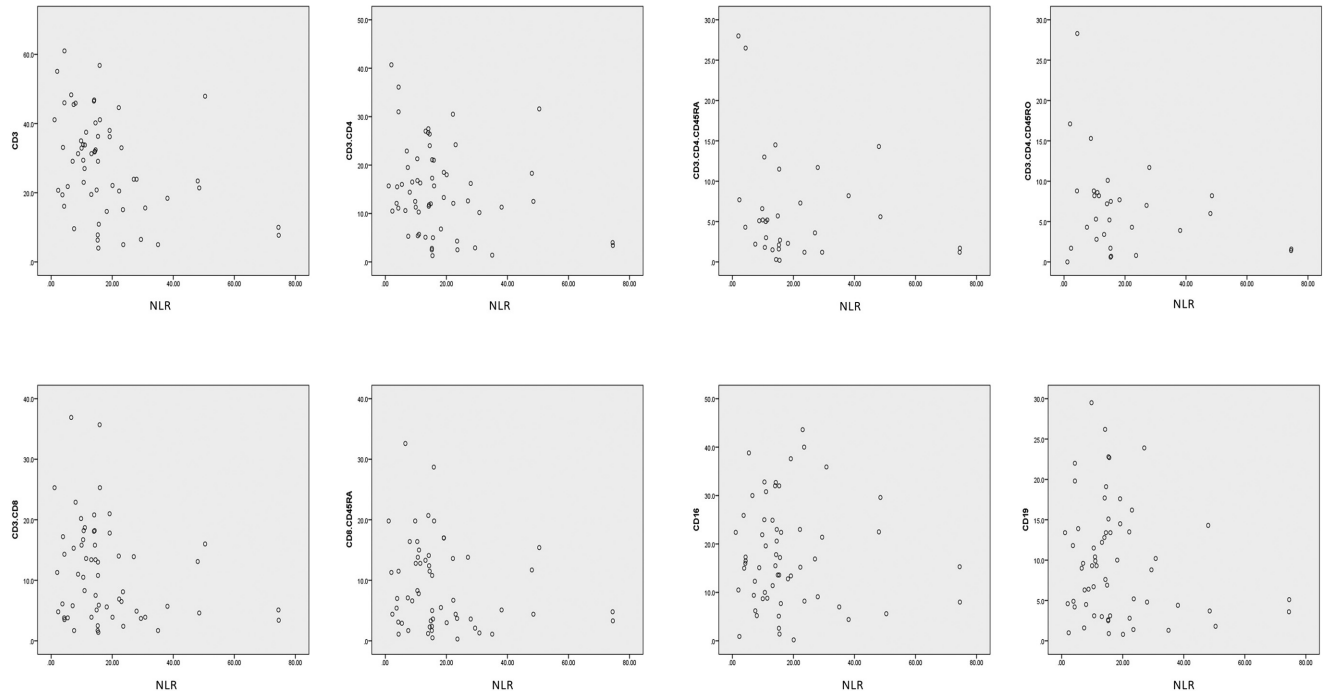
Correlations of platelet count with lymphocyte subsets.

Figure 2



Correlations of PLR with lymphocyte subsets.

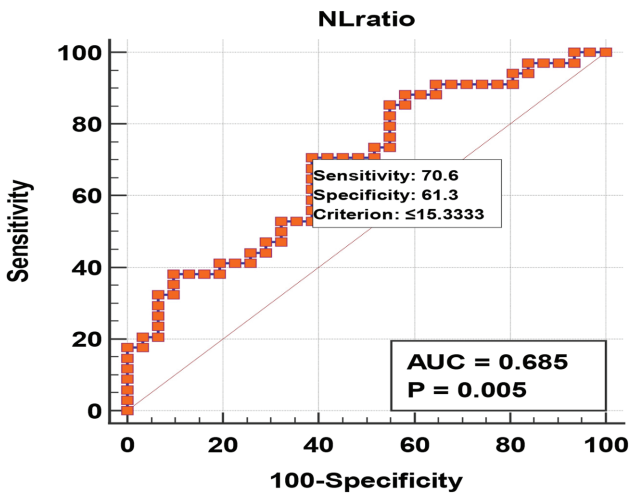
Figure 3



Correlations of NLR with lymphocyte subsets.

relative lymphocyte count, higher absolute neutrophil count and higher NLR compared with survivors. Lymphopenia and neutrophilia were reported as the most common hematological changes in COVID-19 patients [23] and lymphocyte count was proposed as a marker for classification of disease severity besides pulmonary imaging [24]. NLR was associated with all-cause mortality in COVID-19 patients [11,25]. After ROC curve analysis, NLR at a cut off 15.3 had a sensitivity of 70.59% and specificity of 61.29%

Figure 4



ROC analysis showing the performance of baseline NLR in distinguishing non-survivors from survivors.

as an indicator for mortality in COVID-19 patients. Similarly, but in a different ethnic group, Wang found that NLR at admission, with a cut off 3.338, was associated with all-cause mortality; with a sensitivity of 100% and a specificity of 84% [11].

The high NLR results can be explained by the inflammatory response that can stimulate the production of neutrophils and speed up the apoptosis of lymphocytes [25]. Secondary bacterial infection, a common finding in severe COVID-19 infection increases the neutrophil count. Regarding corticosteroid intake and whether or not it can influence the NLR as proposed by Bedel and Korkut, 2021, most of the patients were on steroids [60 (93.7%)] and still NLR was significantly higher in nonsurvivors [26].

The reduction of peripheral lymphocytes, especially of T-cell subsets, in COVID-19 patients can be justified by different mechanisms that might be interacting together in many instances: (1) direct infection of T-lymphocyte by SARS-CoV-2 and subsequent cytolysis or apoptosis (through the cellular receptor Basigin [BSG/CD147]) [27], (2) Migration of lymphocytes to the lungs [28,29], and (3) Functional exhaustion of surviving T-cells in severe COVID-19 patients as marked by their increased PD-1 and Tim-3 expression leading to reduction in their replicative abilities upon stimulation [30].

Similar to previous literature, nonsurvivors showed lower platelet count compared with survivors ($P = 0.023$) [11,31]. The platelet count is a marker of disease severity and prognosis in COVID-19 patients. In COVID-19 patients disseminated intravascular coagulopathy (DIC) is indicated by thrombocytopenia, prolonged PT and high D-dimer level [23]. Reduced platelet count may also be due to increased platelet clearance through the activation of the immune system and an antibody-mediated phagocytic response [32].

However, PLR ratio showed no statistical difference among survivors and non-survivors. This agrees with the results of Wang and his colleagues and Kalabin and his colleagues [11,33]. On the other hand, Qu *et al* 2020 reported that PLR at the peak of platelet count recorded for the COVID-19 patients was associated with severe cases [34].

Relative and absolute counts of total T lymphocytes (CD3+ cells), T helper (CD3+CD4+), T cytotoxic (CD3+CD8+), naïve T helper (CD3+CD4+CD45RA+), memory T helper (CD3+CD4+CD45RO+), naïve T cytotoxic (CD8+CD45RA+), activated T cytotoxic cells (CD3+CD8+CD69+), NK cells (CD16+) and B lymphocytes (CD19+) were higher in survivor group, however they did not show significant difference from nonsurvivors. Our results support the findings of previous literature that estimation of absolute counts of T lymphocytes and T lymphocyte subsets are predictive of in-hospital mortality and concluded that their estimation help in identification of patients at increased risk of unfavorable outcomes. Lack of significance unlike previously reported results by Deng *et al* 2020 and Iannetta *et al* 2021 whose results showed significant increase in the absolute counts of total T lymphocytes (CD3+ cells), T helper (CD3+ CD4+), T cytotoxic (CD3+ CD8+) and relative count of B lymphocytes (CD19+) in the survivor's group may be due to low number of cases in this study in comparison to previously mentioned studies [2,35].

Platelet count positively correlated with absolute counts of total T lymphocytes (CD3+ cells), T helper (CD3+CD4+), T cytotoxic (CD3+CD8+) and B lymphocytes (CD19+), while no correlation was found with NK-cells (CD16+). This is in concordance with Wang *et al* 2021, where CD4+ and CD8+ T-cell counts positively correlated with platelet count [36].

The PLR and NLR were negatively correlated with the total T lymphocytes (CD3+ cells), T helper (CD3+CD4+), naïve T helper (CD3+CD4+CD45RA+), memory T helper (CD3+CD4+CD45RO+),

T cytotoxic (CD3+CD8+), naïve T cytotoxic (CD8 +CD45RA), B lymphocyte (CD19+) and NK (CD16 +) absolute counts. In agreement with our findings, Li and his colleagues also found significant negative correlation between NLR and lymphocyte subsets namely T, T helper, T cytotoxic, NK and B cells [37]. The correlation between platelet count, PLR and lymphocyte subsets can be explained by that platelets are suggested to be highly active in severe cases of COVID-19 leading to formation of platelet–leucocyte aggregates [38] with increased formation of CD4+ T lymphocyte–platelet aggregates, that have a potential role in dampening inflammatory responses in COVID-19 [39].

Regarding other laboratory markers, serum LDH, ferritin, D-dimer, procalcitonin and troponin were significantly higher in the nonsurvivor group. This is in correlation with Zhou *et al* 2020 and Henry *et al* 2020. It is postulated that elevated procalcitonin may be driven by the higher rate of secondary infection rate seen in non-survivors versus survivors [31,40]. Biomarkers of cardiac injury were found to be elevated in patients with fatal COVID-19, suggesting potential for viral myocarditis as well as cardiac injury secondary to progression to multiple organ failure [41].

The D-dimer level was negatively correlated with the total T lymphocytes (CD3+ cells), T helper (CD3 + CD4+), T cytotoxic (CD3 + CD8+), naïve T cytotoxic (CD8+CD45RA+) and B- Lymphocyte (CD19+) absolute counts. This is in concordance with Wang *et al* 2020 where CD4+ and CD8+ T-cell counts negatively correlated with D-dimer and LDH [16].

There were some limitations in this study. Most of the enrolled patients were severe cases which may have influenced statistical judgment upon difference in level of lymphocyte subsets between survivor and nonsurvivor groups. However, it can be an advantage to describe the findings in these severe COVID-19 cases, whose numbers are expected to diminish with the spread of vaccinations.

Conclusion

In conclusion, we identified several candidate variables that may serve as clinical predictors of fatal COVID-19. The NLR, absolute neutrophil count and platelet count may serve as adjuvant predictors of survival. The absolute counts of T helper (CD3+CD4+) and T cytotoxic (CD3+CD8+) lymphocytes positively correlated with platelet counts and correlated negatively with NLR, PLR ratio and D-dimer results. Thus, lower T helper and T cytotoxic counts were associated with lower platelet counts and higher D-dimer levels, justifying the possible use of flowcytometric evaluation of lymphocyte subsets to better understand the pathophysiology of COVID-19 disease.

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Declaration: I confirm that this work is original and has not been published or submitted for publication elsewhere. All authors have approved the manuscript for submission.

Ethics approval and consent to participate: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Ain Shams University (approval number FMASU R 13 2022).

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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