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ORIGINAL ARTICLE

The role of CD4$^+$, CD8$^+$, CD4$^+$/CD8$^+$ and neutrophile to lymphocyte ratio in predicting and determining COVID-19 severity in Indonesian patients

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Background: Biomarkers that are cost-effective and accurate for predicting severe coronavirus disease 2019 (COVID-19) are urgently needed. We would like to assess the role of various inflammatory biomarkers on admission as disease severity predictors and determine the optimal cut-off of the neutrophile-to-lymphocyte ratio (NLR) for predicting severe COVID-19.

Methods: We conducted a cross-sectional study in six hospitals in Bali and recruited real-time PCR-confirmed COVID-19 patients aged >18 y from June to August 2020. Data collection included each patient's demographic, clinical, disease severity and hematological data. Multivariate and receiver operating characteristic curve analyses were performed.

Results: A total of 95 Indonesian COVID-19 patients were included. The highest NLR among severe patients was 11.5±6.2, followed by the non-severe group at 3.3±2.8. The lowest NLR was found in the asymptomatic group (1.9±1.1). The CD4$^+$ and CD8$^+$ values were lowest in the critical and severe disease groups. The area under the curve of NLR was 0.959. Therefore, the optimal NLR cut-off value for predicting severe COVID-19 was ≥3.55, with sensitivity at 90.9% and a specificity of 16.7%.

Conclusions: Lower CD4+ and CD8$^+$ and higher NLR values on admission are reliable predictors of severe COVID-19 among Indonesian people. NLR cut-off ≥3.55 is the optimal value for predicting severe COVID-19.

Keywords: CD4$^+$, CD8$^+$, COVID-19, neutrophil-to-lymphocyte ratio, predictor

Introduction

Coronavirus disease 2019 (COVID-19), which was first reported in December 2019 and determined as a pandemic by the WHO, had infected >420 million people globally and resulted in >5.8 million deaths by February 2022.[1] COVID-19 infected >5.2 million Indonesian people and resulted in >140 000 deaths. Several efforts have been employed to reduce COVID-19 infections, such as enforcing health protocol laws and distributing the COVID-19 vaccine nationally.[2] However, no specific anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) drugs have been developed until now, and SARS-CoV-2 mutation has been spreading. Therefore, prompt diagnosis and identification of COVID-19 patients at risk of developing severe courses are essential. Several signs and symptoms, risk factors, comorbidities and biomarkers have been studied to find the association between those and the severity of COVID-19. Several biomarkers for predicting severe COVID-19 may not be applicable in low- and middle-income countries or with limited resources in healthcare facilities. As COVID-19 is a pandemic, a cost-effective yet readily available and accurate biomarker is needed.[3,4] In this study, we focus on laboratory parameters of $CD4^+$, $CD8^+$ and the neutrophile-to-lymphocyte ratio (NLR) as a predictor of COVID-19 severity in the Indonesian population. We want to explore the role and dynamics of lymphocyte subtypes in COVID-19. We also determine the NLR cut-off value in predicting COVID-19 severity, which may help physicians allocate the type of care for COVID-19 and periodic observation.

Materials and Methods

Study design

We conducted a cross-sectional study in six hospitals in Bali and recruited all confirmed COVID-19 patients aged >18 y based on real-time PCR from June to August 2020. Informed consent was obtained before the study and samples were collected using consecutive sampling methods. Sera were collected on the admission day. Each patient's demographic, clinical and hematological data and disease severity according to the National Guidelines of COVID-19 Management were recorded.[3] The subjects were divided into five subgroups according to clinical typing: asymptomatic, mild, moderate, severe and critically ill. We excluded patients with HIV infection.

Flow cytometry

EDTA anticoagulated whole blood samples were collected from all subjects. Lymphocyte subsets were detected and counted by Fluorescent Flow Cytometry (BD FACS Canto II) using BD Multitest CD3/CD8/CD45/CD4 reagents, and corresponding phenotypes of CD antigens characterized the subsets. Tests were performed according to the product manual.

When whole blood is added to the reagent, the fluorochrome-labeled antibodies bind specifically to leucocyte surface antigens. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter, and fluorescence signals, detected by the instrument, provide information about the cell's size, internal complexity and relative fluorescence intensity. BD Multitest CD3/CD8/CD45/CD4 reagents use fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate. A precise volume of sample is stained directly in a BD Trucount tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads.

Results are reported as the percentage of positive cells per lymphocyte population or as the number of positive cells per microliter of blood (absolute count). During analysis, the absolute number (cells/μl) of gated cells in the sample can be determined by comparing cellular events with bead events (Figure 1).

Statistical analysis

Data analysis was supported with SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, Ill., USA). Continuous variables were denoted as mean±SD to normal data and median (IQR) to skewed data. ANOVA was performed to determine the significant difference of white blood cells (WBC), $CD4^+$ and hemoglobin. Kruskall–Wallis analysis was performed to determine the significant difference of age, gender, total lymphocytes count, NLR, platelet, $CD8^+$, $CD4^+$:$CD8^+$ ratio, ICU admission and ventilator usage, then a post-hoc analysis using the Bonferroni approach. The receiver operating characteristic (ROC) curve was used to

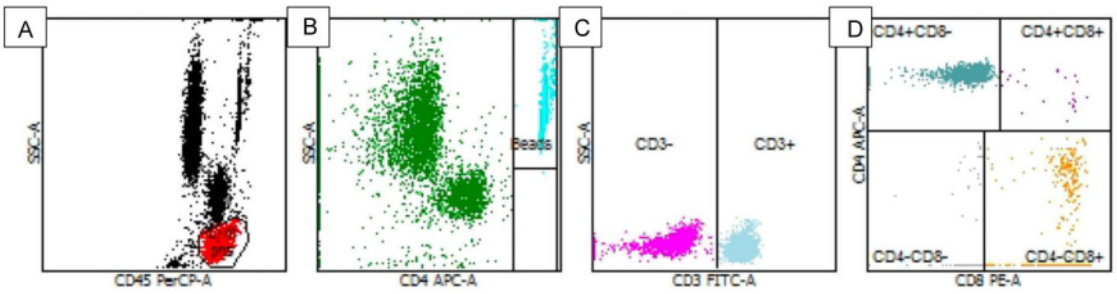

Figure 1. Example flow cytometry gating: FACS dot plot on BD FACS Canto II gating CD4 and CD8 T cells. Panel A depicts $CD45^+$ lymphocytes (1) detected in the dot plot of CD45 PerCP-A vs SSC-A. Panel B shows the CD4 APC-A vs SSC-A dot plot with BD Trucount absolute count bead events (2). Panel C depicts $CD3^+$ T cells in the dot plot of CD3 FITC-A vs SSC-A. In the CD8 PE-A vs CD4 APC-A dot plot, Panel D depicts suppressor/cytotoxic ($CD4^-CD8^+$) and helper/inducer ($CD4^+CD8^-$) T lymphocytes.

assess the diagnostic value of NLR in predicting COVID-19 severity. An ordinal logistic regression test was used for multivariate analysis. A value of $p<0.05$ indicated statistical significance.

Results

From June to August 2020, 95 COVID-19 patients were eligible for the study. The mean age of all participants was 44.9±16.1 y and 52 patients were male (54.7%). Most patients were asymptomatic (35.8%) and the most common comorbidity was diabetes mellitus (15.8%). Shortness of breath (24.2%) was the most common chief complaint among symptomatic COVID-19 patients. ICU admission was seen in 11.6% of patients, ventilator usage was observed in 2.1% of patients and no mortality was found among all groups in this study. The mean WBC of all participants was 7326.8±2864.1/μl, the mean total lymphocyte count was 1945.8±995/μl, the mean $CD8^+$ was 587.9±381.1/μl, the mean $CD4^+$ was 661.5±299.3/μl and the mean $CD4^+$:$CD8^+$ ratio was 1.3±0.6. The patients' characteristics were grouped according to disease severity and are presented in Table 1.

The highest NLR was found among severe COVID-19 patients (the critically ill and severe disease subgroups: 11.5±6.2) followed by the non-severe group (the mild and moderate disease subgroups: 3.3±2.8), while the lowest NLR was found in the asymptomatic subgroup (1.9±1.1). We found that $CD4^+$ and $CD8^+$ values were lowest in the critical and severe disease subgroups: 130.7±46.8/μl and 152.8±131.6/μl, respectively. The highest $CD4^+$ count was found in the asymptomatic subgroup (771.6±241.7/μl), followed by the non-severe group (701.1±246.8/μl). The same trend was displayed by the CD8 count, for which the highest value was found in the asymptomatic subgroup (696.9±317.8/μl) followed by the non-severe group (606.3±398.6/μl). ANOVA was performed to determine the significant difference between NLR, $CD4^+$ and $CD8^+$ according to disease severity and all the analyses showed significant differences ($p<0.001$).

Post-hoc analysis using the Bonferroni approach was performed to further determine the difference signification. The NLR value was significantly different between asymptomatic and severe disease groups ($p<0.001$). NLR analysis between non-severe and severe groups also showed significant differences ($p<0.001$). A similar trend was also displayed comparing $CD4^+$ and $CD8^+$ values of the severe and asymptomatic groups, as well as the non-severe and severe groups. The result was $p<0.001$ for all those analyses.

Ordinal logistic regression was performed to determine the simultaneous effect between $CD4^+$, $CD8^+$ and NLR on disease severity. This analysis showed that each elevation of one NLR unit would increase the severity risk from asymptomatic to non-severe or non-severe to severe group by 35% ($p=0.001$). Conversely, each elevation of one $CD4^+$ decreased the severity risk by 0.2% ($p=0.001$), while increasing the CD8 value was not associated with an increased severity risk ($p=0.816$). Furthermore, the fitting model analysis showed a significance value ($p<0.001$) and the pseudo R-square value was 0.449. Therefore, it was concluded that a 44.9% variation in disease severity between study participants was contributed by the difference value of $CD4^+$, $CD8^+$ and NLR.

Diagnostic testing using ROC curve analysis showed that the area under the curve was 0.959 for the NLR (Figure 2). Therefore, the NLR is an excellent predictor to determine COVID-19 disease severity. We found that an NLR cut-off point of ≥3.550 is associated with the highest sensitivity and specificity to differentiate between the severe and non-severe COVID-19 groups. The sensitivity was described as 90.9% and the specificity as 16.7%.

Discussion

In viral infections, $CD4^+$ and $CD8^+$ T cells fight the acute viral infection at the front in harmony with other immune system components. The naïve $CD4^+$ T cells are activated by peptide viral antigens and differentiated into distinct effector subtypes. They secrete specific cytokines that boost $CD8^+$ T cell activation and activate B-lymphocytes, where the plasma cells produce specific immunoglobulins (Ig). The activated $CD8^+$ T cells have roles in killing virus-infected cells by programming them to undergo self-apoptosis or releasing perforin and granzymes to create holes in the membrane of target cells and initiate apoptosis. This mechanism leads to a decrease in viral loads. Therefore, lymphocytosis is commonly seen in viral infection as an immune response. The study showed that lymphocytopenia is observed in >90% of severe COVID-19 patients and is related to poor prognosis.[5] Lymphocytopenia is typically observed in COVID-19 patients with a drop in $CD8^+$ T cells relative to $CD4^+$ T cells that is proportionate to the disease's severity. As patients start to improve, T-lymphocyte counts begin to increase, indicating a correlation between the count and disease development and regression.[6] This finding draws a comparison with HIV infection, which causes lymphopenia mainly attributed to the depletion of $CD4^+$ and is related to a worse prognosis.[7]

The mechanism of $CD4^+$ depletion has been widely described, consisting of direct attack by HIV, chronic immune activation, accelerated $CD4^+$ apoptosis rate, cytokine storm and dysregulation by regulatory T-cells (T-reg). These mechanisms lead to abrupt $CD4^+$ depletion in the early disease course and persistent low $CD4^+$ in the long term.[8] We found a significantly lower total lymphocyte count, $CD4^+$, $CD8^+$ and $CD4^+$:$CD8^+$ ratio among the critically ill and severe COVID-19 group. A study from Guan et al. supports this finding, that lower median lymphocyte count in the severe group compared with the non-severe group (800 vs 1000/mm^3) and the frequency of lymphopenia (lymphocyte count <1500/mm^3) was higher in the severe group (96.1% vs 80.4%).[9] A review conducted by Jafarzadeh et al. found that lymphopenia is reported in 32.7–96.1% of severe COVID-19 patients and 0.6–80.4% of patients with mild/moderate COVID-19, consistently indicating that lymphopenia is more commonly found in severe disease.[5] A meta-analysis conducted by Zhao et al. showed that lymphocyte count was significantly lower in severe COVID-19 patients. Therefore, lymphopenia was associated with an increased risk of severe COVID-19.[10] Zheng et al. and Liu et al. showed that $CD4^+$ and $CD8^+$ were significantly lower in critically ill COVID-19 patients compared with non-critical patients.[11,12] Therefore, based on those findings, we concluded that lymphopenia and low $CD4^+$ and $CD8^+$ levels were associated with more severe COVID-19 and may predict disease prognosis. In addition,

Table 1. Characteristics of participants grouped according to disease classification

Characteristics	Disease classification					p
	Critically Ill (n=3)	Severe (n=8)	Moderate (n=18)	Mild (n=32)	Asymptomatic (n=34)	
Age, y	47.3±11.9[a]	58.9±11.6[a]	59.8±10.3[a]	41.2±16.6[a]	31.8 (19.1)[b]	<0.001
Gender, n (%)						
Male	3 (3.2)	5 (5.3)	12 (12.6)	18 (18.9)	14 (14.7)	0.185
Chief complaints, n (%)						
Cough	0 (0)	0 (0)	2 (2.1)	11 (11.6)	0 (0)	-
Fever	0 (0)	1 (1.1)	3 (3.2)	17 (17.9)	0 (0)	
Lethargy	0 (0)	0 (0)	0 (0)	2 (2.1)	0 (0)	
Headache	0 (0)	0 (0)	0 (0)	1 (1.1)	0 (0)	
Sore throat	0 (0)	0 (0)	0 (0)	1 (1.1)	0 (0)	
Shortness of breath	3 (3.2)	7 (7.4)	13 (13.7)	0 (0)	0 (0)	
None	0 (0)	0 (0)	0 (0)	0 (0)	34 (35.8)	
Comorbidities, n (%)						
Diabetes	1 (1.1)	3 (3.2)	5 (5.3)	6 (6.3)	0 (0.0)	
Coronary artery disease	0 (0)	0 (0)	1 (1.1)	0 (0)	0 (0)	
Lung comorbidities	1 (1.1)	0 (0)	0 (0)	0 (0)	0 (0)	-
Cancer	0 (0)	0 (0)	1 (1.1)	1 (1.1)	0 (0)	
Chronic kidney disease	0 (0)	0 (0)	1 (1.1)	1 (1.1)	0 (0)	
Autoimmune disease	0 (0)	0 (0)	0 (0)	1 (1/1)	0 (0)	
White blood cells (in $10^3/\mu l$)	6.41±5.52[a]	9.63±2.39[a]	7.53±3.96[a]	7.31±2.49[a]	6.77±2.17[a]	0.141
Total lymphocytes count (in $10^3/\mu l$)	0.83±0.32[a]	0.73±0.23[a]	1.34±0.59[a]	2.15 (1.14)[b]	2.10 (0.77)[b]	<0.001
NLR	13.3±9.04[a]	9.5 (6.7)[b]	3.46 (5.5)[b]	2.15 (1.0)[b]	1.85 (1.0)[b]	<0.001
Hemoglobin (in g/dl)	14.27±1.00[a]	12.9±0.62[a]	13.58±2.51[a]	13.97±1.76[a]	13.9±1.46[a]	0.567
Platelet (in $10^3/\mu l$)	158.00 (-)[b]	220.25±109.18[a]	266.00 (198.75)[b]	280.88±82.84[a]	275.00 (83.00)[b]	0.058
CD8$^+$ (in cell/μl)	205.07±131.58[a]	109.40 (144.73)[b]	409.69±209.79[a]	594.95 (419.43)[b]	613.95 (295.17)[b]	<0.001
CD4$^+$ (in cell/μl)	130.73±46.77[a]	145.44±73.12[a]	554.41±257.58[a]	783.53±201.02[a]	771.56±241.74[a]	<0.001
CD4$^+$: CD8$^+$ ratio	0.84±0.52[a]	1.15 (0.51)[b]	1.52±0.71[a]	1.31±0.55[a]	1.14 (0.52)[b]	0.355
ICU admission, n (%)	3 (3.2)	8 (8.4)	0 (0)	0 (0)	0 (0)	<0.001
Ventilator usage, n (%)	2 (2.1)	0 (0)	0 (0)	0 (0)	0 (0)	<0.001

[a]mean±SD
[b]median (IQR)

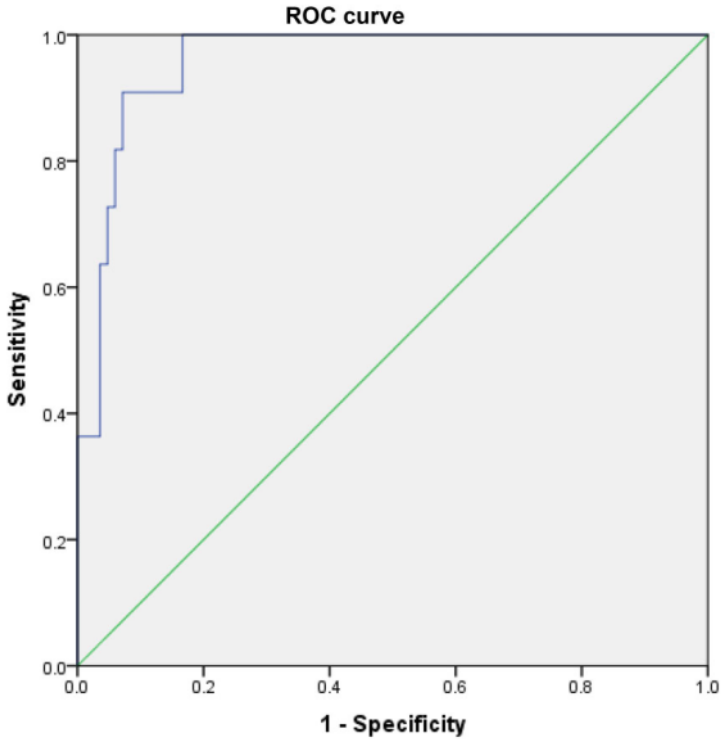

Figure 2. ROC curve analysis was used to determine the NLR cut-off.

among the critical and severe groups, our study reported a lower percentage of comorbidities than the moderate and mild groups. The patients with comorbidities were also fewer compared with previously published studies about CD4 and CD8.

The current study also shows that lymphopenia in COVID-19 is only transient. Full recovery is observed upon resolution of COVID-19.[12] Theoretically, lymphopenia may worsen the disease course due to delayed viral clearance, uncontrollable suppression of macrophage stimulation and cytokine storm, leading to end-organ damage.[6] Several mechanisms of lymphopenia occurrence in COVID-19 have been proposed. Increased cytokine levels in response to SARS-CoV-2 infection, such as TNF-alpha and IL-6, were related to lymphopenia, as proinflammatory cytokine may induce the apoptosis of lymphocytes through Fas–FasL interactions.[13] IL-6 also further inhibits CD8 cytotoxic function and Th1 cell-dependent antiviral responses. The use of an IL-6 antagonist (tocilizumab) in COVID-19 patients was associated with an increased blood lymphocyte count.[14]

We found a proportionally absolute decrease in $CD4^+$ and $CD8^+$ levels among our mild to severe COVID-19 patients, reflecting the $CD4^+$:$CD8^+$ ratio still >1.0. This finding is supported by Ganji et al., who found no significant difference in the $CD4^+$:$CD8^+$ ratio between COVID-19 patients and the control group.[15] Inverted $CD4^+$:$CD8^+$ ratio has been described as immune dysregulation and immunosenescence in HIV-negative people, associated with a higher risk of infection, disease progression and predictor of all-cause mortality.[16,17] Our finding also showed that the inverted $CD4^+$:$CD8^+$ ratio might be related to more critical conditions of COVID-19 patients, although it was not significant. Inversion of the $CD4^+$:$CD8^+$ ratio may have resulted from isolated $CD4^+$ cell destruction, expansion of $CD8^+$ cells or a combination of both mechanisms.[18] In their study, Khan et al. proposed that suppression of the $CD4^+$ population and activation of the cytotoxic $CD8^+$ T cell population in SARS-CoV-2 infection may lead to an inverted $CD4^+$:$CD8^+$ ratio.[19] Response of $CD8^+$ T cells in COVID-19 may be described as hyperactivation compared with $CD4^+$ T cell activation, which is reflected by increased circulated natural-killer cell-related markers and cytotoxicity.[20]

The role of the NLR has been studied widely as a prognostic marker of COVID-19. The NLR may serve as a practical and cost-effective immune-inflammatory parameter, especially in developing countries. The NLR can easily be calculated in an emergency department using routine laboratory tests and may provide an insight regarding the need for more intensive care admission.[21,22] We found that an increasing trend of NLR value was associated with more severe COVID-19 presentation at hospital admission. Our further analysis showed that the NLR cut-off of ≥3.550 was associated with the best sensitivity to predict the severity of COVID-19. Yang et al. found that an NLR cut-off of 3.3 was associated with a poorer outcome with a specificity of 63.6% and sensitivity of 88%.[23] A study from Indonesia also found that the NLR had good prognostic value in predicting adverse

COVID-19 outcomes; they proposed a higher NLR cut-off of 6.0.[24] Liu et al. found that COVID-19 patients with NLR≥3.13 were predicted to develop a critical disease course.[25] Meta-analysis using 38 articles, including 5699 COVID-19 patients, concluded that higher NLR levels on admission were associated with a more severe disease course and higher mortality rate. Elevated NLR was associated with a 2.7 times higher mortality risk than in patients with normal NLR. They also found that an NLR cut-off of 3.3 to 5.9 was reported as an optimal cut-off to predict severity.[4] The rationale for use of the NLR as a potential biomarker for COVID-19 is the excessive immune response to SARS-CoV-2. Cytokine storm reflected by a high IL-6, IL-8 and granulocyte-colony-stimulating factor may increase the proliferation of neutrophils. Also, lymphocyte counts decreased as a result of the mechanisms described above.[4,25,26]

The current study has several limitations. The criteria for categorizing COVID-19 severity may differ among countries, limiting interpretation of the results of our study. Several factors may influence the lymphocyte count and its subsets, such as malnutrition, which this study does not assess.

Conclusion

The current study shows that lymphopenia is common among COVID-19 patients. Lower $CD4^+$ and $CD8^+$ values are associated with severe COVID-19. Higher NLR values upon admission are associated with more severe disease. We found that the NLR cut-off of ≥3.550 was associated with the best specificity and sensitivity to predict the severity of COVID-19. Therefore, the NLR may serve as a practical, cost-effective and valuable biomarker in predicting severe COVID-19, especially in resource-limited settings.

Authors' contributions: SM, AAB, RA, MK and EJN conceived the study; DA, SK, AWP, OP and AS conducted data curation; AAB, RA and WA analyzed the data; SW, PN, JA, DA, PW AAB, SW, LGY, MW, SK, CW, SH and NLP carried out field investigation; SM, JA and LGY provided analysis software; PN, NLP, WA, OP, AWP and RMF conducted project administration; SM, AAB and RA wrote the original draft of the manuscript; RMF, PW, MK and EJN reviewed and edited the manuscript; MK and EJN carried out supervision.

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Competing interests: The authors declare that they have no competing interests.

Ethical approval: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board (or Ethics Committee) of Buleleng Hospital (Document number 070/1119/2020) and Bali Mandara Hospital Ethics Committee (Document number 007/EA/KEPK.RSBM.DISKES/2020). Informed consent was obtained from all subjects involved in the study.

Data availability: All data generated or analyzed during this study are included in the published article.

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