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Original Article

Pathology Section

Multilineage Haematologic Pattern Involvement in COVID-19 - Evaluation from Peripheral Blood Elements

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ABSTRACT

Introduction: Coronavirus disease is a highly contagious disease emerged from the Chinese city of Wuhan in December 2019. The Coronavirus Disease-19 (COVID-19) involves haematopoietic system affecting erythropoiesis, granulopoiesis, lymphopoiesis and thrombopoiesis. Bone marrow is the ultimate source of most cells of innate and acquired immunity. In response to infectious or inflammatory stimuli, bone marrow enhances its output under the influences of growth factors and cytokines. Peripheral blood smear examination is a simple, rapid and cost effective procedure. Thus, examining peripheral blood elements could be helpful, particularly when Real Time Polymerase Chain Reaction (RT-PCR) test is not available at all levels of healthcare. Complete Blood Count (CBC) along with peripheral blood smear examination can reflect the impact of virus on haematopoietic system.

Aim: The goal was to describe the peculiar morphological findings of peripheral blood elements in COVID-19 cases and the immune-inflammatory parameters from CBC that would be helpful to assess the disease severity so that early therapeutic intervention can be made.

Materials and Methods: This was a cross sectional retrospective study carried out in Department of Pathology, Fakir Mohan Medical College and Hospital, Balasore from 05 June 2020 to 10 August 2020. Among the suspected COVID-19 patients admitted to the isolation ward; 200 adult patients (≥18 years of age) were included in the study. Suspected COVID-19 patient with co-morbidities, pregnant women, paediatric population were excluded from the study. Data including clinical features, co-morbidities, CBC and peripheral blood smear examination was received and compared with RT-PCR result of these patients. The immune-inflammatory parameters like Neutrophil Lymphocyte Ratio (NLR), Platelet Lymphocyte Ratio (PLR) and Systemic Inflammatory Index (SII) were evaluated from CBC. Data were evaluated for statistical significance using Statistical Package for Social Sciences (SPSS), version 21.0. The categorical variables of the patients were analysed using Chi-square test and presented as numbers and percentages. Parametric continued variables were analysed using Independent Sample Student's t-test and p-value <0.05 was considered statistically significant.

Results: Monolobate neutrophils and plasmacytoid lymphocytes were seen in peripheral blood smear of COVID-19 patients (p<0.05). Giant platelets with cytoplasmic vacuolations was detected in 77.27% (102) of postive cases. The NLR was greater than 12 (mean 18.44±1.735) in all severe patients that were admitted in Intensive Care Unit.

Conclusion: In addition to the morphological changes in peripheral blood smear examination, evaluation of immune-inflammatory parameters, particularly neutrophil lymphocyte ratio, may be helpful in the screening, diagnosis, predicting prognosis as well as in treatment of COVID-19.

Keywords: Giant platelets, Lymphoplasmacytoid cells, Monolobate neutrophils, Neutrophil lymphocyte ratio

INTRODUCTION

In late 2019, rapidly spreading outbreak of corona virus disease emerged from the Chinese city of Wuhan, abbreviated as (COVID-19). It turned into a global challenge in respect of treatment as well as prevention. Although control and quarantine measures have been applied to prevent a global spread, the infection has gradually increased resulting in a pandemic [1].

The gold standard test for diagnosis of COVID-19 is by RT-PCR. It is relatively a time consuming procedure and requires substantial manpower. Moreover, this procedure focuses over a single compartment that is the pharyngeal samples only. Hence, the percentage of false negative is quite high. Further laboratory investigations which are easily available, less time consuming and cost effective are needed that supports diagnosis and aids for follow up of the course [1].

The disease has systemic manifestations including haematopoietic system. The viral cytopathic effect on the peripheral blood elements can be readily identifiable on peripheral blood film and can be easily and serially monitored [2]. CBC along with peripheral blood smear examination can reflect impact of virus on blood, which can reflect early inflammatory sign. Observation of blood cells can be a simple alternative to first triage and early identification of infection. As the infection might progress rapidly in some patients; thus identifying these risk groups is important for healthcare workers [3]. The goal was to describe the peculiar morphological findings of peripheral blood elements and the immune-inflammatory parameters from CBC in suspected adult patients admitted to isolation ward, that would be helpful for the clinicians in suspecting a diagnosis in the absence of RT-PCR at all levels of healthcare and to assess the severity of illness.

MATERIALS AND METHODS

This cross-sectional retrospective study was carried out in Department of Pathology, Fakir Mohan Medical College and Hospital, Balasore from 05 June 2020 to 10 August 2020 the approval of 4th Institutional Ethics Committee was taken and numbered 2020-05/14. In this study, 200 suspected COVID-19 patients admitted to the isolation ward (≥18 years of age) were included. Suspected COVID-19 patients with co-morbidities like hypertension, diabetes mellitus, chronic kidney disease and immune suppressive conditions, pregnant women, paediatric population were excluded from the study. Data including clinical features, co-morbidities, CBC and peripheral blood smear examination were evaluated and compared with RT-PCR result from

throat swabs of these patients. The positive cases were categorised into mild, moderate and severe disease based on their clinical signs and symptoms. Those patients having only upper respiratory tract symptoms/fever without shortness of breath or hypoxia were categorised into mild disease. Those positive cases having any of the two that is respiratory rate ≥24/min or SpO_2 <94% on room air were categorised into moderate disease. If any of the two features that is either respiratory rate ≥30/min or SpO_2 <90% on room air present, these patients were categorised as severe disease [4]. The decision to obtain a CBC from the patients in the isolation ward was made by the attending physician. The blood samples were collected by the central laboratory technicians and were preserved in Ethylene Diamine Tetra-acetic Acid (EDTA) anticoagulated vials and examined within one hour of collection. CBC was evaluated using five part differential cell counter (haematology auto analyser) in the central laboratory. Peripheral blood smears were prepared in each case and air dried. The slides were stained with Leishman's stain and examined under light microscope and the morphological changes in blood elements were noted. The immune-inflammatory parameters like Neutrophil Lymphocyte Ratio (NLR), Platelet Lymphocyte Ratio (PLR) and Systemic Inflammatory Index (SII) were evaluated from the CBC report. The NLR was calculated by dividing Absolute Neutrophil Count (ANC) and Absolute Lymphocyte Count (ALC). The PLR refers to the ratio of total platelet count to the ALC. Systemic Immune-Inflammation Index was calculated with the formula, SII={Total platelet count×ANC}/ALC [5].

STATISTICAL ANALYSIS

The analysis of the data was made using Software SPSS, version 21. The categorical variables of the patients were analysed using Chi-square test that were presented as numbers and percentages. Independent Sample Student's t-test was used for analysing parametric continued variables that were presented as mean and standard deviation.

RESULTS

Out of 200 symptomatic patients included in the analysis, 132 patients (66%) were positive by RT-PCR for COVID-19. Among the positive cases, 85 (64.39%) were male and 47 (35.60%) were female. The average age of positive cases were 42.8±8.299 years for mild disease, 35.523±4.176 years for moderate disease, and 55.4±8.073 years for severe disease. CBC using five part differential cell counter (haematology auto analyser) was evaluated for all symptomatic patients. CBC and peripheral blood smear findings of positive cases were compared with those of 68 suspected patients tested negative by RT-PCR [Table/Fig-1]. CBC of positive cases revealed anaemia in 14 (10.60%) cases, mean haemoglobin was 13.11 g% (9.2-16.4 g%), mean total leucocyte count was 16.74×10³/µL (11.5-24.6×10³/µL). Total platelet count was in the range between 90,000-425000/cmm. Thrombocytopenia was detected in 16 (12.12%) positive cases and thrombocytosis in 10 (7.57%) positive cases. ANC was between (10.97-18.48)×10³/µL. Absolute lymphopenia was detected in 12 (9.09%) cases who presented with severe disease. Absolute monocyte count was low in 42 cases (31.81%) and within normal limit in rest cases. Absolute eosinophil count was also within normal limit in all patients. The immune-inflammatory parameters like NLR, PLR and SII were calculated from the CBC for mild, moderate and severe disease [Table/Fig-2-4]. CBC of negative cases revealed anaemia in 4 (5.88%) cases, mean total leucocyte count was 11.5×10³/µL and total platelet count was in the range between 1.50000-3.50000/cmm. The ANC was between (4.97-11.48×10³/µL) and NLR was between (2.25-3.82).

Peripheral blood smears were examined under light microscope after staining with Leishman's stain and morphologic changes in the blood elements of COVID-19 positive and negative cases [Table/Fig-5]. Peripheral blood smears revealed Acquired Pelger Huet Anomaly (APHA) characterised by neutrophils having hypolobulated nucleus and hypogranular cytoplasm [Table/Fig-6a]. In 130 out of 132 positive cases (98.48%), APHA detected in greater than 10% of peripheral blood neutrophils in 98 (75.38%) cases; three (23.48%) cases had APHA detected in 5-10% of peripheral blood neutrophils and one (0.75%) case had APHA detected in <5% of peripheral blood neutrophils. Whereas among the 68 patients negative for COVID-19, APHA was detected in <5% of peripheral blood neutrophils in five (7.35%) cases. Monolobate neutrophils [Table/Fig-6b,c] were

Haematological parameters	Positive cases n=132	Negative cases n=68
Haemoglobin (g%) (NR=13-17.5 g%)	13.11	13.68
Total leucocyte count (×10³/µL) (NR=4-11×10³/µL)	16.74	11.50
Absolute neutrophil count (×10³/µL). (NR=2-8×10³/µL)	14.72	8.22
Absolute lymphocyte count (×10³/µL) (NR=1-3×10³/µL)	1.25	2.24
Neutrophil lymphocyte ratio (NR=2-2.66)	11.77	3.03
Total platelet count (NR=1.5-4×10⁹/l)	2.57	2.50
Platelet lymphocyte ratio (NR=100-187.5)	229.14	111.60
Systemic inflammatory index (NR=300-1066)	3151.85	898.4

[Table/Fig-1]: Comparison of haematological parameters according to RT PCR test results.
NR: Normal range

Haematological parameters	Mean	Standard deviation
Absolute neutrophil count (NR=2-8×10³/µL)	12.92×10³/µL	0.409
Absolute lymphocyte count (NR=1-3×10³/µL)	1.068×10³/µL	0.163
Neutrophil lymphocyte ratio (NR=2-2.66)	8.098	0.8166
Platelet lymphocyte ratio (NR=100-187.5)	118.3	17.96
Systemic inflammatory index (NR=300-1066)	1530.65	219.65

[Table/Fig-2]: Immune-inflammatory parameters in 15 positive cases (11.36%) with mild disease.

Haematological parameters	Mean	Standard deviation
Absolute neutrophil count (NR=2-8×10³/µL)	12.524×10³/µL	1.941
Absolute lymphocyte count (NR=1-3×10³/µL)	1.118×10³/µL	0.070
Neutrophil lymphocyte ratio (NR=2-2.66)	11.10	0.456
Platelet lymphocyte ratio (NR=100-187.5)	209.895	48.217
Systemic inflammatory index (NR=300-1066)	2562.867	564.395

[Table/Fig-3]: Shows immune-inflammatory parameters in 105 positive cases (79.54%) with moderate disease.

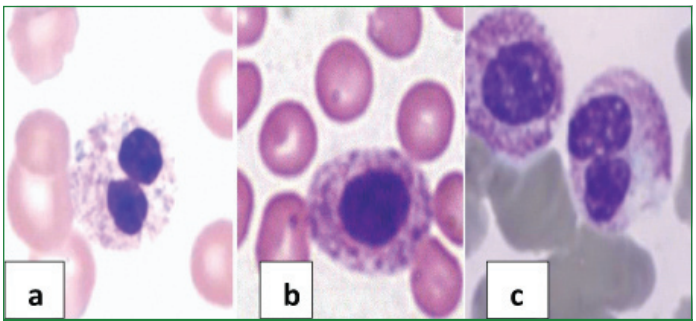
Haematological parameters	Mean	Standard deviation
Absolute neutrophil count (NR=2-8×10³/µL)	15.475×10³/µL	1.805
Absolute lymphocyte count (NR=1-3×10³/µL)	0.84×10³/µL	0.068
Neutrophil lymphocyte ratio (NR=2-2.66)	18.44	1.735
Platelet lymphocyte ratio (NR=100-187.5)	359.25	126.948
Systemic inflammatory index (NR=300-1066)	5362.056	1528.58

[Table/Fig-4]: Immune-inflammatory parameters 12 positive cases (9.09%) with severe disease.

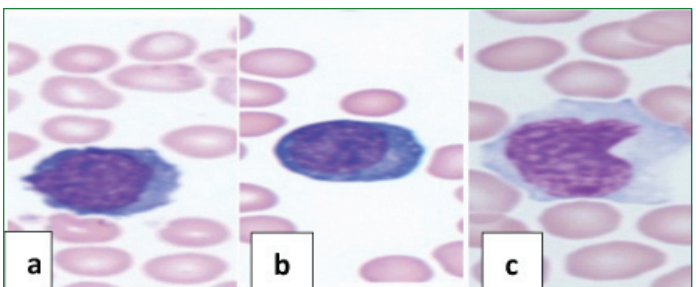
noted in 104 out of 132 positive cases (78.78%) but not seen in any of the patients tested negative for COVID-19 (p<0.05 Chi-square test). None of the cases showing APHA and monolobate neutrophils had a history of myeloid neoplasm or no definite history of medications related to APHA. Morphologcal changes in lymphocyte series were also noted. COVID-19 positive cases showed varying types of atypical lymphocytes constituting <10% of peripheral blood lymphocytes in 118 out of 132 cases (89.39%). Blastoid morphology characterised by round to oval cell with thin rim of cytoplasm and open nuclear chromatin [Table/Fig-7a] was seen in five positive cases (3.78%). Lymphocytes having eccentric nucleus, condensed chromatin, perinuclear clearing with basophilic cytoplasm referred to as plasmacytoid cells [Table/Fig-7b] were detected in 98 (74.24%) positive cases. Monocytoid morphology characterised by lymphocytes with lobulated nucleus and relatively open chromatin [Table/Fig-7c] was noted in 15 positive cases (11.36%). Majority of negative cases (23.52%) 16 out of 68 showed monocytoid lymphocyte. Only two negative cases (2.94%) showed plasmacytoid lymphocyte. So plasmacytoid lymphocytes were seen in greater frequency in COVID-19 positive cases compared to negative cases (p<0.05 Chi-square test). Aberrant morphological changes were also noted in erythrocyte and thrombocyte lineages. Aberrant erythropoiesis in the form of nucleated RBCs [Table/Fig-8a] was seen in 22% (29) positive cases. Giant platelets with cytoplasmic vacuolations was detected in 77.27% (102) postive cases [Table/Fig-8b,c].

Morphology	Positive case (n=132)	Negative case (n=68)
APHA	130 (98.48%)	5 (7.35%)
Monolobate neutrophils	104 (78.78%)	0
Atypical lymphocytes	118 (89.39%)	18 (26.47%)
Blastoid morphology	5 (3.78%)	0
Monocytoid morphology	15 (11.36%)	16 (23.52%)
Plasmacytoid morphology	98 (74.24%)	2 (2.94%)
Aberrant erythropoiesis	29 (22%)	1 (1.47%)
Giant platelets	102 (77.27%)	0

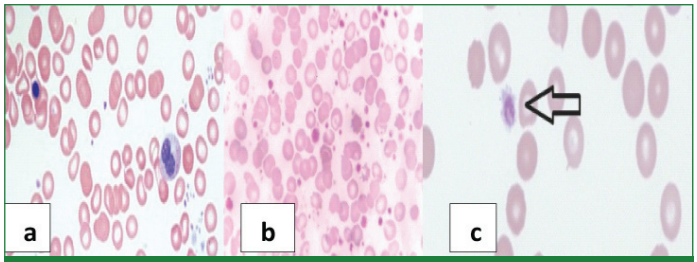
[Table/Fig-5]: Morphologic changes in peripheral blood elements of COVID-19 positive and negative cases.



[Table/Fig-6]: (a) Showing APHA (Leishman's stain x1000); (b) Showing monolobate neutrophil (Leishman's stain x1000); (c) Showing one monolobate neutrophil and one neutrophil with APHA (Leishman's stain x1000).



[Table/Fig-7]: (a) Showing blastoid lymphocyte (Leishman's stain x1000); (b) Showing plasmacytoid lymphocytes; (Leishman's stain x1000); (c) Showing monocytoid lymphocyte (Leishman's stain x1000).



[Table/Fig-8]: (a) Nucleated RBC and APHA in neutrophil; (Leishman's stain x400); (b) Showing giant platelets (Leishman's stain x400); (c) Showing giant vacuolated platelet (Leishman's stain x1000).

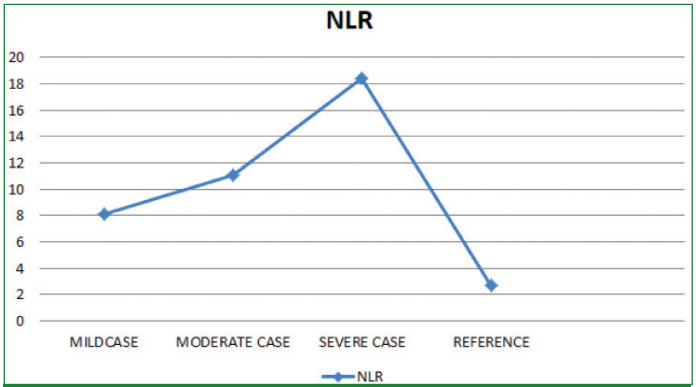
DISCUSSION

The majority of COVID-19 patients had relatively mild symptoms but a considerable number of patients progress to severe pneumonia and eventually develop Acute Respiratory Distress Syndrome (ARDS), septic shock and/or multiorgan failure. The basis for the discrepant symptomatology and their severity lie within the genetic and acquired differences in host immune system. The immunological parameters in peripheral blood are important indicators for assessing prognosis; thus reducing mortality. The present study analysed that COVID-19 positive patients had elevated total leucocyte count (>10×10³/μL) and absolute neutrophilia (>8×10³/μL). Elevated NLR and lymphopenia are the most consistent abnormal haematologic findings (p<0.05 Student's t test). Absolute lymphopenia (<1×10³/ μL) was observed in all severe COVID-19 patients. Rest all other positive patients had lymphocyte count within lower limit of normal range. This is according to Feng X et al., pronounced lymphopenia was strongly associated with increased disease severity [1]. An increased level of neutrophils along with a decrease in lymphocyte numbers resulting in high NLR is seen in patients with COVID-19 [5,6].

The NLR calculated by dividing ANC and ALC of a CBC is a potential marker of the systemic inflammatory response [5]. In present study, NLR was greater than 12 (mean 18.44±1.735) in all severe patients that were admitted in Intensive Care Unit. These patients had either reduced oxygen saturation SpO_2 to <90% on room air or respiratory rate ≥30/min. They were treated with respiratory support, antiviral therapy, anti-inflammatory or immune modulatory therapy and anticoagulation therapy in the form of high dose prophylactic Low Molecular Weight Heparin (LMWH). Patients with moderate disease (105 cases) with either reduced oxygen saturation SpO_2 to <94% on room air or respiratory rate ≥24/minutes needed oxygen support with antiviral or anti-inflammatory therapy. The NLR of these patients prior to starting therapy was between 10 to 12 (mean 11.10±0.456). Patients who manifested mild disease had NLR <10 (mean 8.098±0.8166). These patients had only respiratory tract symptoms and fever without shortness of breath or hypoxia. They were treated with symptomatic management. A higher neutrophil count indicates the intensity of the inflammatory response and simultaneously a falling lymphocyte count indicates damage to the cells of immune system. In this study, we observed that severe patients had a higher NLR than non-severe patients [Table/Fig-9]. Thus, high value of NLR could be an independent marker for predicting the prognosis. Long L et al., reported that NLR was an important risk factor for progression of COVID-19 by multivariate Cox regression analysis [6].

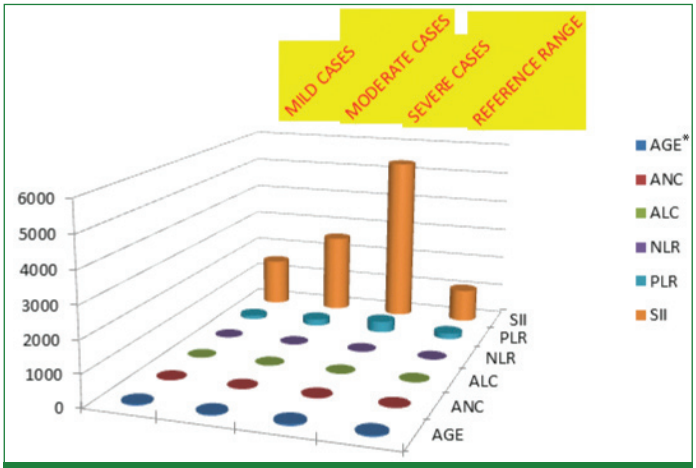
The PLR refers to the ratio of total platelet count to the ALC. As PLR takes into account both platelets and lymphocytes; it reflects both inflammatory pathway and platelet aggregation pathway thus may be more important in predicting the inflammatory status compared to the platelet count or ALC alone. In present study, 12 cases of severe disease had higher PLR >300 (mean 359.25±126.948) compared to mild disease (mean 118.3±17.96) and moderate disease (mean 209.895±48.217) (p<0.05 Student's t-test). Patients with high PLR had chances of platelet activation and hypercoagulation. So they were treated with prophylactic anticoagulant therapy. In addition to the immune dysregulation, a further characteristic clinical feature

of COVID-19 is the high incidence of thromboembolism [7-9]. Inflammation induced endothelial dysfunction is the probable cause of thromboembolism [10].



[Table/Fig-9]: It is showing mean value of NLR in mild, moderate and severe cases.

The present study also analysed that the adverse clinical progress was closely associated with higher value of SII. The high SII resulted from increased neutrophil and platelet count and decreased ALC [11]. In present study, patients with severe disease had higher SII [Table/Fig-10], (mean value 5362.056±1528.58) than non-severe disease ($p<0.05$ student's t test). Fan BE et al., reported SII as a prognostic parameter in COVID-19 that relies on thrombocytes, neutrophils and lymphocytes [5]. Besides these quantitative changes, peculiar morphological changes were also seen in various cellular elements of peripheral blood such as granulocytes, lymphocytes, erythrocytes and platelets. This is consistent with previous sporadic reports describing particular aspects like abnormal lymphocytes, plasmacytoid cells, APHA and leucoerythroblastic blood picture in COVID-19 patients [12-15]. Atypical lymphocytes are reactive lymphocytes which are common in Ebstein-Barr virus (EBV) and cytomegalovirus infections [15]. Wang F et al., reported although atypical lymphocyte morphology is common in viral infections; in general pneumonia causing viruses including influenza A, SARS-1 and swineflu are not commonly associated with atypical lymphocytic morphology [16]. A few viral infections like dengue fever and to a lesser extent rubella are associated with plasmacytoid lymphocytes [17]. Therefore, the results of present study reflects the COVID-19 virus which may be associated to mechanisms of immune dysregulation involving multiple hematologic lineages like granulopoiesis, lymphopoiesis, erythropoiesis and thrombopoiesis; not frequently seen with other viral infections [18,19]. In present study, COVID-19 positive cases (89.39%) showed varying types of atypical lymphocytes constituting <10% of lymphocytes. Lymphocytes having condensed chromatin, perinuclear clearing with basophilic cytoplasm referred to as plasmacytoid cells were detected in 98 (74.24%) positive cases. The frequency of



[Table/Fig-10]: It is showing the mean value of immunoinflammatory parameters in mild, moderate and severe cases.
*Average age of presentation of patients for mild,moderate and severe disease

plasmacytoid lymphocytes was higher in COVID-19 positive cases than COVID-19 negative patients. Foldes D et al., reported the classic Downey II like cells (monocytoid lymphocytes), which are common in viral infections, are less frequent in COVID-19 [19]. Nazarullah et al., reported, plasmacytoid lymphocytes seen more often in COVID-19 patients than in controls [20].

Granulocytes demonstrated APHA characterised by bilobed neutrophils with coarse chromatin in 98.48% cases. Among the positive cases 75.38% revealed APHA in greater than 10% of peripheral blood neutrophils. Monolobate neutrophils were noted in 104 out of 132 positive cases (78.78%) but not seen in any of the patients tested negative for COVID-19. Nazarullah A et al., reported morphologic abnormalties in neutrophil, namely APHA and left shift, that were significantly more common in COVID-19 cases [20]. These morphological changes reflect the involvement of bone marrow in COVID-19 in the form of accelerated and disordered granulopoiesis [21]. Aberrant morphological changes were also noted in erythrocyte and thrombocyte lineages. Aberrant erythropoiesis in the form of nucleated RBCs was seen in 22% (29) positive cases. Giant platelets with cytoplasmic vacuolations was detected in 77.27% postive cases independent of total platelet count. Fan BE et al., reported the high incidence of giant platelets might contribute to hypercoagulability in COVID-19 [5]. These morphologic changes in peripheral blood involving all the three lineages of bone marrow indicate how the hematopoietic progenitor cells are affected in COVID-19 pathogenesis; as a direct target via infection and/or indirectly via pro-inflammatory cytokine burst. Thus systemic long term follow up will be very important to analyse the recovery of the haematopoietic system. The extent of multilineage morphologic changes are not found in any other virus infection [22,23]. Peripheral blood smear examination is a simple, rapid and cost-effective procedure, this approach could be particulary helpful when RT-PCR testing is not available widely. The peripheral blood smears could also easily be used for follow up analysis investigating the impact on the hematopoietic system. COVID-19 infection can activate innate and adaptive immune responses. As NLR takes into account both the levels of neutrophils and lymphocytes, hence it may be a better biomarker for predicting systemic inflammation compared to single neutrophil or lymphocyte count [23]. In this study, we found that increased NLR indicated higher disease severity. The possible explanation for increased NLR may be neutrophils activation by virus related inflammatory factors produced by lymphocytes and endothelial cells (such as interleukin 6, interleukin 8, tumour necrosis factor alpha, granulocyte colony stimulating factor and interferon gamma).The activated neutrophils are able to release Reactive Oxygen Species (ROS) and other cytotoxic mediators that may reduce the viral load [23]. In response to different infectious and inflammatory agents, neutrophils release nuclear material comprising of meshwork of histones and DNA conjugated with antimicrobial peptides and enzymes forming neutrophil extracellular traps (NETs). These NETs are able to capture and kill different pathogens, including viruses thereby preventing the spread of microbes [24-26]. On the other hand, lymphocytes are significantly decreased in severe and critically ill patients. The significant exhaustion of lymphocyte in severe cases may be due to direct damage of target cells by the virus. Viral infection causes immune cells to enter an activated state and participate in the process of elimination of the virus, resulting in severe damage and apoptosis. During systematic inflammation neutrophils are activated and speed up the process of apoptosis of lymphocytes. Increased level of pro-calcitonin and C reactive protein, indicating a potential bacterial co-infection has been observed in severe COVID-19 patients and bacterial co-infection might affect the immune response [27].

Limitation(s)

The major limitation of the study was relatively less number of blood sample from COVID-19 patients. Studies conducted with a large

patient group will better portray the importance of biomarkers from peripheral blood smear test and CBC picture in the diagnosis of COVID-19 patients. Another limitation was the fact that patients who deteriorated were transferred to higher tertiary care hospital, thus the follow-ups of these patients remain unknown.

CONCLUSION(S)

In summary, we came to the cautious conclusion that immunoinflammatory parameters such as ALC, NLR, PLR, and SII were correlated with disease severity and could be used as potentially independent risk factors for disease progression. COVID-19 positive cases also had significant APHA, monolobate neutrophils and plasmacytoid lymphocytes were significantly detected in COVID-19 positive cases compared to negative cases. Increased NLR is a poor prognostic index. Therefore, in addition to these morphologic changes in peripheral blood smear examination that can be easily used for follow up analysis and long term evaluations indicating the impact of virus on the haematopoietic system; surveillance of immune- inflammatory parameters, especially NLR, may be helpful in the screening, diagnosis, prognosis and treatment of COVID-19.

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