Ind J Clin Biochem
https://doi.org/10.1007/s12291-022-01087-z

ORIGINAL RESEARCH ARTICLE

Monocyte HLADR and Immune Dysregulation Index as Biomarkers for COVID-19 Severity and Mortality

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Received: 7 May 2022 / Accepted: 20 August 2022

Abstract
Immune dysregulation in COVID-19 is the major causal factor associated with disease progression and mortality. Role of monocyte HLA-DR (mHLA-DR), neutrophil CD64 (nCD64) and Immune dysregulation index (IDI) were studied in COVID-19 patients for assessing severity and outcome. Results were compared with other laboratory parameters. Antibody bound per cell for mHLA-DR, nCD64 and IDI were measured in 100 COVID-19 patients by flow cytometry within 12 h of hospital admission. Thirty healthy controls (HC) were included. Clinical and laboratory parameters like C - reactive protein (CRP), Procalcitonin (PCT), Absolute Lymphocyte count (ALC), Absolute Neutrophil count (ANC) and Neutrophil to Lymphocyte ratio (NLR) were recorded. Patients were followed up until recovery with discharge or death. Parameters from 54 mild (MCOV-19), 46 severe (SCOV-19) and 30 HC were analysed. mHLA-DR revealed significant and graded down regulation in MCOV-19 and SCOV-19 as compared to HC whereas IDI was lowest in HC with increasing values in MCOV-19 and SCOV-19. For diagnostic discrimination of MCOV-19 and SCOV-19, IDI revealed highest AUC (0.99). All three immune parameters revealed significant difference between survivors (n = 78) and non-survivors (n = 22). mHLA-DR < 7010 and IDI > 12 had significant association with mortality. Four best performing parameters to identify patients with SCOV-19 at higher risk of mortality were IDI, NLR, ALC and PCT. mHLA-DR and IDI, in addition to NLR and ALC at admission and during hospital stay can be utilized for patient triaging, monitoring, early intervention, and mortality prediction. IDI reported for the first time in this study, appears most promising. Immune monitoring of 'in hospital' cases may provide optimized treatment options.

Keywords Monocyte HLADR · Immune dysregulation index · Neutrophil CD64 · Antibody bound per cell · COVID-19 · Biomarkers

A "Take-home message"

Significant down regulation of mHLA-DR coupled with up regulation of nCD64 resulting in high IDI was recorded in severe COVID-19 cases at admission. MHLA-DR and IDI, in addition to NLR and ALC at admission and during hospital stay, can be utilized for patient triaging, monitoring, early intervention, and mortality prediction.

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Introduction

Coronavirus disease 2019 (COVID-19) is a mild to moderate respiratory tract infection in which a subset of patients progress to severe disease and respiratory failure. Systemic inflammation is associated with unfavorable clinical outcome and the development of severe COVID-19 (SCOV-19). Studies have suggested a complex dysregulation of the immune response in SCOV-19 [1–3]. Severe COVID-19 infection with acute respiratory distress syndrome (ARDS)

Published online: 06 October 2022

should be considered viral sepsis as per the international definition of sepsis pertaining to organ dysfunction [2].

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection and is being considered as a prototype critical illness for understanding disease pathogenesis [4]. Low expression of the human leukocyte antigen D related (HLA-DR) on CD14 monocytes (mHLA-DR) is characteristic of sepsis-induced immunoparalysis [5, 6]. Beyond sepsis, mHLA-DR has progressively become a popular immune monitoring tool in illnesses requiring intensive care like trauma, burns, post-surgery, and other clinical areas, including gastroenterology, oncology, hematology, transplantation, and cardiac surgeries,where it also identifies patients at risk of worsening in terms of mortality, secondary infections and cancer relapse [7–10].A unique signature of immune dysregulation has been identified in patients with COVID-19, characterized by normal or high cytokine production with increased circulating cytokines (especially IL-6) and defects in the lymphoid function associated with IL-6- mediated decrease in HLA-DR expression [3].

CD64 is a high-affinity immunoglobulin receptor FcγRI found on normal monocytes, macrophages, dendritic cells, and some resting neutrophils with different effector functions as opsonization and antibody-dependent cellular cytotoxicity. Measurement of CD64 on neutrophils(nCD64)and CD169 on monocytes has been studied to differentiate bacterial from viral infections in the emergency department and recently in COVID-19 [11, 12].

Pradhan et al., 2016 described the utility of 'Sepsis Index' (SI), a derived parameter based on the ratio of median fluorescence intensity (MFI) of nCD64 and mHLA-DR in the setting of neonatal sepsis [13]. Mony et al. improvised on the sepsis index using a more standardized approach of antibody bound per cell (ABC) using commercially available QuantibriteTM kit (BD Biosciences) and demonstrated its utility in detecting sepsis after cardiac surgery{Sepsis Index = (nCD64 ABC / mHLA-DR ABC) x 100} [8].We prefer to consider this index as immune dysregulation index (IDI) rather than limit its usage to sepsis only.

The current study was designed to assess the role of mHLA-DR,nCD64 and IDI in COVID-19 patients to answer the following clinically relevant questions related to severity and outcome:Whether these markers of immune dysregulation are deranged in mild COVID-19 (MCOV-19) as compared to healthy controls?Are the expressions different between clinically MCOV-19 and SCOV-19 patients? Whether expression of these biomarkers at hospital admission is different between patients who recovered vs. those who succumbed to the disease and whether this information is of potential clinical relevance (e.g., identifying patients, within the clinically 'severe' group, who are at higher risk of an adverse outcome/mortality). The immune parameters were compared with other commonly used lab parameters like C-reactive protein (CRP), procalcitonin (PCT), neutrophil to lymphocyte ratio (NLR), absolute lymphocyte count (ALC), and absolute neutrophil count (ANC) to ascertain any added advantage of testing these immune parameters.

Patients and Methods

This is a hospital-based prospective pilot study conducted at a tertiary healthcare center in Northern India. **Case definition**:Positive SARS CoV-2 RNA detection in nasal/nasopharyngeal swab by reverse-transcriptase-polymerase-chain-reaction and admitted to the dedicated COVID-19 hospital. **Inclusion Criteria**: Age > 18 years of both sexes. **Exclusion criteria**:pregnant women and patients on steroids/ immunosuppressant.Written informed consent was obtained for all the cases. Cases were categorized as MCOV-19 and SCOV-19as per WHO guidelines 2020 [14]. The study was carried out in accordance with the declaration of Helsinki and its later amendments and was approved by the Institutional Ethical Committee of Institute (IEC34/21).

Demographics and clinical profiles were recorded, including age, gender, co-morbidities, date of symptom onset, and previous drug treatment. Results of various laboratory parameters, including complete hemogram, liver and renal function tests, coagulation tests, CRP, PCT, and ferritin were recorded at hospital admission (within 12 h). NLR was calculated from ALC and ANC. The mHLA-DR, nCD64, and IDI were measured in all cases at hospital admission by flow cytometry. Patients were followed until the end of the clinical observation, defined as death or complete recovery and discharge from the hospital with $SpO_2 > 94\%$ while breathing in ambient air.

Flowcytometry for mHLA-DR and nCD64 MFI for mHLA-DR and nCD64 was measured as per previously published gating strategy and quantified to ABC (antibodies bound per cell) values by using BD Quantibrite™PE calibration beads (Becton Dickinson) [9, 15]. Briefly, peripheral blood was collected in EDTA vial and processed within 2 h of collection. 50 µl of whole blood was stained with premixed cocktail of anti-CD64PE/ anti-CD45PerCP (clone MD22/2D1) and anti-CD14FITC (clone MφP9) in Tube 1; and premixed cocktail of anti-HLADR PE/anti-CD14PerCP-Cy5.5 (clone L243/clone MφP9) and anti-CD45APCH7 (clone 2D1) in Tube 2 at room temperature for 30 min in the dark. Following incubation, red blood cells were lysed, washed, and resuspended in phosphate-buffered saline. The samples were acquired on a BD FACS CANTO flow cytometer. (Becton Dickinson, San Jose, California, USA). Data were analyzed

Table 1 Laboratory parameters in healthy controls, MCOV-19 and SCOV-19 (n = 100)

	Healthy Controls	MCOV- 19	P value (healthy control vs. MCOV-19)	SCOV-19	P-value (MCOV-19 vs. SCOV-19)
Number of cases	30	54		46	
Absolute Lymphocyte Count($x10^9$/L)	2 (1.82–2.45)	1.2 (0.7–1.7)	< 0.0001	0.9 (0.6–1.5)	0.20
Neutrophil Lymphocyte Ratio	2.2 (1.46–2.72)	3.8 (3-8.6)	< 0.0001	8.5 (5.7–23)	0.002
CRP (mg/L)	Not measured	4.46 (1.36-18.0)	-	18 (14–57)	0.003
PCT (ng/ml)	Not measured	0.08 (0.05–0.30)	-	0.5 (0.2–0.9)	0.0004
nCD64 (ABC)	348 (243–467)	263 (167–429)	0.27	931 (429–1494)	< 0.0001
mHLA-DR(ABC)	14,950 (11,604–19,866)	8235 (3855–10,769)	< 0.0001	2522 (1734–2927)	< 0.0001
Immune dysregulation index (IDI)	2.1 (1.7–2.7)	3.6 (2.3–8.1)	0.0040	38.4 (19.7–73.1)	< 0.0001

using BD FACS Diva software. Antibody bound per cell (ABC) values for mHLA-DR and nCD64 were calculated using the MFI of respective Quantibrite™ PE calibration beads, with known PE molecules bound on their surface.

Healthy Controls Blood samples from thirty healthy adults, both males and females aged 20–43 years, were analyzed for immune parameters.

Statistical Analysis

Data were analyzed with SPSS v25.0 (Chicago, IL, USA). The categorical variables are reported as number (%) and continuous variables as median and interquartile range. Statistical significance was assessed using the chi-square test for dichotomous variables or using two independent sample *t*-test or by Mann–Whitney *U* test when appropriate. *P*-value < 0.05 was considered statistically significant. The survival rate was assessed by Kaplan–Meier (KM) plot. The Log Rank test evaluated differences between survival curves.

Results

A total of 100 COVID-19 patients (54 MCOV-19 & 46 SCOV-19) and 30 healthy controls were included in the study with a median (range) age of 59 years (45–65) for SCOV-19 and 53 years (40–60) for MCOV-19. The patients experiencing mild disease were admitted to the isolation ward (54%), while the remaining 46% with severe disease were admitted to the high dependency unit or intensive care unit, depending on availability. Only two cases of moderate disease were recruited and hence excluded from the cohort and final analysis.

Co-morbidities in COVID-19 patients Hypertension was observed in 50% and 47%, type II diabetes mellitus in 35.7% and 29.4%, coronary artery disease in 7.1% and 5% and chronic kidney disease in 5.2% and 4.8% of SCOV-19 and MCOV-19 respectively. Other comorbid conditions were malignancy and tuberculosis recorded in less than 5% cases of SCOV-19 and none in MCOV-19. Alcoholic liver disease was noted in 11.76% of MCOV-19 and none in SCOV-19. No significant difference was found in the between-group comparison.

Comparison of Parameters in Healthy Control, MCOV-19, and SCOV-19

Representative dot-plots for flow cytometric measurement of immune parameters in the three groups are shown in **Supplementary Fig. 1**. In MCOV-19 patients ALC, NLR, mHLA-DR (p = < 0.0001) and IDI (p = 0.004) were significantly different from healthy controls whereas no difference was found for nCD64 (0 = 0.27).The laboratory parameter results are provided in Table 1. The NLR (p = 0.002), CRP (p = 0.003), PCT (p = 0.0004), nCD64 and IDI (p = < 0.0001) were higher in SCOV-19 whereas mHLA-DR expression was lower (p < 0.0001) as compared to MCOV-19. ALC was lower in SCOV-19 than in MCOV-19 but the difference was not statistically significant.

Diagnostics for discrimination of MCOV-19 and SCOV-19 using ROC curve analysis As the differential expression of mHLA-DR, nCD64, and IDI revealed highly significant results at hospital admission in both the groups, these were

Table 2 Diagnostics for discrimination of MCOV-19 vs. SCOV-19 at admission (n = 100)

Marker	Cut off	AUC (95% CI)	P value	Sensitivity	Specificity
IDI	12.0	0.99 (0.98-1)	< 0.001	100%	96.3%
nCD64 (ABC)	768.0	0.90 (0.83–0.98)	< 0.001	65.2%	92.6%
mHLA-DR (ABC)	7010.0	0.87 (0.77–0.97)	< 0.001	91.3%	63%
PCT (ng/ml)	0.102	0.79 (0.66–0.92)	< 0.001	91.3%	63%
NLR	4.07	0.75 (0.62–0.89)	0.002	91.3	65.6
CRP (mg/L)	14.5	0.74 (0.60–0.88)	0.003	73.9%	70.4%

Table 3 Biomarkers at hospital admission between survivors and non-survivors (n = 100)

	Non survivors (n = 22) Median (Q1-Q3)	Survivors (n = 78) Median (Q1-Q3)	P value*
ALC	0.68 (0.38–0.79)	1.19 (0.79–1.90)	< 0.001
NLR	23.0 (9.5–31.6)	4.52 (3.13–8.49)	< 0.001
CRP (mg/L)	18.0 (15.50–98.80)	6.0 (3.0-45.50)	0.018
PCT (ng/ml)	0.80 (0.25–2.57)	0.11 (0.06–0.44)	< 0.001
nCD64 (ABC)	1187.0 (735–1700)	349.0 (204–747)	< 0.001
mHLA-DR (ABC)	2046.0 (1275–2530)	6801.0 (2927–10,193)	< 0.001
IDI	73.01 (59.16–96.91)	6.13 (2.73–18.33)	< 0.001

**Mann Whitney U test*

further tested for diagnostic discrimination between mild and severe cases by ROC curve analysis. Results were also compared with CRP, PCT, and NLR (Table 2**& supplementary Fig. 2**). IDI has the highest AUC(0.99) with 100% sensitivity and 96% specificity followed by nCD64 (AUC 0.90) and mHLA-DR (AUC 0.87). We suggest that IDI at a cut-off of 12 may be a better parameter for triaging patients into mild and severe instead of CRP, PCT, and NLR.

Outcome Prediction

There was no mortality in MCOV-19, whereas 22/46 SCOV-19 patients expired. We compared the difference in biomarkers at hospital admission between COVID-19 patients who recovered vs. those who succumbed to the disease, as depicted in Table 3. High expression of nCD64, IDI, CRP, PCT, NLR, and low mHLA-DR and ALC were noted in non-survivors at admission (p = < 0.001).

Survival function was tested using Kaplan Meier analysis with cut-offs obtained from ROC curves. The value of mHLA-DR below 7010 and IDI above 12 revealed a significant association with mortality (p 0.03 and 0.008, respectively). Patients with mHLADR > 7010 and IDI < 12 (at admission) performed well, and all survived. The association of nCD64 with mortality was weak (p 0.09), as depicted in Fig. 1.

Prediction of Mortality in SCOV-19 Patients

Of 46 SCOV-19 patients, 24 survived (discharged on a median of 10 days), and 22 expired (median 8 days). We attempted to identify patients with severe disease at higher risk of mortality. Four best performing parameters were IDI (p = 0.0004), NLR (p = 0.0005), ALC (p = 0.001) and PCT (p = 0.03) (**Supplementary Table 1**). The interquartile

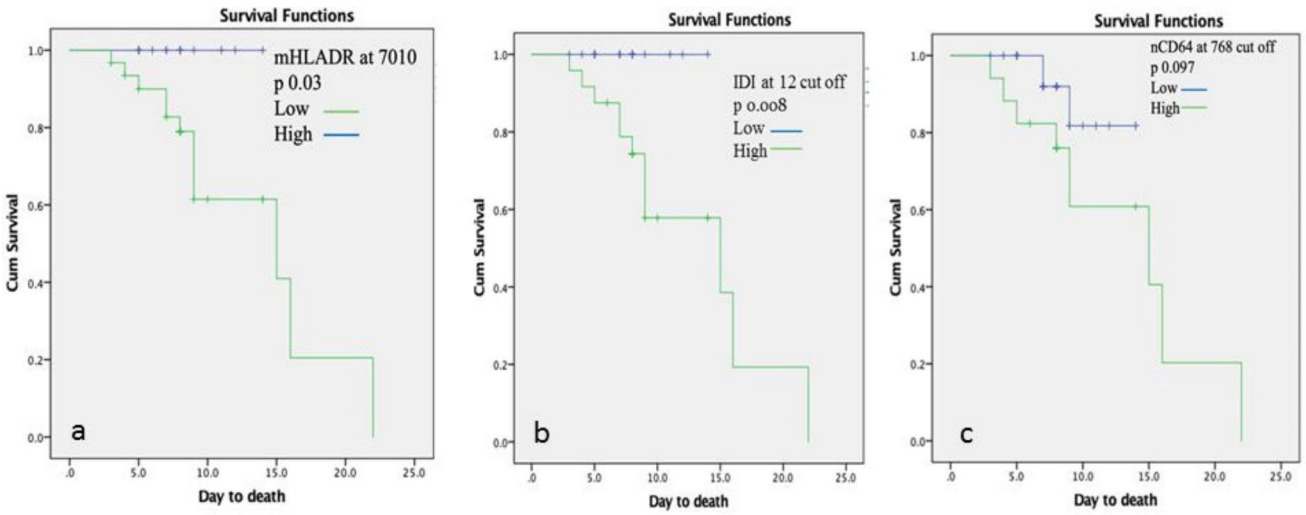

Fig. 1 Kaplan Meier analysis for survival function tested using cut-offs obtained from ROC curves for (a) mHLA-DR, (b) IDI and (c) nCD64

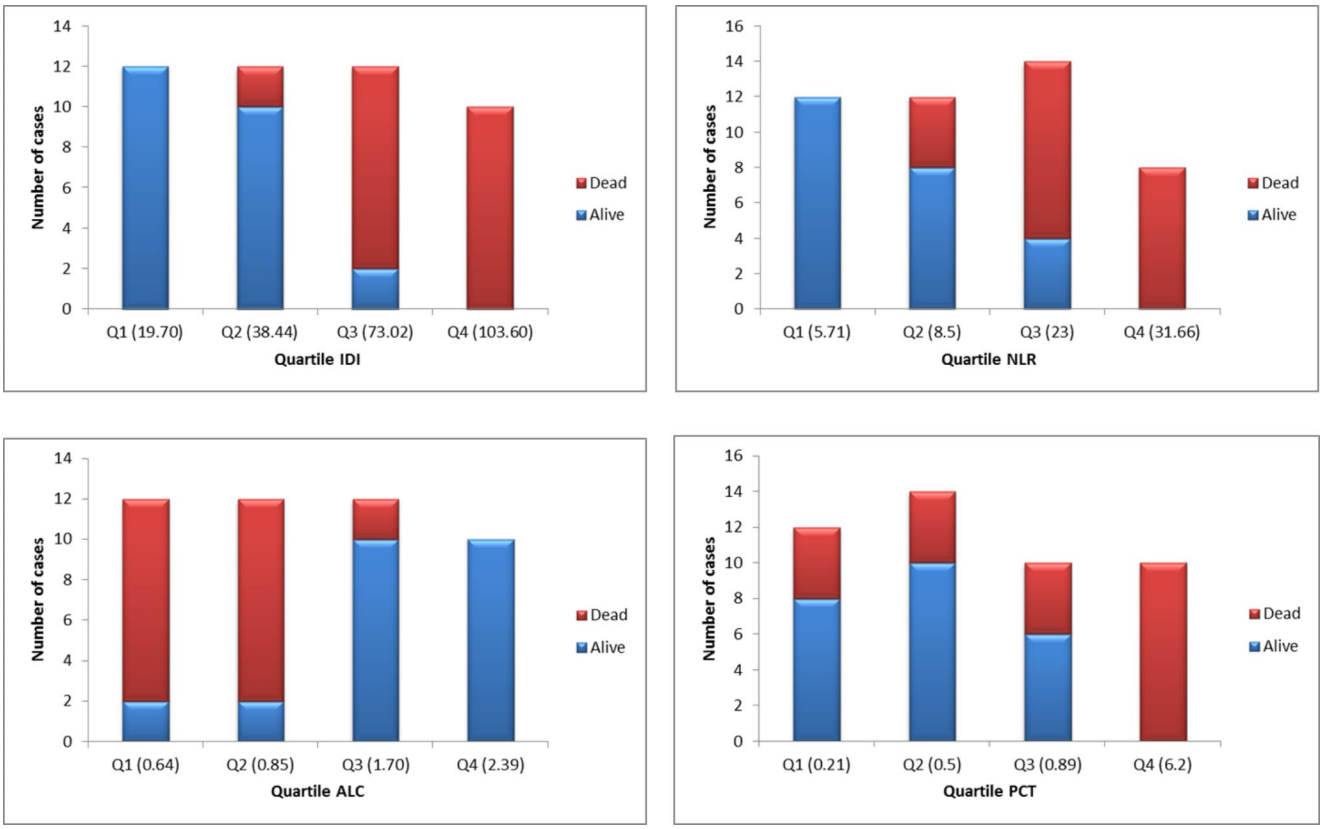

Fig. 2 Interquartile analysis for Severe COVID-19 cases to detect patients with higher risk of mortality: IDI & ALC demonstrate a clear and significant mortality risk in the 3rd and 4th quartile (IDI > 38.44 and ALC < 0.85). NLR and PCT show a similar trend

pattern was analyzed in SCOV-19: IDI & ALC demonstrate a clear and significant mortality risk in the 3rd and 4th quartile (IDI > 38.44 and ALC < 0.85). NLR and PCT show a similar trend, as depicted in Fig. 2.

Discussion

Since the beginning of the COVID-19 pandemic, high mortality in a subset of patients has been a concern. As the information emphasizing systemic inflammation and dysregulated immune response started to pour in, understanding the host immune response to SARS-Cov2 became of foremost importance. COVID-19 patients presenting with severe inflammatory response and lymphopenia are very similar to changes seen in sepsis which is essentially characterized by organ dysfunction resulting from altered host response to infection [16–20]. The complex interplay between pro-and anti-inflammatory mechanisms results in prolonged immunosuppression in sepsis patients who consistently reveal a significant decrease in the number of HLA-DR molecules on CD14 monocytes. However, this has not been fully studied in the COVID-19 outbreak.

Benyalmi et al., in 2020, reviewed 15 studies of COVID-19 in which HLA-DR was assessed by various techniques [2]. Low HLA-DR was a consistent finding in moderate to severe COVID-19 patients with the depth of mHLA-DR fall associated with severity at hospital admission. The current study reveals similar results of low mHLA-DR in SCOV-19 compared to mild disease. Further, we document significant down regulation of mHLA-DR in MCOV-19 compared to healthy controls in the initial phase of the disease, emphasizing the graded immunosuppression in COVID-19 patients. In contrast, Schulte-Schrepping et al., 2020 reported predominance of HLADRhi/CD11chi monocytes by flow cytometry in mild disease, but their cohort comprised only 3 mild and 7 severe cases; hence the results are questionable [21]. We found lowest mHLA-DR levels in non-survivors and levels < 7010 were associated with mortality. Association of low mHLA-DR with mortality has been reported earlier [2, 22, 23].

Neutrophil CD64 (nCD64) is one of the most studied sepsis biomarkers in the context of bacterial infections and has a valuable role in the early diagnosis of sepsis in adults and neonates [13, 24].The expression parallels the extent of the inflammatory response to infection or tissue damage; hence it has been utilized for discrimination between infectious and

noninfectious systemic inflammatory response syndrome in ICU settings [25]. Our study reveals no change in expression of nCD64 in MCOV-19 compared to healthy controls, but significant overexpression with increasing severity owing to systemic inflammation was noted. Higher levels were seen in non-survivors; however,the correlation with mortality was weak. When simultaneously studied with mHLADR as a component of IDI, it was highly informative. IDI was 2.1, 3.6, and 38.4 in healthy controls, MCOV-19 and SCOV-19, respectively. IDI > 12 was associated with a significant risk of mortality, and it was one of the best performing markers to identify patients of SCOV-19 who had a significant risk of mortality. To our knowledge, IDI is being reported for the first time in COVID-19, and it highlights the concomitant immune suppression (low mHLA-DR) with systemic inflammation (high nCD64), leading to adverse outcomes seen in a subset of patients with COVID-19.

Which biomarker needs to be evaluated at what time point and in whom, and how best this information can contribute to patient care are questions that currently lack convincing answers. It has been well established that SCOV-19 is associated with altered leucocyte response (lymphopenia, neutrophilia), high levels of blood proteins (CRP, PCT, ferritin), and cytokine levels [IL-2R, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α] [26–28]. High NLR resulting from neutrophilia and lymphopenia was identified as an early risk factor for SCOV-19 [29–31]. Most of these parameters have been used to stratify and monitor patients of COVID-19 worldwide but have their limitations.

As expected, our results are similar with high CRP, PCT, NLR, and lower level of ALC in SCOV-19 compared to MCOV-19 patients and in non-survivors compared to survivors. None of our patients had received steroids till the time of sampling, and hence the results reflect true disease association. As reported previously, we found NLR, ALC, and PCT were associated with mortality in SCOV-19 cases [32].

There are readily available existing parameters that assist in triaging MCOV-19 from SCOV-19 patients and help in prognostication. To understand any practical utility of adding mHLA-DR, nCD64, and IDI to an already existing armamentarium of tests, we calculated the diagnostics of studied parameters to understand how the differential expression of these biomarkers compares with that of other commonly used lab parameters like CRP, PCT, NLR, and ALC with respect to severity of disease by ROC curve analysis. IDI revealed the highest AUC of 0.99 with 100% sensitivity and 96% specificity, suggesting IDI as a preferred diagnostic marker compared to other established parameters that had an AUC of < 0.75. IDI appears to be a promising tool for triaging COVID-19 patients at the time of admission/ presentation: values > 12 suggest severe disease with increased mortality risk. As IDI continued to increase in the 3rd and 4th quartile (> 38.44), chances of mortality were extremely high, with 20/22 cases ultimately not surviving (death within 3–22 days). Similarly, patients with ALC in the 3rd and 4th quartile (< 0.85 cells/µl) had very high chances of mortality. Together these findings support that low mHLA-DR and lymphopenia act like a double-edged sword for blunting the adaptive immune response seen in COVID-19. There is impaired SARS-CoV-2 antigen presentation due to low mHLA-DR which is IL-6 mediated, with sustained cytokine production and hyper-inflammation [3, 33]. Reduced mHLA-DR expression increases the likelihood of secondary infections and is associated with endotoxin tolerance [34, 35].

With the increasing understanding of COVID-19 associated immune dysregulation, immune modulation therapy appears to be the next step forward. Dickel S et al. reported a single case of superinfected COVID-19 patient with ARDS treated with a single dose of interferon-gamma (IFN-Y) with measurement of mHLA-DR pre and post-therapy; a sharp increase in mHLA-DR was reported [36]. Another case of immunostimulation with IFN-Y in protracted SARS-Cov-2 pneumonia resulted in recovery and discharge in a patient with several comorbid conditions. The level of mHLA-DR together with lymphocyte counts gradually increased over a week [37]. Studies have previously shown that the addition of IL-6 in the growth medium of healthy dendritic cells attenuated HLA-DR membrane expression and decreased the production of IFN-Y by CD4 cells [38]. It was observed by Giamerello-buorbolis et al. that in vitro addition of IL-6 blocker Tocilizumab partially restored the expression of HLA-DR on monocytes of all patients with immune dysregulation [33]. Clinical trials based on immune-modulation therapy are much awaited and may prove to be revolutionary.

COVID-19 is a heterogeneous disease with manifestations depending upon co-morbidities, age and genetic variants of SARS-COV 2. Although it appears that the critical phase of pandemic has subsided and variants with milder virulence are circulating, appearance of a deadly or more virulent strain cannot be ruled out in future. Identifying biomarkers with meaningful information plays an integral role in identifying suspicious cases, triaging, monitoring, and managing severe cases. The addition of immune biomarkers at specific time points to already available biomarkers may provide more reliable information.

The current study has limitation of being a single-center pilot study. Repeat measurement of immune parameters and correlation with IL-6 was not performed. As the follow-up was recorded in terms of mortality, utility of these parameters in predicting progression from mild to severe illness could not be assessed. Cases included in this study belong to both delta and non-delta variants which were prevalent in India at the time of study but sequencing was not performed.

It would be interesting to study the influence of newer variants on the immune parameters. Future studies should be designed to address these concerns and extract more useful information from these immune parameters.

Conclusion

Immune dysregulation in COVID-19 is the major causal factor associated with disease progression and mortality. The key factor is downregulation of mHLA-DR with defective antigen presentation to CD4 cells resulting in compromised adaptive immune response. The mHLA-DR and IDI, in addition to NLR and ALC at admission and during hospital stay, can be utilized for patient triaging, monitoring, early intervention, and mortality prediction. Immune dysregulation index, reported for the first time in this study, appears most promising. Immune monitoring of 'in hospital' cases may provide optimized treatment options. Strategies for Immune modulatory therapy aimed at correcting this dysregulation are needed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12291-022-01087-z.

Acknowledgements Author wish to acknowledge Dr Shikhar Singh Aswal, for statistical analysis of the data and Mr. Abhishek Bajpayee for sample preparation for flow cytometry.

Author Contributions Conceptualization: [Namrata P Awasthi]; Methodology: [Namrata P Awasthi, Paresh Jain, Vandana Tiwari, Jyotsna Agarwal]; Formal analysis and investigation: [Namrata P Awasthi, Sridhar Mishra, Paresh Jain, Pravin Kumar Das], Writing - original draft preparation: [Namrata P Awasthi, Sridhar Mishra, Paresh Jain]; Writing - review and editing: [Paresh Jain, Nuzhat Husain], Supervision: [Namrata P Awasthi, Nuzhat Husain.

Funding No funding was received for conducting this study.

Declarations

Disclosure of Potential Conflicts of Interest None.

Research Involving Human Participants and/or Animals Approval was obtained from the ethics committee of the Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed Consent A written informed consent was obtained from the study participant or legal guardian and was explained about the study procedure.

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Declarations All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Consent to Participate Informed consent was obtained from all the study participants or from legal guardians.

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