Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Reduced IFN-γ levels along with changes in hematologic and immunologic parameters are key to COVID-19 severity in Bangladeshi patients

Mohammed Moinul Islam\textsuperscript{a,b,*}, Shafiqul Islam\textsuperscript{a,b,c,*}, Ridwan Ahmed\textsuperscript{a,b}, Mohit Majumder\textsuperscript{a,b}, Bishu Sarkar\textsuperscript{a,b}, Md. Ejajur Rahman Himu\textsuperscript{a,b}, Md Kawser\textsuperscript{a,b}, Alamgir Hossain\textsuperscript{a,b}, Mohammad Jewel Mia\textsuperscript{a,b}, Rashed Rezwan Parag\textsuperscript{a,b}, Md. Rakibul Hassan Bulbul\textsuperscript{a}, Shakeel Ahmed\textsuperscript{a}, MA Sattar\textsuperscript{a}, Rajdeep Biswas\textsuperscript{a}, Moumita Das\textsuperscript{a}, Md. Mizanur Rahman\textsuperscript{a,b}, Rajib Kumar Shil\textsuperscript{a,b}, Ramendu Paria\textsuperscript{a,b}, Srikanta Chowdhury\textsuperscript{a,b}, Manisha Das\textsuperscript{a}, Abu Shadat Mohammad Noman\textsuperscript{a,b}, and Muhammad Mosaraf Hossain\textsuperscript{a,b,*}

\textsuperscript{a}Department of Biochemistry & Molecular Biology, University of Chittagong, Chittagong, Bangladesh; \textsuperscript{b}EunGEF Research Foundation, Chittagong, Bangladesh; \textsuperscript{c}Stem Cell Genetics, Institute for Life and Medical Sciences, Kyoto University, Kyoto, Japan; \textsuperscript{d}Department of Biochemistry, Rangamati Medical College, Rangamati, Bangladesh; \textsuperscript{e}Bangladesh Institute of Tropical and Infectious Diseases, Chittagong, Bangladesh; \textsuperscript{f}Department of Medicine, Chittagong Medical College and Hospital, Chittagong, Bangladesh; \textsuperscript{g}Anaesthesia & ICU department, General Hospital, Chittagong, Bangladesh

The manifestation of coronavirus disease 2019 (COVID-19) severity and mortality has been associated with dysregulation of the immune response, often influenced by racial disparities and conferred by changes in hematologic and immunologic parameters. These biological and hematologic parameters as well as cytokine profiles were investigated in a cohort of 61 COVID-19-positive patients (categorized into mild, moderate, and severe groups) from Bangladesh using standard analytical methods. The data reported that the interleukin (IL)-4 and IL-6 levels were significantly increased, whereas the levels of interferon (IFN)-γ were significantly reduced in patients with severe COVID-19 (p < 0.05) compared with those in patients with mild and/or moderate COVID-19. The extent of erythrocyte sedimentation rate (ESR), neutrophil count; and levels of ferritin, C-reactive protein (CRP), and D-dimer (p < 0.05) were found to be significantly increased, whereas the white blood cell (WBC), lymphocyte, eosinophil, and platelet counts (p < 0.05) were observed to be significantly reduced in patients with severe COVID-19 compared with those in the patients in other 2 groups. Our study exhibited a significantly higher IL-6-to-lymphocyte ratio in patients with severe COVID-19 than in those with mild and moderate COVID-19. The calculated neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and ferritin-to-ESR ratio were significantly increased in patients with severe COVID-19. The increase in the IL-4 and IL-6 levels along with CRP and D-dimer levels may envisage a hyperinflammatory environment and immune dysregulation, which contribute to prolonged viral persistence, leading to severe disease. However, the reduced level of IFN-γ can be attributed to a less fatality toll in Bangladesh compared with that in the rest of the world. © 2022 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.
The coronavirus disease 2019 (COVID-19) pandemic, engendered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a global disaster since its outbreak in Wuhan, China, in December 2019 [1]. As of March 2022, there were over 507 million confirmed COVID-19 cases and a death toll of more than 62 million, with the case fatality rate ranging from 0.04% to 18.18% reported worldwide; however, Bangladesh experienced approximately 2 million cases and total deaths of less than 30,000 [2]. The disparity in the mortality rates caused by COVID-19 in different regions of the world has been a puzzling conundrum but grossly alluded to demographic variables such as age, sex, ethnicity, presence or absence of comorbidities, human leukocyte antigen (HLA) genotype, genetically inherited immunity, acquired immunity by exposure to a wide variety of pathogens, and a lifestyle reinforcing immunity [3,4]. COVID-19 severity and mortality have been associated with hyperinflammation caused by elevated levels of proinflammatory cytokines, such as interleukin (IL)-6, C-reactive protein (CRP), and ferritin; decrease in T and B lymphocyte counts and their activation; and lymphopenia [5–9]. However, even with mortality risk factors such as dense population, elderly, having comorbidities, poor maintenance of social distancing protocol, unsuccessful lockdown, and failure to implement self-isolation and quarantine [10], Bangladesh has seen a comparatively low mortality rate, which comprised a case-fatality ratio of approximately 1.49% [2].

SARS-CoV-2 acts on the lower respiratory tract in humans and exhibits a myriad of clinical characteristics ranging from no symptoms to severe pneumonia, with acute respiratory distress syndrome and death. The clinical spectrum of the disease has been manifested as asymptomatic; mild, with fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell but the absence of shortness of breath, dyspnea; moderate, with evidence of lower respiratory disease and an oxygen saturation (SpO2) of ≥94%; and severe, with an SpO2 of <94%, an arterial oxygen partial pressure (PaO2)-to-fractional inspired oxygen (FiO2) ratio (with PaO2 in mm Hg) of <300 mm Hg, a rate of respiration of >30 breaths/min, or infiltrates in >50% of the lungs; and perilous, with respiratory failure, septic shock, and/or multiple organ dysfunction, leading to death [11]. However, because of limited resources and unavailability of data from both patients and hospitals in Bangladesh, it was difficult to differentiate between asymptomatic and mild illnesses and between patients with severe and critical illness, which narrowed the category to mild, moderate, and severe illness in our study.

Mild infection causes increased viral replication, which continues to an inflammatory response in case of moderate illness, which becomes predominant in patients with severe-to-critical illness [12]. The 3 phases of immunopathologic progression leading to COVID-19 mortality, namely, initiation phase, amplification phase, and summation phase, are distinguished based on cytokine and chemokine profiles [13]. On that account, for the treatment of COVID-19, it is very important to identify host or viral determinants that are critical for the development of mild or severe disease because inflammatory cell influx and cytokine blockade cannot bring benefit until a good time point or patient selection is not considered [14].

Because of our concern about the disproportionate burden of COVID-19 outcomes, with significant immunopathologic disparities, in our study, we aimed to elucidate the cytokine parameters in mildly, moderately, and severely ill patients with COVID-19, which can help distinguish the severity of the disease by determining the immune signature, explanation of immune regulation that may lead to determining better COVID-19 therapy. In addition, a study of the correlation of biochemical and immunologic parameters with the severity of COVID-19 in Bangladesh is yet to be performed. In line with that, we analyzed biochemical and hematologic parameters for mildly, moderately, and severely ill individuals with COVID-19 to determine the association of cytokine profile and other biological parameters with COVID-19 severity. Our data reported that the IL-4 and IL-6 levels were significantly increased, whereas the levels of interferon (IFN)-γ were significantly reduced in patients with severe COVID-19 compared with those in patients with mild and/or moderate COVID-19. The extent of erythrocyte sedimentation rate (ESR); neutrophil count; and levels of ferritin, CRP, and D-dimer were found to be significantly increased, whereas the WBC, lymphocyte, eosinophil, and platelet counts were observed to be significantly reduced in patients with severe COVID-19 compared with those in patients in the other 2 groups. The reduced level of IFN-γ can be attributed to a less fatality toll in Bangladesh compared with that in the rest of the world. This study will help us to explore the intensity of the severity of the disease caused by SARS-CoV-2 infection and make a defined plan for controlling and medication for such viral infections in the future.

MATERIALS AND METHODS

Study Design

A total of 61 (n = 61) patients with COVID-19, who tested positive for COVID-19 or were admitted with symptoms to Chittagong Medical College Hospital (CMCH), the Bangladesh Institute of Topical and Infectious Diseases (BITID) hospital, or Chittagong General Hospital, were enrolled in this study. The presence of SARS-CoV-2 was confirmed using the real-time reverse-transcription polymerase chain reaction (qRT-PCR) test according to World Health Organization (WHO) interim guidelines. Demographic and epidemiologic characteristics as well as symptoms were recorded using a pretested questionnaire.

Ethics Statement

Blood samples were collected as per the guidelines of ethical permission (Memo No: CMC/PG/2021/183) granted by the Ethical Review Committee of Chittagong Medical College, Chattogram, Bangladesh.

Categorization of the Study Subjects Based on Clinical Symptoms

The selected patients with COVID-19 were categorized as having mild (n = 20), moderate (n = 21), or severe disease (n = 20) based on their clinical symptoms. The patients with mild COVID-19 were not called for hospitalization or had self-recovering disease courses, whereas the patients with moderate COVID-19 were specified as those hospitalized in a nonintensive care unit (non-ICU) setting. Furthermore, severely ill patients experienced cardiac impairment, an exaggerated inflammatory response, and/or aggravation of underlying comorbidities, organ failure, and rapid clinical deterioration, with respiratory failure, necessitating mechanical ventilation and requiring intensive therapy or ICU care.
Sample Collection and Preparation
A total of 5 mL of whole blood was collected from the study subjects in Vacutainer (Invitrogen) blood collection tubes by an expert phlebotomist (Technician) while maintaining proper safety procedures. All the blood samples were collected 5–7 days after symptoms appeared. After collecting blood, the tubes were kept at room temperature for 30 minutes without any disturbance. Finally, serum was collected by centrifuging the tubes at 2,000 g for 10 minutes.

Investigation of Hematologic and Biochemical Parameters
The complete blood count (CBC) test for the estimation of red blood cell (RBC), white blood cell (WBC), and platelet counts as well as a differential count (DC) for the estimation of neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts were performed using Hematology Analyzer Sysmex XT-2000i and checked manually. The Cymmeth and Westergen methods were followed for determining the hemoglobin levels and measuring ESR, respectively. Serum creatinine levels were estimated using the Jaffe alkaline picrate method, and CRP levels were determined using a high-sensitivity assay based on the principle of latex agglutination. In addition, the electrochemiluminescence immunoassay and fluorescence immunoassay were used to perform D-dimer and ferritin tests, respectively.

Investigation of Selected Cytokines
The levels of IL-6, tumor necrosis factor (TNF-α), IFN-γ, and IL-4 were measured in the serum samples using the enzyme-linked immunosorbent assay (ELISA) as per the protocol and instruction of the manufacturer sourced from Demeditec Diagnostics GmbH, Germany (Cat No.: TNF-α [DE4641], IFN-γ [DE4434], IL-6 [DE4640]) and Drug Instruments GmbH, Germany (IL4 [EIA-5539]). In brief, the following procedures were adopted to measure the concentration of the 4 tested cytokines in the serum samples of the study subjects.

After proper labeling of each well (well for the samples, a control, and a calibrator), a volume of 50 μL of buffer (incubation) was aliquoted into each selected well, followed by loading of 200 μL of the samples, a control, and a calibrator in the respective wells. The wells were allowed to incubate at 20°C on a flat shaker at 700 rpm for 120 minutes. After discarding the liquids, the wells were washed thrice using 400 μL of washing buffer. A volume of 50 μL ofhorseradish peroxidase (HRP)-conjugated anti-TNF-α, anti-IFN-γ, anti-IL-6, or anti-IL-4 was aliquoted into the wells of the respective plates. The wells were allowed to incubate at 20°C on a flat shaker at 700 rpm for 120 minutes. The washing steps were repeated. A total volume of 100 μL of a revelation solution was loaded in all the wells. The wells were allowed to incubate at 20°C on a flat shaker at 700 rpm for 15 minutes. Finally, a total volume of 100 μL of a stop solution was aliquoted in all the wells and the absorbance measured at 450 and 490 nm. The ELISA-AID software was used to calculate the concentration of the tested cytokines.

Statistical Analysis
The SPSS 17.00 and Graph pad prism 9 statistical software were used for performing all statistical analyses. Data were expressed as mean ± SD.

RESULTS
Basic and Clinical Characteristics of Patients’ Cohorts
Our study included 20, 21, and 20 patients with mild, moderate, and severe symptoms of SARS-CoV-2 infection, respectively. Among the total of 61 patients (median age, 53 years; interquartile range [IQR], 35.50–60.00 years; range, 24–70 years), the mean ages of the 3 groups (39.3 ± 13.78, 48.67 ± 12.18, and 53.05 ± 11.16, respectively) were significantly different (p = 0.0211), with the moderate and severe groups having higher ages (Table 1 and Supplementary Figure E1). Among all the patients, 31 (50.8%) were men, and the proportions of men and women were almost equally distributed in each group (men:women = 11:10 in the mild, 10:10 in the moderate, and 10:10 in the severe groups). There were no significant differences in the sex ratio of each group.

Among the mild, moderate, and severe groups of patients with COVID-19, the most prominent symptoms were fever (95%, 94.74%, and 95%, respectively), followed by cough (65%, 93.75%, and 100%, respectively), body pain (45%, 60%, and 62.50%, respectively), and headache (60%, 50%, and 37.50%, respectively), accompanied by or without sore throat, vomiting, diarrhea, weakness, loss of taste and smell when the most severe cases manifested fever, cough, body pain, and dyspnea (shortness of breath) as well as an oxygen saturation of less than 80% (Table 1). The oxygen saturation level (%) was significantly lower (p < 0.05) in the severe group (79.44%) than in the mild group (96.2%) of COVID-19-positive patients. A computed tomography (CT) scan and X-ray report of the posteroanterior view of the chest showed normal trachea and diaphragm, free basal angles, normal heart in TD, and a normal lung field in the patients with mild COVID-19, whereas in the patients with moderate COVID-19, lung fibrosis, pulmonary inflammatory lesions, and pneumonitis were seen in many cases. In most patients with severe COVID-19, advanced-stage pneumonia was seen, with more than 50% of lung infection, kidney failure, and heart attack in some cases. Approximately 55% of the mildly ill patients, 76% of the moderately ill patients, and 75% of the severely ill patients with COVID-19 recruited in our study had at least 1 or more comorbidities such as diabetes mellitus, hypertension, hypotension, asthma, chronic kidney disease (CKD), sinusitis, allergy, cancer, lung disease, and liver disease. There were no significant differences in comorbidities observed among the patients with mild, moderate, and severe COVID-19 (p > 0.05) (Table 1). Among the patients included in our study, 15% of those in the mild group, 61.9% of those in the moderate group, and 70% of those in the severe group had diabetes mellitus, whereas approximately 40%, 42.9%, and 55% of the patients of the mild, moderate, and severe groups, respectively, had hypertension. In all the groups, approximately 5% of the patients had heart disease. In addition, among the patients with mild COVID-19, approximately 5% and 10% had CKD and allergy, respectively, with no hypotension, asthma, sinusitis, cancer, lung disease, and liver disease. On the other hand, among the patients with moderate COVID-19, approximately 4.8%, 4.8%, and 10% had asthma, CKD, and cancer, respectively, with no hypotension, sinusitis, allergy, lung disease, and liver disease. Approximately 15% and 10% of the patients severely affected by COVID-19 had heart disease and asthma, respectively, with no CKD, sinusitis, cancer, lung disease, or liver disease (Supplementary Figure E1C).
Hematologic Parameters and Infection-related Biomarkers

Our study showed that the ESR was significantly elevated in the moderate and severe groups compared with that in the mild group (p < 0.05 and p < 0.0001, respectively) and severe-to-moderate group (p < 0.01) of patients with COVID-19. The moderate and severe groups had an ESR of more than the normal range (1%–20%) [15] (Figure 1A). The patients with mild and moderate COVID-19 had normal WBC counts (45,000–100,000 cells/mm3) [16], and less-than-normal levels were present in the patients with severe COVID-19, which were significantly lower in comparison with those in the patients with mild (p < 0.001) and moderate COVID-19 (p < 0.0001) (Figure 1B). The patients with mild COVID-19 had a normal range of lymphocyte count (20%–40%) [15], whereas those with moderate and severe COVID-19 had slightly lower (17.32% ± 8.426%) and far lower (9.714% ± 3.539%) lymphocyte counts than normal, respectively. The moderate and severe groups showed significantly reduced lymphocyte counts compared with the mild (p < 0.0001) and moderate groups (p < 0.00001) (Figure 1C). On the other hand, the neutrophil counts were significantly increased in the moderate (p < 0.01) and severe groups (p < 0.0001) in comparison with those in the mild group and exceeded the normal limit (54%–62%) [15] (Figure 1D). The moderate and severe cases had significantly reduced eosinophil counts compared with the mild cases (p < 0.001 and p < 0.00001), although all the groups manifested a normal range of eosinophil count (0%–3%) [15] (Figure 1F). The patients with severe COVID-19 had significantly lower platelet counts (p < 0.05) than those with moderate COVID-19 (Figure 1H). However, all 3 groups showed a normal range of platelet count (1 × 105–4.5 × 105 cells/mm3) [15]. The patients with mild, moderate, and severe COVID-19 had 0%–0.5% of basophils, which is below normal levels and was found to be statistically insignificant among the groups. We observed the ferritin level to be higher than the normal range (13–300 ng/mL) [16] in both the moderate and severe groups and the D-dimer level to be higher than the normal range (<0.4 μg/mL) [15] in the severe group. The data showed that the severe group had significantly higher (p < 0.05) ferritin levels than the mild group and that the severe group had significantly higher (p < 0.05) D-dimer levels than the mild and moderate groups of patients with COVID-19 (Figure 1, M). There were no significant differences in the monocyte and RBC counts and in the hemoglobin and serum creatinine levels among the patients with mild, moderate, and severe COVID-19 (Table 2; Figure 1E, G, I, L), and all the groups manifested a normal range of monocyte count (3%–7%) [15], RBC count (men: 4.7 × 1012–6.1 × 1012 cells/μL; women: 42 × 1012–5.4 × 1012 million cells/μL), hemoglobin level (men: 13.5–16.5 g/dL; women: 12.0–15.0 g/dL), and serum creatinine level (0.5–1.4 mg/dL) in the patients with COVID-19 included in our study [16]. The CRP level was more than the normal range (>0.3 mg/L) [16] in all the groups of patients with COVID-19, and the severe group had significantly higher CRP levels than the mild (p < 0.001) and moderate groups (p < 0.05) (Figure 1K).

The neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and ferritin-to-
ESR ratio (FER) were observed to increase with disease severity (Figure 2A, C-E). The NLR was significantly higher in the patients with moderate ($p < 0.01$) and severe ($p < 0.0001$) COVID-19 than in the patients with mild COVID-19, and the patients with severe COVID-19 ($p < 0.01$) had a significantly higher ratio than those with moderate COVID-19. Both the patients with moderate and severe COVID-19 exhibited significantly higher FER than those with mild COVID-19.

Table 2 and Figure 1 present a comparative analysis of hematologic and biochemical parameters among patients with mild, moderate, and severe COVID-19.

**Figure 1** Hematologic and biochemical parameters of patients with mild, moderate, and severe coronavirus disease 2019 (COVID-19). The level of (A) erythrocyte sedimentation rate (ESR); (B) white blood cell (WBC), (C) lymphocyte, (D) neutrophil, (E) monocyte, (F) eosinophil, (G) red blood cell (RBC), and (H) platelet counts; levels of (I) hemoglobin (Hb), (J) ferritin, (K) C-reactive protein (CRP), (L) serum creatinine, and (M) D-dimer among the mild, moderate, and severe groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

**Immunologic Signature of Patients with COVID-19 (The Cytokine Profile)**

The levels of IL-4, IL-6, TNF-$\alpha$, and IFN-$\gamma$ were not significantly different among the age groups of the studied COVID-19 patients (Supplementary Figure E2). The most important findings of our study revealed an upward trend in the IL-4 and IL-6 levels and, interestingly, a downward trend in the IFN-$\gamma$ levels from the mild to severe groups of patients with COVID-19 (Table 3). The severe group manifested significantly higher IL-4 levels ($p < 0.001$) than its mild counterpart, whereas the moderate and severe groups manifested significantly higher IL-6 levels ($p < 0.05$ and $p < 0.001$, respectively) than the mild group Figure 3A, B). The IFN-$\gamma$ levels were
Figure 2 Calculated results of neutrophil-to-lymphocyte ratio (NLR): (A) NLR, (B) interleukin (IL)-6-to-lymphocyte ratio, (C) platelet-to-lymphocyte ratio (PLR), (D) lymphocyte-to-monocyte ratio (LMR), and (E) ferritin-to-erythrocyte sedimentation rate (ESR) ratio in patients with mild, moderate, and severe coronavirus disease 2019 (COVID-19). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Table 2 Comparisons of hematologic and biochemical parameters among patients with mild, moderate, and severe coronavirus disease 2019 (mean ± SD)a

| Parameters         | Unit ± SD                  | Mild (n = 20) | Moderate (n = 21) | Severe (n = 20) | p value  | Statistical tests |
|--------------------|---------------------------|---------------|-------------------|-----------------|----------|-------------------|
| ESR (mm/h)         | Mean ± SD                 | 25 ± 16.91    | 46.93 ± 6.686     | 74.07 ± 26.64   | <0.0001  | Ordinary 1-way ANOVA |
| WBC (/mm³)         | Mean ± SD                 | 6,883 ± 2,229 | 9,807 ± 3,609     | 35 ± 21.64      | <0.0001  | Ordinary 1-way ANOVA |
| Neutrophil (%)     | Mean ± SD                 | 58.44 ± 11.82 | 77.26 ± 8.419     | 85.07 ± 3.362   | <0.0001  | Ordinary 1-way ANOVA |
| Lymphocytes (%)    | Mean ± SD                 | 35.56 ± 11.01 | 17.32 ± 8.426     | 9.714 ± 3.539   | <0.0001  | Ordinary 1-way ANOVA |
| Monocyte (%)       | Mean ± SD                 | 2.94 ± 1.056  | 3.778 ± 1.396     | 3 ± 1.519       | 0.1259   | Ordinary 1-way ANOVA |
| Eosinophil (%)     | Mean ± SD                 | 3.056 ± 1.162 | 1.667 ± 0.767     | 1.357 ± 0.633   | <0.0001  | Ordinary 1-way ANOVA |
| RBC (million cells/μL) | Mean ± SD            | 5.624 ± 3.614 | 5.002 ± 2.538     | 5.046 ± 0.9602  | 0.7554   | Ordinary 1-way ANOVA |
| Platelets (/mm³)   | Mean ± SD                 | 341,444 ± 142,308 | 292,706 ± 78,400 | 2,07,000 ± 80,472 | 0.0039  | Ordinary 1-way ANOVA |
| Hb (g/dL)          | Mean ± SD                 | 12.56 ± 1.770 | 11.93 ± 1.702     | 12.75 ± 1.924   | 0.3803   | Ordinary 1-way ANOVA |
| S creatinine (mg/dL) | Mean ± SD               | 1.025 ± 0.3845 | 1.362 ± 0.7571   | 0.8473 ± 0.2448 | 0.0828   | Ordinary 1-way ANOVA |
| Ferritin (ng/mL)   | Mean ± SD                 | 245.6 ± 271.9 | 699.1 ± 485.7     | 982.3 ± 772.6   | 0.2022   | Ordinary 1-way ANOVA |
| D-dimer (µg/mL)    | Mean ± SD                 | 0.267 ± 0.1021 | 0.9936 ± 1.610   | 1.651 ± 2.141   | 0.3851   | Ordinary 1-way ANOVA |
| CRP (mg/L)         | Mean ± SD                 | 9.182 ± 5.43  | 59.83 ± 51.89     | 136.3 ± 74.65   | <0.0001  | Ordinary 1-way ANOVA |

ANOVA=Analysis of variance; CRP=C-reactive protein; ESR=erythrocyte sedimentation rate; S=serum; WBC=white blood cell.  
a GraphPadPrism 9 software was used for data analysis.  
b p < 0.0001.  
c p < 0.01.
progressively reduced by the severity of the disease. Our data showed that the IFN-γ levels were significantly lower ($p < 0.0001$) in the patients with moderate ($p < 0.0001$) and severe ($p < 0.0001$) COVID-19 than in their counterparts with mild COVID-19 (Figure 3C). Although not statistically significant, the TNF-α levels were higher in the moderate and severe groups than in the mild group (Figure 3D). Our study also exhibited significantly higher IL-6 levels in the patients with severe COVID-19 than in those with mild ($p < 0.01$) and moderate ($p < 0.05$) COVID-19 (Figure 3).

## DISCUSSION

The roles of clinical hematological parameters and inflammatory molecules are pivotal in orchestrating host defense against COVID-19 pathogenesis. Various acute phase and proinflammatory markers are associated with disease progression-related mortality in different ethnic populations [17,18]. In this study, most of the patients with severe COVID-19 in Bangladesh tended to have lymphopenia; lower platelet, lymphocyte, and eosinophil counts; higher NLR; and elevated levels of infection-related biomarkers (i.e., ESR, serum ferritin or hyperferritinemia, and CRP) in comparison with those with mild and moderate COVID-19. Therefore, understanding the dysregulation in the immune system and associated hyperinflammation is key to undertaking the disease’s pathologic course of action.

Our study showed leukopenia (lower WBC count) in the patients with severe COVID-19, although those with mild and moderate COVID-19 had a normal WBC count (Figure 1B). Because leukopenia increases the risk of infections when infected [19], higher age and related comorbidities might have caused the lowering of the WBC count, which, in turn, led to more severe COVID-19 pathogenesis. This is also supported by the findings that asymptomatic patients typically have a higher WBC count, particularly lymphocytes, wherein high leukocyte counts help SARS-CoV-2-infected individuals to fight against the virus and prevent the severity of the disease by fast viral clearance [20]. In alignment with this fact, our study also showed a higher lymphocyte count in the patients with mild COVID-19, which was significantly reduced in those with moderate and severe COVID-19 (Figure 1C). The highly reduced lymphocyte counts in the moderate and severe groups in our study compared with those in the mild ($p < 0.0001$) and moderate groups ($p < 0.0001$), respectively, are in alignment with the study results published by Liao et al. [21]. Besides, lymphopenia (lymphocyte count < 18%) is associated with severe COVID-19 manifestation and has been suggested as a prognostic marker for the disease’s severity and mortality [18,22,23]. Lymphocytes (B and T cells) produce antibodies, chemokines, cytokines, and other immune molecules to fight against infections, and a lower lymphocyte count can cause immune system compromise. The eosinophil counts, although in the normal range, were reduced significantly in the patients with moderate and severe COVID-19 compared with those in the patients with mild COVID-19 (Figure 1F). A number of other studies have found reduced WBC, lower lymphocyte (lymphopenia), and lower eosinophil counts (eosinopenia) to be associated with severe illness and death in patients with COVID-19 [21,22,24–26]. In the patients with moderate and severe COVID-19, neutrophilia (higher neutrophil count) was observed, and the neutrophil count was significantly higher than that in the patients with mild COVID-19 (Figure 1D). Although in inflammatory responses, neutrophils are considered the first recruitment to the infection site, conferring protection against viral infection, prolonged and acute neutrophilia caused by viral infection may deteriorate pneumonia and acute respiratory distress syndrome [27]. Therefore, larger activation of neutrophils caused by prolonged viral presence in patients with moderate and severe COVID-19 may be correlated with increased severity of the disease. Our study also exhibited a significant reduction in the platelet count in the patients with severe COVID-19 in comparison with that in the patients with mild COVID-19. This observation is also in alignment with the result of the study by Fan et al. [23], which showed that thrombocytopenia (reduced platelet count) in ICU patients with COVID-19 is associated with hospital admission and death. The basopenia (0%–0.5% of basophil count) observed in our study might have been caused by inflammatory migration of basophils to COVID-19 infection sites.

Furthermore, in this study, the moderate and severe cases had higher NLR (Figure 2A), which indicates a dysregulated host innate immune response. Several other studies have also manifested a strong association between higher NLR and the severity and mortality of COVID-19 [18,28,29] and other infectious diseases, including acute coronary syndrome and pneumonia [30]. In an earlier report, neutrophilia and elevated NLR were shown to be significantly increased in patients with severe COVID-19 and patients requiring ICU care [31]. Additionally, the calculated PLR and LMR, which are 2 important predictors of inflammation and mortality for many diseases [32,33], were significantly higher in the patients with severe COVID-19 than in those with mild and moderate COVID-19 in our study (Figure 2C, D). More importantly, patients severely affected by COVID-19 have been shown to have higher PLR and LMR in several previous studies [34,35]. On the

### Table 3 Cytokines signatures among patients with mild, moderate, and severe coronavirus disease 2019

| Cytokines | Unit | Mild | Moderate | Severe | $p$ value | Statistical test |
|-----------|------|------|----------|--------|-----------|-----------------|
| IL-4 (pg/mL) | Mean ± SD | 0.1583 ± 0.1345 | 0.269 ± 0.3209 | 0.3758 ± 0.2734 | 0.0181 $^b$ | Ordinary 1-way ANOVA |
| IL-6 (pg/mL) | Mean ± SD | 8.419 ± 15.25 | 20.5 ± 26.73 | 50.06 ± 77.29 | <0.0001 $^c$ | Ordinary 1-way ANOVA |
| IFN-γ (IU/mL) | Mean ± SD | 11.83 ± 6.768 | 4.28 ± 8.834 | 0.0595 ± 0.1111 | <0.0001 $^c$ | Ordinary 1-way ANOVA |
| TNF-α (pg/mL) | Mean ± SD | 0.037 ± 0.06681 | 1.834 ± 7.125 | 0.549 ± 1.277 | 0.3812 | Ordinary 1-way ANOVA |

$^a$GraphPad Prism 9 software was used for data analysis.

$^b$ANOVA=Analysis of variance; IFN=interferon; IL=interleukin; TNF=tumor necrosis factor.

$^c p < 0.05$.

$^{c'} p < 0.0001$.
contrary, higher LMR has been shown to be associated with lower mortality in other studies of patients with COVID-19 [36]. Therefore, scrutiny of erythrocytes, lymphocyte subsets, NLR, PLR, and LMR is predictive of the severity and mortality of COVID-19.

Inflammatory or infection markers such as ESR, CRP, and D-dimer have been found to be positively associated with COVID-19 severity [37]. Our study showed progressive and significant elevation of ESR as well as CRP and D-dimer levels with increasing severity of the disease (Figure 1A, K, M). Elevated CRP levels are critically linked with the overproduction of inflammatory cytokines and a hyperactive immune system [38], which may damage respiratory tissues, causing severe COVID-19. In addition, the serum creatinine level was significantly decreased in the patients with severe COVID-19 (Table 1). In our study, the monocyte and RBC counts as well as hemoglobin levels were not significantly different in the mild, moderate, and severe groups (Figure 1E,G, I). Layla et al. [39] did not find any differences in the RBC counts among patients with mild, moderate, and severe COVID-19 in Bangladesh.

We observed the ferritin level (Figure 1J) and FER (Figure 2E) to be higher in the moderate group and significantly inflated in the severe group compared with those in the mild group of patients with COVID-19. A high level of ferritin exerts immune-suppressive and proinflammatory effects, mediating immune dysregulation, which is accompanied by a cytokine storm, and our data are also supported by the findings of previous studies that have suggested that hyperferritinemia (elevated ferritin levels) and FER are associated with increased severity of pulmonary disorders and mortality due to COVID-19 [40,41]. The regulation of proinflammatory and anti-inflammatory mediators is crucial in viral infection, wherein proinflammatory and anti-inflammatory cytokines as
well as ferritin can induce each other to orchestrate the immune system. The most intriguing findings of our study are the increment in the IL-4 and IL-6 levels and, interestingly, a progressive reduction in the IFN-γ levels from the mild to severe groups of patients with COVID-19 (Figure 3A, B,C). Increases in the IL-4 and IL-6 levels, but not reduction in the IFN-γ level, have also been reported by other studies [42]. Previous studies also showed that IL-6 antagonists significantly reduced the respiratory support requirements and the levels of inflammatory markers in all patients with COVID-19 with cytokine storms and implicated benefits for severely ill patients [43]. In addition, IL-6-mediated low expression of HLA-DR and lymphopenia have been shown to be associated with prolonged cytokine production and hyperinflammation in patients with severe COVID-19 [44]. Our study indicates a similar pattern, wherein lymphopenia (Figure 1C) and elevated IL-6 levels (Figure 3B) can be associated with the severity of COVID-19.

IL-6/LY reflects the imbalance of inflammatory response and the disruption of immune function [45]. In this context, IL-6/LY appeared to be crucial in predicting the immune-inflammatory complex index in the prognosis of COVID-19. Our study exhibited significantly higher IL-6 to Lymphocyte ratio in the patients with severe COVID-19 than in those with mild (p < 0.01) and moderate (p < 0.05) COVID-19 (Figure 2B). Furthermore, a negative correlation (Pearson r = -2.51) between IL-6 levels and lymphocytes (Figure 4C) in our study indicates the association of higher cytokine levels and lymphopenia with the severity of COVID-19. In other studies, patients with severe disease were shown to have high levels of IL-6, which are correlated with decreased lymphocyte counts [46]. Macrophages and dendritic cells also manifested high levels of IL-6 and IL-1β, which might directly destroy human spleens and lymph nodes and eventually lead to lymphopenia in vivo [47].

The cytokine environment determines the subsets of pathogenic T helper (Th1) cells by influencing their differentiation. In particular, IFN-γ is critical for determining Th11 development and inhibiting Th12 differentiation, whereas IL-4 is necessary for the development of Th12 proliferation and downregulation of Th11 growth, creating a Th11/Th12 balance that determines the disease outcomes [48]. The Th11/Th12 balance regulates cellular immune responses, which impacts the development of inflammatory dysregulation and susceptibility to infectious diseases [49]. It has also been reported that higher IL-4 and IL-6 levels and lower IFN-γ levels (lower Th11/Th12) mediate diminished cell-mediated immunity and increased production of serum antibodies [48]. The higher IL-4 and IL-6 levels and the lower IFN-γ levels in the patients with severe COVID-19 observed in our study may indicate a preference for an antibody-mediated immune effector response rather than a cell-mediated response. Therefore, a decrease in the lymphocyte counts in our study may cause speculation of a lower Th11/Th12 ratio and a decent increase in B-cell proliferation. IL-6 supports viral persistence by activating IL-4-producing CD4+ T cells and suppressing IFN-γ-producing CD4+ T cells [50]. In addition, IL-
4 has a role in the development of rhinitis, allergic response, and acute inflammation, whereas IFN-γ-mediated Th1 plays a critical role in antigen-specific defense mechanisms at the epithelial surface associated with multiple inflammatory airway diseases [51]. Other reports have stated that IFN-γ acts as a regulator of efficient antigen presentation [52], and lowering the IFN-γ level or deviation in the IFN-γ-IFN-γR system severely impedes host immune responses to infections [53]. Surprisingly, this is the first report to show that the IFN-γ level is extremely reduced in patients with severe COVID-19 within 5−7 days of symptoms appearing. Gadotti et al. [54] showed a higher IFN-γ level within 10 days and a decrease in the IFN-γ level after 10 days of symptoms appearing, wherein a sustained higher IFN-γ level was related to higher mortality. In addition, the patients with severe COVID-19 did not have significantly higher TNF-α levels than those with moderate and mild COVID-19 enrolled in our study. Recent studies also showed IFN-γ and TNF-α levels to be associated with respiratory distress and high mortality in patients with COVID-19 [55]. Other studies showed that the combined effect of IFN-γ and TNF-α induces PANoptosis and a deadly cytokine storm, causing acute lung damage and mortality among patients [56,57]. According to these results, we speculated that in Bangladeshi patients, lower levels of IFN-γ 10 days after symptoms appear could result in worse outcomes through the loss of antiviral and anti-inflammatory mechanisms and exacerbation of COVID-19 pathogenesis but with lower mortality. Therefore, we can posit that increased IL-4 and IL-6 levels with robustly reduced IFN-γ levels in Bangladeshi patients corroborates the enhanced severity of COVID-19, with lower mortality (Figures 5 and 6). However, to prove it entirely, complete immune signatures and genetic disparities are required to be examined in a higher number of samples and wider types of ethnic people worldwide.

Conflicts of Interest Disclosure

The authors do not have any conflicts of interest to declare in relation to this work.

Acknowledgments

This work was supported by the Bangladesh Medical Research Council (BMRC) (BMRC/HPNSP-Research Grant/2020-2021/52 (1-47) and Research and Publication Cell (216/GoBe/PoRi/ProKa/Doptor/cu/2021) University of Chittagong (CU), Bangladesh.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.11.006.
1. Zhu N, Zhang D, Wang W, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020;382:727–33.
2. Johns Hopkins University. COVID-19 dashboard by Johns Hopkins University (JHU). 2022. Available at: https://coronavirus.jhu.edu/map.html. Accessed December 23, 2022.
3. Langton DJ, Bourke SC, Lie BA, et al. The influence of HLA genotype on the severity of COVID-19 infection. HLA 2019;81:947–50.
4. Sze S, Pan D, Nevill CR, et al. Ethnicity and clinical outcomes in COVID-19: a systematic review and meta-analysis. eClinicalMedicine 2020;29:100630.
5. Ling L, Chen G, Liu Y, et al. Longitudinal cytokine profile in patients with mild to critical COVID-19. Front Immunol 2021;12:763292.
6. Wang F, Hou H, Luo Y, et al. The laboratory tests and host immunity of COVID-19 patients with different severity of illness. JCI Insight 2020;5:e137799.
7. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020;395:497–506.
8. Dessie ZG, Zewotir T. Mortality-related risk factors of COVID-19: a systematic review and meta-analysis of 42 studies and 423,117 patients. BMC Infect Dis 2021;21:1–18.
9. Liu K, Yang T, Peng XF, et al. A systematic meta-analysis of immune signatures in patients with COVID-19. Rev Med Virol 2021;31:e2195.
10. Anwar S, Nasrullah M, Hosen MJ. COVID-19 and Bangladesh: challenges and how to address them. Front Public Health 2020;8:154.
11. National Institutes of Health. Clinical spectrum of SARS-CoV-2 infection, Centers for Disease Control and Prevention, 2022. Available at: https://www.covid19treatmentguidelines.nih.gov/. Accessed December 23, 2022.
12. V-kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology of COVID-19. Am J Emerg Med 2021;40:110–4.
13. Qin C, Zhou L, Hu Z, et al. Dysregulation of immune response in patients with Coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis 2020;71:762–8.
14. Tignor A, Ibrahim SA, Murray I. Histology, White Blood Cell. Treasure Island (FL): StatPearls Publishing LLC; 2022.
15. Han H, Xu Z, Cheng X, et al. Descriptive, retrospective study of the clinical characteristics of asymptomatic COVID-19 patients. mSphere 2020;5. e00922-20.
16. Liao D, Zhou F, Luo L, et al. Haematological characteristics and risk factors in the classification and prognosis evaluation of COVID-19: a retrospective cohort study. Lancet Haematol 2020;7:e671–8.
17. Zhang HJ, Qi GQ, Gu X, et al. Lymphocyte blood levels that remain low can predict the death of patients with COVID-19. Medicine (Baltimore) 2021;100:e26503.
18. Sun BE. Hematologic parameters in patients with COVID-19 infection. Am J Hematol 2020;95:E215.
19. Xie G, Ding F, Han L, Yin D, Lu H, Zhang M. The role of peripheral blood eosinophil counts in COVID-19 patients. Allergy 2021;76:771–82.
20. Tong X, Cheng A, Yuan X, et al. Characteristics of peripheral white blood cells in COVID-19 patients revealed by a retrospective cohort study. BMC Infect Dis 2021;21:1–10.
48. Punt JSSAJPPOJA. Kuby Immunology. New York: Macmillan Education; 2019.
49. Romagnani S. The Th1/Th2 paradigm. Immunol Today 1997;18:263–6.
50. Shekhawat J, Gauba K, Gupta S, et al. Interleukin-6 perpetrator of the COVID-19 cytokine storm. Ind J Clin Biochem 2021;36:440–50.
51. Zissler UM, Chaker AM, Effner R, et al. Interleukin-4 and interferon-γ orchestrate an epithelial polarization in the airways. Mucosal Immunol 2016;9:917–26.
52. Vanderbeke L, Van Mol P, Van Herck Y, et al. Monocyte-driven atypical cytokine storm and aberrant neutrophil activation as key mediators of COVID-19 disease severity. Nat Commun 2021;12:1–15.
53. Kak G, Raza M, Tiwari BK. Interferon-gamma (IFN-γ): exploring its implications in infectious diseases. Biomol Concepts 2018;9:64–79.
54. Gadotti AC, de Castro Deus M, Telles JP, et al. IFN-γ is an independent risk factor associated with mortality in patients with moderate and severe COVID-19 infection. Virus Res 2020;289:198171.
55. Costela-Ruiz VJ, Illescas-Montes R, Puerta-Puerta JM, Ruiz C, Melguizo-Rodriguez L. SARS-CoV-2 infection: the role of cytokines in COVID-19 disease. Cytokine Growth Factor Rev 2020;54:62–75.
56. Akamatsu MA, de Castro JT, Takano CY, Ho PL. Off balance: interferons in COVID-19 lung infections. EBioMedicine 2021;73:103642.
57. Karki R, Sharma BR, Tuladhar S, et al. Synergism of TNF-α and IFN-γ triggers inflammatory cell death, tissue damage, and mortality in SARS-CoV-2 infection and cytokine shock syndromes. Cell 2021;184:149–68.
Supplementary Figure E1 (A) Age distribution, (B) oxygen saturation, and (C) comorbidities in patients with mild, moderate, and severe coronavirus disease 2019 (COVID-19). *p < 0.05, **p < 0.01. CKD=Chronic kidney disease; DM=diabetes mellitus.

Supplementary Figure E2 Levels of (A) interleukin (IL)-4, (B) IL-6, (C) interferon (IFN)-γ, and (D) tumor necrosis factor (TNF)-α in different age groups (≤35, 36–45, 46–55, >55 years) of patients with coronavirus disease 2019 (COVID-19).