Received: 1 April 2021 | Revised: 27 April 2021 | Accepted: 29 May 2021

DOI: 10.1002/jmv.27112

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Relative expression of proinflammatory molecules in COVID-19 patients who manifested disease severities

Shireen Nigar[1] | SM Tanjil Shah[2,3] | Md Ali Ahasan Setu[3] | Sourav Dutta Dip[3] | Habiba Ibnat[3] | M. Touhidul Islam[4] | Selina Akter[3] | Iqbal Kabir Jahid[3,5] | M. Anwar Hossain[2,5,6]

[1]Department of Nutrition and Food Technology, Jashore University of Science and Technology, Jashore, Bangladesh

[2]Department of Microbiology, University of Dhaka, Dhaka, Bangladesh

[3]Department of Microbiology, Jashore University of Science and Technology, Jashore, Bangladesh

[4]Department of Cardiology, 250 Bedded General Hospital, Jashore, Bangladesh

[5]Genome Center, Jashore University of Science and Technology, Jashore, Bangladesh

[6]Vice-chancellor, Jashore University of Science and Technology, Jashore, Bangladesh

Correspondence

Iqbal Kabir Jahid, Department of Microbiology, Jashore University of Science and Technology, Jashore 7408, Bangladesh.
Email: ikjahid_mb@just.edu.bd

M. Anwar Hossain, Vice-chancellor, Jashore University of Science and Technology, Jashore 7408, Bangladesh.
Email: hossaina@du.ac.bd

Funding information

This study was funded by the University Grants Commission of Bangladesh through Jashore University of Science and Technology; Grant number 20 (2020-21).

Abstract

Aggressive immune response, due to overexpressed proinflammatory molecules, has been characterized in coronavirus disease 2019 (COVID-19) patients. Some of those mediators have a dual and opposite role on immune systems at play behind differential disease severities. We investigated the expression of some cytokines and chemokines in COVID-19 patients in Bangladesh. We diagnosed the patients by detecting severe acute respiratory syndrome coronavirus 2 RNA in nasal swab samples by the real-time RT-PCR method. Thirty adult patients were preselected based on their disease severities and grouped into mild, moderate, and severe cases. Nine healthy volunteers participated in this study as a control. Relative expression of nine cytokines/chemokine in total leukocytes was semi-quantified in SYBRgreen-based real-time quantitative reverse-transcriptase polymerase chain reaction. We performed statistical tests on transformed log data using SPSS 24.0. At the onset of symptoms (Day 1), angiotensin-converting enzyme 2 (ACE2) ($p < 0.05$) and interleukin (IL)-6 ($p > 0.05$) were upregulated in all COVID-19 groups, although the expression levels did not significantly correlate with disease severities. However, expressions of IL-6, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, tumor necrosis factor-α (TNF-α), RANTES (regulated upon activation, normal T cell expressed and secreted), and ACE2, on Day 14, were positively correlated with disease severities. Relative viral load at Day 1 showed no significant correlation with cytokine expression but had a significant positive correlation with RANTES and ACE2 expression on Day 14 ($p < 0.05$). Male patients had a higher level of IL-6 than female patients on Day 1 ($p < 0.05$). All COVID-19 patients showed upregulated cytokines and chemokines on Day 14 compared to Day 1 except TNF-α. Female patients had a higher expression of ACE2 and IL-12 on Day 14. Upregulated cytokines/chemokines at the convalescent stage, especially IL-6, may help in targeting anticytokine therapy in post-COVID-19 patients' management.

Shireen Nigar and SM Tanjil Shah contributed equally to this study.

Institution where the work has been performed:
Jashore University of Science and Technology.

KEYWORDS
chemokines, COVID-19, cytokines, disease severity, proinflammatory molecules, relative expression

1 | INTRODUCTION

A novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in Wuhan, China, in December 2019 and has lead to over 127 million cases and 2.79 million death worldwide as of March 31, 2021.[1] However, surprisingly in Bangladesh, this period recorded 0.61 million confirmed cases and only 9046 deaths.[1] Coronavirus disease 2019 (COVID-19) related deaths are significantly lower in countries with lower quality of life.[2] Although most SARS-CoV-2 patients demonstrate mild or moderate symptoms, it could also lead to excessive, ineffective and exaggerated immune responses called cytokine release syndrome. The clinical response shows acute respiratory distress syndrome (ARDS), multiple organ failure, and ultimately death.[3] Several authors reported the role of cytokines and chemokines as a double-edged sword. Higher levels of chemokines (such as monocyte chemotactic protein [MCP]-1, macrophage inflammatory protein [MIP]-1α, MIP-1β, RANTES [regulated upon activation, normal T cell expressed and secreted], IP-10, and CCL3) and cytokines (such as interleukin [IL]-6, IL-8, IL-1b, tumor necrosis factor-α [TNF-α], interferon-γ [IFN-γ], and colony-stimulating factors (granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor) have been reported for COVID-19 patients.[3–6] Several authors have been reviewed the importance and function of these chemokines and cytokines[7–9] and demonstrated the role of IL-6, TNF-α, IL-10, and IL-8 for disease severity.

The SARS-CoV-2 virus uses novel Metallo-carboxyl peptidase angiotensin receptor 2 (ACE2) to enter its human host cell.[10,11] In spite of acting as the viral receptor, ACE2 protects against lung injury by degrading vasoconstrictive and proapoptotic protein.[12] ACE2 expressed higher in severe patients than mild and moderate.[13] The authors also found upregulation of several IFNs, cytokines, and immune-related genes for severe patients compared with mild and moderate patients. Biological sex variation influenced the severity of COVID-19 cases[14] as reported in various studies; men are more likely to get severe forms of COVID-19.[15–17]

As cytokines and chemokines play a crucial role in the COVID-19 disease manifestations, theoretical therapeutic strategies should base on those. Several investigations have identified anticytokine therapies targeting overactive proinflammatory cytokines (IL-6 and TNF-α). Correct timing for administrating anticytokine therapies needs to recognize for COVID-19 patients' personalized treatment. Data on circulating viral mutants, disease manifestation to a specific population, relative expression, and comparative analysis of proinflammatory cytokine/chemokine in COVID-19 cases within a said geographical location is vital for making patients' management policy. Moreover, questions remained unanswered about the reasons for reduced severity and mortality in developing countries like Bangladesh. To the best of our knowledge, no data are currently available on cytokine expression either in Bangladesh or surrounding regions. We aimed in this study to explore the relationships between relative cytokine expression levels and disease severity, the effect of biological sex in clinical presentation or viral load at the nasopharynx swap of COVID-19 patients. We hope this work can predict therapeutic strategies in the developing south.

2 | MATERIALS AND METHODS

2.1 | Ethical permission and sample collection

The Ethical Review Committee of the Jashore University of Science and Technology approved this study (ERC No.: ERC/FBST/JUST/2020-49). A total of 30 symptomatic COVID-19 patients were preselected, and patients were categorized into mild cases ($n = 10$), moderate cases ($n = 10$), and severe cases ($n = 10$) of COVID-19 according to the World Health Organization Guidelines for Diagnosis and Treatment of COVID-19 infection.[18] We grouped patients with fever and slight upper respiratory tract symptoms as mild cases; patients with shortness of breathing, constant pain, or pressure in the chest as moderate cases; and patients with respiratory failure requiring intensive care units (ICUs) as severe COVID-19 cases. We informed individual participants (COVID-19 patients) or their family members about the study protocol and took verbal consent. The demographic information of all participants enlisted in Table 1. Ten healthy individuals (who gave their consent and participated in this study) were primarily selected, who were free from recent respiratory diseases, acute or chronic infectious diseases, and had no comorbidities. They maintained home quarantine from the beginning of the COVID-19 outbreak in Bangladesh and tested negative for recent SARS-CoV-2 infection by using All Check COVID-19 immunoglobulin G (IgG)/immunoglobulin M antibody assay (CALTH Inc.). We excluded a sample from one volunteer in the final study due to the appearance of antibodies against SARS-CoV-2. All participants were Bacillus Calmette-Guerin (BCG) vaccinated within 1 month of birth (identified by the skin scar/mark on the left upper arm and their verbal confirmation). We have interviewed patients or their family members to collect demographic data and clinical history from all qualified participants. Hereafter, we refer to the healthy control as HC, mild COVID-19 cases as MIC, moderate COVID-19 cases as MOC, and severe COVID-19 cases as SC.

TABLE 1 Demographic and clinical characteristics of studied participants

Characteristics	HC (*n* = 9)	MIC (*n* = 10)	MOC (*n* = 10)	SC (*n* = 10)
Men	5 (55.55%)	6 (60%)	8 (80%)	6 (60%)
Women	4 (44.45%)	4 (40%)	2 (20%)	4 (40%)
Age (mean [±*SD*]) years	23.89 (±2.435)	37.30 (±4.814)	40.20 (±4.69)	58.40 (±3.361)
Fever (temperature >37°C)	0	8 (80%)	10 (100%)	9 (90%)
Cough	0	4 (40%)	8 (80%)	8 (80%)
Headache	0	5 (50%)	6 (60%)	6 (60%)
Malaise	0	2 (20%)	10 (100%)	7 (70%)
Myalgia	0	2 (20%)	5 (50%)	3 (30%)
Loss of taste or odor	0	0	6 (60%)	4 (40%)
Shortness of breathing	0	2 (20%)	7 (70%)	10 (100%)
Constant pain or pressure in chest	0	3 (30%)	5 (50%)	5 (50%)
SPO_2 at rest ≤94%	0	0	0	6 (60%)
Respiratory failure requiring intensive care unit treatment	0	0	0	10 (100%)
Diabetes	0	0	0	4 (40%)
Hypertension	0	0	1 (10%)	6 (60%)

Abbreviations: COVID-19, coronavirus disease 2019; HC, healthy control; MIC, mild COVID-19 cases; MOC, moderate COVID-19 cases; SC; severe COVID-19 cases.

2.2 | Quantification of SARS-CoV-2 viral load

We performed detection of SARS-CoV-2 at the Genome Center, Jashore University of Science and Technology, Jashore, Bangladesh. We extracted viral RNA from a 10 µl clinical specimen (nasal swab and throat swab) within 12 h of specimen collection using QuickExtract™ RNA extraction kit (Lucigen) following the manufacturer's instructions. Then, 10 µl of each viral RNA extract was amplified by one-step real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using a Novel Coronavirus Nucleic Acid Diagnostic Kit (Sansure Biotech Inc.). The kit detected *n*- and *orf1b* gene of SARS-CoV-2 and human *RNase P* gene as an internal control. In this study, samples with Ct values for both n- and orf1b genes of ≤35.0 were considered positive, that of >35.0 retested, and of >40.0 were considered negative. We used equation $2^{-\Delta Ct}$ to estimate viral load, where $\Delta Ct = Ct_{\text{viral N gene}} - Ct_{RNaseP}$.[19] We have translated all the data as $\log_{10}(1 + 2^{-\Delta Ct})$.

2.3 | Estimating cytokine and chemokine expression levels by qRT-PCR

We collected approximately 4–5 ml of peripheral blood samples, within 24 h (Day 1) of laboratory confirmation of SARS-CoV-2 in nasopharyngeal swabs, from 30 COVID-19 symptomatic and 9 symptomatic patients. After 14 days, we collected blood samples from twenty patients with MIC (*n* = 10) and MOC (*n* = 10) symptoms from their residence while maintaining home quarantine. Three healthy volunteers (out of nine) tested SARS-CoV-2 positive during repeated sampling and were let off from the study. So we had six samples from healthy volunteers on Day 14. We could not collect blood samples from participants of the severe COVID-19 cases as they were either deceased after 14 days of initial sample collection or admitted into the ICU from whom we could not be allowed to collect the blood.

We extracted total messenger RNA (mRNA) from human leukocytes using 1 ml of whole blood from all study participants using the SV total RNA isolation system (Promega), as per the manufacturer's instruction. According to the manufacturer's instructions, we prepared 20.0 µl complementary DNA (cDNA) from 8.0 µl of total mRNAs using the GoScript™ Reverse transcription system (Promega). We diluted 20 µl cDNA into a final volume of 100.0 µl. We performed SYBRgreen intercalation qRT-PCR to detect the expression level of two proinflammatory cytokines (TNF-α and IL-6), two antiviral cytokines (IFN-γ and IL-12), four chemokines (MIP-1α, RANTES, IP-10, and MCP-1), and ACE2. We used the primer sequences to detect these cytokines and chemokines from an earlier study.[20] The qRT-PCR was performed in QuantStudio™ 3 (Applied Biosystems), using the following protocol: denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 10 s, annealing for 30 s, and

extension at 72°C for 30 s. We analyzed the melting curve to determine the assay's specificity (60–95°C, 0.05°C/s). Annealing temperature varied for different primer pairs (such as ACE2: 53°C; IL-6: 45°C; TNF-α: 60°C; IP-10: 45°C; IFN-γ: 48°C; MIP-1α: 58°C; RANTES: 48°C; MCP-1: 52°C; IL-12: 50.5°C; and β-actin: 60°C). We performed each experiment in triplicate and Ct values with less than 10% variance considered for analyses only. In this essay, the human β-actin gene worked as the internal control. We accessed mRNA expression by relative quantification and calculated fold expression change by the 2^{-ddCt} method.[21]

2.4 | Statistical analysis

The distribution of the fold expression change of mRNAs was skewed, so we log-transformed these data for further analyses. Results presented as median (interquartile range). Nonparametric tests such as the Mann–Whitney *U* test and the Kruskal–Wallis test with Dunn multiple comparisons were used to compare relative cytokine expression levels among the different groups where applicable. Spearman's rank correlation coefficient (r) analyzes the correlation between relative cytokine expression levels in total lymphocytes and the close viral load. Spearman rank correlation analyses the association between disease severity and cytokine expression levels. We performed statistical tests based on transformed log data using SPSS 24.0 for Windows (SPSS, Inc.) and GraphPad Prism 8.0 (GraphPad Software) to construct all the figures' logarithmic scales. For all tests, if the two-tailed *p* values were less than 0.05, the test results were considered significant.

3 | RESULT

3.1 | Demographic and clinical characteristics of studied participants

Table 1 lists the demographic characteristics and clinical aspects of 30 COVID-19 patients and 9 healthy volunteers. Mean (±*SD*) age of MIC, MOC, and SC groups were estimated as 37.30 (±4.814), 40.20 (±4.69), and 58.40 (±3.361) years, respectively. HC groups' mean age was 23.89 (±2.435) years. All MOC participants ($n = 10$) and SC ($n = 10$) suffered from shortness of breathing, but only two MIC reported such difficulties. All SC had respiratory failure and thus required ICU support. We recorded no death for mild and moderate groups but one death was reported during the study period in the SC group.

3.2 | Relative expression levels of cytokine and chemokine in studied participants

Figure 1 represented comparing different cytokine and chemokine expression levels on Days 1 and 14 among the study patients' groups. The ACE2 expressed higher ($p < 0.05$) in the MIC group than the HC group on Day 1. The relative expression level of IL-6 was numerically higher in all patients' groups than HC group, although this difference was nonsignificant ($p > 0.05$). For other tested cytokine or chemokine expression on Day 1, no significant differences ($p > 0.05$) was found among the four study groups.

On Day 14, the MIC group had significantly ($p < 0.05$) increased expressions of IL-12 and IFN-γ compared with the HC group. We also observed slightly increased expression of IP-10 and MCP-1 in the MIC group but were nonsignificant. On the other hand, the MOC group had significantly higher ($p < 0.05$) expressions of ACE2, IL-12, MCP-1, TNF-α, MIP-1α, IL-6, RANTES, IP-10, and IFN-γ on Day 14 compared to the HC group. Among the MIC and MOC groups, the latter group had a significantly higher expression ($p < 0.05$) of RANTES and ACE2 with a numerically nonsignificant increased IL-6 expression.

3.3 | Correlation between disease severity and cytokine and chemokine expression level

Spearman rank correlation analyses were performed to determine any correlations between disease severity and expression levels of cytokine and chemokine in COVID-19 patients, separately for both Days 1 and 14. Correlation coefficients (r) for all cytokine and chemokine (both Days 1 and 14) are shown in Figure 2A. Statistical analyses revealed that the relative expression levels of IL-6 ($r = 0.609$), TNF-α ($r = 0.741$), MIP-1α ($r = 0.607$), MCP-1 ($r = 0.486$), RANTES ($r = 0.623$), and ACE2 ($r = 0.845$) on Day 14 were positively correlated ($p < 0.05$) with disease severity (Figure 2B). However, no significant correlation between disease severity with any cytokine and chemokine was observed on Day 1. But expression levels of IL-12 ($r = 0.102$), TNF-α ($r = 0.110$), IFN-γ ($r = 0.166$), IL-6 ($r = 0.295$), IP-10 ($r = 0.210$), and MIP-1α ($r = 0.128$) were weakly positively correlated with severity on Day 1.

3.4 | Correlation between viral load and cytokine and chemokine expression level

Spearman rank correlation analyses were also done to determine any correlations between relative viral load and expression levels of cytokine and chemokine in COVID-19 patients. Correlation coefficients (r) for all cytokine and chemokine (both Days 1 and 14) are shown in Figure 3A. Expression levels of only TNF-α ($r = 0.4469$) had a nonsignificant positive correlation ($p > 0.05$) with relative viral load at the onset of disease. However, IL-6 ($r = -0.4182$), RANTES ($r = -0.2376$), MIP-1α ($r = -0.2078$), and MCP-1 ($r = -0.3111$) had a nonsignificant weak negative correlation with the relative viral load on Day 1. However, after 14 days, expression levels of RANTES ($r = 0.4860$) and ACE2 ($r = 0.6471$) had a significant positive correlation ($p < .05$) with the relative viral load (Figure 3B). Moreover, relative expression levels were nonsignificantly positively correlated with viral load for IL-12 ($r = 0.3516$), TNF-α ($r = 0.4615$), IL-6 ($r = 0.4895$), and MIP-1α ($r = 0.3538$).

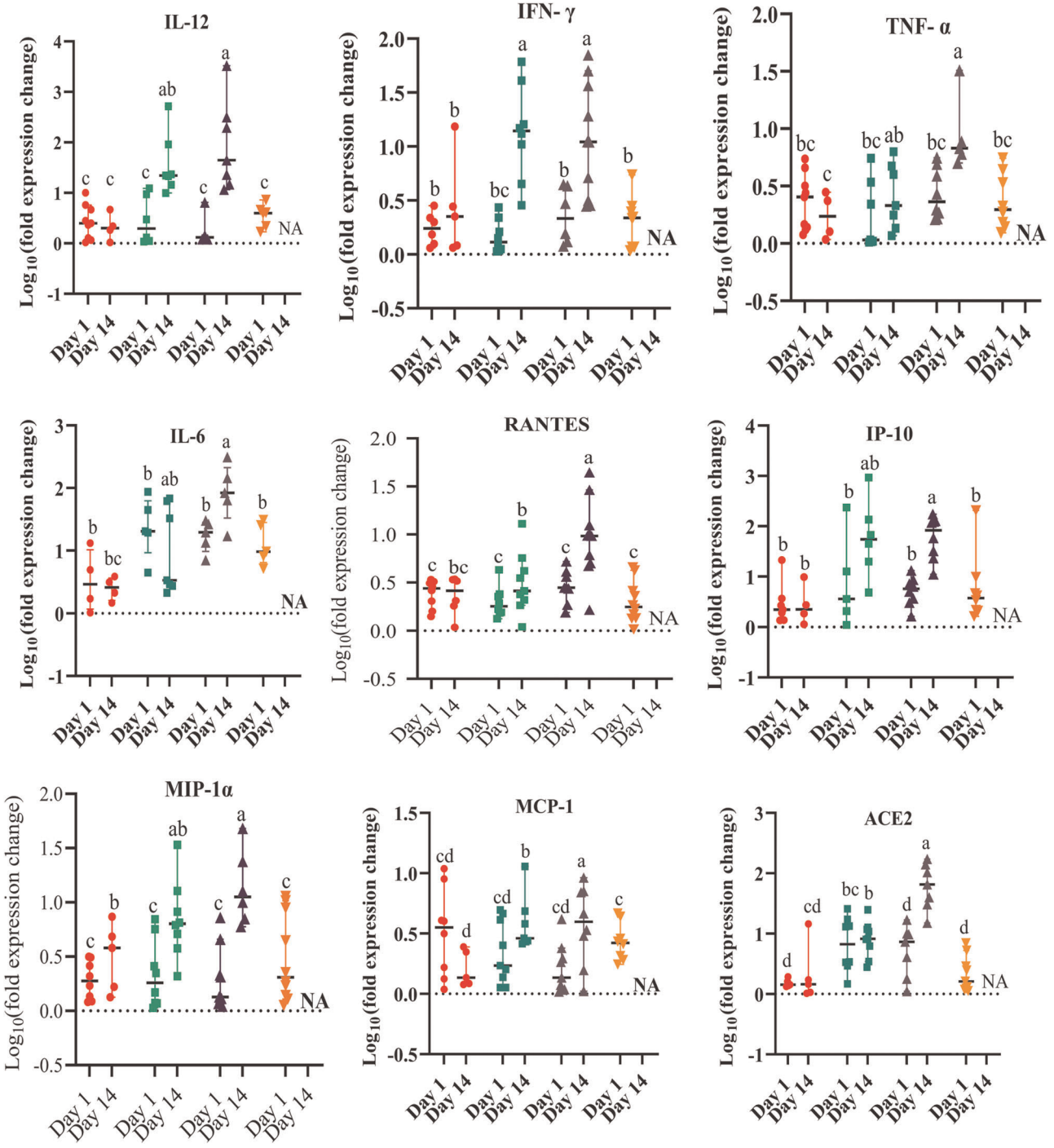

FIGURE 1 Relative expression levels of cytokine and chemokine in healthy control and COVID-19 infected symptomatic patients (mild COVID-19 cases, moderate COVID-19 cases, and severe COVID-19 cases). ● = Healthy Control; ■ = Mild COVID-19 cases; ▲ = Moderate COVID-19 cases; and ▼ = Severe COVID-19 cases. Each RT-PCR was done in triplicate and data that vary less than 10% were taken for analyses. Due to this, the number of samples varies for different cytokines and chemokine. Data are expressed as median with interquartile range (IQR). Separate analyses were performed for Days 1 and 14. For any cytokine, different letters (a, b, c, ...) indicate significant differences at $p < 0.05$. NA, not available (severe COVID-19 cases data on Day 14). ACE, angiotensin-converting enzyme; IL, interleukin; IFN, interferon; IP-10, interferon-inducible protein of 10 kDa; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF, tumor necrosis factor

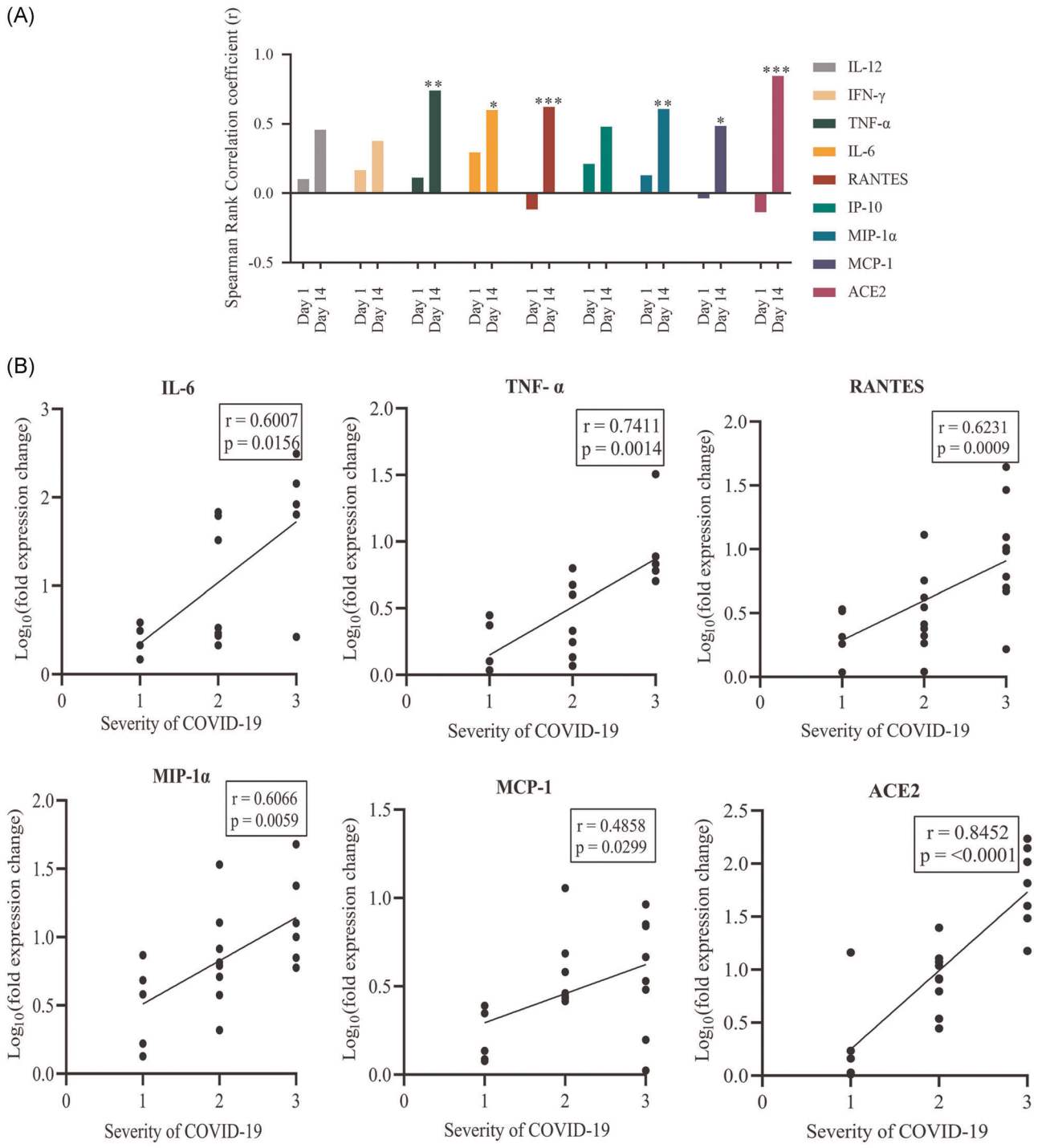

FIGURE 2 Correlation between disease severity and relative expression levels of cytokine and chemokines. (A) Spearman rank correlation analyses between disease severity and cytokine expression levels were done, and correlation coefficient (r) values of IL-12, IFN-γ, TNF-α, IL-6, RANTES, IP-10, MIP-1α, MCP-1, and ACE2 on both Days 1 and 14 are plotted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B). Significant correlation between disease severity and relative expression levels of IL-6, TNF-α, RANTES, MIP-1α, MCP-1, and ACE2 after 14 days are plotted. Here in the X-axis, 1 = healthy volunteer, 2 = mild COVID-19 cases, and 3 = moderate COVID-19 cases. COVID-19, coronavirus disease 2019; IL, interleukin; IFN, interferon; IP-10, interferon-inducible protein of 10 kDa; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF, tumor necrosis factor

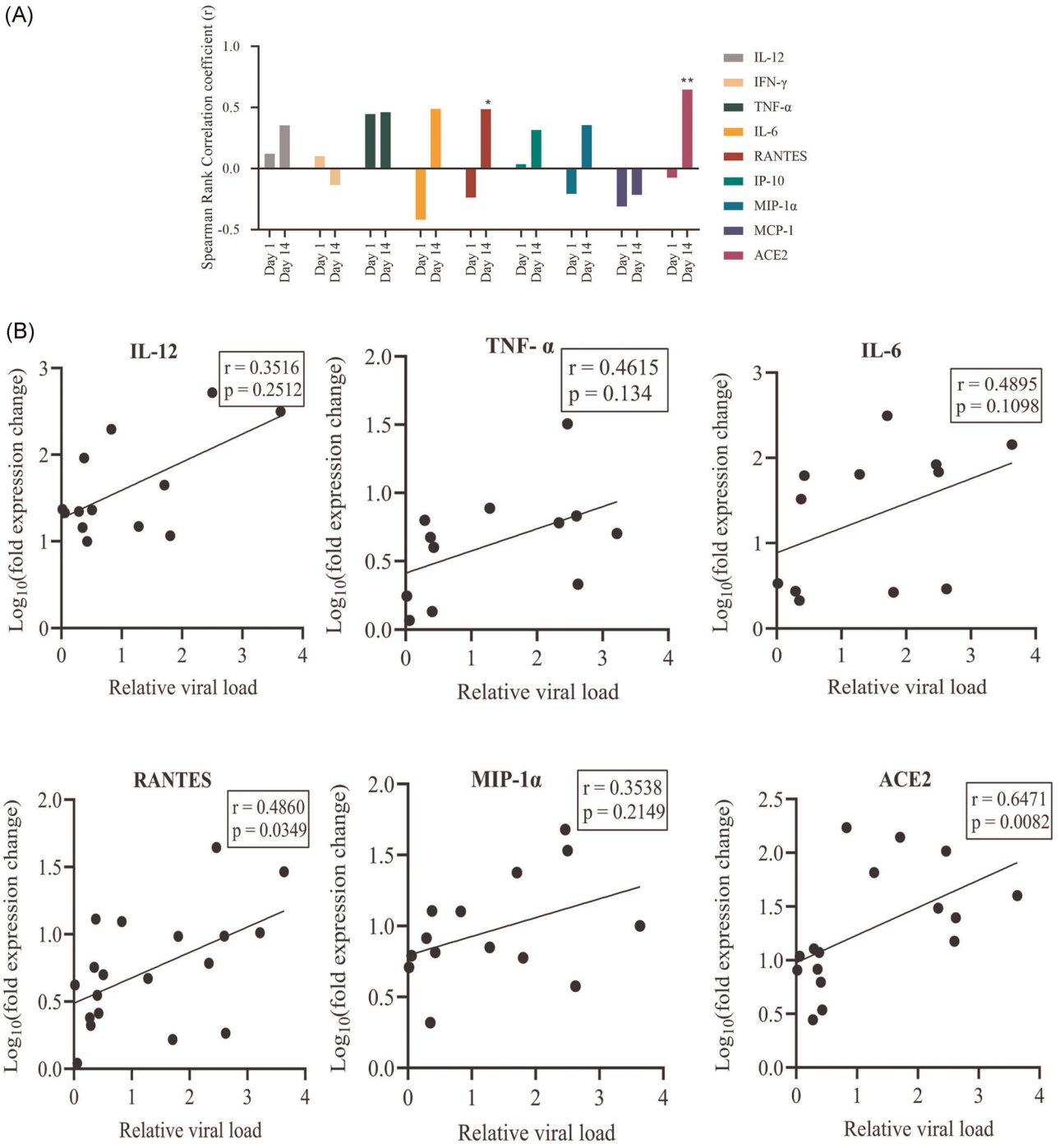

FIGURE 3 Correlation between relative viral load and relative expression levels of cytokine and chemokines in COVID-19 patients. (A). Spearman rank correlation analyses between relative viral load and cytokine expression levels were done, and correlation coefficient (r) values of IL-12, IFN-γ, TNF-α, IL-6, RANTES, IP-10, MIP-1α, MCP-1, and ACE2 on both Days 1 and 14 are plotted. *$p < 0.05$, **$p < 0.01$. (B). Significant correlations between relative viral load and expression levels of IL-12, TNF-α, IL-6, RANTES, MIP-1α, and ACE2 after 14 days are plotted. COVID-19, coronavirus disease 2019; IL, interleukin; IFN, interferon; IP-10, interferon-inducible protein of 10 kDa; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF, tumor necrosis factor

3.5 | Effect of gender on relative cytokine expression levels in COVID-19 patients

To explore any effect of sex on cytokine and chemokine expression levels, we analyzed cytokine's relative expression levels of males and females on both Days 1 and 14 (Figure 4). Irrespective of sex, all cytokine and chemokine were upregulated on Day 14 than on Day 1, except for IL-6 and TNF-α. For males, the IL-6 expression level was similar at both sampling times. TNF-α showed no significant difference in any comparison. The relative expression level of ACE2 and IL-12 were also identical in both gender on Day 1. But both ACE2 and IL-12 were upregulated ($p < 0.05$) in females on Day 14. Additionally, we did not observe a significant difference in the relative expression level of IFN-γ, RANTES, and IP-10 in males and females on both days. On the other hand, MCP-1 and MIP-1α were upregulated ($p < 0.05$) in females on Day 1. But we did not find any sex

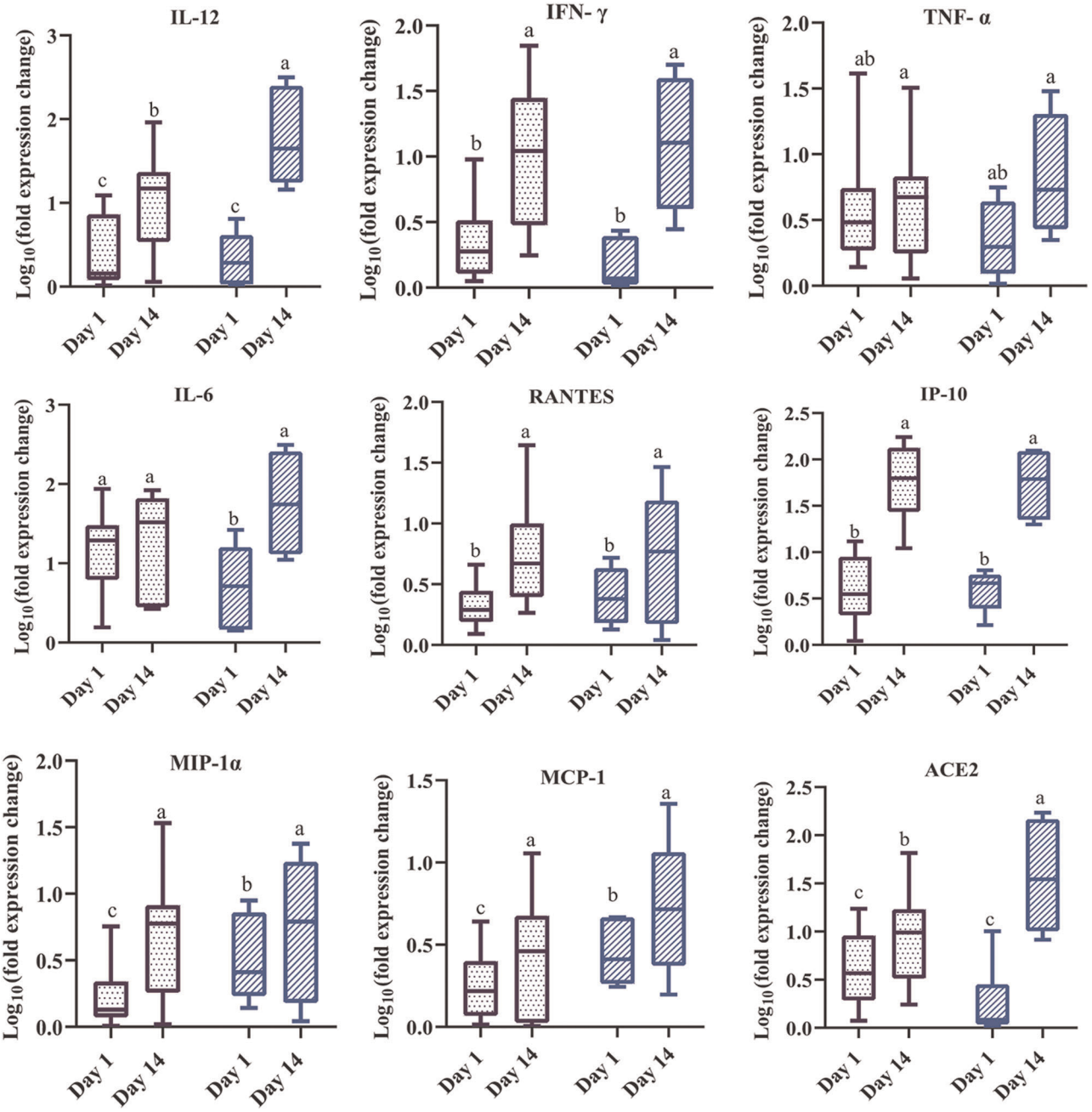

FIGURE 4 Relative expression levels of cytokine and chemokine in male and female COVID-19 patients. Here, ▭ = male and ▭ = female COVID-19 infected patients. Each RT-PCR was done in triplicate and data that vary less than 10% were taken for analyses. Due to this, the number of samples varies for different cytokines and chemokine. Data are expressed as median with interquartile range (IQR). Separate analyses were performed for Days 1 and 14. For any cytokine, different letters (a, b, c,). indicates significant differences ($p < 0.05$). COVID-19, coronavirus disease 2019; RT-PCR, real-time reverse-transcriptase polymerase chain reaction

discrimination for expression of MCP-1 and MIP-1α on Day 14. The relative expression level of IL-6 was higher ($p < 0.05$) in males on Day 1 but did not vary significantly ($p > 0.05$) with gender on Day 14.

4 | DISCUSSION

To our knowledge, this is the first study on cytokine/chemokine expression levels in COVID-19 patients in Bangladesh. We observed upregulation of ACE2 ($p < 0.05$) and IL-6 ($p > 0.05$) in MIC cases compared with HC on Day 1 (Figure 1). Many natural, common ways such as taking Vitamin C, Metformin, Vitamin B3, and Vitamin D can upregulate the ACE2 receptor.[22] Here in Bangladesh, doctors instructed COVID-19 patients to take Vitamin C and Vitamin B3 during their illnesses, increasing ACE2 expression. Although the virus uses ACE2 to enter into its host cell, upregulation of the cellular ACE2 receptor would likely be antiinflammatory and might have contributed to reduced mortality in COVID-19 patients.[22] Proinflammatory cytokines such as IL-6, TNF-α, while upregulated, trigger cytokine storms and ultimately lead to tissue damage and organ failure.[23] Although we observed a slightly increased expression of IL-6, we did not observe TNF-α upregulation at the onset of the disease (Figure 1). Like our study, normal TNF-α levels were reported in COVID-19 patients by Wan et al.[24] However, elevated TNF-α levels in COVID-19 patients' sera were also reported recently.[4,25] Recently some studies reported IL-6 as severity predictors of COVID-19.[26,27] In a meta-analysis including 1426 patients, complicated COVID-19 patients had higher IL-6 expression levels (>3 times) compared with other uncomplicated patients.[28] In our study, COVID-19 patients had no higher expression of either IFN-γ or IL-12 than the HC group, which is indicative of the absence of elevated antiviral immune response at the onset of disease symptoms. Surprisingly, none of the cytokines and chemokines of ICU patients from the SC group shows significant differences compared with others. Zhang et al.[29] found that ICU patients in China had higher levels of IL-2R, IL-6, IL8, IL10, and TNF-α compared with reference ranges of the normal man. In other studies from Finland, ICU patients had higher levels of IL-6, C-reactive protein, and procalcitonin compared with non-ICU patients.[30] Keddie et al.[31] also noted similar findings. The authors demonstrated that CRP, IL-6, IL-10, and lactate dehydrogenase significantly correlated with ARDS and respiratory support. In another study, Lev et al.[32] also noted that IP-10 is associated with ICU patients and act as potential biomarkers. The combination of IL-6 × IL-10 serum levels has shown a predictor of ICU patients.[33] The less expression of most cytokines and chemokines might be probable reasons for less mortality rate (1.5%) of severe COVID-19 patients in Bangladesh.

Moreover, in our study, the relative expression of cytokine and chemokines differs between 2 weeks. After 14 days, significant upregulation of both antiviral cytokines (IFN-γ and IL-12) occurs in MIC and MOC. Although the antiviral immune response was not present in COVID-19 patients at the onset of disease, the antiviral immune response accelerated in those patients after 14 days. This upregulation indicates enhanced host immunity to eliminate viral pathogens. Similar IL-12 upregulation was reported in both SARS-CoV[34] and SARS-CoV-2 infected patients.[4,35] IL-12 has distinctive characteristics and has a vital role in positive and negative feedback.[36] On Day 14, proinflammatory cytokine IL-6 was significantly higher in MOC but not in MIC cases. However another proinflammatory cytokine, TNF-α, was significantly upregulated in all COVID-19 patients than HC. These findings suggest that proinflammatory cytokines had direct effects on the severity and complexity of COVID-19 disease. The antiinflammatory cytokines levels got upregulated compared to proinflammatory cytokines after 14 days. So, it might result in rapid recovery and reduced mortality in COVID-19 patients. On Day 14, RANTES and ACE2 also upregulated with the severity of COVID-19 disease. RANTES, a well-established chemo-attractant, links innate and adaptive immune responses. RANTES decreased significantly in SARS-CoV-2 infected persons in a recent study, where MIP-1α level was higher in ICU patients.[4]

We observed that the relative expression of some cytokines and chemokines varied among COVID-19 cases. Based on the samples' data on Day 1, we could not establish any significant correlation between disease severity and relative cytokine expressions. But after 14 days, estimated expression levels of IL-6, TNF-α, MIP-1α, MCP-1, RANTES, and ACE2 correlated with and differed significantly with COVID-19 severity groups (Figure 2). Several reviews have highlighted the importance of cytokines and chemokines and demonstrated IL-1, IL-6, IL-8, IL-10, IL-2R, IL-18, MCP-1, and TNF-α levels for disease severity.[8,37,38] The cytokines and chemokines regulate the CD4 + T, CD8 + T, monocytes, neutrophils, and macrophages. However, we observed differences in the expressions between studies. Recent work by Qin et al.[25] is also in accord with our observation, where COVID-19 severity was associated with increased levels of IL-6, IP-10, MCP-1, and MIP-1α. Although Costela-Ruiz et al.[7] reported elevated TNF-α and its association with severity in COVID-19 patients, we did not observe such differences. In another study, even the samples from nasopharyngeal swabs revealed the higher expression of IFN-γ and lowered expression of TGF-β1 and RANTES in symptomatic patients compared with negative patients.[39] But our studies demonstrated the higher expression of RANTES for MIC and MOC compared with HC. At the onset of the disease, the relative viral load had no significant correlation with cytokines and chemokines' relative expression (Figure 3A). However, initial viral load positively correlated with expression levels of IL-12, TNF-α, IL-6, RANTES, MIP-1α, and ACE-2, after 14 days. A higher level of IL-6 and RANTES decreased CD8 + T cell and virus.[40] The results from our study agreed with that study.

Although males tend to get more severe forms of COVID-19 diseases, we observed significant gender variation only for IL-6, MIP-1α, and MCP-1 on Day 1 (Figure 4). Our observations of male patients who had more upregulated IL-6 than female patients at the beginning of the disease agreed with China's similar findings. The study reported a higher level of serum IL-6 male compared to female. Increased expression of IL-6 on Day 1 might result in severe COVID-19 diseases in males. Interestingly both

males and females had similar expression levels of proinflammatory cytokines (TNF-α, IL-6) after 14 days. On Day 14, female patients had significant upregulation of ACE2 and IL-12. In response to vaccinations, women induced a robust immune response.[41] In a previous study, estrogens lead to cytokine upregulation in mice treated with coronavirus. Estrogens had a protective role by suppressing the immune response's escalation phase.[42] As women are expressing more ACE2, therefore, less severity and death rate has been attributed in Bangladesh. A recent study showed that, compared with male patients, severe female patients had a greater IgG production level in weeks of the COVID-19 disease onset.[6] The protective role of IgG in female COVID-19 patients might be associated with the upregulation of IL-12.

There were a few limitations due to the small sample size in our study. We could not collect repeated blood samples after 14 days from severe patients admitted to ICU. We also estimated the relative expression levels of cytokines and chemokines instead of serum cytokine levels. We could not collect samples from lungs and could not also target the cytokine expression in alveolar leukocytes. Modulators of cytokine expression released from the injured lungs may also spill and diffuse from the source to enter the systemic circulation, which might also contribute to significant upregulation of cytokine expression in the peripheral leukocytes and hence contributed to COVID-19 disease manifestations. However, live vaccines such as the BCG are known to induce trained immunity (enhanced innate immune response to subsequent infections). COVID-19 cases were reported as being lower in countries with universal BCG vaccination programs (such as Bangladesh, Nepal, Bhutan, Japan) compared to those without the programs (such as the United States, Spain, Canada, Italy).[43] We hypothesized that BCG vaccination might induce an immune response and reduce SARS-CoV-2 viremia and the severity of COVID-19 infections,[44] and cause rapid recovery of SARS-CoV-2 infection in the country.

5 | CONCLUSION

This study describes cytokine and chemokine expression among COVID-19 patients with different disease severity in a developing country, Bangladesh. We found that IL-6 could be targeted for anticytokine therapy here in Bangladesh. Although we could not prove any direct effect of BCG vaccinations on reduced severity of COVID-19, our data provide cytokine expression levels in BCG vaccinated COVID-19 patients. Knowledge of the underlying mechanisms of differential expressions and the associations of these cytokines with disease severity could help to target the choice for therapies.

ACKNOWLEDGMENTS

This work was supported by a research grant (UGC/JUST/2020-21/20) from the University Grant Commission, Bangladesh, through the Jashore University of Science and Technology (JUST), Jashore. We thank all volunteers and COVID-19 patients who actively participated in this study. We also acknowledge the efforts of all doctors, nurses, healthcare workers in fighting SARS-CoV-2.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Shireen Nigar conceived the idea; SM Tanjil Shah, Ali Ahsan Setu, Sourav Datta Dip, Habiba Ibnat, and Selina Akter performed the experiment; M Touhidul Islam collected and performed initial processing of samples; S. M. Tanjil Shah and Iqbal Kabir Jahid performed statistical analysis; Shireen Nigar and S. M. Tanjil Shah wrote the draft manuscript; Selina Akter, Iqbal Kabir Jahid, and M Anwar Hossain finalized the manuscript; Shireen Nigar, Iqbal Kabir Jahid, and Md Anwar Hossain supervised the whole work and submitted the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/jmv.27112

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

SM Tanjil Shah https://orcid.org/0000-0001-8010-3365
Selina Akter http://orcid.org/0000-0002-6219-4328
Iqbal Kabir Jahid https://orcid.org/0000-0003-0717-0806
M. Anwar Hossain http://orcid.org/0000-0001-9777-0332

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. 2020;20(5):533-534.
2. Escobar LE, Molina-Cruz A, Barillas-Mury C. BCG vaccine protection from severe coronavirus disease 2019 (COVID-19). *Proc Natl Acad Sci*. 2020;117(30):17720-17726.
3. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. 2020;395(10229):1033-1034.
4. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395(10223):497-506.
5. Blanco-Melo D, Nilsson-Payant BE, Liu W-C, et al. Imbalanced host response to SARS-CoV-2 drives development of COVID-19. *Cell*. 2020;181(5):1036-1045.e9.
6. Zeng F, Dai C, Cai P, et al. A comparison study of SARS-CoV-2 IgG antibody between male and female COVID-19 patients: a possible reason underlying different outcome between sex. *J Med Virol*. 2020;92:2050-2054.
7. Costela-Ruiz VJ, Illescas-Montes R, Puerta-Puerta JM, Ruiz C, Melguizo-Rodríguez L. SARS-CoV-2 infection: the role of cytokines in COVID-19 disease. *Cytokine Growth Factor Rev*. 2020;54:62-75.
8. Mulchandani R, Lyngdoh T, Kakkar AK. Deciphering the COVID-19 cytokine storm: systematic review and meta-analysis. *Eur J Clin Invest*. 2021;51(1):e13429.

9. Mauvais-Jarvis F. Aging, male sex, obesity, and metabolic inflammation create the perfect storm for COVID-19. *Diabetes*. 2020; 69(9):1857-1863.
10. Turner AJ, Tipnis SR, Guy JL, Rice GI, Hooper NM. ACEH/ACE2 is a novel mammalian metallocarboxypeptidase and a homologue of angiotensin-converting enzyme insensitive to ACE inhibitors. *Can J Physiol Pharmacol*. 2002;80(4):346-353.
11. Li W, Moore MJ, Vasilieva N, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. 2003; 426(6965):450-454.
12. Gopallawa I, Uhal BD. Angiotensin-(1–7)/mas inhibits apoptosis in alveolar epithelial cells through upregulation of MAP kinase phosphatase-2. *Am J Physiol Lung Cell Mol Physiol*. 2016;310(3): L240-L248.
13. Jain R, Ramaswamy S, Harilal D, et al. Host transcriptomic profiling of COVID-19 patients with mild, moderate, and severe clinical outcomes. *Comput Struct Biotechnol J*. 2021;19:153-160.
14. Wei X, Xiao Y-T, Wang J, et al. Sex differences in severity and mortality among patients with COVID-19: evidence from pooled literature analysis and insights from integrated bioinformatic analysis. *arXiv preprint arXiv*. 2003:13547.
15. Yang X, Yu Y, Xu J, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet. *Respir Med*. 2020;8(5):475-481.
16. Abate BB, Kassie AM, Kassaw MW, Aragie TG, Masresha SA. Sex difference in coronavirus disease (COVID-19): a systematic review and meta-analysis. *BMJ Open*. 2020;10(10):e040129.
17. Liu T, Balzano-Nogueira L, Lleo A, Conesa A. Transcriptional differences for COVID-19 Disease Map genes between males and females indicate a different basal immunophenotype relevant to the disease. *Genes*. 2020;11(12):1447.
18. Jin Y-H, Cai L, Cheng Z-S, et al. A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). *Mil Med Res*. 2020; 7(1):4.
19. Chi Y, Ge Y, Wu B, et al. Serum cytokine and chemokine profile in relation to the severity of coronavirus disease 2019 in China. *J Infect Dis*. 2020;222(5):746-754.
20. Law HK, Cheung CY, Ng HY, et al. Chemokine up-regulation in sars-coronavirus–infected, monocyte-derived human dendritic cells. *Blood*. 2005;106(7):2366-2374.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*. 2001;25(4):402-408.
22. McLachlan CS. The angiotensin-converting enzyme 2 (ACE2) receptor in the prevention and treatment of COVID-19 are distinctly different paradigms. *Clin Hypertens*. 2020;26(1):1-3.
23. Tanaka T, Narazaki M, Kishimoto T. Immunotherapeutic implications of IL-6 blockade for cytokine storm. *Immunotherapy*. 2016;8(8): 959-970.
24. Wan S, Yi Q, Fan S, et al. Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus pneumonia (NCP). *MedRxiv*. 2020. https://doi.org/10.1101/2020.02.10.20021832
25. 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(15):762-768.
26. Han H, Ma Q, Li C, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. *Emerg Microbes Infect*. 2020;9(1):1123-1130.
27. Liu Y, Zhang C, Huang F, et al. Elevated plasma level of selective cytokines in COVID-19 patients reflect viral load and lung injury. *Natl Sci Rev*. 2020;7(6):1003-1011.
28. Aziz M, Fatima R, Assaly R. Elevated interleukin-6 and severe COVID-19: a meta-analysis. *J Med Virol*. 2020;92:2283-2285.
29. Zhang D, Zhou X, Yan S, et al. Correlation between cytokines and coagulation-related parameters in patients with coronavirus disease 2019 admitted to ICU. *Clin Chim Acta*. 2020;510:47-53.
30. Broman N, Rantasärkkä K, Feuth T, et al. IL-6 and other biomarkers as predictors of severity in COVID-19. *Ann Med*. 2020:1-5.
31. Keddie S, Ziff O, Chou M, et al. Laboratory biomarkers associated with COVID-19 severity and management. *Clin Immunol*. 2020;221:108614.
32. Lev S, Gottesman T, Sahaf Levin G, et al. Observational cohort study of IP-10's potential as a biomarker to aid in inflammation regulation within a clinical decision support protocol for patients with severe COVID-19. *PLoS One*. 2021;16(1):e0245296.
33. Nagant C, Ponthieux F, Smet J, et al. A score combining early detection of cytokines accurately predicts COVID-19 severity and intensive care unit transfer. *Int J Infect Dis*. 2020;101:342-345.
34. Wong CK, Lam CW, Wu AK, et al. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clin Exp Immunol*. 2004;136(1):95-103.
35. Chen N, Zhou M, Dong X, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. 2020;395(10223):507-513.
36. Jones LL, Vignali DA. Molecular interactions within the IL-6/IL-12 cytokine/receptor superfamily. *Immunol Res*. 2011;51(1):5-14.
37. Noroozi R, Branicki W, Pyrc K, et al. Altered cytokine levels and immune responses in patients with SARS-CoV-2 infection and related conditions. *Cytokine*. 2020;133:155143.
38. Bindoli S, Felicetti M, Sfriso P, Doria A. The amount of cytokine-release defines different shades of Sars-Cov2 infection. *Exp Biol Med*. 2020;245(11):970-976.
39. Montalvo Villalba MC, Valdés Ramírez O, Muné Jiménez M, et al. Interferon gamma, TGF-β1 and RANTES expression in upper airway samples from SARS-CoV-2 infected patients. *Clin Immunol*. 2020; 220:108576.
40. Patterson BK, Seethamraju H, Dhody K, et al. Disruption of the CCL5/RANTES-CCR5 pathway restores immune homeostasis and reduces plasma viral load in critical COVID-19. *MedRxiv*. 2020. https://doi.org/10.1101/2020.05.02.20084673
41. Lotter H, Altfeld M. Sex differences in immunity. *Seminars in Immunopathology*. Springer; 2019.
42. Taghizadeh-Hesary F, Akbari H. The powerful immune system against powerful COVID-19: a hypothesis. *Med Hypotheses*. 2020; 140:109762.
43. Sharma AR, Batra G, Kumar M, et al. BCG as a game-changer to prevent the infection and severity of COVID-19 pandemic. *Allergol Immunopathol*. 2020;48:507-517.
44. Curtis N, Sparrow A, Ghebreyesus TA, Netea MG. Considering BCG vaccination to reduce the impact of COVID-19. *Lancet*. 2020; 395(10236):1545-1546.

How to cite this article: Nigar S, Tanjil Shah SM, Setu MAA, et al. Relative expression of proinflammatory molecules in COVID-19 patients who manifested disease severities. *J Med Virol*. 2021;93:5805-5815. https://doi.org/10.1002/jmv.27112