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Chemokine CCL2 and its receptor CCR2 in different age groups of patients with COVID-19

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Abstract

Background Despite the development of various antiviral drugs, most of them are not effective in the treatment of coronavirus disease 2019 (COVID-19) as a hyperinflammatory disorder. Chemokine (C-C motif) ligand 2 (CCL2) is one of the critical CC chemokines involved in the pathogenesis and severity of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. This study aimed to investigate the expression of CCL2 and CC chemokine receptor 2 (CCR2) in COVID-19 patients.

Methods Peripheral blood samples were collected from 60 confirmed COVID-19 patients and 60 age-matched healthy subjects. The ages of the subjects were categorized as follows: up to 20 years, 20 to 40 years, 40 to 60 years, and more than 60 years. CCL2 serum levels were measured using the enzyme-linked immunosorbent assay (ELISA). *CCR2* gene expression in peripheral blood mononuclear cells (PBMCs) was measured employing real-time polymerase chain reaction (PCR).

Results In all age groups, CCL2 serum levels were significantly elevated in patients compared to healthy controls ($P < 0.0001$). CCL2 levels were higher in severe patients than in moderate patients. Moreover, *CCR2* expression by PBMCs was higher in patients compared to control subjects. However, a significant difference between patients and controls over 60 years of age was identified ($P = 0.0353$). There was no significant difference in *CCR2* expression between moderate and severe COVID-19 patients.

Conclusions Taken together, the findings demonstrate that CCL2 and *CCR2* are upregulated in COVID-19 patients at protein and mRNA levels, respectively. Therefore, the CCL2/CCR2 axis may be a potential therapeutic target in order to improve patient outcomes.

Keywords CCL2, CCR2, COVID-19, SARS-CoV-2 infection, Chemokine

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Background

Coronavirus disease 2019 (COVID-19) was first observed in Wuhan City, Hubei Province, China, in December 2019. Then, the outbreak of COVID-19 rapidly spread to other provinces in China and Asian countries, including Japan, Korea, and Thailand. Due to international transmission, the disease was discovered in different continents, such as Europe and North America [1, 2]. Due to the rapid spread and pandemic potential, COVID-19 has turned into one of the most severe public health problems in recent years [3]. Over two years after the beginning of COVID-19, up to now, there are more than 704,753,890 confirmed cases and more than 7,010,681 confirmed deaths globally (https://www.worldometers.info/coronavirus/). The causative agent responsible for COVID-19 infection is a novel coronavirus (CoV) designated as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [4, 5].

SARS-CoV-2 binds to the host cell receptor angiotensin-converting enzyme 2 (ACE2). In addition to binding to ACE-2, the transmembrane serine protease 2 (TMPRSS2) is needed for the priming of the viral spike (S) protein [6]. Following SARS-CoV-2 infection, the virus replicates and activates immune effector cells resulting in the release of many pro-inflammatory cytokines and chemokines, which is called cytokine storm. The cytokine storm leads to acute respiratory distress syndrome (ARDS) that is the major cause of mortality in patients with COVID-19 [7, 8]. ARDS is observed in some patients and could result in decreased blood oxygen saturation and life-threatening hypoxemia [9].

The cytokine storm is marked by the unrestrained release of pro-inflammatory cytokines and chemokines, resulting in systemic hyperinflammation. In COVID-19 patients, the cytokine storm is initiated by an exaggerated immunological response to SARS-CoV-2 infection, leading to the recruitment and activation of immune cells, including macrophages, neutrophils, and T cells. Essential cytokines implicated in this process comprise interleukins (e.g., IL-1β, IL-6, IL-8), tumor necrosis factor-alpha (TNF-α), and chemokines such as CCL2, CXCL10, and CCL3. These chemicals enhance the inflammatory response by facilitating additional recruitment of immune cells to the infection site, especially in the lungs. Excessive infiltration of immune cells and cytokine production in the lungs result in significant tissue damage and respiratory failure. Uncontrolled systemic inflammation may also impact other organs, leading to multi-organ failure in severe instances [10–13]. Although cytokines help the immune system against infections in normal conditions, they have potential harmful effects on COVID-19 course. Therefore, targeting cytokines (e.g., IL-6) and cytokine-like molecules (e.g., high-mobility group box 1 (HMGB-1)) can be attractive therapeutic option to alleviate the cytokine storm and reduce COVID-19 mortality [14, 15]. Additionally, it has been suggested that inflammatory cytokines polymorphisms may be used to identify the therapeutic response to COVID-19-induced ARDS [16].

Chemokines are a family of small proteins that have a crucial role in leukocyte recruitment to the site of infection during inflammatory responses and other biological phenomena [17–19]. These chemotactic cytokines contribute to the fight against viral infections by recruitment of innate and adaptive immune cells and the production of antiviral mediators [20]. Several studies have reported upregulation of chemokines such as CXCL8, CXCL10, CCL2, and CCL3 in COVID-19 patients [21, 22]. Chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) acts as a chemoattractant for various immune cells such as monocytes, T cells, natural killer cells, and dendritic cells. CCL2 exerts its functions by binding to its receptor CC chemokine receptor 2 (CCR2). The CCL2/CCR2 axis is associated with the pathogenesis of inflammatory diseases such as atherosclerosis, rheumatoid arthritis (RA), multiple sclerosis, asthma, and diabetic nephropathy [23–28].

Given the crucial role of the CCL2/CCR2 axis in regulating monocyte/macrophage trafficking during infection and inflammation, this study was designed to examine CCL2 serum levels and CCR2 gene expression in peripheral blood mononuclear cells (PBMCs) from COVID-19 patients across different age groups. Additionally, CCL2 levels and CCR2 expression were analyzed concerning patients' gender and disease severity.

Methods

Subjects

In this case-control study, patients with SARS-CoV-2 infection ($n=60$) were recruited from Ali-Ibn Abi-Talib Hospital in Rafsanjan between July and December 2021. During this period, the predominant SARS-CoV-2 strain was the Delta variant, which significantly impacts immune responses and chemokine levels. COVID-19 patients were diagnosed with clinical symptoms and confirmed by real-time polymerase chain reaction (RT-PCR) on nasopharyngeal swabs. All patients were divided into four age groups: group 1 (0–19 years), group 2 (20–40 years), group 3 (40–60 years), and group 4 (˃ 60 years). None of the subjects in the patient and control groups had received a COVID-19 vaccine. Patients with a history of inflammatory diseases, autoimmune diseases, chronic lung diseases, cancer, patients with co-infection by other pathogens, and patients on immunosuppressive therapies were excluded from the study. The healthy donors included in this study had not a history of COVID-19 infection. In this study, we also divided the confirmed COVID-19 patients into two groups (moderate and

Table 1 Demographic and laboratory parameters of controls and patients in different age groups

Age of groups (years)		Subjects	Age (years)	Sex (m/f)	WBC (/μL)	CRP (mg/mL)	LDH (IU/L)
Under 20		Control $n=15$	8.53 ± 6.25	4/11	6249.25 ± 1562.1	6.32 ± 2.46	198.24 ± 41.53
		Patients $n=15$	6.87 ± 4.34	6/9	7028.34 ± 2761.41	26.27 ± 3.19	652.56 ± 271.14
	P value		0.45	-	0.39	< 0.0001*	< 0.0001*
20–40		Control $n=15$	32.54 ± 5.42	5/10	6359.15 ± 1658.94	6.13 ± 2.45	182.35 ± 46.71
		Patients $n=15$	31.55 ± 4.62	8/7	9242.56 ± 5686.2	43.21 ± 18.78	704.43 ± 285.88
	P value		0.82	-	0.07	< 0.0001*	< 0.0001*
40–60		Control $n=15$	46.12 ± 7.11	5/10	5852.36 ± 1283.4	4.72 ± 1.35	248.62 ± 72.91
		Patients $n=15$	46.98 ± 6.47	7/8	6692.27 ± 2135.74	42.47 ± 12.63	793.33 ± 318.64
	P value		0.75	-	0.82	< 0.0001*	< 0.0001*
Over 60		Control $n=15$	70.25 ± 6.68	9/6	6147.2 ± 1554.16	5.84 ± 1.61	252.63 ± 64.15
		Patients $n=15$	71.12 ± 7.34	11/4	7758.29 ± 3934.52	35.56 ± 12.74	776.43 ± 255.36
	P value		0.68	-	0.14	< 0.0001*	< 0.0001*

Table 2 Clinical data of patients in different age groups

Age of groups (years)	< 20	20–40	40–60	> 60	*P* value
Oxygen saturation (SpO_2) (%)	0.92 ± 0.05	0.92 ± 0.04	0.90 ± 0.02	0.88 ± 0.05	0.001*
Severity (moderate/severe)	13/2	12/3	7/8	3/12	-
Body temperature (C°)	36.74 ± 0.56	36.51 ± 0.62	37.68 ± 0.52	37.56 ± 0.57	0.11
Respiratory rate (breaths/minute)	17.65 ± 3.22	18.55 ± 3.18	18.69 ± 4.13	20.65 ± 4.25	0.143
Heart rate (beats/minute)	105.65 ± 12.42	100.32 ± 12.53	98.71 ± 12.46	99.50 ± 12.32	0.3
Systolic BP (mmHg)	12.45 ± 1.33	12.56 ± 1.25	12.43 ± 2.18	13.24 ± 2.62	0.6
Diastolic BP (mmHg)	7.44 ± 0.63	7.19 ± 1.35	8.1 ± 1.21	8.32 ± 1.52	0.7
HCO_3 (mEq/L)	19.37 ± 2.75	23.81 ± 2.25	25.14 ± 3.56	20.62 ± 3.57	0.005 *
pCO_2 (mmHg)	29.25 ± 4.36	33.54 ± 6.34	33.43 ± 7.28	30.54 ± 7.58	0.14

severe) according to the diagnosis and treatment protocol for novel coronavirus pneumonia (Trial Version 7) released by the National Health Commission of China [29]. Moderate cases show fever and respiratory symptoms with radiological findings of pneumonia. Severe cases meet one of the following criteria: (1) respiratory distress (≥30 breaths/min); (2) oxygen saturation ≤ 93% at rest; (3) arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ≤ 300mmHg. In addition to patients, 60 healthy individuals with no COVID-19 symptoms were selected as healthy controls. Demographic, clinical, and laboratory data were summarized in Tables 1 and 2. All participants in the study completed a written informed consent form before enrollment into this study. The research protocol was approved by the Ethics Committee at Rafsanjan University of Medical Sciences.

Chemokine assay

CCL2 serum levels were measured using a human CCL2 DuoSet ELISA kit (R&D Systems, Catalog No: DY279) in serum samples from patients and healthy controls. The ELISA assay was performed according to the manufacturer's instructions. Briefly, 96-well plates were coated overnight with diluted mouse anti-human CCL2 capture antibody (100 μL/well). After washing, plates were blocked by adding 300 μL of Reagent Diluent to each well and incubating at room temperature for one hour. Samples were added to wells (100 μL/well), and plates were incubated at room temperature for two hours. Then, a biotinylated goat anti-human CCL2 detection antibody, diluted in Reagent Diluent, was added (100 μL) to each well and incubated at room temperature for two hours. The next step was carried out by adding the working dilution of Streptavidin-HRP to each well. After washing, the working dilution of Streptavidin-HRP was added (100 μL) to each well and incubated at room temperature for 20 min. Substrate solution was added (100 μL) to each well, and plates were again incubated at room temperature for 20 min. In the final step, a stop solution was added (50 μL) to each well, and the optical density of each well was read using a microplate reader at 450 nm

with wavelength correction set to 540–570 nm. Each standard or sample was assayed in duplicate.

RNA extraction, cDNA synthesis, and real-time PCR (RT-PCR)

The peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples of patients and healthy controls using Ficoll-Paque PREMIUM (GE Healthcare, USA). Total RNA was extracted using an RNA extraction kit (KPG, Kerman, Iran) according to the manufacturer's instructions. The integrity, quality, and quantity of isolated RNA were determined by agarose gel electrophoresis and spectrophotometry. Then, complementary DNA (cDNA) synthesis was conducted using a cDNA synthesis kit (KPG, Kerman, Iran) according to the manufacturer's instructions. The reaction was incubated at 42 °C for 60 min (cDNA synthesis) and terminated by heating at 90 °C for 5 min. In order to evaluate the expression of *CCR2* gene, real-time PCR was performed using qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems, UK) on a Rotor-Gene Q 2plex System (Qiagen, Germany). The thermal cycling program was as follows: 2 min at 95 °C, followed by 40 cycles of denaturation (5 s at 95 °C) and annealing/extension (30 s at 60 °C). A housekeeping gene (β-actin) was used as an internal control to normalize the expression of the target gene. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used for real-time PCR are shown in Table 3.

Statistical analysis

All statistical analysis was carried out using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Independent sample t-test, Mann-Whitney U, and ANOVA/Tukey's test were used to evaluate differences among groups. The correlation matrix test was performed to assess the association between variables. Data are shown as mean ± standard error of the mean (SEM). All P values < 0.05 were considered significant.

Results

Serum levels of CCL2 in COVID-19 patients

To evaluate CCL2 serum levels in COVID-19, peripheral blood samples were collected from healthy individuals ($n=60$) and patients who were PCR-positive for COVID-19 ($n=60$). Our results demonstrated that the serum concentrations of CCL2 were elevated in confirmed SARS-CoV-2-infected patients compared to healthy controls (Fig. 1). In all age groups (0–19 years, 20–40 years, 40–60 years, and ˃ 60 years), statistical analysis of data showed that there was a significant difference between patients and controls ($P<0.0001$). These data indicate that CCL2 may play a significant role in the host response to SARS-CoV-2 infection.

There was no statistically significant difference between male and female patients regarding serum levels of CCL2, and CCL2 levels were also not significantly different between moderate and severe COVID-19 patients (Fig. 2C and D). Serum levels of CCL2 did not correlate with CCR2 expression, age, WBC, fever, oxygen saturation (SpO_2), and CRP (Fig. 3).

Table 3 The sequences of primers used in the study

Gene	Forward	Reverse
β-actin	AAACTGGAACGGTGAAGGTG	AGAAGTGGGGTGGCTTTTAG
CCR2	TCTGTTTATGTCTGTGGCCCT	GCCTCTTCTTCTCGTTTCGAC

Expression of CCR2 in COVID-19 patients

In addition to serum levels of CCL2, the expression of its receptor, CCR2, was quantified by real-time PCR in PBMCs from COVID-19 patients ($n=60$) and healthy individuals ($n=60$). Our findings revealed that the expression level of *CCR2* gene in PBMCs from all COVID-19 patients (0–19 years, 20–40 years, 40–60 years, and ˃ 60 years) was higher than that in PBMCs from control subjects (Fig. 4). However, we observed a significant difference between patients and controls only in group 4 (˃ 60 years) ($P=0.0353$). These results show that CCR2 may be an essential chemokine receptor in inflammation during COVID-19.

Similar to CCL2 levels, CCR2 expression was not significantly different between male and female patients. Moreover, we observed no significant difference in CCR2 expression between moderate and severe COVID-19 patients (Fig. 2A and B). The correlation matrix showed weak or near-zero correlations between most variables, with no significant associations. WBC had a slight positive correlation with fever, while oxygen saturation (SpO_2) and C-reactive protein (CRP) exhibited minimal correlation with other variables. Chemokines CCR2 and CCL2 also showed negligible correlations across the dataset. Overall, the variables appeared largely independent of one another based on this matrix (Fig. 3).

Discussion

Chemokines have a crucial role in combating viral infections through the recruitment of innate and adaptive immune cells to the site of infection and inducing the production of antiviral mediators. On the other hand, a significant recruitment of immune cells to the site of infection and increased antiviral responses can result in hyperinflammation and tissue damage [30]. There is some evidence that CC chemokines are more critical than CXC chemokines in response to respiratory viral infections [31]. CCL2 is a potent chemoattractant chemokine able to recruit monocytes and macrophages and initiate inflammation [32]. Numerous studies have suggested the role of CCL2 in the pathogenesis of viral infections, such as those caused by human cytomegalovirus

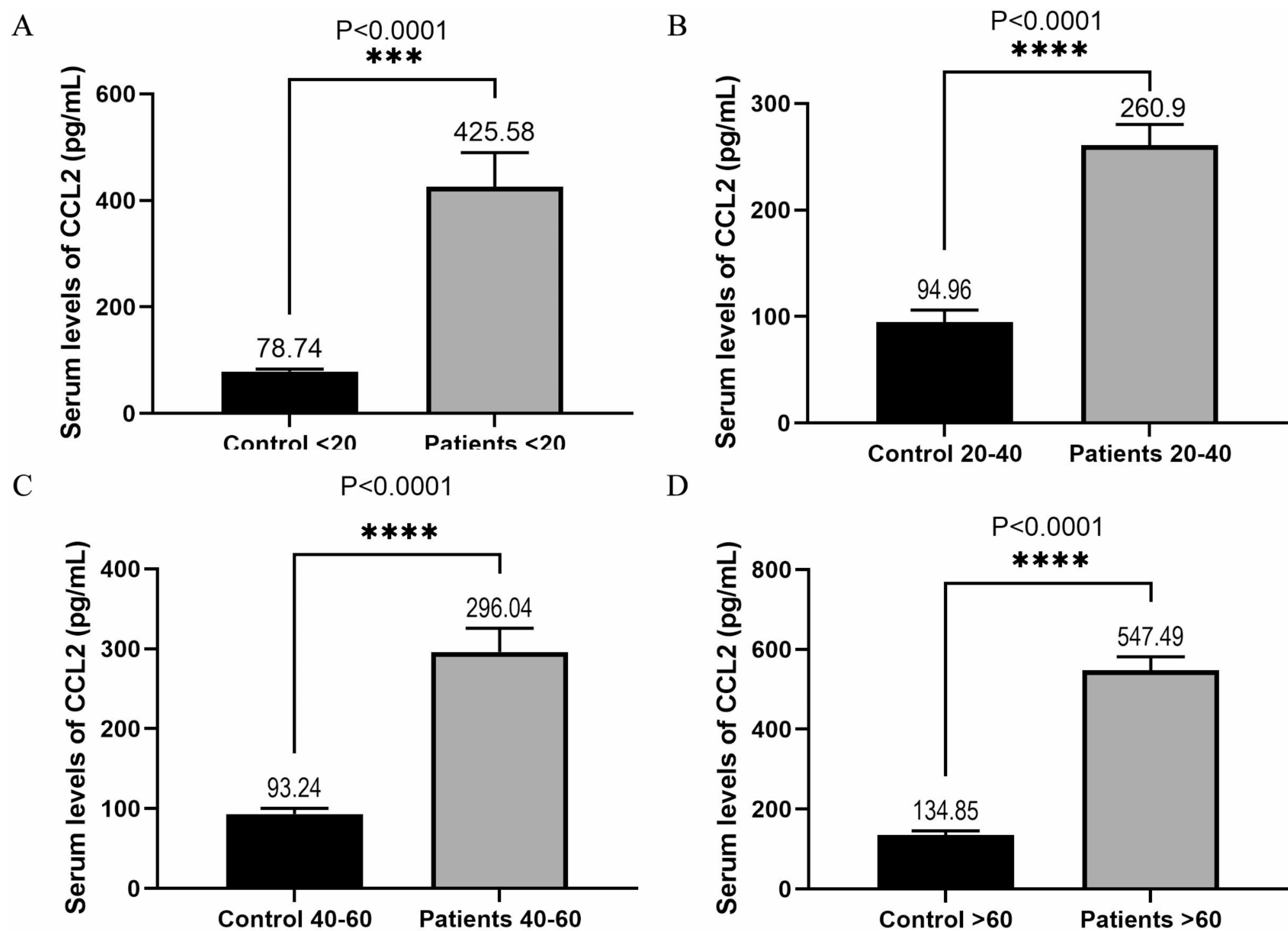

Fig. 1 Serum levels of CCL2 in COVID-19 patients and healthy controls of different age groups. (**A**) < 20 years (**B**) 20–40 years (**C**) 40–60 years (**D**) > 60 years. Differences were considered statistically significant when $P < 0.05$ ($P < 0.0001$)

(CMV), human rhinovirus (HRV), HIV, and influenza [33–36].

Our previous study showed that there was no significant difference between serum levels of interleukin-1β (IL-1β) in COVID-19 patients and normal subjects [37]. In the present study, we found significantly elevated serum concentrations of CCL2 in confirmed COVID-19 patients compared to the age-matched healthy control groups. Several chemokines (e.g., CCL2, CCL3) are among proinflammatory mediators involved in SARS-CoV-2 infection. It has been shown that infection of mice with murine coronaviruses (mouse hepatitis virus) leads to similar chemokine responses [38]. In patients with severe COVID-19, lung macrophages expressed high levels of several chemokines, such as CCL2 and CCL3 [39]. Transcriptome analysis of bronchoalveolar lavage fluid (BALF) from COVID-19 patients identified several chemokines (e.g., CCL2, CCL8) induced by SARS-CoV-2 infection [40]. Serum levels of chemokines have been investigated in COVID-19 patients (asymptomatic, symptomatic) [41]. A higher level of CCL2 was found in symptomatic COVID-19 patients than in healthy controls.

Additionally, severe COVID-19 patients showed higher serum levels of CCL2 compared to mild cases. Another study showed that fatal COVID-19 patients had a significantly elevated plasma level of CCL2 compared to severe and mild COVID-19 patients [42]. Transcriptional analysis of lung samples from COVID-19 patients showed upregulation of chemokines, including CCL2, CCL8, and CCL11. A significant increase was also observed in serum levels of chemokines such as CCl2 and CCL8. This increase in these chemokines was associated with generalized inflammation in patients with COVID-19 [43]. It was also revealed that ICU patients showed an increased plasma level of CCL2 in comparison to non-ICU patients [44]. It should be noted that increased levels of CCL2 could be used as a biomarker for mortality in COVID-19 patients [45].

All these data indicate that CCL2 is upregulated in different samples from COVID-19 patients, such as serum, plasma, and BALF. Our findings are consistent with these studies and show that serum concentrations of CCL2 are

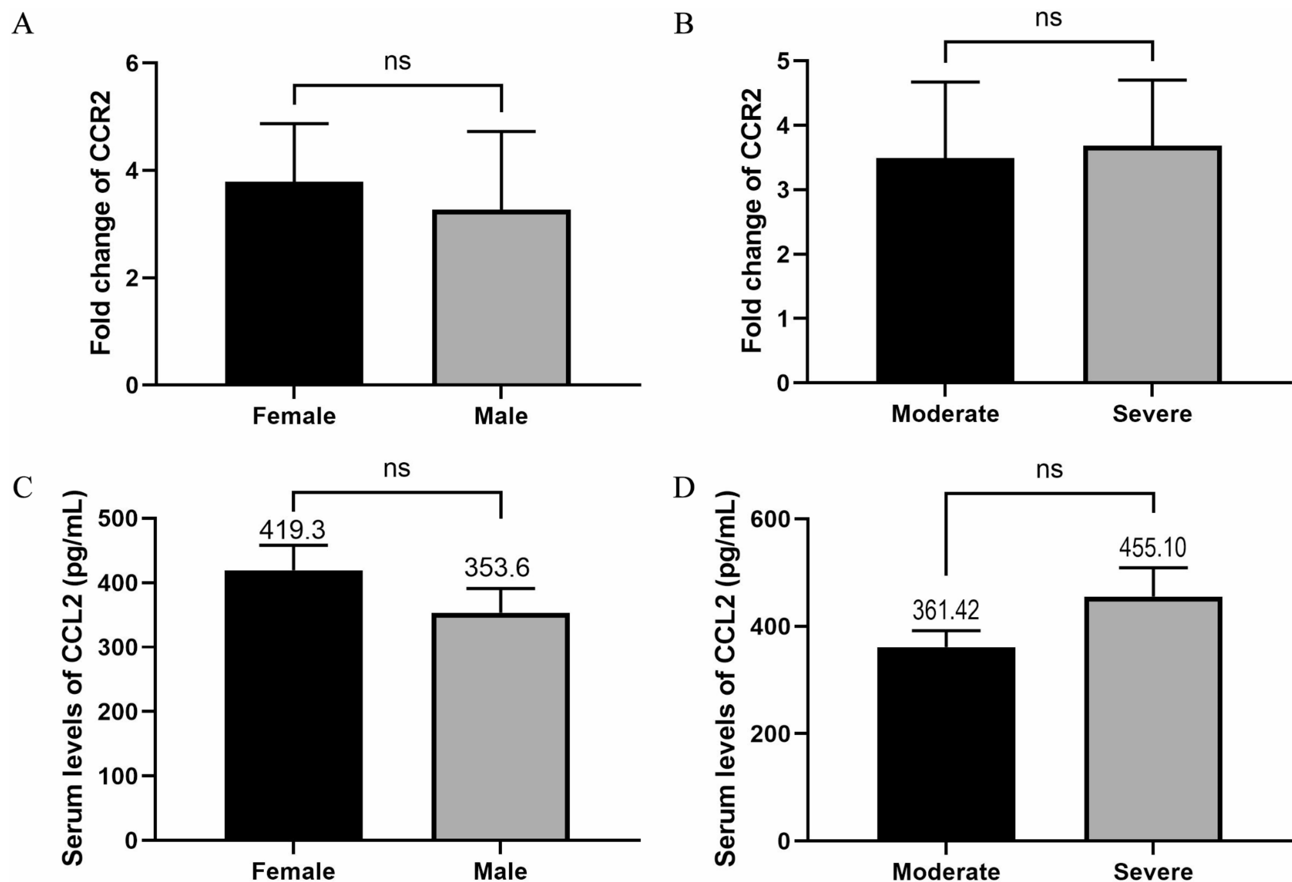

Fig. 2 CCR2 expression and CCL2 levels in COVID-19 patients according to gender and stages of disease. (**A**) The expression level of *CCR2* gene in male and female COVID-19 patients. (**B**) The expression level of *CCR2* gene in moderate and severe COVID-19 patients. (**C**) Serum level of CCL2 in male and female COVID-19 patients. (D) Serum level of CCL2 in moderate and severe COVID-19 patients. ns: not significant

significantly elevated in COVID-19 patients of different age groups. We also observed an increase in CCL2 levels in severe COVID-19 patients compared to moderate patients, but this increase was not significantly different between both groups. Similar to our results in the present study, Tincati et al. showed that severe COVID-19 patients had higher plasma levels of CCL2 (without statistical significance) than mild patients [46]. Among chemokines involved in COVID-19, CCL2 and CXCL10 show the most substantial upregulation. According to the available studies, CCL2 is a chemokine that has a vital role in the initiation of the COVID-19 cytokine storm [47]. In addition to COVID-19 lung pathology, CCL2 may have a pathogenic role in heart damage. It was found that cardiomyocytes infected with SARS-CoV-2 can secrete CCL2 and recruit CCR2$^+$ monocytes [48]. Taken together, these outcomes suggest that the inflammatory chemokine CCL2 is of great importance in the pathogenesis of SARS-CoV-2 infection.

CCL2 functions are mediated through its receptor, CCR2, which is expressed in various cells, including monocytes, dendritic cells (DC), and T cells. CCR2 is associated with several disorders, including atherosclerosis, central nervous system (CNS) inflammation, and diabetes [49]. The absence of CCR2 in influenza A virus infection has been examined using CCR2-deficient mice. The study showed that defective migration of monocytes/macrophages led to protection against influenza-induced tissue damage [50]. Excessive accumulation of CCR2$^+$ inflammatory monocytes in the lungs has been reported during influenza A virus infection. Moreover, CCR2-deficient mice showed a reduction in leukocyte infiltration and cytokine storm [51]. During influenza virus infection, CCR2 also contributes to the migration of NK cells [52]. An increase in the transcription of CCR2 and CCR5 (CCL3 receptor) has been observed in BALF samples of COVID-19 patients [40]. High expression of CCR2 was shown to be involved in severe COVID-19 using transcriptome-wide association in lung tissue [53]. The expression of *CCR2* at mRNA levels was increased in peripheral blood samples of patients with COVID-19, and severe COVID-19 patients had higher expression than moderate and critical patients [54].

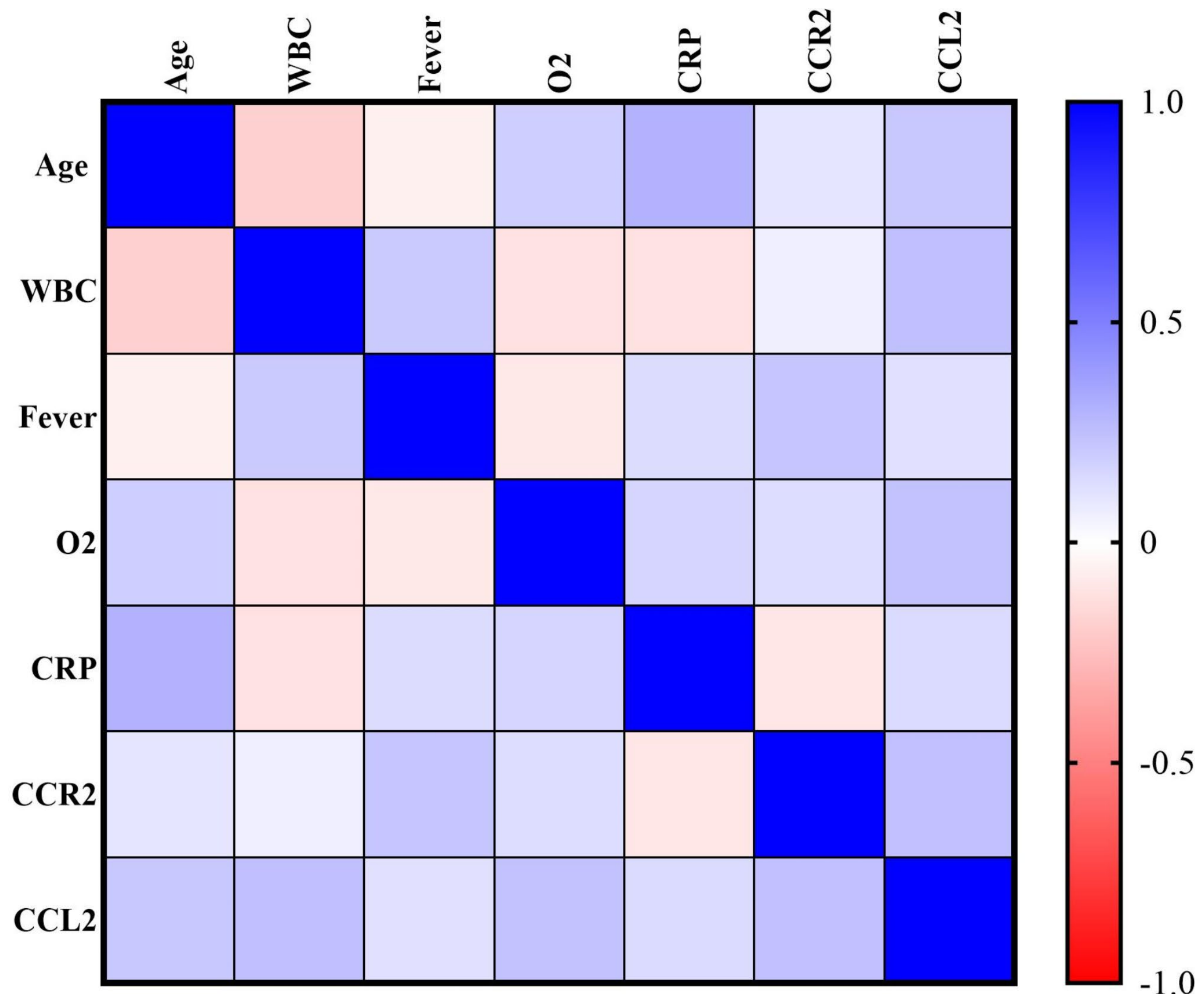

Fig. 3 The heatmap displays the correlation coefficients between various parameters, including Age, White Blood Cell count (WBC), Fever, Oxygen saturation (O2), C-reactive protein (CRP), CCR2, and CCL2. The color scale on the right represents the strength of the correlation, ranging from −1 (red, strong negative correlation) to +1 (blue, strong positive correlation). Most variables showed weak or negligible correlations with one another, indicating minimal associations across the dataset

In our study, we also reported that the expression level of *CCR2* in PBMCs from COVID-19 patients was upregulated compared with control subjects. It is interesting to note that we observed a significant difference between patients and controls above 60 years of age. Furthermore, there was no significant difference in *CCR2* expression between moderate and severe COVID-19 patients. Targeting the CCL2/CCR2 axis has been investigated in several diseases, such as cancer and atherosclerosis. For example, blocking the CCL2/CCR2 axis by a CCR2 antagonist or CCL2 neutralizing antibody could have therapeutic effects on hepatocellular carcinoma [55, 56]. Pharmacological targeting of the CCL2/CCR2 axis also could inhibit atherosclerosis [57]. In the context of COVID-19, we suggest that the CCL2/CCR2 axis may have beneficial or detrimental roles depending on the stage of the disease. A CCR2 and CCR5 antagonist (cenicriviroc) was able to inhibit SARS-CoV-2 replication [58]. On the other hand, CCR2 has been demonstrated to control SARS-CoV-2 infection and inflammation in the lung through the infiltration of monocytes into the lung and the expansion of monocyte-derived cells [59].

This study has various limitations that must be recognized. The sample size was limited, impacting the generalizability of the findings. This limitation stemmed mainly from the rigorous inclusion criteria, which mandated unvaccinated persons devoid of a history of inflammatory or autoimmune illnesses, alongside the difficulties of participant recruitment during the pandemic. Secondly, although the study detected increased levels of CCL2 and CCR2 in COVID-19 patients, it needed more protein-level validation by techniques such as Western blot owing

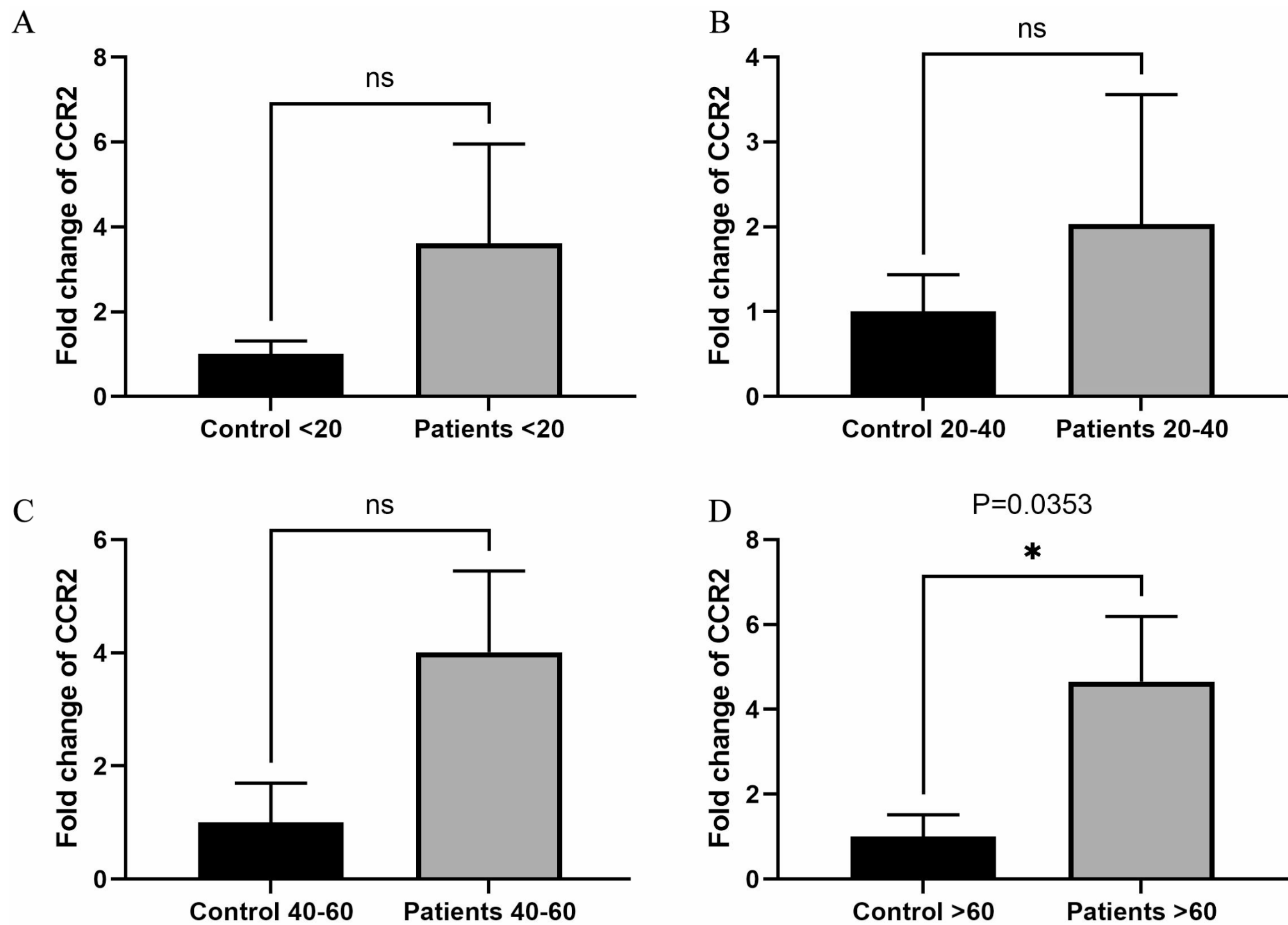

Fig. 4 CCR2 expression in PBMCs from COVID-19 patients and healthy controls. (**A**) < 20 years (**B**) 20–40 years (**C**) 40–60 years (**D**) > 60 years. Ns: not significant. * Significant difference between the two groups ($P = 0.0353$). The expression of CCR2 was measured by real-time PCR

to financial and resource limitations. The investigation was undertaken during a period when the Delta variation was prominent, perhaps restricting the application of our findings to other SARS-CoV-2 variants.

Subsequent studies should endeavor to incorporate a more significant, more heterogeneous cohort to corroborate and enhance these findings. Integrating protein-level analysis, such as Western blotting, with gene expression studies would yield a more thorough comprehension of the functions of CCL2 and CCR2 in COVID-19. Furthermore, analyzing these chemokines in relation to various viral variations and vaccinated cohorts may provide insights into the evolution of the immune response to SARS-CoV-2. Additional research on the molecular pathways connecting CCL2 and CCR2 to illness severity and progression will be beneficial for the development of targeted treatment methods.

Conclusions

In conclusion, the findings of our study showed that the circulating levels of CCL2 were significantly elevated in patients with COVID-19. The expression of its receptor, CCR2, was also higher in PBMCs of patients. Our results emphasize the importance of the CCL2/CCR2 axis in SARS-CoV-2 infection among different age groups. This chemokine axis could have a protective role in the early stage of COVID-19 by recruitment of monocytes/macrophages into the lung. However, excessive recruitment of immune cells into the lungs may cause hyperinflammation and tissue damage at the late stage of the disease. Therefore, targeting the CCL2/CCR2 axis should be investigated in the different stages of SARS-CoV-2 infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12865-024-00662-8.

Supplementary Material 1

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Author contributions
MA and HK designed the study. VB and MK performed the experiments. HK analyzed the data. VB wrote the manuscript. MA reviewed the final version of the manuscript. All authors read and approved the final manuscript.

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Data availability
The data supporting the findings of this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All participants signed a written informed consent form. The study protocol was approved by the Ethics Committee at Rafsanjan University of Medical Sciences (Ethics Code: IR.RUMS.REC.1400.031). This research was conducted in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Clinical trial number
Not applicable.

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