

T-Cell Immune Responses and Immunological Factors Associated with Coronavirus Disease 2019 Progression as Predictors for the Severity of the Disease in Hospitalized Patients

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Keywords

COVID-19 · SARS-CoV-2 · T-bet · GATA3 · RORγt · FoxP3

Abstract

Introduction: The prevalence of coronavirus disease 2019 (COVID-19) has rapidly increased worldwide. More investigation is needed to progress toward understanding the exact role of immune responses in the pathology of the disease, leading to improved anticipation and treatment options.

Methods: In the present study, we examined the relative expression of T-bet, GATA3, RORγt, and FoxP3 transcription factors as well as laboratory indicators in 79 hospitalized patients along with 20 healthy subjects as a control group. In order to make an exact comparison between various degrees of severity of disease, patients were divided into critical ($n = 12$) and severe ($n = 67$) groups. To evaluate the expression of genes of interest by performing real-time PCR, blood samples were obtained from each participant. **Results:** We found a significant increase in the expression of T-bet, GATA3, and RORγt and a reduction in the expression of FoxP3 in the critically ill patients compared to the severe and control groups. Also, we noticed that the GATA3 and RORγt expressions were elevated in the severe group in

comparison with healthy subjects. Additionally, the GATA3 and RORγt expressions showed a positive correlation with elevation in CRP and hepatic enzyme concentration. Moreover, we observed that the GATA3 and RORγt expressions were the independent risk factors for the severity and outcome of COVID-19. **Discussion:** The present study showed that the overexpression of T-bet, GATA3, and RORγt, as well as a decrease in the FoxP3 expression was associated with the severity and fatal outcome of COVID-19.

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Introduction

Coronavirus disease 2019 (COVID-19) is an emergent public health problem globally caused by SARS-CoV-2, a virus widely known for excessive mutagenic ability, epidemiological rapid growth, and high capacity to infect hosts from different species [1, 2]. COVID-19 exhibits a wide range of clinical manifestations, and in terms of severity, it varies from an asymptomatic course of infection to severe respiratory failure, ultimately needing an inten-

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sive care unit (ICU) [3]. The disease severity and its outcome are associated with many factors including age, gender, obesity, and underlying medical conditions (e.g., cancer, immunodeficiency, diabetes, hypertension, and cardiovascular disease) [4].

Studies have reported infected epithelial cells with SARA-CoV-2 by secretion of inflammatory cytokines like IL-8 can recruit and activate both innate and adaptive immune cells to start the antiviral responses [5]. Basically, in antiviral responses, the adaptive immune system polarizes T-cell differentiation to the Th1 and Th17 populations to neutralize the infection by expressing IFN- γ , TNF- α , IL-22, and IL-17 [6]. Additionally, the Th2 population by coordination of humoral responses via producing IL-4 and IL-6 has a role in the clearance of infection [7]. In contrast, in such conditions, Treg cells have key roles in the maintenance of immune hemostasis by producing TGF- β and IL-10 [8].

Moreover, recent studies have shown uncontrolled and exacerbated antiviral responses can enhance the recruitment of neutrophils, monocytes, and other immune cells which leads to the creation of cytokine storms by overproduction of IFN- γ , TNF- α , GM-CSF, MCP-1, IL-6, IL-2, and IL-8 [6]. The cytokine storm is a major life-threatening condition associated with COVID-19 [9]. These features highlighted the magnitude of the effect of immunological response, particularly T helper cell responses in the determination of the severity and outcome of the disease.

We carried out this experiment due to contradictory results obtained from recent studies on immunological responses against SARA-CoV-2 and also the lack of a proper classification of patients. The present study has aimed to investigate the role of T helper polarizations in the severity of COVID-19 as well as the correlation between T helper polarizations and laboratory indicators and its association with the outcome of the disease in hospitalized patients with COVID-19.

Materials and Methods

Study Design and Participants

In this study, the 79 hospitalized patients suffering from COVID-19 (67 non-ICU hospitalized and 12 ICU hospitalized) were recruited from Kosar Hospital, Semnan, Iran, from 7 April to 3 May 2021. All enrolled patients were confirmed positive for COVID-19 by the combination of checking clinical symptoms and RT-PCR. A group of healthy individuals ($n = 20$) was enrolled as the control group.

The inclusion criteria for enrolling patients were laboratory and clinical evaluation for confirming positive COVID-19 and being over 18 years old. Additionally, patients with a history of cancer and infectious disease including hepatitis, HIV, and brucellosis were excluded from this experiment. Moreover, healthy participants were all over 18 years old, had no underlying medical conditions and infectious diseases. Of note, all patients and healthy individuals were not vaccinated for COVID-19.

The present study aimed to describe differences in demographics, clinical presentation, and immunological factors between the severe and critical spectrum of COVID-19 severity as well as comparison with healthy subjects. Consequently, patients were divided into severe and critical groups. Patients who exhibited more severe weakness, persistent pressure in the chest, the onset of confusion or altered level of consciousness, needed a ventilator machine and intensive critical care were placed in the critical group ($n = 12$). Patients who showed shortness of breath, respiratory rate ≥ 30 beats/min, and progression of lung lesions $\geq 50\%$ were placed in the severe group [10]. Informed written consent was obtained from all participants, and the Ethical Committee of the Semnan University of Medical Science approved the study (IR.SEMUMS.REC.1399.8). The clinical characteristics and laboratory findings were shown in Table 1, and also, comorbidities and symptoms were provided in Table 2.

Blood Sampling and PBMC Isolation

In order to indicate laboratory data and PBMC isolation, 10 mL peripheral blood samples were taken from all subjects on the first day of hospitalization. The Ficoll-Hypaque density method was used to isolate PBMCs from whole blood. First, the PBS-diluted blood samples were laid over the Ficoll-Hypaque (Histosep, Iran) and centrifuged for 30 min, 400g at RT. Subsequently, the supernatants were taken out; then the mononuclear cells were transported to another tube and finally washed twice using PBS (300 g, 10 min, RT).

RNA Extraction, cDNA Synthesis, and qRT-PCR

In order to assess the relative expression of genes of interest, total RNA isolation from PBMCs was performed using BIOzol reagent (Stem Cell Technology Research Center, Iran) according to instructions provided by the manufacturer. The quality and quantity of purified RNA were determined by gel electrophoresis and NanoDrop spectrophotometer, respectively. All purified RNA samples were reserved at -80°C until further use for cDNA synthesis and real-time PCR. Afterward, 1 μg of RNA from each sample was reverse transcribed to cDNA using a cDNA synthesis kit (Parstous, Iran). SYBR green master mix (Parstous, Iran) and specific primers were used to perform real-time PCR. 40 cycles of PCR were carried out on a real-time PCR detection system (Applied Biosystems, USA). Briefly, reaction mixture contained 10 μL 2 $\times$ SYBR green master mix, 0.4 μL 50 $\times$ ROX dye, 0.35 μL specific forward primer, 0.35 μL specific reverse primer, and 8 μL DEPC water for each well; at last, 1 μL of cDNA was added to 19 μL of the reaction mixture, giving a final volume of 20 μL . Subsequently, the PCR tube was placed in the real-time PCR detection system and went through a cycling program as follows: one cycle of denaturation at 94°C for 10 min followed by 40 cycles of amplification at 95°C for 15 s, specific annealing temperature for 30 s and 72°C for 60 s. The level of gene expression was normalized to the housekeeping gene (GAPDH), and the comparative threshold cycle ($\Delta\Delta\text{CT}$) method was used to interpret the results.

Table 1. Clinical characteristics and the laboratory indicators of participants

Variables	Severe patients (n = 67)	Critical patients (n = 12)	Healthy controls (n = 20)	p value*	p value**	p value***
Age, years	55±16	70±15.5	61.8±12.0	0.01	0.22	0.34
Gender						
Male, %	31 (46.2)	6 (50)	11 (55)			0.78 ^a
Female, %	36 (53.7)	6 (50)	9 (45)			
TOH, days	3.34±1.3	2.91±0.7	–	0.29	–	–
Lymphocyte, %	23.3	11.1	29.0	0.001	0.03	<0.0001
Neutrophils, %	71.5	84.4	55.2	0.003	<0.0001	<0.0001
Monocytes, %	4.7	3.7	5.0	0.62	0.89	0.51
Eosinophils, %	1.3	1.2	2.75	0.90	<0.0001	0.002
Platelet count, ×10 ⁹ /L	174.8	226.4	186.8	0.002	0.56	0.06
Hemoglobin, g/L	142.5	126.6	126.9	0.92	0.88	1.00
ALT, U/L	36.8	70.5	22.7	0.003	0.017	<0.0001
AST, U/L	34.4	60.9	26.1	0.001	0.28	<0.0001
ALP, IU/L	140.9	182.8	103.6	0.002	0.007	<0.0001
ESR, mm/h	40.0	53.0	15.1	0.16	<0.0001	<0.0001
CRP, mg/L	49.8	96.5	8.75	<0.0001	<0.0001	<0.0001
BUN, mg/dL	16.4	21.2	16.3	0.07	0.99	0.12
Creatinine, mg/dL	1.4	1.0	1.0	0.63	0.53	0.99
O ₂ saturation	90.4	83.0	95.8	<0.0001	<0.0001	<0.0001
Body temperature, °C	36.6	36.3	36.9	0.45	0.63	0.22

Data are presented as numbers (%) or mean ± SD, unless indicated otherwise. TOH, time from symptom onset to hospitalization; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen. *p**, severe group versus critical group. *p***, severe group versus healthy control group. *p****, critical group versus healthy control group. ^aComparison between groups using χ^2 test on other comparisons performed using one-way ANOVA.

Statistical Analysis

All data were expressed as the number (%) and mean ± SEM. Data comparison was conducted between groups using independent *t* test, Mann-Whitney test, and the one-way ANOVA for multiple comparisons. Categorical variables were expressed as numbers (%) and compared using the χ^2 test. Furthermore, the correlations between different variables were tested by Spearman's correlation analysis. Additionally, the odds ratio was used to express the relative measure of an effect between a binary outcome variable and a predictor variable. Also, multivariate analysis was performed using logistic regression model. Data analysis was performed with StepOne Software v2.1, Prism 8.0.2 (GraphPad v7, USA), and SPSS (SPSS, v22, USA). The *p* value of less than 0.05 was considered to be statistically significant.

Results

Clinical Characteristics of Study Population

Our study comprised 79 hospitalized COVID-19 patients (37 males, 42 females) who were divided into severe and critical groups, along with 20 healthy subjects as the control group. The severe group consisted of 31 male and 36 female patients with a mean age of 55 ± 16 years, while

the critical group consisted of 6 male and 6 female patients with a mean age of 70 ± 15.5 years. The intergroup comparison showed that subjects in the severe group were notably younger than those who were in the critical group (*p* < 0.05). The control group included 11 male and 9 female participants with a mean age of 61.8 ± 12.0 which had no differences in terms of age compared with both critical and severe groups. Additionally, there were no significant differences in the time from symptom onset to hospitalization between the severe and critical groups (3.34 ± 1.3 days and 2.91 ± 1.7 days, respectively). It was noted that participants in the control group did not have any underlying medical issues or comorbidities. In addition, as shown in Table 2, patients in the severe and critical groups were similar in having comorbidities such as hypertension, diabetes, cardiovascular disease, asthma, and chronic kidney disease. Moreover, the rate of obesity was significantly higher in the critical group compared to the severe group (*p* < 0.01). Hypertension (38.8% severe, 50% critical cases), coronary heart disease (23.8% severe, 33.3% critical cases), and diabetes (28.8% severe, 16.6% critical cases) were the most common comorbid condi-

Table 2. Comorbidities and symptoms in study participants

Variables	Severe patients (n = 67)	Critical patients (n = 12)	Healthy controls (n = 20)	p value ^a
Past medical history, n (%)				
High blood pressure				0.53
Negative	41 (61.1)	6 (50)	20 (100)	
Positive	26 (38.8)	6 (50)	0 (0)	
Diabetes				0.49
Negative	48 (71.6)	10 (83.3)	20 (100)	
Positive	19 (28.3)	2 (16.6)	0 (0)	
Obesity				0.004
Negative	65 (97.0)	8 (66.6)	20 (100)	
Positive	2 (2.98)	4 (33.3)	0 (0)	
Coronary heart disease				0.48
Negative	51 (76.1)	8 (66.6)	20 (100)	
Positive	16 (23.8)	4 (33.3)	0 (0)	
Asthma				0.22
Negative	63 (94.0)	10 (83.3)	20 (100)	
Positive	4 (6.0)	2 (16.6)	0 (0)	
Chronic kidney disease				0.22
Negative	63 (94.0)	10 (83.3)	20 (100)	
Positive	4 (6.0)	2 (16.6)	0 (0)	
Immune compromise				0.71
Negative	65 (97.0)	12 (0)	20 (100)	
Positive	2 (3.0)	0 (0)	0 (0)	
Symptoms, n (%)				
Fever				0.02
Negative	20 (29.8)	8 (66.6)	N/A	
Positive	47 (70.2)	4 (33.3)		
Headache				<0.0001
Negative	31 (46.2)	12 (100)	N/A	
Positive	36 (53.8)	0 (0)		
Dry cough				0.49
Negative	15 (22.3)	2 (16.6)		
Positive	52 (77.7)	10 (83.4)	N/A	
Fatigue				0.01
Negative	10 (14.9)	6 (50)	N/A	
Positive	57 (85.1)	6 (50)		
Vertigo				0.53
Negative	36 (53.7)	8 (66.6)	N/A	
Positive	31 (46.3)	4 (33.3)		
Shortness of breath				0.03
Negative	37 (55.2)	2 (16.6)	N/A	
Positive	29 (44.8)	10 (83.4)		
Sore throat				0.72
Negative	49 (73.1)	8 (66.6)	N/A	
Positive	18 (26.9)	4 (33.3)		
Sputum				0.11
Negative	38 (56.7)	10 (83.3)	N/A	
Positive	29 (43.3)	2 (16.7)		
Sweating				<0.0001
Negative	24 (35.9)	0 (0)	N/A	
Positive	43 (64.1)	12 (100)		
Anorexia				0.20
Negative	20 (29.8)	6 (50)	N/A	
Positive	46 (70.2)	6 (50)		
Nausea				0.49
Negative	41 (61.1)	8 (66.6)	N/A	
Positive	26 (38.9)	4 (33.3)		

Table 2 (continued)

Variables	Severe patients (n = 67)	Critical patients (n = 12)	Healthy controls (n = 20)	p value ^a
Stomachache				
Negative	53 (79.1)	12 (100)	N/A	0.11
Positive	14 (20.9)	0 (0)		
Diarrhea				
Negative	51 (76.1)	12 (100)	N/A	0.11
Positive	16 (23.9)	0 (0)		
Myalgia				
Negative	9 (13.4)	4 (33.3)	N/A	0.10
Positive	58 (86.6)	8 (66.6)		
Hemoptysis				
Negative	65 (97.0)	8 (66.6)	N/A	0.004
Positive	2 (3.0)	4 (33.3)		

^a Comparison of the probiotic group versus the placebo group using the χ^2 test.

tions. In terms of symptoms, as shown in Table 2, the percentage of patients who experienced hemoptysis, sweating, and shortness of breath in the critical group was dramatically higher than that in the severe group. On the contrary, other symptoms such as fever, headache, and fatigue were more common in the severe group.

Laboratory Findings

The laboratory findings of all participants in our study were analyzed upon admission. We observed that many laboratory results were significantly different between participating groups. In comparison with patients in the severe group, critical subjects had a higher level of CRP, AST, ALT, ALP, ESR, an increase in counts of neutrophils and platelets, and a lower count of lymphocytes. Moreover, as shown in Table 1, both hospitalized groups had a significant decrease in the level of oxygen, a lower percentage of lymphocytes, and a notable increase in neutrophil percentage, hepatic enzymes, and CRP level compared to the control group. The intergroup comparison showed that there were no differences in the percentage of monocytes, level of hemoglobin, BUN, creatinine, and body temperature between participating groups (Table 1).

The Assessment of Th1, Th2, Th17 and Treg Transcription Factors in Patients with Severe and Critical Forms of COVID-19

We evaluated the expression of transcription factors related to the differentiation of Th1, Th2, Th17, and Treg populations in PBMCs from all participants at the time of admission. T-bet expression, the key regulator of Th1

population development, was notably increased in critical and severe groups compared to the control group ($p < 0.001$, $p < 0.05$, respectively). There were no significant differences in T-bet expression between the severe and critical groups (Fig. 1a). It was further noted that the relative expression of FoxP3, the master regulator of Treg cell differentiation, was significantly decreased in the critical subjects in comparison with the severe and control groups ($p < 0.05$, $p < 0.0001$, respectively), while there were no differences between the severe and control groups (Fig. 1b). In terms of expression of ROR γ t and GATA3, Th17 and Th2 differentiation factors, ROR γ t was expressed at a significantly higher rate in the critical group compared to the severe and control groups ($p < 0.05$, $p < 0.0001$, respectively). Also, patients who had critical conditions showed upregulation in GATA3 expression compared to the severe and control groups ($p < 0.05$, $p < 0.0001$, respectively). Additionally, the expressions of ROR γ t and GATA3 were notably higher in the severe group compared to healthy subjects ($p < 0.05$, $p < 0.001$, respectively) (Fig. 1c, d).

Correlation of T-Cell Transcription Factors with Hepatic Enzymes, CRP, and O₂ Level

To investigate the impact of expression of transcription factors related to the differentiation of Th1, Th2, Th17, and Treg populations on the hepatic enzymes and CRP levels in all participating subjects, the Pearson correlation coefficient was performed. As shown in Table 3, the results revealed that GATA3 expression showed a significant positive correlation with AST, ALP, ESR, and CRP levels. Likewise, the T-bet expression showed a positive

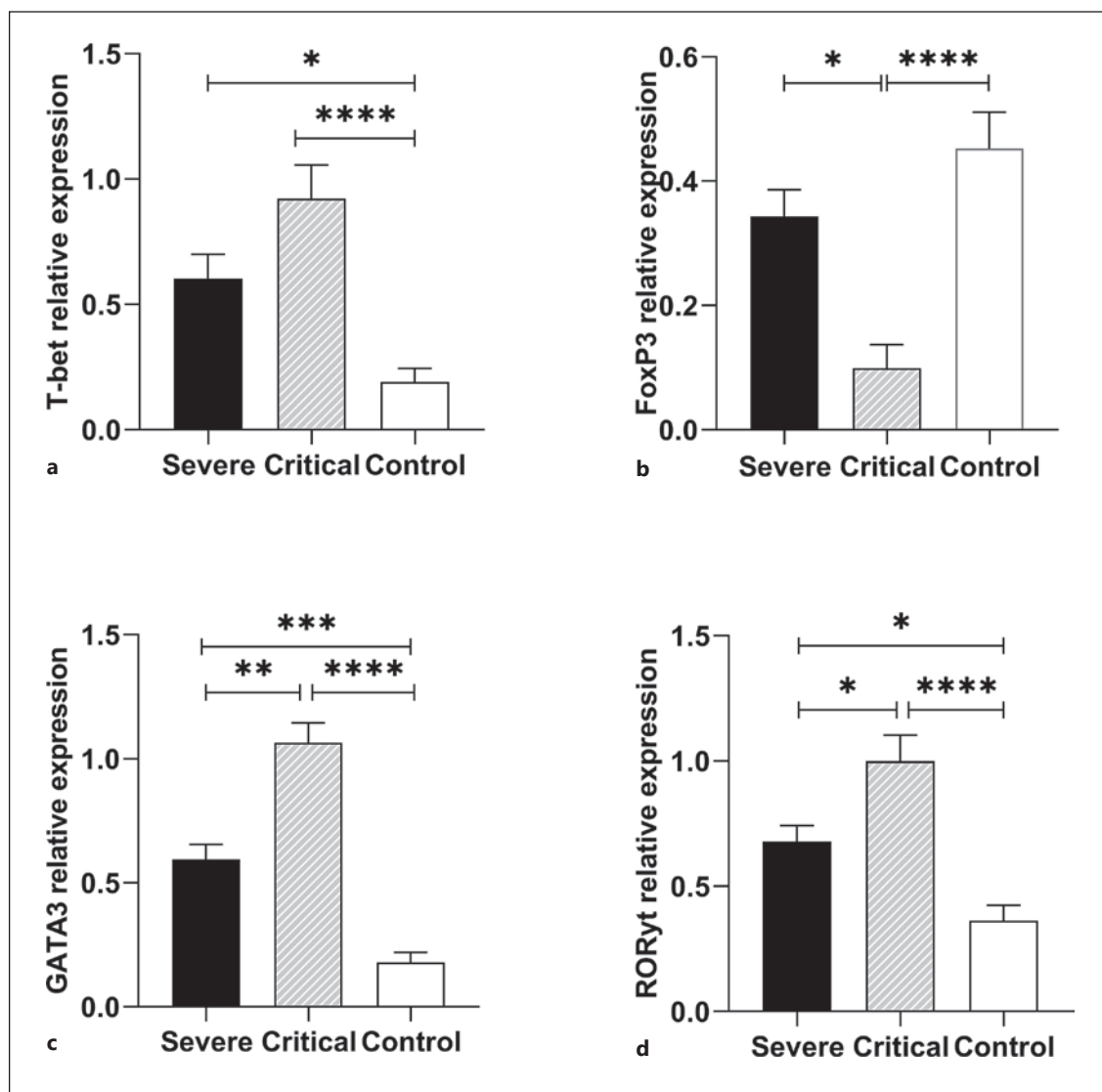


Fig. 1. Progressive COVID-19 is associated with upregulation of T-bet, GATA3, and RORγt and downregulation of FoxP3 genes. The gene expression of (a) T-bet, (b) FoxP3, (c) GATA3, and (d) RORγt in PBMCs from participants in severe, critical, and control groups. Bars represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

correlation with the ALP level. Also, a positive correlation was observed between RORγt expression and CRP and ALT levels. Regarding O₂ saturation levels, RORγt, T-bet, and GATA3 expression levels demonstrated a negative correlation with it; however, FoxP3 showed a positive correlation with the O₂ saturation level. Taken together, the results showed that GATA3 expression was the most efficient variable that affected liver damage, inflammation, and O₂ level in blood which made this factor the most determinative factor affecting the outcome of the disease.

Severity Prediction of COVID-19

The univariate logistic regression analysis showed that the severity of COVID-19 (whether patients needed critical care or not) was associated with GATA3 (OR = 9.09, $p < 0.05$), lymphocyte counts (OR = 0.74, $p < 0.01$), and RORγt (OR = 3.63, $p < 0.05$). The multivariate logistic regression analysis, after adjusting potential factors including GATA3, RORγt, and lymphocyte counts, indicated that RORγt (OR = 10.32, $p < 0.05$) and GATA3 (OR = 50.15, $p < 0.05$) were the independent risk factors for

Table 3. Spearman correlation of T-cell transcription factors with hepatic enzymes, CRP, and O₂ level in the participants

	ALT level	AST level	ALP level	ESR level	CRP level	O ₂ level
GATA3						
Correlation coefficient	0.17	0.21	0.23	0.33	0.37	-0.27
<i>p</i> value	0.08	0.03	0.017	0.0008	<0.0001	0.005
T-bet						
Correlation coefficient	0.07	0.006	0.24	0.06	0.13	-0.223
<i>p</i> value	0.45	0.95	0.01	0.52	0.18	0.02
FoxP3						
Correlation coefficient	-0.12	-0.15	-0.14	-0.14	-0.19	0.29
<i>p</i> value	0.2	0.13	0.15	0.14	0.05	0.002
RORyt						
Correlation coefficient	0.20	0.16	0.13	0.13	0.27	-0.13
<i>p</i> value	0.04	0.11	0.19	0.20	0.006	0.19

Table 4. Univariate and multivariate logistic analysis of T-cell transcription factors related to the severity of COVID-19

Predictors	Univariate analysis			Multivariate analysis		
	B	OR (95% CI)	<i>p</i> value	B	OR (95% CI)	<i>p</i> value
GATA3	2.20	9.09 (1.65–49.99)	0.01	3.91	50.15 (1.55–1,616.64)	0.02
T-bet	0.45	1.58 (0.74–3.35)	0.23			
RORyt	1.29	3.63 (1.07–12.33)	0.03	2.33	10.32 (1.37–93.82)	0.03
FoxP3	-4.75	0.09 (0.00–1.88)	0.08			
Lymphocytes	-0.29	0.74 (0.61–0.89)	0.001	-0.318	0.72 (0.57–0.99)	0.01

Univariate and multivariate analyses were carried out using a logistic regression model. $p < 0.05$ was considered statistically significant.

progression of the disease. In contrast, lymphocyte count (OR = 0.72, $p < 0.05$) was the independent protective factor against disease progression (Table 4).

Discussion

Due to an increase in the incidence of COVID-19, a heavy burden is imposed on medical sources. More investigations are necessary for the identification of the exact pathogenic mechanisms as well as the immunological factors that significantly affect the progression of the disease [11]. Epidemiological studies have reported that patients with underlying medical issues including hypertension, cardiovascular disease, diabetes, cancer, obesity, and immunodeficiencies showed a higher risk of developing severe and critical forms of COVID-19 [4, 12].

In the present study, we noticed that participants in the critical group were significantly older than patients in the severe group. Furthermore, the rate of obesity was

higher in the critical group compared to the severe group. On the other hand, the critical and severe groups were similar in having comorbidities. It has been shown that age and underlying medical issues can affect the outcome of COVID-19 through immune responses. Indeed, these factors were associated with high levels of pro-inflammatory mediators and reactive oxygen species that resulted in development of uncontrolled immune responses, tissue injuries, and impaired viral clearance, contributing to the poor prognosis of the disease in the elderly [13, 14]. Moreover, the localized inflammatory responses in the adipose tissue led to the production of leptin and the reduction of adiponectin resulted in an increase in the systemic level of TNF- α and IL-6. These factors caused systemic inflammation and affect the severity of COVID-19 [15].

Multiple studies have demonstrated that COVID-19 may cause varying degrees of liver injury, particularly in critical cases [16]. These experiments indicated that hepatic enzymes including ALT and AST in critically ill pa-

tients were dramatically higher than those in the general ward [17, 18]. We observed similar results: the levels of ALT, AST, and ALP were higher in the critical group compared to the severe subject and these enzymes were elevated in both hospitalized groups in comparison to the healthy subject which were the signs of liver injury. The possible mechanisms of this damage to the liver might be the direct viral invasion of hepatocytes through ACE2r that expresses on the healthy hepatocytes, immune-mediated injury caused by cytokine storm, or underlying liver conditions like alcohol-related liver disease [19, 20].

Our results showed that the CRP level, neutrophil-lymphocyte ratio (NLR), platelet and neutrophil counts were notably higher in the critical group compared to the severe subjects as well as in both hospitalized subjects compared to the control group. Consistently, Feng and colleagues reported that inflammatory mediators such as IL-6, CRP, LDH, and NLR were significantly elevated in patients with COVID-19, leading to worsening of the disease, and they can also be useful predictors for the prognosis of the COVID-19 infection [21]. It has been shown that excessive inflammatory responses induced by neutrophils led to the creation of cytokine storms which have been implicated in COVID-19. Furthermore, the presence of NLR was associated with a more severe form of the disease [22].

T-cell responses have a prominent role in immunity against COVID-19 as well as the progression of this disease. Interestingly, evidence on COVID-19 has shown that dysfunction or excessive immune response can contribute to the creation of cytokine storms, leading to rising in inflammatory mediators including IL-6, IL-8, MIP-1A, GM-CSF, and IFN- γ which caused massive damage to the respiratory system and tissue to break down [7, 23, 24].

Studies have reported that Th1 responses play a crucial role in cell-mediated immunity against intracellular pathogens [25]. Our results demonstrated that T-bet relative expression was increased in both hospitalized groups compared to the control group. Thereby, it can be concluded that this type of response could augment inflammation by overproduction of IFN- γ in severe and critical forms of COVID-19. Consistent with this result, Berejo-Martin et al. [26] found that in subjects with the severe form of COVID-19, T-bet, IFN- γ , and Th1 populations were significantly increased. The study conducted by Lin and colleagues reported that cytokines like IFN- γ , IL-12, IL-2, and TNF- α were notably raised in patients with COVID-19 compared to healthy subjects [27]. It has been suggested that SARS-CoV-2 could induce polyclonal Th1

responses that led to uncontrolled immune responses and cytokine storms [28].

In the present investigation, we found that GATA3 and ROR γ t expressions were dramatically increased in the severe and critical groups in comparison with the control group. In addition, expressions of these two factors were significantly elevated in the critical patients compared to the severe group. It has been suggested that the Th2 responses might be triggered by excessive Th1 and Th17 responses [28]. It has been reported that Th2 responses can trigger bronchoconstriction and dyspnea by producing a large number of cationic protein cytokines like IL-6, a key mediator for causing cytokine storms, in critical forms of COVID-19 [29]. Furthermore, Roncati and colleagues [30] demonstrated evidence that Th2 responses could lead to type 3 hypersensitivity taking place inside the vascular walls which is life-threatening. In consistence with our research, Khoshmirsafa and colleagues demonstrated that the gene expressions of ROR γ t, IFN γ , IL-17, and T-bet were higher in patients with COVID-19 compared to healthy individuals. Khoshmirsafa et al. [31] also showed that ROR γ t, IL-6, and IL-17 gene expressions in the severe group were higher than in the moderate group, which supported our findings. Multiple studies have indicated that the population of Th17 cells was dramatically increased in COVID-19 patients; this population by production of IL-23 and IL-17 caused enhanced neutrophil migration to the airways, which resulted in edema, pneumonia, and serious respiratory damage in severe and critical patients with COVID-19 [7, 23].

We presented evidence that FoxP3 expression was significantly reduced in the critical group compared to the severe and control groups, although no significant differences were observed between the severe and critical groups. Consistently, Sadeghi et al. [32] reported that the Treg population, FoxP3 expression, TGF- β and IL-10 production were notably reduced in patients with COVID-19. Multiple studies have also shown Treg cell percentage and their suppressive activity were significantly decreased in SARS-CoV-2 patients [33]. It has been suggested that TNF- α and IL-6 could reduce suppressive activity in Tregs by preventing of TGF- β and FoxP3 expression [34, 35].

In the present study, we found that GATA3 and ROR γ t expressions were positively correlated with CRP concentration in SARS-CoV-2 patients. Recent studies showed that patients with elevated CRP levels were more likely to have critical illness and worst clinical outcomes [36, 37]. Furthermore, we observed that there was a positive cor-

relation between GATA3 expression and levels of hepatic enzymes including AST, ALP, and ESR; also, we found that T-bet and ROR γ t had a positive correlation with ALT and ALP, respectively. It has been demonstrated that acute liver injury, as manifested by the elevated level of hepatic enzymes, was associated with severity and poor prognosis of COVID-19 [18]. Based on these results, elevation in the expression of T-bet, ROR γ t, and GATA3 could be associated with acute liver injury and poor prognosis of COVID-19.

Additionally, we indicated that lymphocyte percentage, ROR γ t, and GATA3 relative expression levels were independent predictors for the severity and outcome of COVID-19. It has been reported that lymphopenia, which could be a consequence of pulmonary recruitment and entrapment of lymphocytes or even infection of lymphocytes with SARS-CoV-2 via dipeptidyl peptidase-4 (DPP-4) receptor, was associated with poor prognosis of COVID-19 [38]. Additionally, Khoshmirsafa and colleagues found that IL-17, IL-6, and IL-10 were the risk factors for the progression of the disease [31]. In addition, Th2 and Th17 hyperactivation due to polyclonal activation possibly with superantigens led to high antibody titers and the creation of cytokine storms [34, 38]. On the basis of these findings, lymphopenia and high expression of ROR γ t and GATA3 could be independent risk factors associated with COVID-19 severity. Consequently, based on the results obtained from the present study besides studies by other researchers in the field of COVID-19, it can be implied whether elevation in Th1, Th2, and Th17 responses or a decrease in Treg counts lead to uncontrolled inflammatory responses, severity, and poor prognosis of COVID-19.

Conclusion

To summarize, we provided evidence that supported the role of excessive and inappropriate T-cell responses in the pathogenesis of COVID-19. Such uncontrolled immune responses were strongly associated with the critical form of illness, serious tissue damage, and organ failure. Given the limitation of the present study, further research is needed to clarify T-cells dynamic in patients during the infection period as well as the memory phase. Analysis of antigen-specific T-cell infiltrate and immune checkpoint molecules involved in T-cell responses against SARS-CoV-2 will provide extensive information and reveal novel opportunities for anticipating and treating COVID-19.

Statement of Ethics

The study complied with the guidelines for human studies and was conducted in accordance with the World Medical Association Declaration of Helsinki. The study protocol was approved by the Ethical Board of the Semnan University of Medical Sciences (IR.SEMUMS.REC.1399. 8). Written informed consent to participate in the study and publication of their clinical details was obtained from all participants.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Rasoul Baharlou conceived and Farhad Malek planned the experiments. Tannaz Abbasi-Dokht and Arefe Vafaiezhad carried out the experiments. Tannaz Abbasi-Dokht wrote the manuscript. Negin Khalesi contributed to sample preparation. Tannaz Abbasi-Dokht, Rasoul Baharlou, and Dariush Haghmorad contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis, and the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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