

Research Article

Study of Regulatory Immune Responses, Including Serum FOXP3 Levels and Clinical Severity of COVID-19: A Cross-Sectional Study

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Article Info

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Abstract

Background: Respiratory diseases can be severe and are caused by dysregulated inflammatory and immune responses due to COVID-19. Forkhead box P3 (FOXP3) is an important transcription factor of regulatory T cells and may play a role in regulating excessive inflammation during viral infection. In this study, we measured serum FOXP3 levels in COVID-19 patients with severe and non-severe disease and explored the relationship between the severity of COVID-19 and clinical and demographic parameters. **Materials and Methods:** A cross-sectional study was conducted in November–December 2021, with 76 patients with confirmed COVID-19 in Al-Hillah, Babylon Province, Iraq. Patients were categorized into severe (n=38) and non-severe (n=38) groups based on their respiratory status, oxygen saturation, and chest computed tomography. We used a sandwich enzyme-linked immunosorbent assay (ELISA) to measure serum FOXP3 concentration. Demographic variables included age, sex, geographic residence, and vaccination status. The data were analyzed using SPSS version 27, with independent-samples t-tests and chi-square tests. Serum FOXP3 was evaluated for severe disease using receiver operating characteristic analysis. **Results:** Severe cases were significantly older than non-severe cases (42.15 ± 19.69 versus 25.66 ± 6.39 years; $p < 0.0001$). The rural population (68.4%) and non-vaccinated patients (76.4%) had more severe disease. However, there was no significant difference between severe and non-severe cases in the mean serum FOXP3 level (2.507 ± 2.742 versus 1.827 ± 1.697 ; $p=0.1977$). No differences were detected between the mean FOXP3 concentrations in either severity group in relation to sex. **Conclusion:** Serum FOXP3 levels showed no significant correlation with COVID-19 severity in this cohort and may not prove to be a standalone severity marker. Factors independently associated with severe disease were older age, rural residence, and non-vaccination status.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) produces a wide range of clinical illnesses, from asymptomatic infection and upper airway symptoms to severe viral pneumonia, acute hypoxemic respiratory failure, and acute respiratory distress syndrome [1–3]. Vaccination and better clinical care have decreased the incidence of severe respiratory illness; however, a subset of infected patients develops progressive respiratory illness with impaired gas exchange, widespread infiltrates in the lungs, and systemic inflammation [2]. While viral

factors are important, host-related factors, such as age, sex, comorbidities, vaccination status, and the magnitude and regulation of the immune response, all play a role in determining the severity of COVID-19. Recent clinical guidance still focuses on the early identification of patients at risk of clinical deterioration [4, 5].

One of the hallmarks of severe COVID-19 is a poorly regulated immune response. An antiviral response involves complementing innate and adaptive immune mechanisms to clear the infection and avoid unnecessary tissue damage. However, in severe disease, there is the production of pro-inflammatory mediators, disturbance of lymphocyte homeostasis, endothelial injury, and recruitment of inflammatory cells that may lead to diffuse pulmonary inflammation and respiratory impairment [4, 6]. A decrease in the number of lymphocytes and an imbalance of T-cell subpopulations have been repeatedly correlated with disease severity. Additionally, recent blood-based immunophenotyping efforts have found that COVID-19 patients have decreased numbers of regulatory T-cell subsets, such as FOXP3+ Tregs, with the lowest numbers seen in severe COVID-19 [6].

Regulatory T cells (Tregs) are a specific type of CD4+ T cells that regulate and prevent excessive immune activation. These cells require the transcription factor forkhead box P3 (FOXP3) to develop, remain stable, and have immunosuppressive activity. FOXP3+ Tregs could contribute to the prevention of the switch from protective antiviral inflammation to immune-mediated tissue damage by regulating effector T cells, antigen presenting cells, inflammatory cytokines, and tissue repair responses. This role is especially important in COVID-19, as pulmonary inflammation and damage due to cytokines are key factors of the severe respiratory symptoms. Recent reviews suggest that decreases in Treg cell numbers and functional activity of FOXP3 are associated with increasing severity of systemic inflammation and respiratory disease during acute SARS-Cov-2 infection, and may occur in parallel [7].

However, the role of FOXP3 in the severity of COVID-19 remains unclear. Conflicting data from published studies show some reporting a decrease in circulating Tregs or FOXP3 expression in the most severe phases of the disease, while others report a relatively increased proportion of FOXP3+ cells in some disease phases. These differences may stem from differences in the time of sampling, stage of the disease, age, treatment exposure, and/or differences in the testing platform and testing method, whether by looking at Treg-cell frequency, absolute count of Treg cells, FOXP3 gene expression, or concentration of circulating serum FOXP3 [7]. This uncertainty was accentuated by a recent longitudinal study that showed that absolute Treg and FOXP3+ cell numbers were reduced early in severe disease, but with variable relative cell frequencies within and between cohorts and time points [8].

Notably, little evidence exists to elucidate the clinical relevance and diagnostic value of serum FOXP3 concentration, particularly in localized Middle Eastern populations with severe nasal and respiratory symptoms of COVID-19. The majority of studies performed have been on flow cytometric Treg counts or on measuring the expression of FOXP3 mRNA, as opposed to measuring it in serum by ELISA. Hence, additional studies are required to assess whether there are differences in serum FOXP3 between severe and non-severe disease and whether the serum level of FOXP3 can help to differentiate between disease severity. This study aimed to determine the levels of serum FOXP3 in COVID-19 patients with severe and non-severe infections, explore the association between serum FOXP3 levels and demographic and clinical parameters, and test the discriminatory potential of serum FOXP3 in differentiating between severe COVID-19 infection and non-severe infection using receiver operating curve (ROC) analysis.

2. Methods

2.1. Study design and setting

This cross-sectional comparative study was performed to determine the level of serum Forkhead box P3 (FOXP3) in patients with confirmed coronavirus disease 2019 (COVID-19) and to investigate the correlation between the level of serum FOXP3 and clinical severity and selected demographic characteristics. Patients were recruited in the study during November and December 2021 in Al-Hillah area, Babylon Province, Iraq. Blood samples were collected from patients at the Marjan Medical City and Al-Mustaqbal Laboratory under the guidance of trained doctors. This study population comprised 76 patients with confirmed COVID-19 infection, either clinically or by laboratory testing, and was divided evenly between severe and non-severe patients (38 each).

2.2. Case definition and classification of severity

Diagnosis of COVID-19 was confirmed based on clinical symptoms, combined with laboratory findings. Patients were grouped as having severe or non-severe COVID-19 based on respiratory conditions and chest imaging. Severe COVID-19 was defined as patients with significant nasal and lower respiratory tract symptoms, plus one or more of the following:

- A respiratory rate ≥ 30 breaths per minute.
- Low peripheral oxygen saturation (P.B.S.) $< 93\%$
- Pulmonary infiltrates in chest CT that encompass $>50\%$ of the lung fields.

Patients whose COVID-19 infection was confirmed but did not meet the above criteria were classified as belonging to the non-severe group.

2.3. Participants and eligibility criteria

Seventy-six patients (38 severe and 38 non-severe) were enrolled. The age range of the participants was wide (18–96 years). Demographic and clinical information gathered for every patient comprised age, sex, rural or urban residence, vaccination status, and severity of COVID-19 disease. Patients with other illnesses that might significantly impact inflammatory or immune parameters were excluded. These conditions include hepatitis, anemia, renal failure, type 1 diabetes mellitus, and type 2 diabetes mellitus. These conditions were excluded to minimize possible confounding of the serum FOXP3 concentration and severity of COVID-19.

2.4. Blood sampling and serum preparation

Fresh venous blood was aseptically drawn from each participant in approximately 3 mL volume. Samples were placed in plain, non-anticoagulant tubes to clot and separate the serum. The serum was then used to quantify the amount of FOXP3.

2.5. Measurement of serum FOXP3

Serum FOXP3 levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit based on the sandwich ELISA principle. Human FOXP3 pre-coated micro-ELISA plates were used. After adding patient serum and standards to the wells, FOXP3 molecules (where present) bind to the immobilized capture antibodies. Avidin-IR was then added, as were a biotinylated detection antibody specific for human FOXP3. Following the incubation, the unbound material was washed off. The substrate solution was then added to the wells. The wells with the antigen–antibody complex for FOXP3 developed a blue color and turned yellow after the addition of the stop solution. The optical density was read at a wavelength of 450 nm using a spectrophotometer with a maximum deviation of ± 2 nm. The serum FOXP3 level was determined by measuring the optical density of the serum sample and comparing it with the value obtained from the standard curve.

2.6. Demographic and clinical variables

Continuous and categorical age groups were also analyzed: 18–25, 26–40, 41–60, and 61–96 years. Sex was determined as male or female. Geographic place of residence was defined as rural or urban. Vaccination status was defined as vaccinated (two doses) or unvaccinated (0–1 doses) for the children. To describe and compare, the distribution of these variables was analyzed in severe and non-severe cases. In addition, reported data indicated that the mean age for the severe group was higher than that for the non-severe group, and vaccination status and geographic residence were also different between the two groups.

2.7. Statistical analysis

The data were analyzed using the IBM SPSS Statistics version 27 package software. All continuous variables, such as age and serum FOXP3 concentration, were presented as mean $\pm$ standard deviation (SD), while categorical variables were presented as frequencies and percentages. The means for the severe and non-severe COVID-19 groups were compared using an independent-samples t-test. The chi-square test was used to evaluate the relationship between categorical variables (sex, geographic residence, and vaccination status) and disease severity. Receiver operating characteristic (ROC) curve analysis was used to assess the performance of serum FOXP3 concentration in distinguishing severe COVID-19 cases from non-severe COVID-19 cases. The area under the curve (AUC), 95% confidence interval (CI), optimal cut-off value using the Youden indicator, sensitivity, specificity, positive and negative predictive value, and diagnostic accuracy were computed. A two-tailed P value of < 0.05 was deemed to be statistically significant.

2.8. Ethical considerations

All subjects or legal guardians gave verbal informed consent prior to enrollment and blood draw. All the study procedures were carried out under medical supervision and the information of the participants was processed in a confidential manner. The study was carried out after obtaining approval from Research Ethics Committee of Hammurabi College of Medicine University of Babylon – Babylon – Iraq. The study did not have a conflict of interest.

3. Results

Severe and non-severe COVID-19 patients were included in this study, and the total number of patients was 76, of which 38 were severe and 38 were non-severe. The demographic and clinical features of the patients based on disease severity are summarized in Table 1.

The mean ages of patients with severe and non-severe COVID-19 were 42.15 ± 19.69 and 25.66 ± 6.39 years, respectively. Non-severe cases were most common in the youngest age group (18–25 years, 24/38, 63.2%), while severe cases were more common in older age categories. In particular, all individuals aged 61–96 years were deemed to have severe disease (9/9, 100%).

As shown in Figure 4, the distribution of severe cases increased with age. Severely and non-severely ill patients were mostly male. Among the severe cases, 23 patients (60.5%) were male and 15 (39.5%) were female. Similarly, 24 non-severe patients (63.2%) were male and 14 (36.8%) were female.

The distribution of cases by sex and severity is shown in Figure 2, in which the sex distributions of the two groups were broadly similar. The correlation between residence and disease severity was significant. Twenty-six severe cases were in a rural setting (68.4%), and 12 were in an urban setting (31.6%). In contrast, urban residents had more non-severe cases than rural residents, with 29 non-severe cases (76.3%) and 9 non-severe cases (23.7%). There was also some separation of severity groups by vaccination status. 29 (76.3%) of patients had no vaccination, and 9 (23.7%) had received vaccination among severe COVID-19 cases. Of the 26 patients in the non-severe group, 26 (68.4%) had received vaccination, and 12 (31.6%) had not been vaccinated. ROC curve analysis was conducted to determine the ability of serum FOXP3 concentration to distinguish severe from non-severe COVID-19 cases Figure 1.

When aggregated, severe disease was more frequently seen in those who were not vaccinated. Serum FOXP3 levels for each disease severity and sex are presented in Table 2 and Figure 3. Although the mean total serum FOXP3 level was higher in severe cases than non-severe cases (2.5 ± 2.7 vs 1.8 ± 1.7), this difference did not reach the level of statistical significance. ($p=0.19$ $p=0.19$). A significant difference was, however, not seen in the mean FOXP3 concentration in the severe group (2.9 ± 3.3) compared to female patients (1.8 ± 4.5), $p=0.4$. Likewise, the level of FOXP3 in non-severe cases did not differ between males and females (1.9 ± 2.1 vs. 1.8 ± 0.9 ; $p=0.80$, $p=0.80$). When serum FOXP3 levels were compared between the severe and non-severe cases, there was no significant difference between the levels of the protein in males ($p=0.18$, $p=0.18$), females ($p=0.93$, $p=0.93$), or the total study population ($p=0.19$, $p=0.19$).

Table 1: Demographic and clinical characteristics of patients with COVID-19 according to disease severity

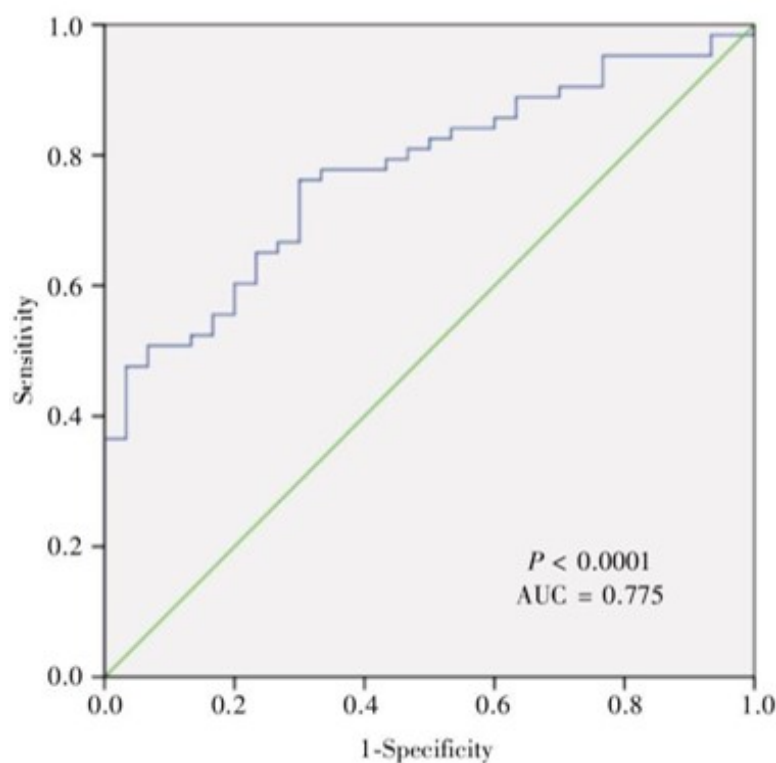
Characteristic	Severe (n = 38)	Non-severe (n = 38)	Total (N = 76)
Age (years), mean $\pm$ SD	42.15 $\pm$ 19.69	25.66 $\pm$ 6.39	-
Age group, n (%)			
18-25 years	13 (34.2%)	24 (63.2%)	37 (48.7%)
26-40 years	8 (21.1%)	11 (28.9%)	19 (25.0%)
41-60 years	8 (21.1%)	3 (7.9%)	11 (14.5%)
61-96 years	9 (23.7%)	0 (0.0%)	9 (11.8%)
Sex, n (%)			
Male	23 (60.5%)	24 (63.2%)	47 (61.8%)
Female	15 (39.5%)	14 (36.8%)	29 (38.2%)
Residence, n (%)			
Rural	26 (68.4%)	9 (23.7%)	35 (46.1%)
Urban	12 (31.6%)	29 (76.3%)	41 (53.9%)
Vaccination status, n (%)			
Vaccinated	9 (23.7%)	26 (68.4%)	35 (46.1%)
Unvaccinated	29 (76.3%)	12 (31.6%)	41 (53.9%)

- Values are presented as mean $\pm$ standard deviation or numbers (percentages).
- Percentages were calculated within each severity group, except in the Total column.

Table 2: Serum FOXP3 concentrations by severity and sex

Severity	Sex	Number	FOXP3 mean $\pm$ SD	Within-group p-value
Severe	Male	22	2.9 $\pm$ 3.3	0.4
	Female	16	1.8 $\pm$ 4.5	
	Total	38	2.5 $\pm$ 2.7	
Non-severe	Male	23	1.9 $\pm$ 2.1	0.8
	Female	15	1.8 $\pm$ 0.9	
	Total	38	1.8 $\pm$ 1.7	

Additional reported P-values: Male comparison = 0.18; Female comparison = 0.93; Total comparison = 0.19.

**Figure 1:** Receiver operating characteristic (ROC) curve of serum FOXP3 concentration for discrimination between severe and non-severe COVID-19 cases

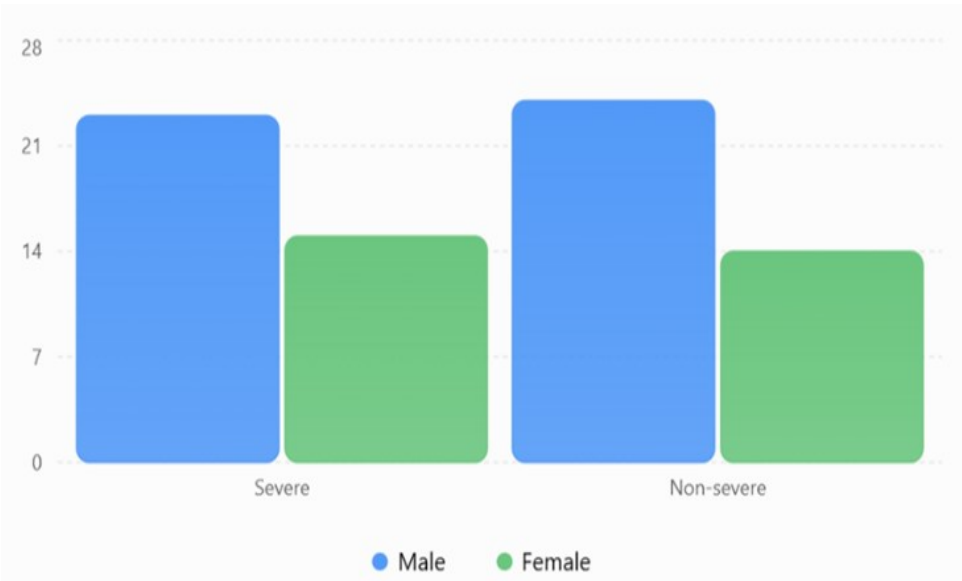


Figure 2: Distribution of COVID-19 cases by sex and severity

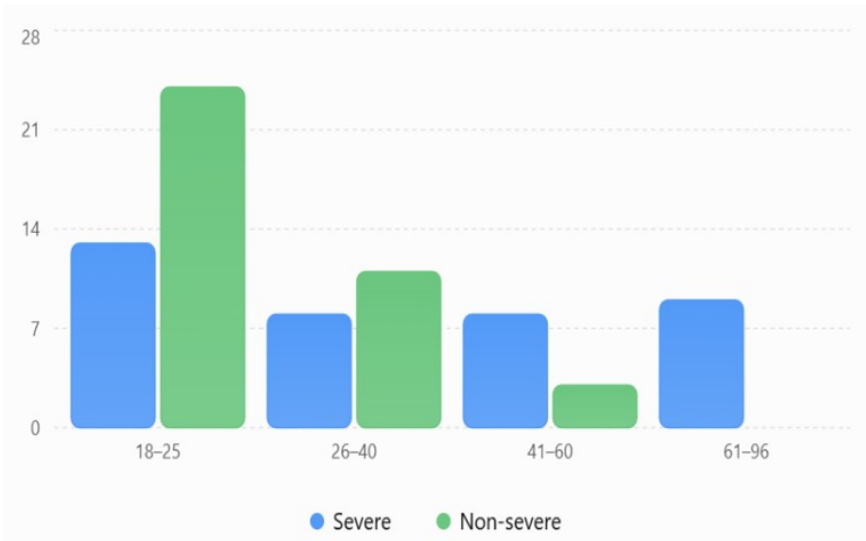


Figure 3: Distribution of COVID-19 cases by age group and severity

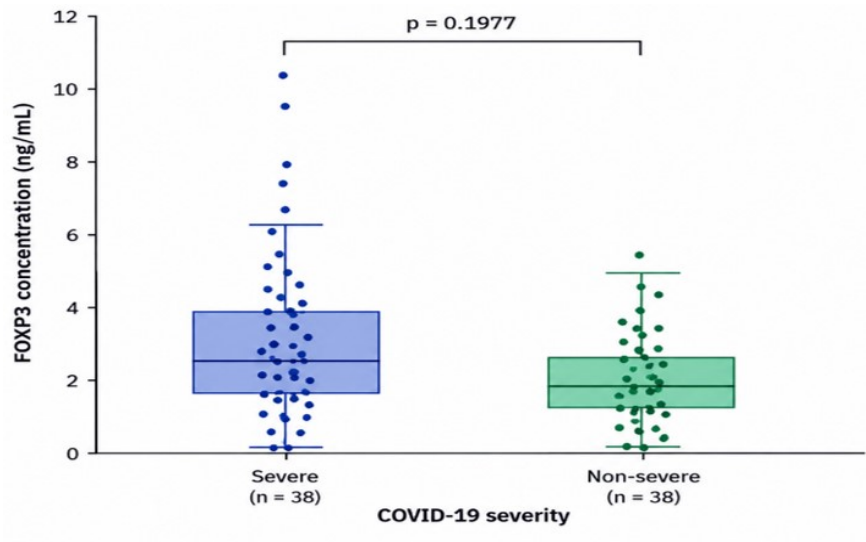


Figure 4: Distribution of serum FOXP3 concentrations by COVID-19 severity

4. Discussion

Cross-sectional evaluation of the serum level of FOXP3 in 76 confirmed COVID-19 patients, including 38 severe and 38 non-severe cases. Patients older than 18 years, rural residents, and those who were not vaccinated had higher rates of developing severe disease, but the mean serum FOXP3 was not significantly different between the severe and non-severe disease groups ($p=0.19$). Both severe and non-severe cases had a mean concentration of FOXP3, but in severe cases, the mean FOXP3 was numerically higher than in the non-severe cases (2.507 ± 2.742 versus 1.827 ± 1.697 , respectively). Nevertheless, this difference was not statistically significant as the principal transcription factor that supports the development, stability, and suppressive function of regulatory T cells (Tregs) that limit excessive immune activation and maintain immune homeostasis [9]. During acute SARS-CoV-2 infection, lower levels of circulating Tregs and decreased FOXP3 expression have been noted, which may be associated with increased systemic inflammation, especially in severe disease [7].

The association between FOXP3-mediated regulation and the severity of COVID-19 is inconsistent among studies. Apparent discrepancies between these results may occur due to several factors, including differences in sampling time, disease stage, age, treatment exposure, viral variant, and laboratory method, since both the concentration of the serum FOXP3 and the intracellular expression of FOXP3, as well as the frequency of Tregs and their suppressive activity, are distinct biological processes [8].

A recent longitudinal study identified variations in Treg and FOXP3-related patterns based on age, sex, and stage of the disease, but did not show a consistently higher percentage of FOXP3⁺ cells in the mild and severe COVID-19 groups [8]. This finding provides support for the interpretation that a non-significant difference in the present study might be due to the dynamic and heterogeneous nature of regulatory immune responses in acute COVID-19. The higher mean serum FOXP3 level in severe cases should therefore not be viewed as an increase in effective immune regulation, but rather as a measure of the severity of the disease. It could also be an indicator of compensatory immune activation, release of intracellular material in response to tissue injury, the timing of blood collection, or analytical variation of the protein circulating in blood rather than of direct FOXP3 expression of cells.

4.1. Age and sex

In the present study, age was a significant factor related to severity. The mean age of severe COVID-19 was significantly higher than non-severe COVID-19 (42.15 ± 19.69 years versus 25.66 ± 6.39 years; $p < 0.0001$), and all patients aged ≥ 61 years were classified as severe. Severe COVID-19, hospitalization, and mortality were strongly associated with advanced age. Recent real-life data further support that the risk increases significantly with age, especially from 60 years onward, due to immunosenescence, loss of physiologic reserve, accumulation of comorbidities, and dysregulated inflammatory response [10]. Additionally, age-associated changes in the immune system may affect the number and function of Tregs associated with FOXP3. The loss of circulating Treg subsets during acute SARS-CoV-2 infection is associated with systemic inflammation and severity [7].

In this study, males were overrepresented in both disease-severity groups, and individuals with severe disease had a higher mean level of FOXP3 than those with severe disease, but this difference was not statistically significant. In the current study sample, disease severity did not significantly affect the relationship between sex and serum FOXP3 levels. Factors other than sex (immune system regulation, cardiometabolic risk, smoking exposure, and health care factors) may influence the risk of having severe COVID-19 in a sex-specific manner. When assessing clinical outcome differences, biological age and host inflammatory responses may be more important than sex, as suggested by recent analyses, where relevant confounding factors have been taken into account [10].

4.2. Geographic residence and vaccination

Rural residents had a higher rate of severe COVID-19, whereas urban residents had a higher rate of non-severe COVID-19. In terms of distribution by area, the severity of the case was associated with living in a rural area (68.4% vs. 23.7%), with the difference being statistically significant. This is not a direct biological factor in having severe COVID-19 living in rural areas. Rather, the differences observed with rural location can be attributed to variations in access to diagnostic testing, timely clinical evaluation, oxygen therapy, hospital referral, vaccination services, socioeconomic resources, and burden of underlying disease. The consistently higher post-infection mortality rates among people living in rural areas have been recently reported in population-based samples, even after several baseline and community-level risk factors were accounted for [11].

In the current study, severity was significantly associated with vaccination status. Of those who suffered severe illness, 76.4% were not vaccinated, while 68.4% of those with non-severe illness were vaccinated. This finding is in line with existing data showing that COVID-19 vaccines lower the risk of disease severity following infection. Recent systematic reviews and meta-analyses conducted worldwide have demonstrated that clinical severity was reduced in breakthrough infections with vaccines, and updated vaccine effectiveness estimates have maintained significant protection against COVID-19-associated hospitalization and critical outcomes [12, 13].

The association in the present study should be interpreted with caution because information on vaccine product, dose number, time since vaccination, previous infection, and treatment history were not reported. These factors may significantly affect the level of protection and should be included in future multivariable analyses [13].

4.3. Implications and limitations

The lack of significance of the comparison between serum FOXP3 levels suggests that it is not an adequate marker of COVID-19 severity in clinical practice. The ROC analysis further confirms the conclusion that serum FOXP3 has poor independent diagnostic value in this cohort, as the AUC was not significant and the sensitivity and specificity were rather low. A clinically relevant prediction model could include age, oxygen saturation, inflammatory markers, the ratio of lymphocytes to neutrophils, vaccination status, and pertinent comorbidities along with FOXP3. There is current evidence favoring the use of multiple clinical and laboratory risk factors instead of a single biomarker for risk stratification of severe COVID-19 outcomes [14].

These are the limitations of the study due to its cross-sectional design, small sample size, and recruitment from one region, which makes it difficult to interpret cause and effect and to generalize. Furthermore, rather than measuring the expression of FOXP3 in the serum at one time point, it was measured using a single time point, and the expression of intracellular FOXP3, Treg count, Treg phenotype, or functional

suppressive capacity were not serially measured. Limited data on disease duration, viral variant, medication exposure, corticosteroid use, comorbidities, previous infection, vaccine type, vaccine dose, and time since vaccination may have resulted in residual confounding. Longitudinal immune profiling and adjustment for multivariable models in prospective, multicenter studies are needed to determine if serum FOXP3 is a prognostic marker in addition to known clinical factors [7, 8].

5. Conclusion

This study aimed to assess the serum FOXP3 concentration in patients with severe and non-severe COVID-19 and its correlation with demographic and clinical parameters. The difference in the mean serum FOXP3 level between severe and non-severe cases was not statistically significant. Thus, serum FOXP3 concentration measured at one time point does not seem to be a good independent biomarker to distinguish patients with severe and non-severe COVID-19 in this cohort. Older age, rural location, and not being vaccinated were significantly associated with severe disease. These results highlight the need for immunization and prompt clinical evaluation of patients with a high risk of deterioration of their respiratory status. The observed variations in levels of FOXP3 could be due to the dynamic nature of regulatory T cell responses during acute infection. However, larger prospective multicenter studies are needed to further elucidate the prognostic significance of FOXP3 expression in the severity of COVID-19, which should include serial measurements of FOXP3 expression, flow-cytometric analysis of regulatory T cells, and multivariable prediction models.

Article Information

Ethical Approval: Ethical approval was obtained from the Research Ethics Committee of Hammurabi College of Medicine, University of Babylon, Babylon, Iraq, prior to commencement of the study.

Informed Consent: Written informed consent was obtained from all participants before their inclusion in the study.

Consent for Publication: Not applicable.

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Clinical Trial Registration: Not applicable.

Conflict of Interest: The authors declare that they have no conflict of interest.

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AI Use Disclosure: No generative artificial intelligence tools were used in the preparation of this manuscript.

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