ORIGINAL ARTICLE

Toxoplasma gondii as a possible risk factor for COVID-19 severity: a case-control study

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ABSTRACT

Background: *COVID-19 is a worldwide pandemic that stroke almost all countries of the world causing thousands of deaths and disabilities and burdened the economy of countries. One of the main criteria of the immune response against COVID-19 is the "immune exhaustion", due to increased expression of T cell suppressor molecules e.g. programmed death-1 (PD-1), that leads to flaring of viral multiplication and disastrous clinical outcomes. This immune exhaustion is not restricted to COVID-19 but is also a common complication of chronic infections with the widely spreading protozoan, Toxoplasma (T.) gondii. Thus, theoretically, the toxoplasmosis-associated immune exhaustion can worsen that of COVID-19 and consequently increases its severity. However, the studies on this theory are still insufficient.* ***Objective:*** *this work was designed to answer two questions. Does T. gondii co-infection affect the severity of COVID-19 manifestations? Is this action related to T. gondii-induced PD-1 changes?* ***Methodology:*** *Covid-19$^+$patients with moderate and severe conditions were screened for T. gondii IgG and compared to healthy controls. Serum levels of IL-1β, IL-6, TNFα, IFN-γ, IL-1α cytokines were assessed to evaluate COVID-19 severity and prognosis. Lymphocytic expression of PD-1 was assessed by flowcytometry.* ***Results:*** *We recorded a higher incidence of toxoplasmosis among COVID-19 patients especially patients with severe/critical manifestations. T. gondii positive cases exhibited a statistically significant increase in lymphocytic expression of PD-1 that correlated positively with the proinflammatory and bad prognosis cytokines. With fixation of other risk factors for severity, toxoplasmosis still scored a significant value.* ***Conclusion:*** *toxoplasmosis increased the severity of COVID-19. These effects can be related to the Toxoplasma-associated increased lymphocytic PD-1 expression. So, toxoplasmosis can be considered as an unrecognized independent risk factor for COVID-19 severity.*

INTRODUCTION

The coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has raised a global health emergency. This pandemic has affected millions of world's populations and resulted in thousands of deaths all-over the world. Moreover, depression and economic crisis became common sequelae to the lockdowns which were applied as a trial to "flatten the curve" of the rising cases[1,2].

The clinical manifestations of COVID-19 are a reflection to the behavior of the immune system. The immune response which diverses between individuals is the cause of the diverse clinical manifestations of the disease. While in some individuals, the COVID-19 disease remains asymptomatic, other individuals present severe complications, such as interstitial pneumonia and respiratory failure [3].

The immune response to COVID-19 is mainly driven by T lymphocytes especially CD8+ ones. The massive activation of lymphocytes that occurs in the early stage of infection is usually rapidly changed into a lymphopenia state due to the occurrence of apoptosis and cell senescence, a phenomenon that is called "lymphocyte exhaustion". It occurs due to increased expression of apoptosis inducing molecules e.g. programmed death-1 (PD-1). PD-1 expression is a critical checkpoint for T cell exhaustion. It is a crucial molecule for the induction and maintenance of peripheral tolerance, and for keeping T cells stable and healthy but its increased expression mediates progressive inhibitory signals to proliferation and function of T effector cells, metabolic dysregulation, poor memory and homeostatic self-renewal. These losses that occur in the first line of defense against the virus is the cause of the massive increase in the viral load that is presented clinically as severe clinical manifestations which may end in loss of patient's life[4,5,6].

Although most of cases range between mild to moderate manifestations, the percentage of severe cases

and deaths is still high. Many demographic criteria and comorbidities were claimed to be risk factors for disease severity and even death of COVID-19 positive patients. Patients having one or more risk factors usually receive more care because they are more vulnerable to complications and even death. The main risk factors concluded by many studies are, old age, male gender, cardiovascular disease, hypertension, diabetes, obesity, respiratory diseases, renal impairment, pregnancy, cancers, autoimmune and immune-suppressive diseases [7]. Till now, co-infections with protozoa were not included as risk factors even if some of them is associated with a similar immune exhausting condition like *Toxoplasma gondii* infections.

Toxoplasma gondii is a world-wide obligatory intracellular protozoan that affects more than 30% of world's population. It can infect any vertebrate animal and remains quiescent in his tissues until his immunity is suppressed [8,9]. Chronic infection is associated with gradually increasing lymphocytic expression of PD-1 which leads to subsequent apoptosis of memory T lymphocytes. This can lead to reactivation of latent infections [10].

Because both COVID-19 and *T. gondii* share a common point of immune reaction which is "lymphocytic exhaustion", *T. gondii* can aggravate COVID-19 manifestations theoretically. The current work was designed to answer two questions. Does *T. gondii co*-infection affect the severity of COVID-19 manifestations? Is this action related to *T. gondii*-induced PD-1 changes?

METHODOLOGY

Ethical considerations:

This study was approved by the Research Ethics Committee of Faculty of Medicine, Menoufia University. The aim of the study was explained to all participants and informed consents were obtained from patients or their guardians according to their condition.

Subjects and study design:

The present study was a case-control study. It was performed on 100 COVID-19 patients admitted to the Isolation Department of Shibin El-Kom Educational Hospital with moderate or severe/critical manifestations. Duration of the study extended from April 2020 to June 2020. All patients had a positive throat swab PCR for COVID-19. Exclusion criterion was the negative PCR even if there is a radiological and clinical suspicion. All patients underwent full history taking, clinical examination and they were classified according to the National Health Commission of the People's Republic of China into moderate and severe/critical patients[11]. Moderate patients had fever, respiratory tract symptoms, and their imaging showed pneumonia. Severe patients met any of the following: a) respiratory distress i.e. respiratory rate ≥ 30 beats/min; b) means oxygen saturation in the resting state ≤ 93%; c) arterial blood oxygen partial pressure/ fractional inspired oxygen ≤ 300 mmHg (PaO2/FiO2); d) pulmonary imaging showed that the lesion progressed more than 50% within 24–48 h. Critical patients were diagnosed when they had one of the following conditions: a) respiratory failure and requirement of mechanical ventilation; b) Shock c) ICU admission is required for combined organ failure. A control group of 50 healthy non-COVID-19 participants with matched age and sex was included.

Participants were divided into three main groups, 50 participants each. GI: healthy controls (HC). GII: COVID-19$^+$ patients with moderate severity. GIII: COVID-19$^+$ patients with severe or critical manifestations. Each group was further subdivided into two subgroups. All the "a" subgroups were negative for *T. gondii* IgG. All the "b" subgroups were positive for *T. gondii* IgG.

Assessment of T. gondii positivity:

Serum samples of all participants were analyzed for anti-*T. gondii* IgG antibodies using commercial enzyme immunoassay, Human Anti-*T. gondii* IgG kit (ab108776, Abcam, USA) in accordance with manufacturer's recommendations. Positive samples were analyzed for anti-*T. gondii* IgM antibodies using Human Anti-*T. gondii* IgM Kit (ab108778, Abcam, USA) to exclude acute infection. Anti-*T. gondii* IgG antibody levels were expressed as U/ml.

Assessment of cytokines in sera of the participants:

The serum levels IL-1β, IL-6, TNFα, IFN-γ, IL-1α, and IL-10 were quantified using Multiplex ELISA Kit for Human Cytokine Panel 1 (Bosterbio, Pleasanton, California). Samples were run in duplicates. Data represented the mean of two technical replicates. The Protocol was recommended by the manufacturer.

Assessment of PD-1 expression on T lymphocytes by flow cytometry:

25 µl of EDTA-anti-coagulated blood was mixed thoroughly with 2 µl of the following, mouse anti-human monoclonal antibodies, CD279 (PD-1) phycoerythrin, PE (Miltenyi Biotec, USA) and CD3 fluorescein isothiocyanate, FITC (BD Biosciences, USA). Blood samples were incubated for 20 min at room temperature in the dark. Red blood cells were lysed by adding 1 ml of lysing solution for 5 min. Then, samples were washed twice using phosphate buffered saline (PBS) and finally, the cells were suspended in 200 µl of PBS for flow cytometric analysis.

PD-1$^+$CD3$^+$ cell percentage was determined by analysis on FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), gating was done on lymphocytes using side versus forward scatter and at least 10,000 events were acquired.

Statistical analysis:

SPSS was used for data analysis (SPSS 20.0 for Windows (SPSS, Inc, Chicago, Ill). The descriptive

statistics were presented as mean and standard deviation for continuous data. The statistics for categorical variables were expressed as counts and percentages. ANOVA test was performed for comparison of more than two groups having normally distributed continuous variables, and the chi square test and was used for categorical variables. Pearson correlation was used to evaluate the linear relationship between two continuous variables. Multivariable binary logistic regression analyses were used to assess the association between age, gender, comorbidities and *T. gondii* infection, and the dependent variable of COVID-19 severity. The odds ratio (OR) along with the 95% CI were reported. The level of significance of the present data was 95%, so, p-value >0.05 was considered a non-statistically significant difference, while p-value < 0.05 was considered a statistically significant difference.

RESULTS

All the recorded cases were over 50 years old. Most of severe manifestations occurred in patients over 60 years while most of patients with moderate manifestations were below 60 years. The difference between mean ages of both groups was a statistically significant one (p<0.001). No statistically significant difference was detected between groups regarding either gender or co-morbidities (Figures 1 & 2).

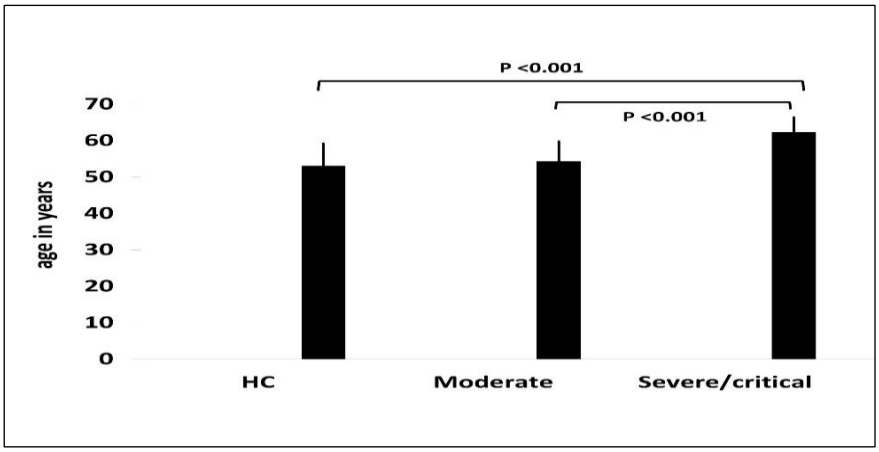

Fig. 1: Comparison between mean ages of the studied groups.

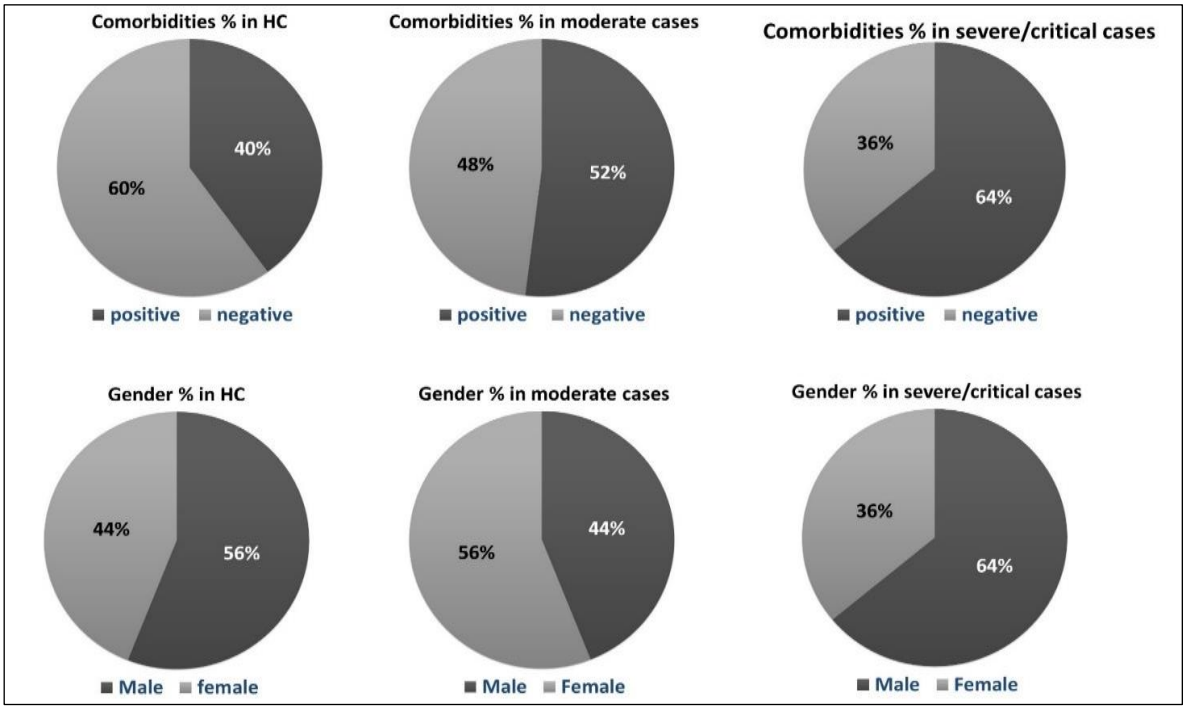

Fig. 2: Comparisons between percentages of comorbidities and gender differences of the studied groups

Prevalence of toxoplasmosis was higher in COVID-19+ groups. A statistically significant difference was present when comparing severe cases to HC. Also, the number of positive cases was higher in the severe than the moderate group with a statistically significant difference between them ($p< 0.05$). Regarding serum levels of *T. gondii* IgG, the IIIb group (severe/critical cases) ranked the 1st followed by the group of moderate cases (IIb) with a statistically significant difference between both groups ($p<0.001$). HC scored the lowest values with statistically significant differences compared to both COVID-19 ($p<0.001$) (Figure 3).

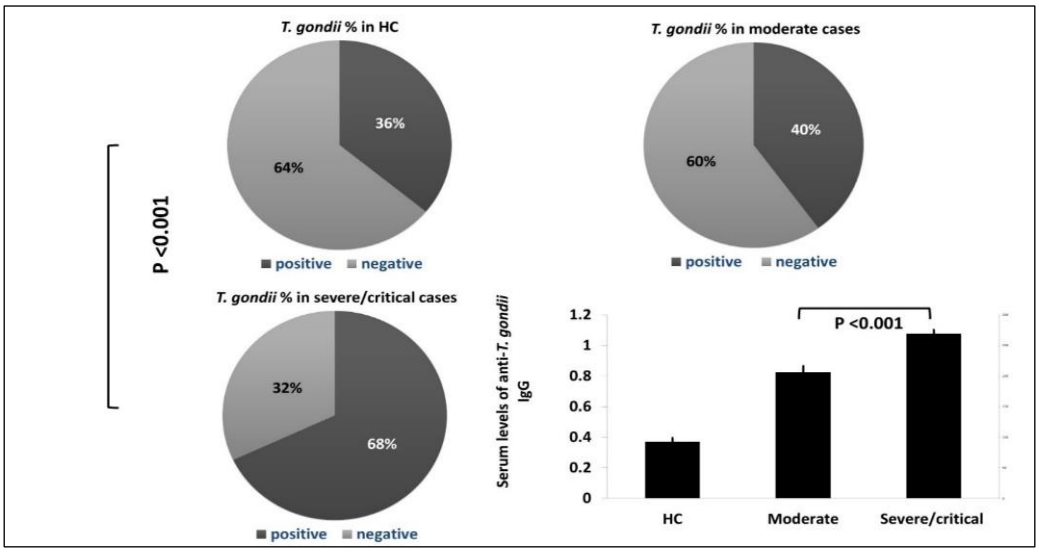

Fig. 3: Comparisons between percentages of *T. gondii* infections and its serum levels of the studied groups.

The serum levels of IL-1β, IL-6, TNFα, IFN-γ, IL-1α, and IL-10 cytokines were the highest in the group of severe/critical cases compared to other studied groups ($p<0.001$). Moreover, the highest records belonged to the *T. gondii* positive subgroup, IIIb that showed a statistically significant higher difference compared to the *T. gondii* negative subgroup, IIIa ($p<0.001$). The same goes for the moderate cases where the *T. gondii* positive subgroup, IIb showed a statistically significant higher difference compared to the *T. gondii* negative one, IIa ($p<0.001$) (Figure 4).

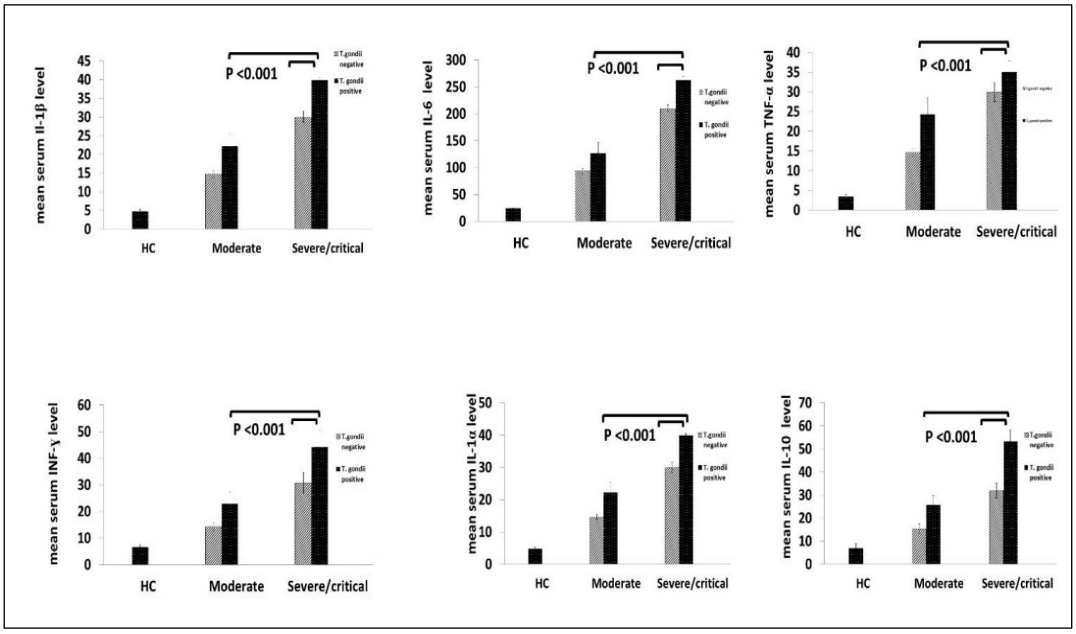

Fig. 4: Comparison between mean serum levels of cytokines of the studied groups

The group of severe cases achieved the highest score of PD-1 expression on their T lymphocytes especially with *T. gondii* positive patients (i.e. GIIIb) with a statistically significant difference compared to the negative one, GIIIa (p<0.001). Also *T. gondii* positive subgroup, IIb of the moderate cases showed a statistically significant higher difference compared to the negative subgroup, IIa (p <0.001) (Figure 5).

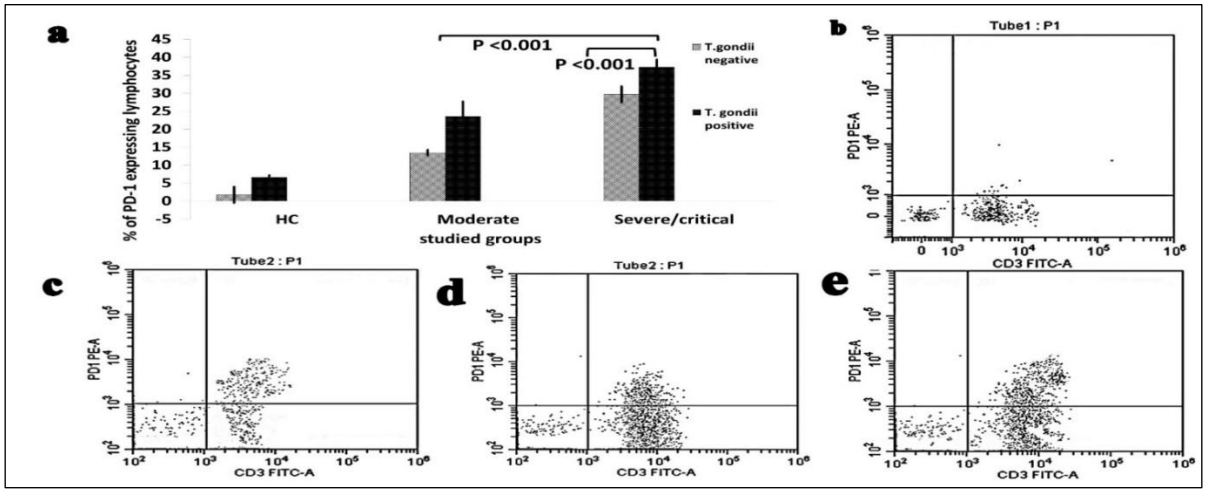

Fig. 5: Lymphocytic expression of PD-1.

a. Comparison between percentage of lymphocytic PD-1 expression in the studied groups.
b. Flowcytometry graph of lymphocytic PD-1 expression in a normal participant.
c. Flowcytometry graph of lymphocytic PD-1 expression in a moderate COVID-19/*T. gondii* positive patient.
d. Flowcytometry graph of lymphocytic PD-1 expression in a severe COVID-19/*T. gondii* negative patient.
e. Flowcytometry graph of lymphocytic PD-1 expression in a severe COVID-19/*T. gondii* positive patient.

A statistically significant positive correlations were detected between lymphocytic PD-1 % and serum levels of anti-*T. gondii* IgG, IL-1β, IL-6, TNF-α, IFN-γ, IL-1α, and IL-10 cytokines. (Figure 6).

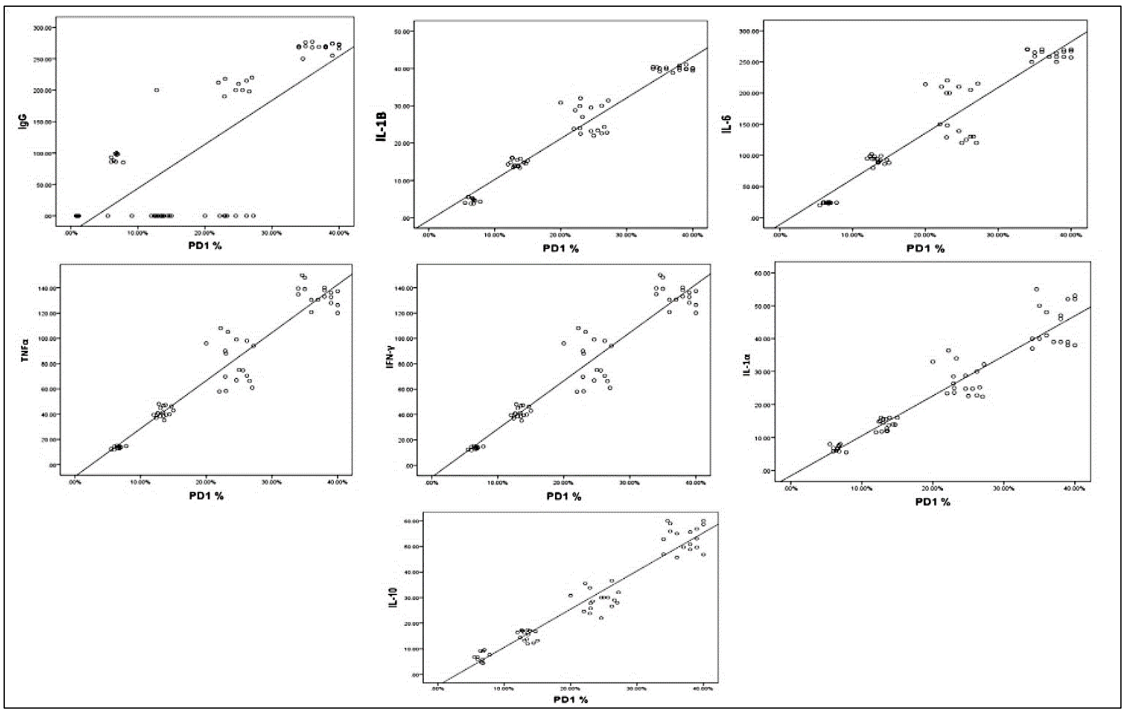

Fig. 6: Graphical correlation between lymphocytic PD-1 expression and anti-*T. gondii* IgG and bad prognosis cytokines

Toxoplasma achieved significant results even after control of demographic variables and other comorbidities by the Wald test. In the multivariate analyses, old age over 60 years, and other comorbidities were also positively associated with the risk of severity of COVID-19 infection. (Table 1).

Table 1: Binary logistic regression analysis for severity among patients with COVID-19

Variables	Wald	P value	Odds Ratio	95 % CI	
				Lower	Upper
Age ≥ 60 years **vs** < 60 years	11.87	0.001**	4.93	2.74	9.25
Gender Males vs females	0.12	0.73	0.79	0.21	3.02
Comorbidities Present vs absent	3.14	0.04*	2.75	1.06	5.32
***Toxoplasma* infection** Positive vs negative	4.94	0.02*	4.83	1.21	19.42

DISCUSSION

The current work aimed to investigate the impact of *T. gondii* co-infection on the severity of COVID-19 manifestations and the possible mechanisms of its action to decide if this widely spreading protozoan can be considered as a risk factor for COVID-19 severity. Its wide prevalence can explain the danger if this pathogen can really affect COVID-19 severity. Only moderate and severe cases were included in the study population because mild cases were isolated at their homes according to the protocol of the Ministry of Health [12]. Chronicity of toxoplasmosis was diagnosed by presence of specific IgG with absence of IgM [13].

The noticed increased incidence of severe manifestations with increased age can be explained by the associating aging of the immune system and increased liability to malfunction and apoptosis of immune cells with subsequent increase in growth and multiplication of the virus [3].

The increased prevalence of toxoplasmosis in COVID-19 patients compared to the HC, especially the severe cases, and the statistically significant difference between severe and moderate groups refer to the presence of a link between toxoplasmosis and COVID-19. This link appeared clearly on comparing the estimates of serum anti-*T. gondii* IgG that increased in COVID-19 patients and even was statistically higher in severe cases. This finding can be a sequala of the "immune exhaustion" induced by COVID-19 that enhanced the reactivation of chronic toxoplasmosis [14,15].

The serum levels of cytokines are important markers of prognosis of COVID-19 and development of the "cytokine storm". We've chosen the proinflammatory cytokines IL-1β, IL-6, TNFα, IFN-γ, IL-1α because of their importance as markers of severity and bad prognosis [16]. For example, IL-6 was proved to be one of the causes of rapid disease progression. Drugs that block its action are used as therapeutic candidates that proved efficiency in many COVID-19 patients [17]. Also, increased levels of both IL-6 and TNF-α are used as a prediction to a bad prognosis [18]. The choice of IL-10 was because its production is increased in severe conditions and its direct correlation to lymphocytic exhaustion and apoptosis [19,20].

The potentiating action of toxoplasmosis on COVID-19 severity can be explained by the statistically significant increase in all these measured cytokines that occurred in *T. gondii* positive groups and was the highest in the severe one.

Our theory depended on the presence of a shared point between *T. gondii* and COVID-19. This point was the increased lymphocytic expression of PD-1 and the subsequent cellular exhaustion that leads to increased activity of both pathogens [21,6]. Flowcytometric analysis was performed using CD3 gating to include both CD4 and CD8+ lymphocytes that are commonly involved in both infections[10,22]. The recorded increased lymphocytic expression of PD-1 with combined infections that was the highest in severe patients is similar to the findings of Diao et al. [23] and De Biasi et al. [6] who related lymphocytic PD-1 expression to the severity of COVID-19 manifestations. Also, the increased PD-1 in *T. gondii* groups was similarly reported by Bhadra et al. [24,14], Moretto et al. [25], Hwang et al. [21] and Xiao et al. [26]. So, supposing the presence of additive or synergistic effects of these PD-1-enhancing pathogens can explain the statistically significant increase in clinical and immunological markers of COVID-19 severity that associated *T. gondii* infection. Also, the positive significant correlation detected between increased lymphocytic expression of PD-1 and serum levels of the prognostic cytokines explained the potentiating effect of the PD-1-enhancing pathogen, *T. gondii* on COVID-19 severity and prognosis. This point was further

confirmed by the multivariate analysis where *T. gondii* achieved significant results even after control of demographic variables and other comorbidities.

Conclusion: we concluded that, *T. gondii* prevalence and activity were higher in severe/critical cases and were associated with increased serum levels of the bad prognosis cytokines. These effects can be regarded to the PD-1-dependent lymphocytic exhaustion that worsened the already present COVID-19-associated lymphocytic exhaustion. Severity depended on presence of *T. gondii* even after fixation of other risk factors. So, *T. gondii* infection can be considered as an unrecognized independent risk factor for severity.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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