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# Expression of Programmed Cell Death 1 (PD-1) as a Marker of T-Cell Exhaustion and Its Correlation with Interleukin-10 Serum Level in Patients with COVID-19

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## Abstract

**Coronavirus disease 2019 (COVID-19), which is a major global concern, is characterized by a progressive disease pattern involving diverse host immune responses. Programmed cell death marker-1(PD-1) expression, a critical checkpoint for T cell exhaustion, can be modulated by interleukin-10, which also mediates apoptotic T cell cytopenia. We aimed to measure the level of PD-1 expression and to investigate its correlation with IL-10 serum levels in modulating T cell effector function, correlating the results with the level of severity of the disease. This study involved 40 patients with COVID-19 and 20 healthy controls. Using flow cytometry, the expression of PD-1 was determined on $CD8^+$ T lymphocytes and $CD4^+$ T lymphocytes. ELISA was used to determine the levels of IL-10 in the serum. We found a remarkable decrease in T cell counts with functionally exhausted surviving T cells in the patient groups, especially in patients with severe disease. PD-1 expression increased significantly in $CD4^+$, $CD8^+$, and total T cells, showing a higher expression in $CD8^+$ T cells. The patient groups had significantly higher serum IL-10 levels than the control group. The ROC analysis demonstrated the predictive role of IL-10 levels in disease severity (65% sensitivity, 80% specificity, and AUC = 0.806). IL-10 serum levels and PD-1 expression in total T cells were positively correlated, suggesting that IL-10 participates in T cell exhaustion.**

**Keywords:** T cell exhaustion markers, PD-1, IL-10, COVID-19, SARS-CoV-2

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## INTRODUCTION

Coronavirus disease 2019 (COVID-19) is considered to be a worldwide pandemic with 2,597,381 deaths and 116,874,912 confirmed COVID-19 cases globally according to the report of the World Health Organization (WHO) dated March 9, 2021[1,2]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the cause of this pandemic, is a new beta corona virus belonging to the order Nidovirales, with a positive single-stranded RNA[3,4].

A greater number of COVID-19 cases have mild symptoms including fever and cough, which usually go away after 2–3 weeks[1]. Unfortunately, critical cases rapidly develop acute respiratory distress syndrome, metabolic acidosis, multiorgan failure, coagulation disorders, septic shock, and finally death[1,5].

This heterogeneous disease pattern of acute SARS-CoV-2 infection presents with differential cytokine patterns that may be directed by variable immunological responses to the virus and investigating these responses is a critical step toward precision medicine in this disease[4].

T cell exhaustion refers to impaired T cell function, occurring throughout infections and malignancies, described as continuously expressed inhibitory receptors and weak effector function, which is distinct from functional effector T cells in terms of transcriptional state[6,7].

Programmed cell death-1(PD-1) (CD279), which belongs to the CD28 superfamily, is considered one of the most commonly studied immune checkpoints. Its expression on the surface of B and T lymphocytes is chromosomally encoded by the programmed cell death 1 gene[8].

Once PD-1 binds to its ligands, it delivers inhibitory signals to adjacent cells and exerts immune-regulatory effects on T cells by down regulating T cell activity[8,9]. As an important checkpoint, it plays a significant role in T cell exhaustion[6].

The dramatic increase in interleukin-10 (IL-10) serum levels is a distinctive feature of the cytokine storm that occurs with COVID-19[10]. Researchers know that IL-10 can suppress inflammation through a negative feedback mechanism. Even so, many clinical studies have proposed that early pro-inflammatory IL-10 elevation plays a pathological role in COVID-19 severity[10,11].

In this study, we aimed to measure the level of PD-1 expression and to investigate its correlation with IL-10 serum levels in modulating T cell effector function, correlating the results with the level of severity of the disease.

## MATERIALS AND METHODS

Our study involved 40 patients with COVID-19, who were classified as 20 severe cases and 20 moderate cases according to the WHO guidelines for the clinical management of COVID-19[12], from Ain Shams University Hospitals. Twenty healthy control subjects were included in this study. The study was conducted from July - October 2020.

All the patients enrolled in this study were clinically and radiologically diagnosed with COVID-19 and confirmed based on a positive RT-PCR performed on their respiratory samples.

None of the participants included in this study had chronic infections, cancers, immunological disorders, or were on immunosuppressive therapy.

Under the complete standard aseptic technique, 5 mL of blood was withdrawn from each participant, collected as 3 mL blood in an EDTA tube for flow cytometric analysis and 2 mL blood in a tube without additives for enzyme-linked immunosorbent assay (ELISA).

The two tubes were transferred immediately to the Ain Shams Hospital laboratory for the separation of mononuclear cells and serum for further steps.

### Flow cytometric analysis

CD3, CD4, CD8, and CD279 monoclonal antibodies were used to detect $CD279^+$ $CD4^+$ T cells and $CD279^+$ $CD8^+$ T cells as follows:

Separation of peripheral blood mononuclear cells from the blood was done using density gradient centrifugation; then purification of the isolated cells was performed using anti-human CD3-PC5 (Beckman Coulter, USA), anti-human CD4-FITC (Beckman Coulter, USA), anti-human CD8-PE (Beckman Coulter, USA), and in addition, PD1 expression was measured using anti-human CD279-PC7 (Beckman Coulter, USA).

The labeled samples were analyzed using flow cytometry and the NAVIOS CXP software (Beckman Coulter, USA).

**Separation of the serum for ELISA**

The second part of the sample collected for ELISA was left for 10–20 min at 20 – 22°C to clot before centrifugation at 500 – 1000 × g for 20 min. The serum was collected in a tube and preserved at -80°C until analysis using the human IL-10 ELISA kit (Biotech, LTD, China) according to the manufacturer's instructions.

**Data management and analysis**

Statistical analysis was conducted using SPSS 23.0 statistical software. The mean, standard deviations, and ranges were used to presenting the parametric quantitative data, although the interquartile range (IQR) and median were accustomed to present non-parametric quantitative data. The numbers and percentages were used to represent qualitative variables. The qualitative data were compared between the two groups using the chi-square test. In addition, the independent t-test was used to compare the quantitative parametric data between the two groups, and the non-parametric distributions were tested using the Mann-Whitney test. Quantitative parametric data between more than two categories were compared using the one-way ANOVA test, while the Kruskal-Wallis test was used for non-parametric distribution. The correlation of paired data was analyzed using Spearman's correlation coefficient. For moderate and severe COVID-19 cases, the ROC AUC of IL-10 serum levels was calculated. Statistical significance was set at P <0.05.

## RESULTS

Our study was conducted on 40 patients with COVID-19 admitted to isolation wards at the Ain Shams University Hospitals. The numbers of males and females were 21 (52.5%) and 19 (47.5%), respectively, while there were 20 healthy control subjects in the control group; they were equally divided into both sexes with a mean age of 44.35 ± 14.47. The study was conducted from July–October 2020. All the studied groups were subjected to blood sample collection, and the expression of PD-1 was measured using flow cytometric analysis, and IL-10 serum levels were measured using ELISA.

A full history was taken and patients with predisposing diseases were categorized as follows; diabetes mellitus 10 (25.0%), hypertension 16 (40.0%), chronic kidney disease 7 (17.5%), hepatic diseases 5 (12.5%), ischemic heart disease 3 (7.5%), bronchial asthma 2 (5.0%) and chronic obstructive pulmonary disease 1 (2.5%).

We first compared the healthy control, moderate, and severe groups regarding the predisposing factors, and they were significantly different regarding chronic kidney disease only, without any other significant differences in the other predisposing factors.

To understand the effect of COVID-19 on T cells, we analyzed cell counts and the expression of PD-1 on $CD4^+$, $CD8^+$, and total T cells. A highly

**Table 1.** Comparison between the healthy control, moderate, and severe groups regarding Total T cell count, $CD4^+$ T cell count, and $CD8^+$ T cell count

| T cell count (number/µl) | | Healthy control group (No. = 20) | Moderate group (No. = 20) | Severe group (No. = 20) | P-value[a] |
|---|---|---|---|---|---|
| Total T cell | Mean ± SD | 1348.22 ± 281.92 | 687.78 ± 326.03 | 337.15 ± 101.06 | 0.000 |
| | Range | 808.6 – 1677.5 | 303.6 – 1468.7 | 184.8 – 513.9 | |
| $CD4^+$ T cell | Mean ± SD | 797.78 ± 239.90 | 402.33 ± 181.70 | 191.10 ± 64.56 | 0.000 |
| | Range | 445.9 – 1159.2 | 155.6 – 866 | 71.1 – 291 | |
| $CD8^+$ T cell | Mean ± SD | 501.74 ± 137.06 | 254.08 ± 142.04 | 153.05 ± 70.93 | 0.000 |
| | Range | 327.6 – 736.8 | 77.5 – 491.4 | 31.4 – 275.2 | |

There was a high statistically significant decrease in total T cell counts (P-value: 0.000), $CD4^+$ T cell counts (P-value: 0.000), and $CD8^+$ T cell counts (P-value: 0.000) in patient groups especially in patients with severe disease.

[a]: P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant

The used test for statistical analysis is the One Way ANOVA test.

significant decrease in total T cell counts (p = 0.000), $CD4^+$ T cells (p = 0.000), and $CD8^+$ T cells (p = 0.000) was found in patient groups, particularly in the severe group (Table 1). In addition, a highly significant increase in the expression of PD-1 on $CD4^+$, $CD8^+$, and total T cells (p = 0.000) was detected among the three groups. Both the moderate and severe groups expressed higher levels of PD-1 on $CD8^+$ T cells, as shown in Table 2.

The severe group had a highly significant increase in CRP (p = 0.000) and D-dimer levels (p = 0.001) than the moderate group.

Serum IL-10 levels in the patient groups were significantly higher than those in the control group (p = 0.000) (Fig. 1).

Furthermore, we examined the relationship between IL-10 and other inflammatory markers that have been used as markers for the diagnosis and prognosis of COVID-19. We found a highly significant positive correlation among IL-10 serum levels, CRP levels (p = 0.003) and IL-10 serum levels, D-dimer levels (p = 0.018).

We compared the sensitivity and specificity of IL-10 in the moderate and severe

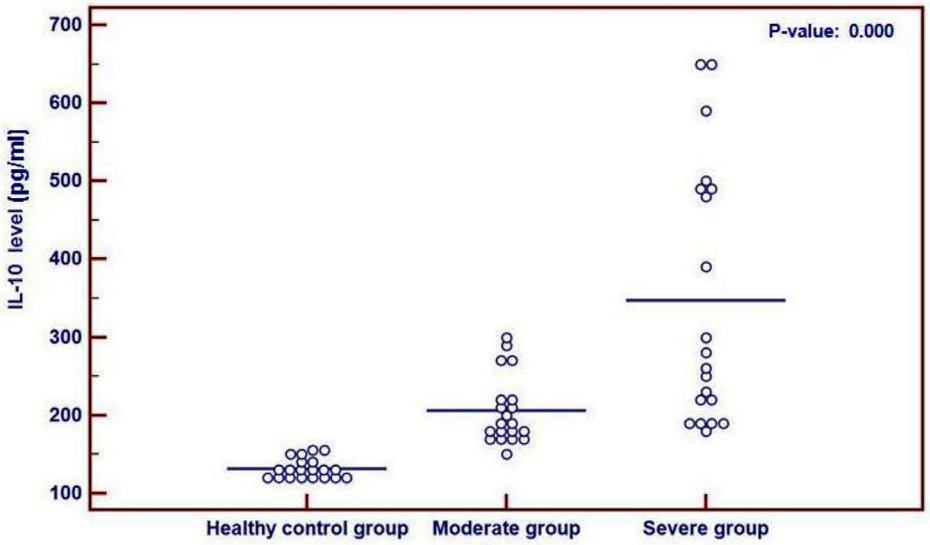


**Fig. 1.** Comparison between the healthy control group, moderate group and severe group regarding IL-10 serum level revealing a higher level in patient groups than in the control group. The used test for statistical analysis is the One Way ANOVA test.

**Table 2.** Comparison between the healthy control group, moderate group, and severe group regarding % PD1 expression

| | | Healthy control group (No. = 20) | Moderate group (No. = 20) | Severe group (No. = 20) | P-value[a] |
|---|---|---|---|---|---|
| PD1 on total T cells | Median (IQR) | 0.9 (0.8 – 1.6) | 2.25 (1.75 – 3.15) | 7.8 (4.65 – 15.3) | 0.000 |
| | Range | 0.6 – 1.8 | 1.4 – 4.6 | 3.1 – 39.2 | |
| PD1 on CD4 | Median (IQR) | 1.45 (1.2 – 1.9) | 3.05 (2.45 – 4.95) | 15.7 (8.25 – 24.9) | 0.000 |
| | Range | 0.7 – 4.1 | 1 – 12.3 | 4 – 45.9 | |
| PD1 on CD8 | Median (IQR) | 1.7 (1.4 – 2.7) | 4.6 (2.55 – 5.9) | 18.4 (7.45 – 35.55) | 0.000 |
| | Range | 1 – 3.2 | 1.7 – 15.5 | 2.2 – 91.7 | |

A high statistically significant increase in % PD1 expression on $CD4^+$, $CD8^+$, and total T cells (p-value: 0.000) was detected between the three groups, with higher expression on $CD8^+$ T cells in both moderate and severe groups.
[a]: P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant
The used test for statistical analysis is Kruskal-Wallis test.

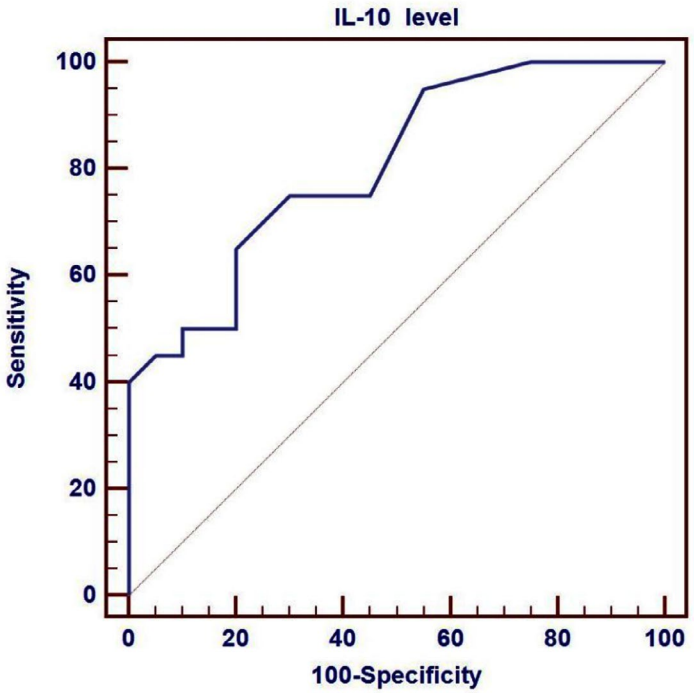


**Fig. 2.** ROC curve of plasma IL-10 levels to identify the severe COVID-19 patients from moderate patients. Performance of ROC curve of IL-10 for predicting severe COVID-19 patients. Receiver operating characteristic curve (ROC) was used to assess the best cut off point with its sensitivity, specificity, positive predictive value, negative predictive value and area under the curve (AUC) of IL-10.

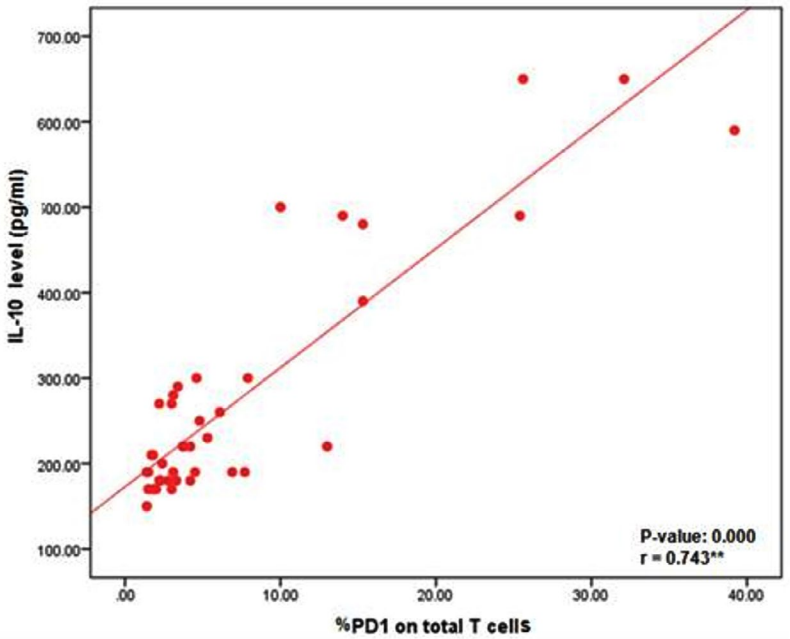


**Fig. 3.** Positive correlation between IL-10 serum level and % PD1 expression on total T cells. The used test for statistical analysis is the Spearman correlation coefficient.

cases to assess its predictive role in the severity of the disease, and found that the sensitivity of IL-10 was 65%, specificity was 80%, and AUC was 0.806, as shown in Fig. 2.

To illustrate the pathological role of IL-10 in the exhaustion of T cells, which may affect the overall count and function of T lymphocytes, we correlated IL-10 serum levels with the percentage of PD-1 expression on total T cells, revealing a positive correlation between them ($p = 0.000$) (Fig. 3).

## DISCUSSION

As the cases of COVID-19 are increasing dramatically globally, and there is limited availability of intensive care units (ICU) for treatment, early detection of severe cases is critical for prompt patient triaging[13].

In this study, we demonstrated that T cell exhaustion and inflammation reflected by a cytokine storm may have a significant effect on the progression of COVID-19.

Cytotoxic T cells (CTLs), which can secrete a number of molecules such as interferon-gamma, perforins, and granzymes, play a significant role in viral clearance during infections[14]. Simultaneously, helper T cells (Th) help CTLs to clear up any pathogens[15]. However, prolonged viral stimulation can exhaust T cells, resulting in the loss of cytokine production[16].

The current study revealed that the patient groups had a significant decrease in $CD4^+$, $CD8^+$, and total T cell counts compared to the control group. These results were similar to those of Zhang et al.[17], who found that patients with COVID-19 had significantly lower $CD4^+$, $CD8^+$, and total T cell counts, particularly in those suffering from severe disease. Furthermore, Henry[18] reported that lymphopenia is related to disease severity, and lymphocyte counts were significantly lower in nonsurvivors than in survivors. Therefore, lymphocyte replenishment is crucial for recovery. Qin et al.[19] stated that both The cells and CTLs were below average in patients with COVID-19, with Th cells being lower in severe cases.

Cytotoxic cells, including CTLs and natural killer (NK) cells, have a great effect on the limitation of viral infection, and their functional exhaustion is linked to disease progression[20]. During high-grade chronic viral infections causing $CD8^+$ T cell exhaustion, there is high expression of inhibitory receptors, such as PD-1, in exhausted cells, which are important negative immune checkpoints in suppressing cell-mediated immune responses, as in COVID-19 and malignancies[21]. In the current study, % PD-1 expression was high in $CD4^+$, $CD8^+$, and total T cells, but both moderate and severe groups expressed higher PD-1 levels on $CD8^+$ T cells. Diao et al.[6] found that in COVID-19, the percentages of $PD\text{-}1^+CD4^+$ and $PD\text{-}1^+CD8^+$ cells were significantly higher in ICU patients than in healthy controls, suggesting the role of SARS-CoV-2 in T cell exhaustion, especially in those who need ICU treatment. In addition, Bellesi et al.[22] also demonstrated that $CD4^+$ and $CD8^+$ T cells in 42 patients with COVID-19 had significantly higher PD-1 and CD95 (Fas) expression than the controls. PD-1 expression may increase to avoid uncontrolled inflammation. Consequently, anti-PD-1 therapy could have beneficial therapeutic effects, but should be used with caution. As a result, inhibition of the PD-1 pathway alters the strength and efficiency of cytotoxic cell attack, allowing viral clearance with limited collateral tissue damage[23].

Patients with COVID-19 have high levels of C-reactive protein (CRP), which is higher in nonsurvivors than in survivors, suggesting that the severity and prognosis of the disease are strongly correlated with it[24]. Moreover, Tang et al.[25] reported that significantly higher D-dimer plasma levels were found in nonsurvivors than in survivors. Since coagulopathy and disseminated intravascular coagulation seem to be linked to high mortality rates, D-dimer was found to be the most powerful independent predictor of mortality[26]. These results are consistent with our study as the severe group had a highly significant increase in CRP and D-dimer levels compared with the moderate group.

Cytokine storm is a known phenomenon that occurs during a severe inflammatory response to any microbial infection, in which large amounts of cytokines are released, which play a significant pathological role in multiple organ dysfunction syndromes and acute respiratory distress syndrome[27]. During sepsis, different types of cells at inflammation sites release IL-6 and IL-10 into the bloodstream, which is similar to acute organ injuries. Thus, these cytokines contribute

to COVID-19 pathogenesis by inducing a strong inflammatory response[11]. In our study, the patient groups had significantly higher serum IL-10 levels than the control group. Furthermore, Huang et al.[4] found that IL-10 and other markers, including IL2, IL7, IP-10, and TNF-$\alpha$, were highly significantly different in patients with COVID-19.

Han et al.[11] revealed that patients with COVID-19 had higher cytokine levels (IL-2, 4, 6, 10, and TNF-$\alpha$) and CRP levels in their blood than healthy controls.

Additionally, our study showed that there were highly significantly different positive correlations among the serum levels of IL-10, CRP and D-dimer. This is similar to the results of Han et al.[11] who demonstrated that the levels of IL-10 and CRP were positively correlated. The significantly different elevated IL-10 serum levels in the patient groups prompted us to assess the predictive role of IL-10 in disease severity. Therefore, we compared the sensitivity and specificity of IL-10 in moderate and severe cases, and our results revealed that the sensitivity of IL-10 was 65%, specificity was 80%, and AUC was 0.806. Inconsistent with our results, Dhar et al.[28] found that the sensitivity of IL-10 was 61%, but the specificity was 100%. In addition, Liu et al.[29] reported that the AUC for IL-10 between moderate and severe cases was 0.838, suggesting that this cytokine could be a biomarker for disease severity. The cost of measuring IL-10 levels in the blood is low, and it is crucial to identify patients who are more likely to progress to serious disease so that appropriate precautions can be taken[28].

Regarding the role of IL-10 in the expression of PD-1, Diao et al.[6] demonstrated that following infection with SARS-CoV-2, high serum IL-10 levels, together with high levels of TIM-3 and PD-1 exhaustion markers, may suggest that IL-10 is mechanically responsible. Similarly, Lu et al.[10] reported findings similar to those reported by Diao et al.[6] which revealed the role of IL-10 in the exhaustion of T cells, but his explanation of the role of IL-10 was different, as he proposed that after SARS-CoV-2 infection, there are three steps in the immunopathological process: initiation, expansion, and completion.

Early induction of IL-10 occurs in the lung during the initiation stage of infection with SARS-CoV-2, which is considered a negative feedback process to protect against inflammation induced by other pro-inflammatory mediators. Even so, increased endogenous IL-10 production could activate the immune system, cause inflammation, and pro-inflammatory cytokine that stimulate the release of other cytokine storm mediators.

New therapeutic lines for ICU patients with COVID-19 are required as soon as possible to prevent disease progression in patients with lymphopenia who are at higher risk[6]. Thus, IL-10 might represent an important target for lowering the mortality of COVID-19 by blocking its pathological pro-inflammatory function. Accordingly, it may be important to select the right time to block the activity of IL-10 in severe and critical cases[10].

## CONCLUSION

In patients with COVID-19, there is a marked decrease in lymphocytic counts, while PD-1 expression increases in surviving T cells. PD-1 expression is positively correlated with IL-10 serum levels, suggesting that IL-10 plays a pathological role in T cell exhaustion. Thus, blockade of the PD-1 pathway alone or in combination with IL-10 could be a key in the development of a new therapeutic approach for nullifying functional exhaustion in T cells, and restoring vigorous T cell cytotoxicity against the viral antigen.

## ACKNOWLEDGMENTS

None.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

AH and DM assisted in the collection of samples and patients' data. FM and DM contributed to laboratory work. All authors contributed significantly to the study's conception, design, manuscript drafting, revision, and final approval of the manuscript.

## FUNDING

None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

The Ethical Committee of Scientific Research of the Faculty of Medicine, Ain Shams University, Cairo, Egypt, gave its approval to this study (FWA 000017585 / MD 134 / 2020).

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