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Original article

Interferon- gamma-induced protein-10 (IP-10) and natural killer cell count as predictive factors for the severity of COVID-19

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ABSTRACT

Background: The immune mechanisms underlying the pathology of COVID-19 are currently under immense scrutiny. Following NK and T cell activation, a surge of proinflammatory cytokines, and chemokines such IP-10, are found to increase. These cytokines stimulate the adaptive immune response and recruit other immune cells to the site of infection. Paradoxically, NK cells are known to be decreased in the peripheral circulation of severely ill COVID patients. It is not clear if this decrease in number is due to cell depletion or due to recruitment to sites of inflammation. The purpose of this study was to determine if there is a correlation between IP-10 levels and peripheral NK cell numbers and if this correlates with diseases severity. **Methods**: The study involved 30 patients divided into 2 groups (moderate and severe) depending on disease severity, as well as 15 age- and gender-matched controls. Serum IP-10 levels were assessed by ELISA and determination of NK cell numbers were determined by flow cytometry. **Results:** The results demonstrate an inverse relationship between NK cell numbers and IP-10 levels. Moreover, severely ill patients characteristically displayed decreased peripheral NK cell numbers and increased serum IP-10 levels in a manner that correlates with disease severity. **Conclusion:** The results demonstrate that disease severity is associated with increased production of chemokines such as IP-10 in the serum which may be involved in the recruitment of NK cells to peripheral tissues such as the lung, which may partially explain the decreased NK cell number associated with severe pathology.

Introduction

Since it was declared as a global pandemic by WHO, coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in thousands of deaths worldwide. The symptoms vary from mild to severe and critically ill patients often develop acute respiratory distress syndrome (ARDS) and multiorgan injuries [1]. It rapidly became a critical target of research to determine predictors of severity to guide therapeutic intervention. It was quickly recognized that the main cause of morbidity and mortality is the dysregulated immune response and its inflammatory component, mainly manifested in the now well-recognized cytokine storm [2]. Several studies have shown that increased amounts of proinflammatory cytokines in serum were associated with pulmonary inflammation and extensive lung damage in COVID-19 [3]. Lymphopenia has also been shown to be a characteristic sign and may correlate with disease severity and outcome [4].

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The immune response to viral infections initiates with the production by infected cells of type I interferons, that perform an antiviral effect and activate cells such as natural killer (NK) cells [5]**.** Natural killer cells, known for their crucial role in combatting and controlling viral infections, destroy virally infected cells and secrete cytokines such as IFN-γ, which activates other cells. Natural killer cells are innate immune responders critical for viral clearance and immunomodulation. Despite their vital role in viral infection, the contribution of NK cells in fighting SARS-CoV-2 has not yet been directly investigated [6]**.**

Following NK and T cell activation, a surge of proinflammatory cytokines and chemokines are found to increase. These cytokines stimulate the adaptive immune response and recruit other immune cells to the site of infection. The release of large amounts of pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNFα, TGFβ and chemokines such as IP-10, CXCL8, CXCL9, CCL2, CCL3, CCL5, precipitates and sustains the aberrant systemic inflammatory response [7].

More recently, interest has been shown in the chemokine interferon γ-induced protein 10 kDa (IP-10) /CXCL10, which belongs to the CXC chemokine family. It is secreted from leukocytes, epithelial cells, endothelial and stromal cells in response to IFN-γ [8]. It binds to CXCR3, which is predominantly expressed on activated T lymphocytes and natural killer cells [9]. IP-10 induces chemotaxis, apoptosis and cell growth inhibition. Abnormal levels of IP-10 have been observed in body fluids of individuals infected with some viruses [10]. Recently, IP-10 was detected in COVID-19 patients and was highly associated with disease severity and could be used as an excellent predictor of COVID-19 progression. Therefore, detecting the expression levels of IP-10 in patients with COVID-19 in the early stage of the moderate disease may provide useful information for the formulating of a specific strategy of treatment [11]. Moreover, it is possible that IP-10 may play a role in recruiting NK cells to the lungs resulting in NK cell depletion and increasing lung infiltration and pathology thus contributing to ARDS [6].

The aim of this work was to correlate the levels of serum IP-10 to the severity of the disease and to determine if NK cell depletion correlates inversely with IP-10 in COVID-19 patients.

Patients and Methods

A cross-sectional study was conducted on 30 (17 males, 13 females) confirmed COVID-19 patients admitted to El Demerdash Geriatrics Isolation Hospital. The control group was comprised of 15 (8 males, 7 females) healthy, age- and gender-matched controls. The study group was divided into 2 subgroups. The first subgroup included 15 moderate cases and the second group included 15 severe and critical cases. Classification was performed according to the Ain Shams Guide for COVID-19 [12]. (The criteria for classifying patients according to disease severity are included in appendix 1). The control group included 15 age- and sex- matched apparently healthy individuals.

After obtaining the patient's informed consent, 3 ml EDTA anti-coagulated blood samples were collected. Following centrifugation, serum samples were obtained and serum IP-10 levels were measured using human IP-10 ELISA kit (bioassay technology lab, Cat. No: E3746Hu, China) as described in the manufacturer's instructions [13, 14].

Natural killer cell identification was performed by flow cytometry using a NAVIOS Six-Color Flowcytometer (Beckman Coulter, USA). Briefly, blood samples were mixed with 2ml lyse solution. After incubation for 5 minutes at room temperature, PBMCs were obtained by Ficoll gradient centrifugation. Fresh PBMCs were then treated by addition of 5 µL of each of anti-CD56 R-PE-conjugated monoclonal antibody (mouse anti-human CD56-PE, Beckman Coulter; ref IM2073U) and anti- CD3 FITC -conjugated monoclonal antibody (mouse anti-human CD3-FITC, Beckman Coulter, ref IM1281U). Antibody-treated cells were then vortexed and incubated for 15 minutes at room temperature in the dark. After a final wash and centrifugation, cells were resuspended in PBS and acquired through the flow cytometer.

Lymphocyte populations were gated using a forward scatter vs side scatter dot plot and gated cells were analysed by a dot plot of CD56-PE vs CD3-FITC. NK cells were identified as CD3-CD56+ lymphocytes. Data was analyzed using a Navios software version 1.2.

For statistical analysis, the collected data was revised, coded and tabulated using the statistical package for Social Science (SPSS 25). Analytical statistics used in the different analyses included Student t-test, Mann-Whitney test, Chi-squared test,

and correlation analysis was performed using Pearson and Spearman's rho methods.

Results

The study group included 30 cases: 17 males and 13 females. The control group included 15 age- and gender-matched healthy controls. The mean age for all participants in the study was 53.62 (±19.21).

Comparison of laboratory results revealed significant increase in total leucocytic count in the cases in comparison with the control group (*p* value 0.033), while there was significant decrease in absolute lymphocytic count in the cases in comparison with the control group (*p* value< 0.001) **(Table 1)**.

The comparison between the IP-10 level and NK cell count in the peripheral blood of patients and controls shows that there was a significant increase in the IP-10 level in cases compared to controls and there was a significant decrease in the peripheral NK cell count in cases compared to controls **(Table 2)**. Thus, there is an inverse relationship between NK cell count and IP-10 level. The higher the IP-10 level, the lower the NK cell count and vice versa.

Comparison of NK cell count and IP-10 level in moderate and severe cases revealed that there was a significant decrease in NK cell count in the severe group in comparison with the moderate group (*p* value <0.001), while there was a significant increase in the IP-10 level in the severe group in comparison with the moderate group **(Figure 1)**.

A representative dot plot, obtained from flowcytometric data acquisition and analysis is shown in **figure (3)**, representing the difference between NK cell counts in a moderate vs a severe case. The figure demonstrates that severe cases are associated with a significantly smaller peripheral NK cell count compared to moderate cases.

By correlation between the IP-10 level and the other laboratory parameters and markers measured, there was a significant correlation observed between the IP-10 level and the D-dimer level, absolute lymphocytic count and the NK cell count, thus higher levels of IP-10 are associated with higher D-dimer levels, lower NK cell count and lower lymphocytic count **(Figure 4)**.

Finally, a correlation between disease severity and the two markers assessed in this study, peripheral NK cell count and IP-10 levels, explicitly shows that severe cases experience a decrease in NK cell counts that directly correlates with an increase in IP-10 levels, while moderate cases demonstrate a higher NK cell count and a lower IP-10 level **(Figure 5)**.

Table 1. Leucocytic and lymphocytic count in cases and controls.

	Group		Test of significance		
	Controls (n=15)	**Cases (n=30)**			
	Median (IQR) Mean ± SD	Median (IQR) Mean ± SD	Value	*p*-Value	Sig.
TLC (*10^3) (4-10)	6100 (5200 - 7100)	10000 (5100 - 14100)	***U***= 136.5	0.033$^{(M)}$	S
Lymph. Abs (*10^3) (1.5-3.5)	2.1 (2 - 2.4)	1.37 (0.9 - 1.5)	***U***= 5.244	<0.001$^{(M)}$	S
Neutr. Abs (*10^3) (2-7)	3.4 (2.7 - 10.9)	7.09 (3.81 - 10.6)	***U***= 177	0.316$^{(M)}$	NS

$^{(M)}$ Mann-Whitney test of significance (U= Mann-Whitney test value).

TLC= Total leucocytic count, Neutr. Abs= absolute neutrophil count, Lymph. Abs= absolute lymphocytic count.

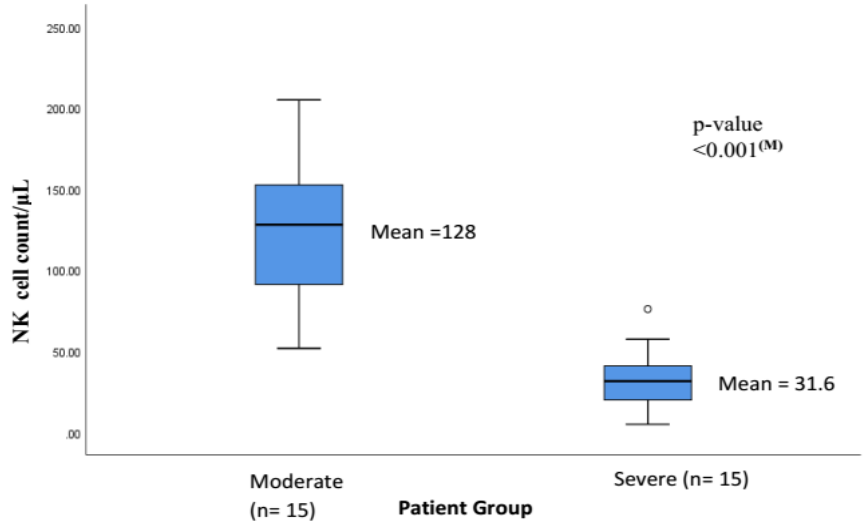
Table 2. Comparison of NK cell count and IP-10 level in cases and controls.

	Group		Mann-Whitney test		
	Controls (n=15)	Cases (n=30)			
	Median (IQR)	Median (IQR)	***U***	p-Value	Sig.
NK count (cells/μL)	224 (222 - 238.8)	66.7 (31.6 - 128)	0.0	<0.001	S
IP-10 (pg/mL)	50 (50 - 53)	125 (100 - 155)	0.0	<0.001	S

NK Count= Natural Killer cell count (cells/μL)

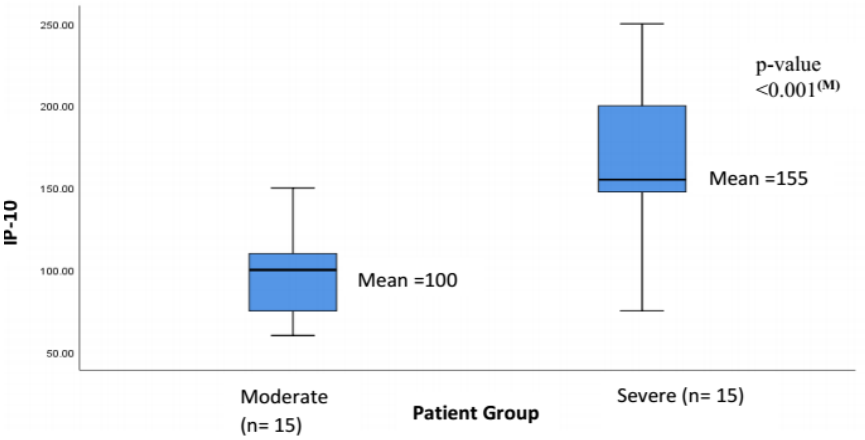
IP-10= serum interferon-gamma-inducible protein-10 level (pg/ml)

Figure 1. Comparison of the NK cell count between moderate and severe cases.



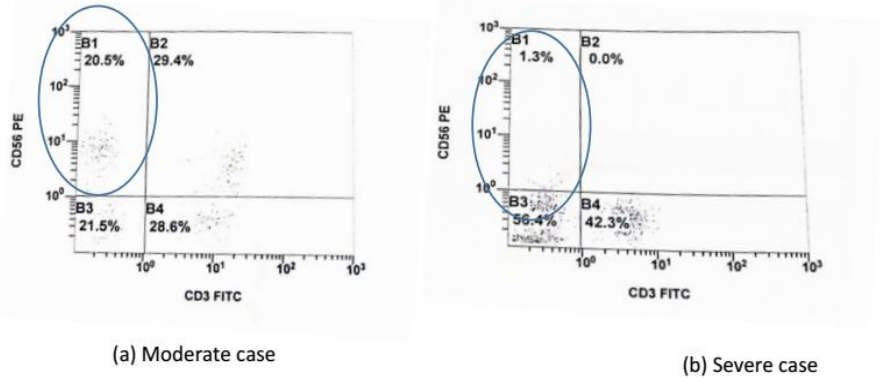
(M) Mann-Whitney test of significance

Figure 2. Comparison of the IP-10 level between moderate and severe cases.



(M) Mann-Whitney test of significance

Figure 3. Flowcytometric representative dot plots from one moderate case (a) and one severe case (b), showing the difference in the peripheral blood CD56+ CD3- population of NK cells.



(a) Moderate case

(b) Severe case

Figure 4a Correlation between the IP-10 level and the D-Dimer level.

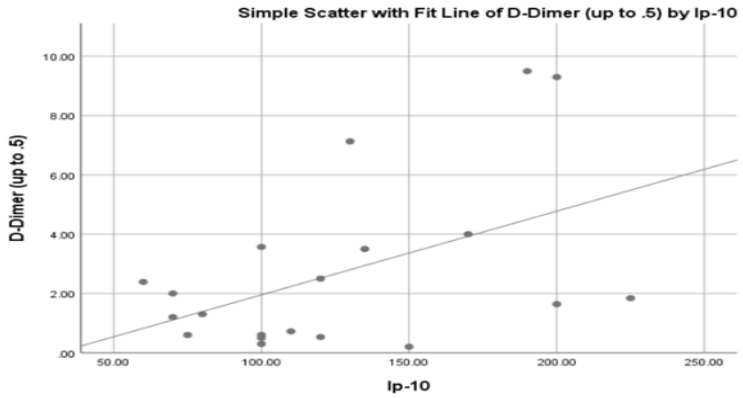


Figure 4b Correlation between the IP-10 level and absolute lymphocytic count

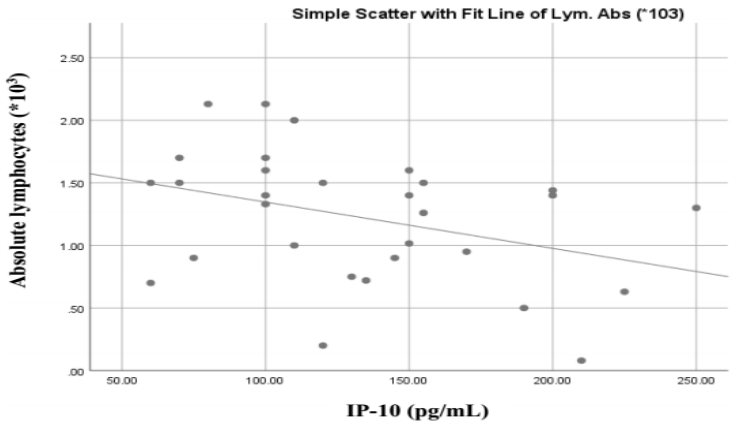
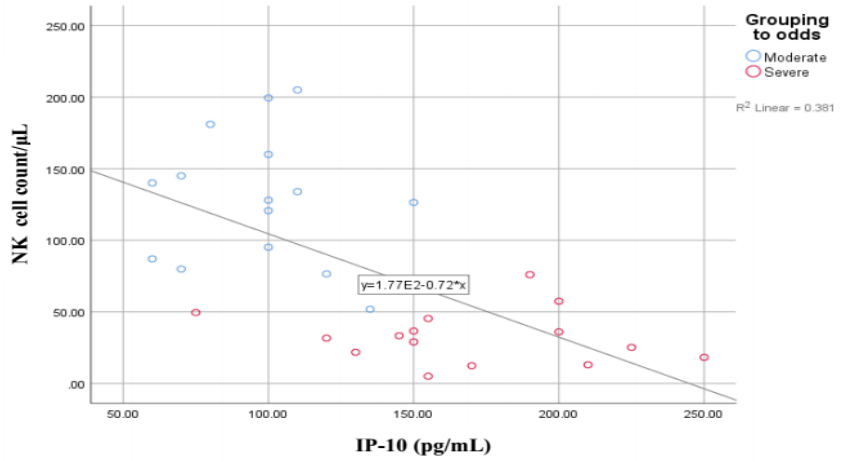


Figure 5 Correlation between the IP-10 level, NK cell count and the severity of COVID-19.



Discussion

The current study sheds light on a critical area of COVID-19 disease pathogenesis and provides novel insight into potential methods of intervention. The association between IP-10, a well-established chemokine with a critical role in the pathogenesis, as well as the quantity of NK cells, which are indispensable innate immune system cells with a prominent role in the anti-viral immune response, was determined. Moreover, disease progression and severity were compared with these important markers. The results presented herein demonstrate a decrease in NK cell numbers in the peripheral blood of COVID-19 patients in a manner that correlates with disease severity **(Table 2, Figure 2, Figure 3)**. The study additionally demonstrates an increase in the level of IP-10 in patient serum in a manner that siilarly correlates with disease severity and inversely correlates with NK cell numbers in the peripheral blood of patients **(Table 2, Figure 2, Figure 5)**. The evidence thus strongly suggests a role for NK cells in promoting lung pathology and hence disease severity and that such activated NK cells may be recruited from the blood under the effect of chemokines, such as IP-10.

It is clear that NK cells play a vital role in controlling viral infections [6,15,16]. The role in the control of SARS CoV-2 was also specifically demonstrated by **Zheng et al.** [17], where numbers of blood NK cells in COVID-19 patients were shown to inversely correlate with disease severity. Furthermore, circulating NK cells in severely ill patients were found to display a hyporesponsive phenotype with increased expression of the inhibitory receptor NKG2A and lower levels of IFN-γ secretion [17]. The decrease in circulating NK cell number is consistent with the currently presented study. Indeed, NK cell cytopenia seems to be a consistent characteristic among SARS CoV-2-infected patients.

The finding that a decrease in NK cell numbers is associated with disease severity and progression can be explained by the redistribution of NK cells and other lymphocytes under the effect of chemokines, such that NK cells are recruited to the lungs and sites of inflammation [18]. This remains a postulation due to the difficulty to assess NK cell migration in COVID-19 patients. It is also possible that NK cells undergo apoptotic cell death as a result of the increased viral load [6].

Histochemical examination of lung biopsy specimens from patients who died as a result of COVID-19 have shown intense CD4+ and CD8+ T cell infiltration, indicating that the involvement of immune-mediated injury is plausible in the pathogenesis of COVID-19. In fact, it has been proposed that the lymphopenia associated with COVID-19 may be related to activation-induced apoptosis of lymphocytes or aggressive migration from peripheral blood to the lungs, where robust viral replication occurs [1]. Furthermore, depletion of NK cells alleviated lung pathology in high dose-infected mice in a mouse model infected with SARS CoV-1[19]. It is therefore highly likely that NK cell infiltration plays an important role in inducing lung pathology in SARS CoV infections as is suggested by the results of the current study.

The results presented herein demonstrate that IP-10 is increased in a manner that is proportional to diseases severity **(Table 2, Figure 2)**. IP-10 is a chemokine, known to be specifically increased in viral infections [9]. Indeed, IP-10, also known as CXCL10, was shown to be lower in bacterial ARDS when compared with non-COVID 19 viral ARDS, indicating that this chemokine may be used as a viral biomarker [20]. This is consistent with several studies. **Huang et al.**[21] have shown a significant increase in the peripheral blood of COVID-19 patients of proinflammatory cytokines, especially IL-1 β. IFN-γ, IP-10 and MCP-1. IP-10 is secreted by many cell types in response IFN-γ and shows a strong positive correlation with other inflammatory cytokines, such as IL-1 β, IL-6, IL-8, IL-1 and TNF-α. In addition to its inflammatory and chemotactic activities, it has been shown to inhibit endothelial recovery [22] and to play a role in the thrombosis commonly associated with critical illness and mortality of COVID-19 patients [8]. The increase in IP-10 secretion is commonly reported as part of the cytokine storm, which is central in the immunopathologic mechanism in COVID-19-associated complications and is associated with disease severity [23]. Our study further corroborates these findings and demonstrates a clear relationship between IP-10 expression and disease severity. Severely ill patients demonstrated significantly higher levels of IP-10.

Research has shown that IP-10 plays an important role in the recruitment of NK cells to the brain and that such recruitment was essential in the control of infection therein [24]. Several animal models have also demonstrated the presence of infiltrates of immune system cells to the lungs and associated lung pathology [25-27], although there

has not been a specific study of NK cell infiltration. This area of research has been largely overlooked despite the clearly significant role played by NK cells in COVID-19 and other viral immune pathogenesis [6].

The significant morbidity and mortality observed in COVID-19 patients is due to acute lung injury (ALI) and ARDS [28,29]. The pathologic features of this inflammatory process may be due to the enhanced cytopathic effect and increased production of proinflammatory cytokines by infected cells [17]. The existing evidence is consistent with the hypothesis that NK cells are involved with the cytokine storm associated with CoV infection and that this contributes significantly to the disease severity and inflammation-mediated lung damage [6].

Collecting the evidence provided by this study and others [1,6,8,30], a model is proposed in which the cytokine storm associated with SARS CoV-2 infection results in IFN-γ -induced upregulation of IP-10, which is potentially responsible for NK cell and other inflammatory cell recruitment to lung tissues in an attempt to control the infection. Massive infiltration of activated NK cells to lung tissue may be at least partially responsible for inducing an aggressive immune response in an attempt to control the infection and destroy virally-infected cells, resulting in marked respiratory symptoms, ARDS and potentially death. Clearly, this hypothesis cannot be definitively proven in the current study and warrants further research, with emphasis on lung biopsy and autopsy in severe and fulminant lung pathology.

There are clear limitations to this study, as is likely the case for many research studies conducted during these unprecedented times. Handling severe cases in a stressful clinical environment, where health systems are forcefully impounded and where clear information is lacking, renders saving the patients' lives the primary goal, with little time and resources to work on obtaining research specimens from critically-ill patients. Indeed, lung biopsy and immunohistochemical analysis of lung tissue in critically-ill patients will be important to determine NK cell infiltration and to provide a correlation with NK cell decrease in the periphery and to provide a correlation to demonstrate the dependence of such infiltration on IP-10, now clearly established as a central marker of disease severity. Natural killer cell recruitment to lung tissue can be one of the major mechanisms of disease progression to the critical stage and may provide exceptionally important insight into specific methods of blockade and intervention.

Another limitation in this study is that long-term follow up and correlation of disease state with lung pathology was not possible. In the future, this can potentially provide important insight.

The results of this study warrant a recommendation to further pursue research into this area by increasing the number of patients involved in the study, including immunohistochemical studies of lung infiltrates and bronchoalveolar lavage specimens and to develop a follow up mechanism for cases after convalescence. It will also be important to determine if targeting IP-10 for therapeutic intervention will be sufficient to decrease NK and other inflammatory cell recruitment to the lungs, and to mitigate the consequences of severe disease, thus decreasing mortality.

Conflicts of interests: none.

Financial disclosure: none.

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Appendix 1

Clinical classification of COVID-19 patients according to disease severit.
Extracted from the Ain Shams Guidelines [12].

Table 1: COVID-19 disease severity

Mild disease		Symptomatic patients meeting the case definition for COVID-19 without radiological evidence of pneumonia or hypoxia
Moderate disease	Pneumonia	Adolescent or adult with clinical signs of non severe pneumonia (e.g. fever, cough, dyspnea) and radiological evidence of pneumonia
Severe disease	Severe pneumonia	Adolescent or adult with clinical signs of pneumonia (e.g. fever, cough, dyspnea, fast breathing) plus one of the following: • Respiratory rate > 30 breaths/min; severe respiratory distress; orSpO_2 < 93% on room air and radiological evidence of pneumonia • Patients with more than 50% lesions progression within 24 to 48 hours in lung imaging
Critical disease	Acute respiratory distress syndrome (ARDS)	***Meeting any of the following criteria:*** Occurrence of respiratory failure requiring mechanical ventilation; Presence of shock; Sepsis, other organ failure that requires monitoring and treatment in the ICU Critical cases are further divided according to the degree of hypoxemia as categorized by the P/F ratio (PaO2/FiO2 *100) or S/F ratio • Early stage: PO2/FiO2 (P/F ratio) 200-300, or Oxygen saturation by pulse oximetry/ Fraction of inspired oxygen (S/F ratio) 181-235; without organ failure other than the lungs. The patient has a great chance of recovery through active antiviral, anti-cytokine storm, and supportive treatment. • Middle stage: P/F ratio 100-200, or S/F ratio 118-181; may be complicated by other mild or moderate dysfunction of other organs. • Late stage: P/F ratio less than 100, S\F ratio less than 118; diffuse consolidation of both lungs; or failure of other vital organs. The mortality risk is significantly increased. ***Sepsis:*** acute life-threatening organ dysfunction caused by a dysregulated host response to suspected or proven infection. Signs of organ dysfunction include: altered mental status, difficult or fast breathing, low oxygen saturation, reduced urine output, fast heart rate, weak pulse, cold extremities or low blood pressure, skin mottling, laboratory evidence of coagulopathy, thrombocytopenia, acidosis, high lactate, or hyperbilirubinemia. ***Septic shock:*** persistent hypotension despite volume resuscitation, requiring vasopressors to maintain MAP ≥ 65 mmHg and serum lactate level > 2 mmol/L