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Upregulation of *FOXP3* is associated with severity of hypoxia and poor outcomes in COVID-19 patients

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ABSTRACT

The levels of messenger RNA (mRNA) transcription of *FOXP3, IFN-γ, TNF, IL-6* and *COX-2* from both COVID-19 infected and control subjects were evaluated using SYBR™ green real-time polymerase chain reaction (RT-PCR). Severe/critical cases showed significantly lower lymphocyte counts and higher neutrophil counts than the mild or moderate cases. There were significantly lower levels of mRNA expressions of *IFN-γ, TNFα* and *FOXP3* in COVID-19 patients than in the control group. On the other hand, *IL-6* and *COX-2* expressions were significantly higher in patients suffering from severe disease. *FOXP3* expressions were correlated with the severities of hypoxia and were excellent in predicting the disease severity. This was followed by the *IL-6, COX-2 and TNFα* expressions. *FOXP3* expression was the only biomarker to show a significant correlation with patient mortality. It was concluded that SARS-CoV-2 infection is associated with the downregulation of *FOXP3* and upregulations of *IL-6* and *COX-2*.

1. Introduction

In December 2019, SARS-CoV-2, a new member of the family *Coronaviridae*, emerged in Wuhan, China. The virus resulted in a harsh pandemic with more than 203,000,000 laboratory-confirmed cases and more than 4,3 million deaths worldwide as of August 11, 2021. Although the majority of patients who get the coronavirus disease 2019 (COVID-19) have good prognoses, 5–10% develop critical symptoms that may require mechanical ventilation and have fatal consequences (Guan et al., 2020; Hadjadj et al., 2020; Lai et al., 2020).

The immune system adopts different mechanisms in response to various pathogens. During the immune response, several inflammatory pathways are activated; however, an exaggerated response can cause a severe and sometimes uncontrolled inflammatory reaction (Dinarello, 2000; Hadjadj et al., 2020; Jamilloux et al., 2020). Pro-inflammatory cytokines, including interleukin 1 beta (IL-1β), interleukin 6 (*IL-6*), tumour necrosis factor (*TNF*) and gamma interferon (*IFN-γ*) (Dinarello, 2000), are important in the development of innate antiviral immune responsiveness. However, exaggerated responses may lead to severe inflammatory reactions. IL-1β induces the synthesis of cycloxygenase-2 (*COX-2*) with a subsequent increase in prostanoid production that induces inflammation (Duque et al., 2006). *IL-6* mainly induces pro-inflammatory signalling and modulates massive cellular processes, and it has been found to be associated with many viral diseases, inflammatory diseases and multiple cancers (Bruzzese and Lazzarino, 2020; Luo and Zheng, 2016). Homeostasis of the immune responsiveness is controlled by the regulatory T cells (Tregs) that work through downregulations and suppression of the host immune responses. The forkhead box P3 (*FOXP3*) protein is the gene expressed on the surface of Tregs (Bacchetta et al., 2007; Sakaguchi et al., 2010). The latter play an important role in the suppression of different inflammatory, allergic and autoimmune disorders, including pulmonary infections (Adamzik et al.,

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2013; Aggarwal et al., 2010).

A cytokine storm results from a sudden increase in the pro-inflammatory cytokines with subsequent chemotaxis of macrophages, neutrophils and lymphocytes to the site of infection. The concentrations of some of these cytokines in the blood allow the discrimination between mild, moderate and severe cases (mainly IL-1 β, IL-1Ra, *IL-6*, IL-7, IL-10, IP-10, *IFN-γ* and *TNF-α*) (Jamilloux et al., 2020). During moderate COVID-19 infection, the immune responsiveness reacts normally to viral infection in a robust manner. In contrast, with severe COVID-19 infection, the complications occur as a sequel to dysregulated and excessive immune responses (Ye et al., 2020). The innate immune response is exaggerated in an uncontrolled manner, while the adaptive immunity is impaired with subsequent deterioration of pulmonary functions due to severe tissue damage (Chen et al., 2020; Tan et al., 2020; Thevarajan et al., 2020). The exaggerated immune responses that lead to pulmonary tissue damage in patients with severe COVID-19 disease are still obscure. Most patients who are critically ill initially show only mild symptoms. However, their conditions deteriorate suddenly 9–12 days after the first onset of symptoms and sometimes during the process of recovery when the patients develop acute respiratory distress syndrome (ARDS) and multiple-organ failure, which may be followed by death within a short period of time (Hadjadj et al., 2020).

Although several studies have been published about the pathogenesis of COVID-19, our knowledge regarding the immunological features and the molecular mechanisms involved with respect to COVID-19 severity is still limited (Hadjadj et al., 2020). Understanding the pathogenesis of this severity is the first step toward designing therapeutic interventions that can prevent the progression of COVID-19 infection and save the patients' lives (Ye et al., 2020). We aim in this study to evaluate the levels of expression of five genes; namely, *FOXP3, IFN-γ, TNF, IL-6 and COX-2*, in adults who are diagnosed with COVID-19 and to correlate these levels of expression with disease outcomes and clinical and laboratory data, especially those known to have prognostic value.

2. Methods

2.1. Ethical approval and consent to participate

All patients and control subjects signed informed consents, and the study was approved by the ethical committee of the National Cancer Institute (NCI), Cairo University. Clinical, radiological and laboratory data were collected from patients' files.

2.2. Patients

This study included 111 hospitalized patients who were admitted to Cairo University hospitals. All patients presented to the hospitals between April and July 2020. The control group included 32 age-matched normal subjects. Sample size was calculated using SigmaPlot software 12.5.0.38 for Windows (SigmaPlot, Systat Software Inc. UK, 2011). Infection with the SARS-CoV-2 virus was confirmed by real-time polymerase chain reaction (RT-PCR) through mixed throat and nasal swabs.

2.3. Clinical and laboratory investigations

Patients were classified into mild, moderate and severe/critical cases according to Wu et al. (Wu and McGoogan, 2020), and the outcomes were recorded. Mild disease was defined as the presence of clinical symptoms and no changes seen in computed tomography (CT) chest scans, and moderate cases included all those with respiratory symptoms associated with changes found in CT scans and oxygen saturation above 92%. Severe cases were defined by the presence of the following criteria: respiratory distress, with respiratory rate (RR) $\geq$ 30/minutes, resting blood oxygen saturation $\leq$93% or partial pressure of arterial blood oxygen (PaO2)/oxygen concentration (FiO2) $\leq$ 300 mmHg and chest radiography showing more than 50% lesion or progressive lesion within 24–48 h. Critically ill cases included all severe cases that deteriorated who possessed respiratory rate (RR) $>$ 30, oxygen saturation$<$92% at room air, partial pressure of arterial blood oxygen (PaO2)/oxygen concentration (FiO2) $<$ 200 mmHg despite oxygen therapy with a chest radiography showing more than 50% lesion or progressive lesion within 24–48 h. Routine laboratory investigations, including complete blood counts (CBC) and measurements of liver and kidney functions were ordered for all patients. Other tests were ordered according to the clinical condition of the patient.

2.4. RNA extraction

Peripheral blood samples were collected in tubes containing ethylene diamine tetra acetic acid (EDTA) under complete aseptic conditions. Erythrocytes were lysed using buffer supplied by QIAGEN and used according to the manufacturer's instructions. Total RNA was extracted from the lymphocytic cell pellets using the total RNA purification kit (Direct-Zol RNA Kit, Zymo Research, Germany), as described in the manufacturer's instructions.

2.5. cDNA synthesis and RT-PCR

Total RNA (200 ng) was used as a template for synthesis of cDNA using the RevertAid First Strand cDNA synthesis kit (ThermoFisher, UK), according to the manufacturer's instructions. The expression levels of the five genes were assessed using quantitative RT-PCR (qRT-PCR), which was conducted according to manufacturer's instructions using SYBR^{TM} Green real-time PCR (qPCR) master mix (Applied Biosystems, USA). The previously designed reverse and forward primers flanking mRNA transcripts of *FOXP3*, *IFN-γ*, *TNF*, *IL-6* and *COX*-2 were used (Alexandre-Ramos et al., 2018; Daneshmand et al., 2016; Fu et al., 1999; Madec et al., 2009; Mencarelli et al., 2009). β-actin was used as a reference gene and as a control in the relative quantification method. Data were analysed using relative quantification of the cycle threshold (CT), and results were expressed using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Statistical analysis was performed using Minitab 17.1.0.0 for Windows (Minitab Inc., 2013; Pennsylvania, USA). Continuous data were presented as means and standard deviations (SD), and categorical data as numbers and percentages (%). The normality of the data was examined using the Shapiro-Wilk test. Comparison between the two groups of continuous data was performed using the independent *t*-test or Mann Whitney test for parametric and non-parametric variables, respectively, while more than two categorical groups were statistically analysed using the chi square test. Factors influencing the gene expressions of *IFN-γ, TNFα, FOXP3, IL-6* and *COX-2* were assessed using general linear models (GLM) with stepwise backward elimination. The accuracy of gene expression for predicting COVID-19 infection was assessed with receiver operating curve (ROC) analysis, assuming that the area under the ROC = 0.9 was significant with margins of type I error = 0.05 and type II error = 0.1. The validity of *FOXP3* gene expression for predicting mortality in COVID-19 patients was evaluated with ROC analysis, assuming that the area under the ROC = 0.8 was significant with margins of type I error = 0.05 and type II error = 0.1. Simple linear regression equations were estimated to predict *FOXP3* expressions from oxygen saturation percentage (SO2%). All tests were two-sided; P was considered significant if $<$ 0.05.

3. Results

3.1. Patients' characteristics

Among the 111 laboratory-confirmed COVID-19 positive patients,

mild and moderate cases represented 55% (n: 61), while the severe/critical cases represented 45% (n: 50) (Table 1). The severe/critical group was significantly older, with a mean age of 54 years. About 96% (n: 48) of those in the severe/critical group were diabetic and hypertensive. Cough and dyspnea were the significant clinical signs in patients in the group with the severe cases, while fever, headache and bone pain were more common among the mild and moderate cases.

3.2. Blood pictures and chemistry

Severe cases showed significantly lower levels of haemoglobin (Hb), total leucocytic (TLC) and lymphocyte counts, while having higher neutrophil counts in comparison to the mild and moderate cases. The blood chemistry was significantly higher in the group with the severe/critical cases than in those with mild and moderate cases and showed significantly higher levels of alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatinine ($P < 0.001$) (Table 1).

3.3. Gene expressions of FOXP3, IFN-γ, TNF, IL-6 and COX-2

A significantly greater downregulation of mRNA expression of *IFN-γ*, *TNFα* and *FOXP3* was detected in COVID-19 patients in comparison to the control group ($P < 0.001$) (Fig. 1). On the other hand, *IL-6* and *COX-2* were significantly upregulated ($P < 0.001$). *FOXP3* was found to be significantly unregulated in patients suffering from severe disease ($P < 0.001$) (Fig. 1, Table 1).

The possible role of *FOXP3, IFN-γ, TNF, IL-6* and *COX-2* in predicting COVID-19 is illustrated in Fig. 2. *IFN-γ* gene expressions were significantly different in the patient group than in the healthy control group, with the area under the curve (AUC) at 1 ($P < 0.001$). Furthermore, at a cut-off point of 9.02, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were equal to 100%. *FOXP3* expression was excellent in predicting the disease, where the AUC was 0.9 ($P < 0.001$). The best cut-off point value was 11.62. Accordingly, sensitivity, specificity, PPV and NPV were 87%, 88%, 61% and 97%, respectively (Table 2, Fig. 2).

TNFα also has a good power of prediction, since the area under the ROC curve (AUC) is 0.76 ($P < 0.001$). The best cut-off point with excellent (100%) specificity was 0.37 and the sensitivity, PPV and NPV were 56%, 100% and 91%, respectively. The ability of the *IL-6* and *COX-2* gene expressions for predicting COVID-19 infection was very good, since the AUCs were 76% and 81%, respectively. Also, at cut-off points of >11.26 *IL-6* expression, the sensitivity, specificity, PPV and NPV were 70%, 91%, 62% and 93%, respectively, and at cut-off points of >4.27 *COX-2* expression, the sensitivity, specificity, PPV and NPV were 70%, 84%, 50% and 93%, respectively (Table 2, Fig. 2).

3.4. Factors influencing the gene expressions of different biomarkers

While the correlations of the expression of *IFN-γ* were significant with age and respiratory rates, as well as the presence of fever ($P = 0.03$, 0.04 and 0.04, respectively), the correlation of the expression of *TNFα* was significant with platelet counts, total leukocyte counts and the presence of headache ($P < 0.001$, <0.001 and 0.05, respectively) (Table 3). There was a significant association between the expression of *FOXP3* and SO2%, RR, Hbs and creatinine levels ($P < 0.001$, 0.02, 0.04 and <0.001, respectively). The *IL-6* expression was significantly influenced by being female and also by the presence of headache ($P = 0.03$). Finally, *COX-2* expression was affected by age and lymphocytic counts, the presence of fever and SO2% ($P = 0.001$, 0.01 and 0.02, 0.01)

Table 1
Patients' clinical and laboratory characteristics.

Factors	Mild/Moderate (n = 61)		Severe/critical (n = 50)		P
	Mean/Median/N	SD/IQR/%	Mean/Median/N	SD/IQR/%	
Age	45.39	15.97	53.06	16.36	0.01$$
Sex (Male)	38	62.29	27	54	0.49#
Comorbidities					
Diabetes mellitus	10	16.39	24	48	<0.001#
Hypertension	18	29.50	24	48	0.07#
Clinical and lab data					
Cough	10	16.39	46	92	<0.001#
Dyspnea	1	1.63	26	52	<0.001#
Chest pain	3	4.91	2	4	0.98#
Fever	26	42.62	11	22	0.03#
Headache	16	26.23	1	2	0.001#
Bone ache	13	21.31	0	0	0.001#
Fatigue	12	19.67	10	20	0.84#
Vomiting	7	11.47	1	2	0.21#
Diarrhea	7	11.47	1	2	0.21#
Loss of smell	2	3.27	0	0	0.56#
SO2%	96	(95–97)	84.5	(70.75–90)	<0.001$
Respiratory rate	21	(20–23)	31	(27–34)	<0.001$
Laboratory					
HB	12	(11.25–13.25)	11.3	(10–13.2)	0.02$
PLT	196	(167.5–210)	200.5	(177.25–299)	0.33$
TLC	6	(5–7.8)	5	(3.9–7.9)	0.02$
LYMPH%	20	(13–33.5)	10	(6.75–18.25)	<0.001$
SEG%	74	(60–80)	81.5	(75–87)	<0.001$
ALT	30	(25–35.5)	39	(32–41.5)	<0.001$
CREATININE	0.78	(0.6–0.9)	1	(0.9–1.3)	<0.001$
LDH	217	(159.5–287.75)	405	(298–486.5)	<0.001$
IFNγ	0.71	(0.29–1.75)	0.84	(0.39–2.26)	0.44$
TNFα	0.3	(0.2–0.62)	0.37	(0.15–0.77)	0.68$
FOXP3	0.28	(0.06–0.595)	0.775	(0.17–14.44)	<0.00$1
IL-6	27.6	(0.23–65.25)	25.695	(11.29–48.55)	0.81$
COX2	11.551	(1.22–22.94)	13.727	(4.44–22.67)	0.2$
Outcome (Death)	2	3.27	25	50	<0.001#

$: Mann Whitney test, $$: Independent *t*-test, #: Chi square test, P considered significant if < 0.05.
Continues data represented as mean (SD) or median (IQR), and categorical data as number (%).

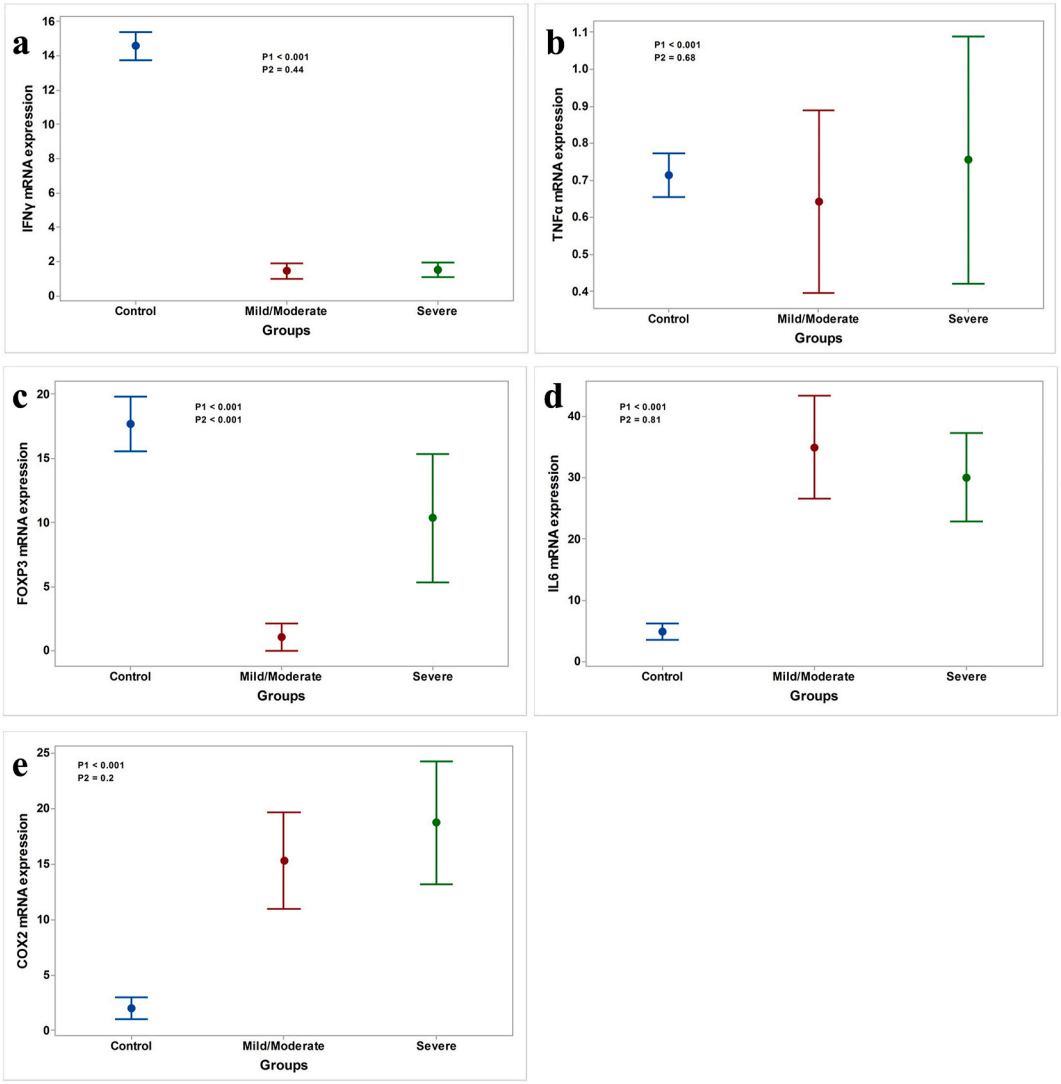

Fig. 1. IFNγ, TNFα, FOXP3, IL-6 and COX2 mRNA expression in control and COVID-19 patients. a) IFNγ, b) TNFα, c): FOXP3 d): IL-6 and e) COX2.

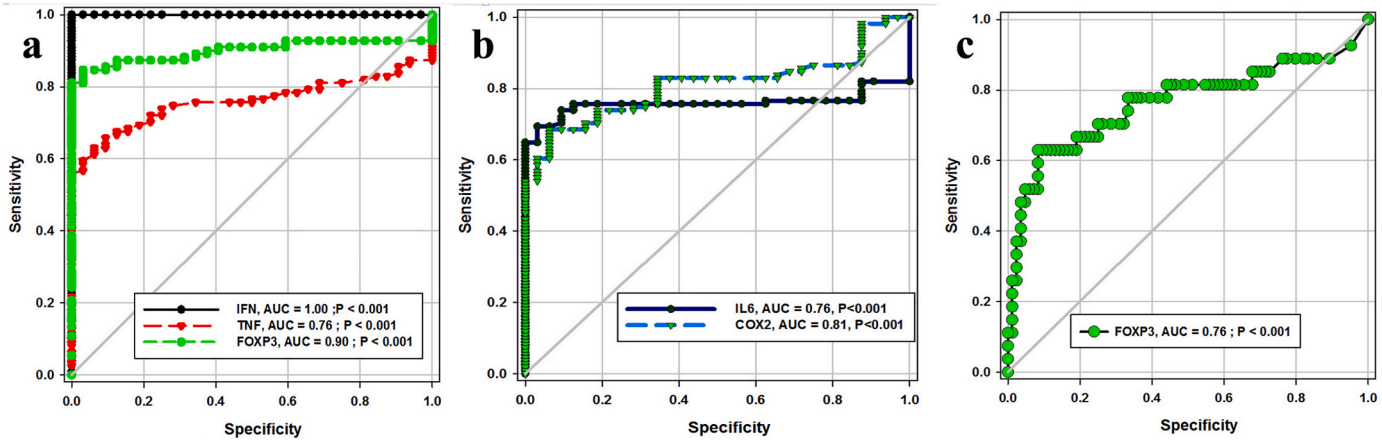

Fig. 2. ROC curve of the mRNA expression in COVID-19 infected patients. a) IFNγ, TNFα and FOXP3 mRNA expression in predicting COVID-19 disease, b) IL-6 and COX2 mRNA expression in predicting COVID-19 disease, c) FOXP3 mRNA expression in predicting mortality among COVID-19 patients.

Table 2
Performance of the expression of the different biomarkers in predicting COVID-19 disease.

Markers	Cut off	Sensitivity	95% CI	Specificity	95% CI	PPV	NPV
IFNγ	<9.02	100%	0.8911 to 1.000	100%	0.9673 to 1.000	100%	100%
TNFα	<0.37	56%	0.4612 to 0.6527	100%	0.8911 to 1.000	100%	91%
FOXP3	<11.62	87%	0.7974 to 0.9293	88%	0.7101 to 0.9649	61%	97%
IL6	>11.26	70%	0.6085 to 0.7857	91%	0.7498 to 0.9802	62%	93%
COX2	>4.278	70%	0.6085 to 0.7857	84%	0.6721 to 0.9472	50%	93%

PPV: Positive predictive value, NPV: Negative predictive value, CI: Confidence interval.

Table 3
Factors influencing the gene expression of different biomarkers in COVID-19 patients.

	IFNγ			TNFα			FOXP3			IL-6			COX-2		
	Coef	F-Value	P-Value[1]	Coef	F-Value	P-Value[§]	Coef	F-Value	P-Value[§]	Coef	F-Value	P-Value	Coef	F-Value	P-Value
Age	-0.03	5.07	0.03	0.01	0.48	0.49	-0.09	0.71	0.40	0.11	0.17	0.68	0.55	11.46	0.001
Sex	0.16	0.91	0.34	0.00	0.00	0.98	1.31	0.70	0.41	6.91	4.64	0.03	1.39	0.53	0.47
Diabetes mellitus	-0.44	2.89	0.09	0.10	0.46	0.50	3.06	2.42	0.12	-0.49	0.01	0.92	3.05	1.17	0.28
Hypertension	-0.05	0.06	0.81	-0.04	0.12	0.73	-2.47	2.16	0.15	-1.69	0.17	0.68	2.34	0.97	0.33
SO2%	0.00	0.00	0.96	-0.02	1.15	0.29	-0.86	13.31	<0.001	0.65	1.32	0.25	-1.10	7.10	0.01
Respiratory rate	-0.11	4.32	0.04	-0.02	0.34	0.56	-0.92	5.67	0.02	0.65	0.46	0.50	-0.49	0.79	0.38
Severity	-0.43	2.73	0.10	-0.03	0.04	0.84	-1.49	0.56	0.46	0.99	0.04	0.84	1.89	0.33	0.57

§: General linear model (GLM) with stepwise backward elimination, Coef: Coefficient, the negative sign denotes a reverse relationship, $P < 0.05$ is considered significant.

(Table 3).

3.5. Comparison of mRNA expression of the different biomarkers in survivors and patients who died

The total mortality rate in the cohort that was studied was 24.32% (27 of 111) (Table 1). The mortality was significantly higher in the group with severe disease (P < 0.001). *FOXP3* expression was the only biomarker in which there was a significant association between upregulation and mortality (P < 0.001) (Table 4). Moreover, *FOXP3* expression was found to be a good predictive biomarker for mortality among COVID-19 patients, where the AUC was 0.76 ($P < 0.001$) (Fig. 2). The best cut-off point was >0.63, with sensitivity, specificity, PPV and NPV at 70%, 75%, 38% and 92%, respectively.

4. Discussion

In the current study, there was a significant decrease in the lymphocyte counts, especially in the severe cases that were also correlated to *COX-2* expression and SO2%. Pronounced lymphopenia and high serum pro-inflammatory cytokines had previously been reported to be associated with COVID-19 (Chen et al., 2020; Tan et al., 2020). Deterioration of the condition is usually accompanied by decreased lymphocyte counts and high levels of D-dimer, as well as extensions of ground-glass lung opacities on chest CT scans (Huang et al., 2020; Liu et al., 2020; Tan et al., 2020; Yu et al., 2020). Lung injury is one of the major threatening conditions associated with COVID-19 that result from the cytokine storm that may precede acute lung injury or ARDS (Gallelli et al., 2020; Ragab et al., 2020; Shimizu, 2019). Replacement of the FABP4+ macrophages with the inflammatory FCN1+ macrophages was detected in the lungs of patients who were severely affected by SARS-CoV-2. However, clonal expansion of $CD8^+$ T cells, especially those specific for conserved coronavirus epitopes cells, was also detected in mild or moderate disease (Liao et al., 2020; Mallajosyula et al., 2021), and the presence of a greater number of these cells was associated with improved survival in patients with hematologic cancer who had COVID-19 (Bange et al., 2021).

Table 4
Comparison of mRNA expression of the different biomarkers in dead and survivors.

Markers	Dead (n = 27)		Survived (n = 84)		P[$]
	Median	IQR	Median	IQR	
IFNγ	0.79	(0.41–2.25)	0.775	(0.31–2.05)	0.9
TNFα	0.42	(0.20–0.80)	0.3	(0.19–0.61)	0.13
FOXP3	6.32	(0.51–32.42)	0.28	(0.10–0.68)	<0.001
IL-6	27.07	(11–50.4)	25.19	(4.37–51.99)	0.9
COX2	14	(3.45–40.05)	12.27	(1.60–19.49)	0.18

The data represented as median and inter quartile rang (IQR), $: Mann Whitney test, P considered significant if < 0.05.

In our cohort, *FOXP3* expression was found to be significantly downregulated in COVID-19 patients. This proves that some degree of loss of the suppressive and regulatory functions of Tregs is associated with COVID-19 infection. However, progression occurs only in a proportion of patients who may have other risk factors. It is interesting to note that, similar to previous reports, older ages, males and the presence of comorbidities were associated with the development of severe forms of the disease (Conti and Younes, 2020; Harrison et al., 2020; Palaiodimos et al., 2020; Tan et al., 2020).

In addition, *FOXP3* expression was found to be a good predictive biomarker for mortality, since it was upregulated in severe cases and coincides with the development of severe hypoxia and the deaths of patients. In a recent study, the ratio of Tregs to all $CD4^+$ lymphocytes was three times higher in patients who had acute respiratory distress syndrome (ARDS) and did not survive and twice as high in survivors, compared to the control group (Adamzik et al., 2013). Moreover, the ratio of T regulatory lymphocytes to all $CD4^+$ lymphocytes in the bronchoalveolar lavage (BAL) was found to be an independent prognostic factor for 30-day survival in this study group (Adamzik et al., 2013). Another study showed that blood Tregs/CD4+ percentages were higher in patients who developed ARDS than in those who did not, and that a threshold of 10.4% for the blood Tregs/CD4+ percentages during the first week of ARDS was able to distinguish survivors from non-survivors (Halter et al., 2020).

While *FOXP3* was generally downregulated in COVID-19, it was upregulated in severe cases, with the development of severe hypoxia. The presence of hypoxia in association with upregulation of *FOXP3* and the production of Tregs was previously reported (Ben-Shoshan et al.,

2008; Neildez-Nguyen et al., 2015). This effect depends mainly on hypoxia-inducible factor-1α (HIF-1 α), which drives the abundance and the function of Treg during inflammation (Ben-Shoshan et al., 2008; Clambey et al., 2012; Wu et al., 2014). However, a report by Hsu and Lai did not find a strong evidence that HIF-1 α is needed for FOXP3 expression (Hsu and Lai, 2018).

Epithelial proliferation in the lungs after acute lung injury (ALI) is associated with an increase in FOXP3+ Treg cells in the lung during resolution. This role can be mediated through integrin CD103, which plays a role in retaining FOXP3 Treg cells in the lung after injury (Mock et al., 2014). We hypothesize that the upregulation of *FOXP3* in patients with severe hypoxia is a desperate attempt by the body to modify the immune response and overcome and repair the lung injury associated with severe hypoxia and decreased haemoglobin levels. However, this trial is usually too late, and death follows. A previous report suggested that the levels of functional Tregs are reduced as severity increases in COVID-19 patients (Stephen-Victor et al., 2020). Based on our findings, we suggest that the opposite is correct, since *FOXP3* showed a generalized downregulation in COVID-19 patients. However, the levels of functional Tregs tend to increase with the development of hypoxia and with increased disease severity. *FOXP3* expression was correlated with increased creatinine levels, which is also an indicator for the severity and progression of the disease.

The development of auto-immune and auto-inflammatory disorders such as paediatric multi-systemic inflammatory syndrome, Guillain–Barré syndrome, auto-immune haemolytic anaemia, auto-immune thrombocytopenic purpura and Graves' disease was reported among patients after they were infected with COVID-19 (Galeotti and Bayry, 2020; Mateu-Salat et al., 2020). Downregulation of *FOXP3*, the marker of Tregs, might be among the possible leading causes for this.

On the other hand, *IL-6* was significantly more upregulated in COVID-19 patients than in the control group. This finding indicates that there are generalized changes in the immune system of COVID-19 patients, but that the progression and severity might result from a combination of different factors in addition to the initial immune regulation associated with virus infection. In our cohort, although the expression of *IL-6* was not associated with deteriorated outcomes for patients infected with COVID-19, the median gene expression in patients who did not survive was more than double that of the median expression in COVID-19 patients in general. This indicates a progression of immune dysregulation and the possibility of using *IL-6* as a progression and prognostic marker. An early meta-analysis conducted in April 2020 demonstrated that higher mean levels of serum *IL-6* on admission were associated with increased likelihoods of death (Aziz et al., 2020). Similar results were reported with subsequent recommendations to use *IL-6* as a prognostic marker of disease progression and mortality (Grifoni et al., 2020; Wang et al., 2020).

The *COX-2* gene was also significantly upregulated in COVID-19 patients in the current study. Two studies on SARS-CoV showed that both the nucleocapsids and the spike proteins play important roles in virus-stimulated *COX-2* expression (Liu et al., 2007; Yan et al., 2006). *COX-2* was also hyper-induced in macrophages and in the epithelial cells of autopsied lung tissues of patients infected in vitro with the H5N1 influenza virus, and *COX-2* inhibitors suppressed the H5N1 hyper-induced cytokines in the pro-inflammatory cascades (Lee et al., 2013). A previous report recommended using *COX-2* inhibition medications, among others, in high doses early in the course of COVID-19 disease, post infection and/or at symptom presentation (Verrall, 2020).

The spectrum of COVID-19 cases varies from asymptomatic to severe and fatal. In general, the clinical deterioration of patients infected with SARS-CoV-2 is thought to be the result of cytokine storms, and/or the renin-angiotensin-aldosterone system imbalance (Abdel-Moneim et al., 2021; Liu et al., 2020; Rysz et al., 2021). In Egypt, the treatment protocol for COVID-19 patients includes the use of the macrolide antimicrobial azithromycin (AZM), which has been suggested to have anti-inflammatory properties through inhibiting the production of pro-inflammatory cytokines. It was found that AZM significantly increased the production of IL-10 which strongly impedes IL-1, IL-8 and TNF-α production from the inflammatory cells (Sugiyama et al., 2007). In addition, the use of AZM was associated with reductions of IL-1α and TNF-α in 100% of the stimulated human monocytes collected from different individuals (Khan et al., 1999). AZM use was also associated with the reduction of IL-8, IFN-γ and TNF-α levels in the cervical secretions in female patients infected with *Chlamydia trachomatis* (Srivastava et al., 2009). Both in vitro and in vivo studies have shown the efficacy of macrolides in respiratory viral infections including influenza virus, where their use was associated with decrease in the release of proinflammatory cytokines (Min and Jang, 2012). Immunomodulatory effects of azithromycin are multifactorial, and some authors thought that through its effect on immune cells and cytokine release, AZM could be beneficial in some COVID-19 patients (Venditto et al., 2021). Collectively, the use of AZM in COVID-19 patients could be one of the mechanisms that contributed to the downregulation of IFN-γ and TNF-α in our patients. However, our results don't present a solid evidence for this effect, and more studies are needed to confirm or disprove the direct effect of AZM on the inflammatory cytokines in COVID-19.

5. Conclusion

Here, we concluded that that SARS-CoV-2 infection is associated with the downregulation of *FOXP3*, with the latter being the marker of T regulatory lymphocytes, and upregulation of the inflammatory genes *IL-6* and *COX-2*. As the disease progresses, upregulation of *FOXP3* follows, and this is associated with the severity of hypoxia and the impending death of patients infected with COIVD-19. This upregulation could, then, be used as a biomarker for disease deterioration. Using anti-inflammatory drugs early in the course of the disease could be useful to limit disease progression. The kinetic curve for gene expression of immune and inflammatory markers is also recommended to gain a better understanding of the interaction of SARS-CoV-2 with the host immune system.

5.1. Limitations of the study

Our study has two major limitations Firs, collection of a single sample form each patient is considered as a limitation of this study. We recommend collection of serial samples from each patient at different stages of the disease to monitor the change in the expression of these markers along with the progress of the disease. Second, we collected two types of clinical data about the patients: the clinical condition of the patient at the time of samples collection, and whether the disease led to survival or mortality. We haven't studied the evolution of clinical condition of the patients during the course of the disease.

CRediT authorship contribution statement

Ahmed S. Abdelhafiz: Conceptualization, methodology, and writing-original draft preparation. **Mariam A. Fouad:** Conceptualization, methodology, formal analysis, and laboratory analysis. **Mohamed M. Sayed-Ahmed:** Conceptualization, methodology, and laboratory analysis. **Mahmoud M. Kamel:** Conceptualization, supervision, and validation. **Asmaa Ali:** Data processing, statistics, and formal analysis. **Merhan Fouda:** Data curation, samples, data collection and curation. **Mahmoud A. Khalil:** Data curation, samples, data collection and curation. **Ahmed S. Abdel-Moneim:** Writing – review & editing. **Lamyaa M. Kamal:** Data curation, samples, data collection and curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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References

Abdel-Moneim, A.S., Abdelwhab, E.M., Memish, Z.A., 2021. Insights into SARS-CoV-2 evolution, potential antivirals, and vaccines. Virology 558, 1–12.
Adamzik, M., Broll, J., Steinmann, J., Westendorf, A.M., Rehfeld, I., Kreissig, C., Peters, J., 2013. An increased alveolar CD4+ CD25+ Foxp3+ T-regulatory cell ratio in acute respiratory distress syndrome is associated with increased 30-day mortality. Intensive Care Med. 39, 1743–1751.
Aggarwal, N.R., D'Alessio, F.R., Tsushima, K., Sidhaye, V.K., Cheadle, C., Grigoryev, D. N., Barnes, K.C., King, L.S., 2010. Regulatory T cell-mediated resolution of lung injury: identification of potential target genes via expression profiling. Physiol. Genom. 41, 109–119.
Alexandre-Ramos, D.S., Silva-Carvalho, A., Lacerda, M.G., Serejo, T.R.T., Franco, O.L., Pereira, R.W., Carvalho, J.L., Neves, F.A.R., Saldanha-Araujo, F., 2018. LL-37 treatment on human peripheral blood mononuclear cells modulates immune response and promotes regulatory T-cells generation. Biomed. Pharmacother. Biomed. pharmacother. 108, 1584–1590.
Aziz, M., Fatima, R., Assaly, R., 2020. Elevated interleukin-6 and severe COVID-19: a meta-analysis. J. Med. Virol. 92, 2283–2285.
Bacchetta, R., Gambineri, E., Roncarolo, M.G., 2007. Role of regulatory T cells and FOXP3 in human diseases. J. Allergy Clin. Immunol. 120, 227–235 quiz 236-227.
Ben-Shoshan, J., Maysel-Auslender, S., Mor, A., Keren, G., George, J., 2008. Hypoxia controls CD4+ CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1α. Eur. J. Iimmunol. 38, 2412–2418.
Bruzzese, V., Lazzarino, A.I., 2020. The paradoxical effect of IL-6 and implications for the use of Tocilizumab in Covid-19 patients. Med. Hypotheses 144, 110284-110284.
Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T., Zhang, X., Chen, H., Yu, H., 2020. Clinical and immunological features of severe and moderate coronavirus disease 2019. J. Clin. Invest. 130.
Clambey, E.T., McNamee, E.N., Westrich, J.A., Glover, L.E., Campbell, E.L., Jedlicka, P., de Zoeten, E.F., Cambier, J.C., Stenmark, K.R., Colgan, S.P., 2012. Hypoxia-inducible factor-1 alpha–dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. Proc. Natl. Acad. Sci. U.S.A. 109. E2784-E2793.
Conti, P., Younes, A., 2020. Coronavirus COV-19/SARS-CoV-2 affects women less than men: clinical response to viral infection. J. Biol. Regul. Homeost. Agents 34, 71.
Daneshmand, P., Saliminejad, K., Dehghan Shasaltaneh, M., Kamali, K., Riazi, G.H., Nazari, R., Azimzadeh, P., Khorram Khorshid, H.R., 2016. Neuroprotective effects of herbal extract (Rosa canina, Tanacetum vulgare and Urtica dioica) on rat model of sporadic alzheimer's disease. Avicenna J. Med. Biotechnol. (AJMB) 8, 120–125.
Dinarello, C.A., 2000. Proinflammatory cytokines. Chest 118, 503–508.
Duque, J., Díaz-Muñoz, M.D., Fresno, M., Iñiguez, M.A., 2006. Up-regulation of cyclooxygenase-2 by interleukin-1beta in colon carcinoma cells. Cell. Signal. 18, 1262–1269.
Fu, S., Ramanujam, K.S., Wong, A., Fantry, G.T., Drachenberg, C.B., James, S.P., Meltzer, S.J., Wilson, K.T., 1999. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in Helicobacter pylori gastritis. Gastroenterology 116, 1319–1329.
Galeotti, C., Bayry, J., 2020. Autoimmune and inflammatory diseases following COVID-19. Nat. Rev. Rheumatol. 1–2.
Gallelli, L., Zhang, L., Wang, T., Fu, F., 2020. Severe acute lung injury related to COVID-19 infection: a review and the possible role for escin. J. Clin. Pharmacol. 60, 815–825.
Grifoni, E., Valoriani, A., Cei, F., Lamanna, R., Gelli, A.M.G., Ciambotti, B., Vannucchi, V., Moroni, F., Pelagatti, L., Tarquini, R., 2020. Interleukin-6 as prognosticator in patients with COVID-19: IL-6 and Covid-19. J. Infect. 81 (3), 452–482.
Guan, W.-j., Ni, Z.-y., Hu, Y., Liang, W.-h., Ou, C.-q., He, J.-x., Liu, L., Shan, H., Lei, C.-l., Hui, D.S., 2020. Clinical characteristics of coronavirus disease 2019 in China. N. Engl. J. Med. 382, 1708–1720.
Hadjadj, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Pere, H., Charbit, B., Bondet, V., Chenevier-Gobeaux, C., Breillat, P., 2020. Impaired type I interferon activity and exacerbated inflammatory responses in severe Covid-19 patients. Science 369, 718–724.
Halter, S., Aimade, L., Barbié, M., Brisson, H., Rouby, J.-J., Langeron, O., Klatzmann, D., Rosenzwajg, M., Monsel, A., 2020. T regulatory cells activation and distribution are modified in critically ill patients with acute respiratory distress syndrome: a prospective single-centre observational study. Anaesth. Crit. Care Pain Med. 39, 35–44.
Harrison, S.L., Fazio-Eynullayeva, E., Lane, D.A., Underhill, P., Lip, G.Y., 2020. Comorbidities associated with mortality in 31,461 adults with COVID-19 in the United States: a federated electronic medical record analysis. PLoS Med. 17, e1003321.
Hsu, T.S., Lai, M.Z., 2018. Hypoxia-inducible factor 1α plays a predominantly negative role in regulatory T cell functions. J. Leukoc. Biol. 104, 911–918.
Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., Zhang, L., Fan, G., Xu, J., Gu, X., 2020. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 395, 497–506.
Jamilloux, Y., Henry, T., Belot, A., Viel, S., Fauter, M., El Jammal, T., Walzer, T., François, B., Sève, P., 2020. Should we stimulate or suppress immune responses in COVID-19? Cytokine and anti-cytokine interventions. Autoimmun. Rev. 19, 102567.
Khan, A., Slifer, T., Araujo, F., Remington, J., 1999. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. Int. J. Antimicrob. Agents 11, 121–132.
Lai, C.-C., Shih, T.-P., Ko, W.-C., Tang, H.-J., Hsueh, P.-R., 2020. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and corona virus disease-2019 (COVID-19): the epidemic and the challenges. Int. J. Antimicrob. Agents 55 (3), 105924. https://doi.org/10.1016/j.ijantimicag.2020.105924, 105924.
Lee, M., Cheung, C., Peiris, J., 2013. Role of cyclooxygenase-2 in H5N1 viral pathogenesis and the potential use of its inhibitors. Hong Kong Med. J. 4 (19), 29–35.
Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., Cheng, L., Li, J., Wang, X., Wang, F., Liu, L., Amit, I., Zhang, S., Zhang, Z., 2020. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. Nat. Med. 26, 842–844.
Liu, M., Yang, Y., Gu, C., Yue, Y., Wu, K.K., Wu, J., Zhu, Y., 2007. Spike protein of SARS-CoV stimulates cyclooxygenase-2 expression via both calcium-dependent and calcium-independent protein kinase C pathways. Faseb. J. 21, 1586–1596.
Liu, Y., Qi, G., Bellanti, J.A., Moser, R., Ryffel, B., Zheng, S.G., 2020. Regulatory T cells: a potential weapon to combat COVID-19? MedComm 1, 157–164.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25, 402–408.
Luo, Y., Zheng, S.G., 2016. Hall of fame among pro-inflammatory cytokines: interleukin-6 gene and its transcriptional regulation mechanisms. Front. Immunol. 7, 604.
Madec, A.M., Mallone, R., Afonso, G., Abou Mrad, E., Mesnier, A., Eljaafari, A., Thivolet, C., 2009. Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia 52, 1391–1399.
Mallajosyula, V., Ganjavi, C., Chakraborty, S., McSween, A.M., Pavlovitch-Bedzyk, A.J., Wilhelmy, J., Nau, A., Manohar, M., Nadeau, K.C., Davis, M.M., 2021. CD8(+) T cells specific for conserved coronavirus epitopes correlate with milder disease in COVID-19 patients. Sci. Immunol. 6.
Mateu-Salat, M., Urgell, E., Chico, A., 2020. SARS-COV-2 as a trigger for autoimmune disease: report of two cases of Graves' disease after COVID-19. J. Endocrinol. Invest. 43, 1527–1528.
Mencarelli, A., Renga, B., Distrutti, E., Fiorucci, S., 2009. Antiatherosclerotic effect of farnesoid X receptor. Am. J. Physiol. Heart Circ. Physiol. 296, H272–H281.
Mock, J.R., Garibaldi, B.T., Aggarwal, N.R., Jenkins, J., Limjunyawong, N., Singer, B.D., Chau, E., Rabold, R., Files, D.C., Sidhaye, V., 2014. Foxp3+ regulatory T cells promote lung epithelial proliferation. Mucosal Immunol. 7, 1440–1451.
Neildez-Nguyen, T.M.A., Bigot, J., Da Rocha, S., Corre, G., Boisgerault, F., Paldi, A., Galy, A., 2015. Hypoxic culture conditions enhance the generation of regulatory T cells. Immunology 144, 431–443.
Palaiodimos, L., Kokkinidis, D.G., Li, W., Karamanis, D., Ognibene, J., Arora, S., Southern, W.N., Mantzoros, C.S., 2020. Severe obesity, increasing age and male sex are independently associated with worse in-hospital outcomes, and higher in-hospital mortality, in a cohort of patients with COVID-19 in the Bronx. New York Metabolism 108, 154262.
Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R., Salem, R., 2020. The COVID-19 cytokine storm; what we know so far. Front. Immunol. 11, 1446.
Rysz, S., Al-Saadi, J., Sjöström, A., Farm, M., Campoccia Jalde, F., Plattén, M., Eriksson, H., Klein, M., Vargas-Paris, R., Nyrén, S., Abdula, G., Ouellette, R., Granberg, T., Jonsson Fagerlund, M., Lundberg, J., 2021. COVID-19 pathophysiology may be driven by an imbalance in the renin-angiotensin-aldosterone system. Nat. Commun. 12, 2417.
Sakaguchi, S., Miyara, M., Costantino, C.M., Hafler, D.A., 2010. FOXP3+ regulatory T cells in the human immune system. Nat. Rev. Immunol. 10, 490–500.
Shimizu, M., 2019. Clinical Features of Cytokine Storm Syndrome, Cytokine Storm Syndrome. Springer, pp. 31–41.
Srivastava, P., Jha, H.C., Salhan, S., Mittal, A., 2009. Azithromycin treatment modulates cytokine production in Chlamydia trachomatis infected women. Basic Clin. Pharmacol. Toxicol. 104, 478–482.
Stephen Victor, E., Das, M., Karnam, A., Pitard, B., Gautier, J.F., Bayry, J., 2020. Potential of regulatory T-cell-based therapies in the management of severe COVID-19. Eur. Respir. J. 56.
Sugiyama, K., Shirai, R., Mukae, H., Ishimoto, H., Nagata, T., Sakamoto, N., Ishii, H., Nakayama, S., Yanagihara, K., Mizuta, Y., Kohno, S., 2007. Differing effects of clarithromycin and azithromycin on cytokine production by murine dendritic cells. Clin. Exp. Immunol. 147, 540–546.
Tan, L., Wang, Q., Zhang, D., Ding, J., Huang, Q., Tang, Y.Q., Wang, Q., Miao, H., 2020. Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. Signal Transduct. Target Ther. 5, 33.
Thevarajan, I., Nguyen, T.H.O., Koutsakos, M., Druce, J., Caly, L., van de Sandt, C.E., Jia, X., Nicholson, S., Catton, M., Cowie, B., Tong, S.Y.C., Lewin, S.R., Kedzierska, K., 2020. Breadth of concomitant immune responses prior to patient recovery: a case report of non-severe COVID-19. Nat. Med. 26, 453–455.
Verrall, G.M., 2020. Scientific rationale for a bottom-up approach to target the host response in order to try and reduce the numbers presenting with adult respiratory distress syndrome associated with COVID-19. Is there a role for statins and COX-2 inhibitors in the prevention and early treatment of the disease? Front. Immunol. 11, 2167.
Wang, C., Fei, D., Li, X., Zhao, M., Yu, K., 2020. IL-6 may be a good biomarker for earlier detection of COVID-19 progression. Intensive Care Med. 46, 1475–1476.

Wu, J., Cui, H., Zhu, Z., Wang, L., Li, H., Wang, D., 2014. Effect of HIF1α on Foxp3 expression in CD4+ CD25− T lymphocytes. Microbiol. Immunol. 58, 409–415.
Wu, Z., McGoogan, J.M., 2020. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese center for disease control and prevention. JAMA 323, 1239–1242.
Yan, X., Hao, Q., Mu, Y., Timani, K.A., Ye, L., Zhu, Y., Wu, J., 2006. Nucleocapsid protein of SARS-CoV activates the expression of cyclooxygenase-2 by binding directly to regulatory elements for nuclear factor-kappa B and CCAAT/enhancer binding protein. Int. J. Biochem. Cell Biol. 38, 1417–1428.
Ye, Q., Wang, B., Mao, J., 2020. The pathogenesis and treatment of the Cytokine Storm'in COVID-19. J. Infect. 80, 607–613.
Yu, H.H., Qin, C., Chen, M., Wang, W., Tian, D.S., 2020. D-dimer level is associated with the severity of COVID-19. Thromb. Res. 195, 219–225.