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Potential relationships between circulating gene expression of *ACE2*, *TLR4*, and *IL-17* and disease severity and outcome of hospitalized patients with COVID-19

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Abstract: Acute respiratory distress syndrome (ARDS) is a form of progressive hypoxemia that can be brought on by a variety of cardiorespiratory or systemic disorders, such as coronavirus disease 2019 (COVID-19). The binding of a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus spike protein to the cell membrane is mediated through its binding to angiotensin-converting enzyme 2 (ACE2) receptors, resulting in viral entry, replication, and induction of a signaling cascade inducing pro-inflammatory responses that are linked to a higher mortality rate and the progression of ARDS, leading to multi-organ failure in these patients. We aimed to analyze the relationships between circulating gene expression levels of *ACE2*, Toll-like receptor 4 (*TLR4*), and interleukin-17 (*IL-17*) and the clinical severity of COVID-19, as well as the associated pathogenic conditions, in hospitalized patients. Sixty COVID-19 patients (34 mild/moderate COVID-19 and 26 COVID-19 with severe ARDS manifestation) and 60 healthy controls were included. The patient group was also subdivided according to outcomes into 32 recoveries and 28 deaths. *ACE2*, *TLR4*, and *IL-17* levels were assessed by quantitative polymerase chain reaction (qPCR) in addition to all routine baseline laboratory investigations, including complete blood count (CBC) with differential analysis and the levels of C-reactive protein (CRP), ferritin, and D-dimer. ACE2, TLR4, and IL-17 serum expression levels were significantly higher in the COVID-19 group and subgroups and were correlated with different laboratory and clinical parameters. The serum expression levels of

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ACE2, TLR4, and IL-17 were accurate in differentiating between the patient groups and controls, with 86.7%, 91.7%, and 95.0% sensitivity and 96.7%, 98.3%, and 98.3% specificity, respectively, and correlated with more severe disease courses in COVID-19 patients. Higher levels are associated with overwhelmingly distressing outcomes. Our results allow us to conclude that increased circulating gene expression levels of *ACE2*, *TLR4*, and *IL-17* are important in assessing the severity of COVID-19. Consequently, targeting these biomarkers may offer additional therapeutic options for COVID-19 patients in the future.

Key words: Acute respiratory distress syndrome (ARDS); Coronavirus disease 2019 (COVID-19); Intensive care unit (ICU); Angiotensin-converting enzyme 2 (ACE2); Toll-like receptor 4 (TLR4); Interleukin-17 (IL-17); Polymerase chain reaction (PCR)

1 Introduction

Acute respiratory failure (ARF) or acute respiratory distress syndrome (ARDS) is a type of acute and progressive hypoxemia brought on by a variety of systemic cardiorespiratory disorders or traumas that involve bilateral lung infiltration (Fujishima, 2023). Histological analysis of lung tissue from ARDS patients reveals acute inflammation such as neutrophil dominance and diffuse alveolar damage to hyaline membranes. Increased pulmonary microvascular permeability, which leads to pulmonary edema as a result of tissue damage and vascular regulatory system disturbance, is part of the pathogenesis of ARDS. Although ARDS was once thought to be a single-organ malfunction, it is now understood to be one part of multiple-organ dysfunction syndrome (Liang et al., 2020; Fujishima, 2023).

By July 16, 2020, nearly 13 378 853 confirmed cases of coronavirus disease 2019 (COVID-19) had been reported in 216 countries, with 580 045 confirmed deaths (Azkur et al., 2020; Liang et al., 2020). The majority of patients had no symptoms (Sohn et al., 2020). Only 10% of all the affected patients progressed to a severe state characterized by dyspnea, lymphopenia, and extensive chest X-ray findings. Of these, half developed critical illness with respiratory and multi-organ failure (Fu et al., 2020; Pascarella et al., 2020).

With a genomic size ranging from 27 to 32 kb, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the largest RNA virus. It is an enclosed, positive-sense, single-stranded virus. It has 16 non-structural proteins (nsp1–nsp16) and 4 structural proteins (nucleocapsid (N), membrane (M), spike (S), and envelope E). The N protein forms a helical capsid that contains the genome firmly, and this capsid is encased in a lipid bilayer envelope. The M, S, and E proteins combine to create this envelope. Due to its requirement for virus entry and its role in providing microbiological underpinnings of viral tropism, the S protein is of therapeutic importance and may be a target for antiviral drugs (Chee et al., 2023).

In terms of the proteomics of the S protein, its total length is 1273 amino acids; it has an extracellular N-terminus attached with a signal peptide chain of 1–13 amino acids and a short intracellular C-terminal segment with a transmembrane domain. This metastable prefusion conformation permits the S protein to undergo significant structural reorganization when the virus engages in host–cell interactions. Additionally, S proteins have polysaccharide covers to blend in and avoid being noticed by the host immune system when they are first introduced (Alabsi et al., 2023).

The initial stage of viral infection is marked by entry into the host cell; the S protein is subjected to proteolysis and divided into S1 and S2 subunits; these two sections are responsible for receptor binding and membrane fusion, respectively (Zheng, 2022). Increased protein proteolysis can be facilitated by furin from the host cell, serine proteases such as transmembrane serine protease (TMPRSS) or transmembrane protease, or cathepsin proteases found in endolysosomes (Sodhi et al., 2023). Type II transmembrane serine protease (TMPRSS2) is abundantly expressed in respiratory epithelial cells, gastrointestinal tract (GIT), and the urogenital tract (Au Yeung et al., 2023).

Once the S1/S2 site is cleaved, the S1 protein links to the host cell membrane by binding to angiotensin-converting enzyme 2 (ACE2). It uses the receptor-binding domain to gain access, whereas the S2 portion connects with the membrane. A second cleavage site then occurs at S2 to catalyze fusion to cellular membranes (Oudit et al., 2023). ACE2, a homolog of ACE, is a type I transmembrane glycoprotein. The renin–angiotensin system, which transforms angiotensin II (Ang II) into Ang-(1–7), is thought to be negatively regulated by ACE2. Nevertheless, ACE1 is a catalyst for the biogenesis of Ang II from Ang I (Liu et al.,

2022). The degree of epithelial-cell ACE2 expression affects susceptibility to COVID-19. Some have suggested that children have a lower risk of contracting COVID-19 because they have fewer ACE2 receptors than adults do (Bartolák-Suki et al., 2022). Numerous investigations have discovered a link between the ACE2 G8790A polymorphism and COVID-19 risk. In Asians, the G allele of ACE2 G8790A is linked to an increased risk of COVID-19 severity. The ACE2 G allele has been linked to a COVID-19 cytokine storm, which is one possible explanation (Pan, 2023).

A panel of conserved pattern-recognition receptors (PRRs), including toll-like receptor 4 (TLR4), forms a type I transmembrane protein. It is activated by various pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which in turn cause inflammation and an innate immune response in higher animals (Kuzmich et al., 2017). It plays a vital part in the pathophysiology of SARS-CoV-2. Remarkably, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), the primary cytokines implicated in severe COVID-19 cases, are located downstream of the TLR4 signaling pathway (Brandão et al., 2021). The activated signaling pathway of TLR4 leads to higher production of pro-inflammatory cytokines and chemokines in lung cells, increasing the severity of disease, especially of developing ARDS (Manik and Singh, 2022).

The pro-inflammatory cytokine (IL-17) is active in host defense mechanisms, immune modulation, tissue damage, physiological stress, stimulation of inflammation, and neutrophil activation (Sultan et al., 2022). IL-17 increases inflammatory reactions that initiate cytokine storms by inducing the production of IL-1, IL-6, IL-8, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) (Montazersaheb et al., 2022); this explains its pathogenic role in alveolar inflammation and destruction of the lung parenchyma in ARDS (Wu et al., 2022). It also leads to viral persistence by affecting the replication of the virus through the inhibition of apoptosis in infected cells (Darif et al., 2021).

Considering the pivotal roles of ACE-II, TLR4, and IL-17 in inflammation, we aimed to assess the potential associations of TLR4 and ACE-II with IL-17 levels and thus with COVID-19 severity and outcomes and with ARDS complications together with other different clinical and laboratory parameters.

2 Patients and methods

This case–control research was carried out on COVID-19-positive patients at the Assiut University Quarantine Hospital, Egypt. The participants enrolled in the study were divided into Group 1, which included sixty polymerase chain reaction (PCR)-positive COVID-19 patients, and Group 2, which included sixty completely healthy, age- and sex-matched volunteer controls who showed no evidence of respiratory disease following a medical check.

We further subdivided the patient group as follows:

(1) According to the severity of the disease, Group 1A included 34 mild to moderate COVID-19 patients (mild/moderate), and Group 1B included 26 COVID-19 patients with severe ARDS manifestation.

Pandemic severity-assessment scales (PSAS) provide information to estimate the timing, magnitude, and intensity of pandemics. The World Health Organization (WHO) Pandemic Influenza Severity Assessment (PISA) characterizes pandemic severity (World Health Organization, 2020) in terms of three indicators: transmissibility, seriousness of disease, and effect. For COVID-19, we modified the PISA approach and classified the severity of the disease as critical, severe, or non-severe. Our severity evaluation was based on intensive care unit (ICU) admission and indications of ARDS as follows:

Critical: people who had ever been hospitalized, admitted to the ICU; ever on invasive ventilation, ever on oxygen and on high-flow nasal oxygen, ever on extracorporeal membrane oxygenation (ECMO), or ever on oxygen alone;

Severe: people who had ever been on oxygen alone;

Non-severe (mild/moderate): if none of the preconditions listed above were satisfied.

(2) During their admission period, we further subdivided the patient group according to their outcome into 32 recoveries and 28 deaths.

Inclusion criteria: hospitalized individuals diagnosed with COVID-19 were enrolled. Diagnosis was based on positive chest computed tomography (CT) features and confirmed by positive quantitative reverse transcriptase-PCR (qRT-PCR) analysis of patients' nasal-swab specimens, in accordance with WHO recommendations.

Exclusion criteria: patients who were pregnant; age under 18 years; a diagnosis of cancer; concurrent

sepsis, septic shock, or confirmed bacterial infection; the use of immunosuppressive medication; or a history of autoimmune or chronic inflammatory conditions.

2.1 Sample collection

Under aseptic conditions, 5 mL of whole blood samples were drawn from all participants. At the time of hospitalization, 2 mL were collected in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 8000*g* for 5 min, and the other 3 mL were collected in an empty Wasserman tube and centrifuged for the serum collection needed for other laboratory investigations.

2.2 History and biochemical parameters

A detailed medical history was obtained from all participants, including age, gender, and comorbid conditions, as well as the presenting clinical symptoms at the time of hospital admission, during the inpatient period, and at release. Patients also underwent chest CT examinations.

Baseline laboratory investigations, including complete blood count (CBC) with differential analysis, C-reactive protein (CRP), ferritin, and D-dimer, were performed at the Central Laboratories of Assiut University Hospitals. All demographic and clinical data of the patients were recorded from patient files.

2.3 Evaluation of circulating mRNA expression levels of *ACE2*, *TLR4*, and *IL-17*

Total RNA extraction was performed on all included samples using the Thermo Scientific Gene JET RNA Purification Kit (catalog No. #K0731). Total RNA (500 ng) from each sample was then transcribed into complementary DNA (cDNA) using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (catalog No. #K1622). Finally, qRT-PCR was performed using the Thermo Scientific Maxima SYBR Green qRT-PCR Master Mix (catalog No. #K0251). *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as housekeeping genes for *ACE2* and *TLR4*, respectively.

All the reactions were performed at the Medical Research Center, Assiut University, in an RNase-free environment, and all steps were performed at room temperature. The primers used are shown in Table 1.

3 Results

3.1 Demographic and clinical characteristics of COVID-19 patients and controls

The mean age of all studied participants was (58.97±17.36) years, and 90.0% of the COVID-19 patients had significant comorbidities such as diabetes mellitus (DM) (26.7%), hypertension (HTN) (13.3%), combined DM and HTN (33.3%), and hepatic disease (16.7%). Among the patients who presented symptoms, 53.3% presented with fever, 66.7% with cough, 70.0% with dyspnea, and 40.0% with headache and fatigue.

The mean respiratory rate (RR in breaths/min) among patients was 30.17±4.72, while it was 18.02±2.94 among controls, with a statistically significant difference of $P<0.001$. For patients reported to have hypoxia, the mean O_2 saturation was (90.40±6.93)%, while in controls it was (97.18±1.16)%, which was also statistically significant ($P<0.001$). Assessment of electrocardiogram (ECG) changes among the COVID-19 patients showed normal sinus rhythm (NSR) in 66.7%, sinus tachycardia in 23.3%, and left bundle branch block (LBBB) in 10.0% (Table 2).

3.2 Biochemical parameters of the studied groups

The serum levels of random blood glucose (RBG), CRP, ferritin, D-dimer, international normalized ratio (INR), prothrombin time (PT), total (T) bilirubin, direct (D) bilirubin, white blood cells (WBCs), neutrophil,

Table 1 List of genes with accession number and sequences of gene-specific primers used for qRT-PCR

Gene	Forward (5'→3')	Reverse (5'→3')
ACE2 (NM_001389402.1)	CAGGGAACAGGTAGAGGACATT	CAGAGGGTGAACATACAGTTGG
TLR4 (NM_0032663)	CTTATAAGTGTCTGAACTCCC	TACCAGCACGACTGCTCAG
IL-17 (NM_002190.3)	CAAGACTGAACACCGACTAAG	TCTCCAAAGGAAGCCTGA
β-actin (NM_001101.5)	AGGAAGGAAGGCTGGAAGAG	GGAAATCGTGCGTGACATTA
GAPDH (NM_001289746.2)	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction; *ACE2*: angiotensin-converting enzyme 2; *TLR4*: Toll-like receptor 4; *IL-17*: interleukin-17; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Demographic and clinical characteristics and laboratory investigations of COVID-19 patients and controls

Parameter	Mean±SD or number (percentage)		*P*-value
	COVID-19 patients (*n*=60)	Controls (*n*=60)	
Age (years)	58.97±17.36	56.67±14.98	0.261
Sex			
Male	32 (53.3%)	35 (58.3%)	0.581
Female	28 (46.6%)	25 (41.6%)	
SBP (mmHg)	127.17±16.03	122.00±9.17	0.074
DBP (mmHg)	78.13±11.09	76.00±4.94	0.382
Respiratory rate (breaths/min)	30.17±4.72	18.02±2.94	<0.001
O_2 saturation (%)	90.40±6.93	97.18±1.16	<0.001
Presenting symptoms			
Fever	32 (53.3%)		
Cough	40 (66.7%)		
Dyspnea	42 (70.0%)		
Headache & fatigue	24 (40.0%)		
Associated comorbidities			
DM	16 (26.7%)		
HTN	8 (13.3%)		
DM & HTN	20 (33.3%)		
Hepatic disease	10 (16.7%)		
None	6 (10.0%)		
ECG changes			
NSR	40 (66.7%)		
Sinus tachycardia	14 (23.3%)		
LBBB	6 (10.0%)		
RBG (mg/dL)	200.85±87.25	152.49±31.89	0.015
CRP (mg/L)	70.44±32.40	9.35±3.22	<0.001
Ferritin (ng/mL)	556.83±182.38	104.01±48.48	<0.001
D-Dimer (μg/mL)	1.62±0.89	0.42±0.15	<0.001
INR	1.19±0.22	0.92±0.16	<0.001
PT (s)	14.29±2.20	12.25±0.97	<0.001
Creatinine (μmol/L)	127.62±41.41	102.65±24.19	0.005
BUN (mmol/L)	15.61±4.42	8.64±1.25	<0.001
ALP (IU/L)	116.72±45.68	88.67±19.96	0.002
ALT (IU/L)	47.45±17.84	38.20±7.15	0.022
AST (IU/L)	48.18±20.16	34.72±6.44	0.005
TP (g/L)	62.15±8.52	67.82±10.53	0.021
Albumin (g/L)	34.67±6.22	37.68±7.31	0.023
T bilirubin (μmol/L)	16.22±8.03	12.80±3.75	0.010
D bilirubin (μmol/L)	7.77±2.54	3.42±0.95	<0.001
WBC count ($\times10^9$ L^{-1})	10.15±4.95	6.89±1.79	0.002
Neutrophil ($\times10^9$ L^{-1})	6.39±2.72	4.55±1.59	<0.001
Lymphocyte ($\times10^9$ L^{-1})	1.26±0.36	1.73±0.54	<0.001
Hemoglobin (g/dL)	11.32±2.34	12.21±2.02	0.037
Platelet ($\times10^9$ L^{-1})	202.53±69.13	238.12±8.21	0.014

t-test and Mann-Whitney test were used to compare different markers between the two groups. *P*-value≤0.05 is considered significant. SBP: systolic blood pressure; DBP: diastolic blood pressure; DM: diabetes mellitus; HTN: hypertension; ECG: electrocardiogram; NSR: normal sinus rhythm; LBBB: left bundle branch block; RBG: random blood glucose; CRP: C-reactive protein; INR: international normalized ratio; PT: prothrombin time; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; TP: total protein; T bilirubin: total bilirubin; D bilirubin: direct bilirubin; WBC: white blood cell; SD: standard deviation.

alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine were significantly higher among COVID-19 patients than among controls, and among severe COVID-19 cases than among mild cases. Meanwhile, lymphocyte, hemoglobin (Hb), platelet, total protein (TP), and albumin levels were significantly lower among COVID-19 patients than among controls, and among severe COVID-19 cases than among mild cases (Tables 2 and 3).

The results showed exacerbation among all COVID-19 patients who unfortunately did not recover (deaths), whereas all the investigated parameters, including RBG, WBC count, and neutrophil count, and the levels of CRP, ferritin, D-dimer, INR, PT, T bilirubin, D bilirubin, ALT, AST, ALP, BUN, and creatinine, were significantly increased among deaths compared to recovered patients, while the lymphocyte, Hb, platelet, TP, and albumin levels showed opposite correlations (Table 4).

3.3 *ACE2*, *TLR4*, and *IL-17* fold-change gene expression in the studied groups

The mean fold-change gene expression levels of *ACE2*, *TLR4*, and *IL-17* were 8.58±0.47, 29.26±1.38, and 5.64±0.35, respectively, in COVID-19 patients; and 1.49±0.23, 1.56±0.49, and 1.063±0.044, respectively, in controls (P<0.001), as shown in Fig. 1.

The expression levels of the studied parameters were elevated in severe COVID-19 patients compared to mild cases. The levels were 10.77±0.64 vs. 6.91±0.51 for *ACE2*; 37.98±0.57 vs. 22.59±1.65 for *TLR4*; and 8.29±0.29 vs. 3.62±0.20 for *IL-17* (P<0.001), as shown in Fig. 2.

We found increased fold-change gene expression levels for *ACE2*, *TLR4*, and *IL-17* among deaths compared to recoveries; the gene expression levels were 9.39±0.81, 33.46±2.25, and 7.13±0.39 for deaths compared to 7.87±0.50, 25.58±1.42, and 4.35±0.43 for recoveries for *ACE2*, *TLR4*, and *IL-17*, respectively (P=0.006, P<0.001, and P<0.001, respectively; Fig. 3).

Table 3 Laboratory investigations of COVID-19 patients as regards severity

Parameter	Mean±SD		P-value
	Mild/moderate COVID-19 patients (n=34)	Severe COVID-19 patients (n=26)	
RBG (mg/dL)	179.70±82.11	228.50±87.53	0.020
CRP (mg/L)	53.21±17.66	92.98±33.67	<0.001
Ferritin (ng/mL)	496.15±115.27	636.2±222.27	0.012
D-Dimer (μg/mL)	1.12±0.37	2.28±0.96	<0.001
INR	1.10±0.19	1.30±0.21	<0.001
PT (s)	13.76±2.12	14.99±2.14	0.020
Creatinine (μmol/L)	117.15±36.84	141.31±43.71	0.001
BUN (mmol/L)	14.12±3.35	17.56±4.94	0.014
ALP (IU/L)	105.41±49.31	131.50±36.27	0.011
ALT (IU/L)	40.38±10.29	56.69±21.31	0.011
AST (IU/L)	41.65±18.04	56.73±19.89	0.003
TP (g/L)	64.91±7.99	58.55±7.95	0.007
Albumin (g/L)	36.15±6.64	32.73±5.13	0.035
T bilirubin (μmol/L)	14.91±8.08	17.92±7.79	0.005
D bilirubin (μmol/L)	7.27±2.43	8.44±2.57	0.028
WBC count ($\times10^9$ L^{-1})	7.76±3.51	13.26±4.86	<0.001
Neutrophil ($\times10^9$ L^{-1})	5.02±1.87	8.19±2.63	<0.001
Lymphocyte ($\times10^9$ L^{-1})	1.41±0.37	1.07±0.24	0.001
Hemoglobin (g/dL)	11.90±1.99	10.56±2.58	0.033
Platelet ($\times10^9$ L^{-1})	229.03±68.92	167.88±52.95	0.001

t-test and Mann-Whitney test were used to compare different markers between the two groups. P-value≤0.05 is considered significant. RBG: random blood glucose; CRP: C-reactive protein; INR: international normalized ratio; PT: prothrombin time; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; TP: total protein; T bilirubin: total bilirubin; D bilirubin: direct bilirubin; WBC: white blood cell; SD: standard deviation.

Table 4 Laboratory investigations of COVID-19 patients as regards outcome

Parameter	Mean±SD		*P*-value
	Recovery (*n*=32)	Death (*n*=28)	
RBG (mg/dL)	181.12±80.74	223.39±90.35	0.038
CRP (mg/L)	57.58±25.54	85.14±33.54	<0.001
Ferritin (ng/mL)	487.56±142.67	636.01±192.69	0.003
D-Dimer (μg/mL)	1.17±0.59	2.14±0.91	<0.001
INR	1.14±0.25	1.24±0.18	0.004
PT (s)	13.69±2.33	14.99±1.84	0.005
Creatinine (μmol/L)	121.59±40.34	134.5±42.26	0.014
BUN (mmol/L)	14.27±3.74	17.14±4.71	0.039
ALP (IU/L)	106.16±48.38	128.79±39.87	0.023
ALT (IU/L)	42.44±13.59	53.18±20.47	0.039
AST (IU/L)	41.78±17.37	55.5±20.93	0.004
TP (g/L)	64.89±8.17	59.02±7.93	0.014
Albumin (g/L)	36.34±6.57	32.75±5.28	0.036
T bilirubin (μmol/L)	15.01±8.35	17.60±7.57	0.025
D bilirubin (μmol/L)	7.31±2.47	8.31±2.55	0.044
WBC count ($\times10^9$ L^{-1})	8.69±3.90	11.81±5.53	0.019
Neutrophil ($\times 10^9$ L^{-1})	5.49±1.83	7.43±3.20	0.028
Lymphocyte ($\times10^9$ L^{-1})	1.39±0.37	1.11±0.29	0.003
Hemoglobin (g/dL)	11.94±2.34	10.61±2.16	0.390
Platelet ($\times10^9$ L^{-1})	228.94±70.51	172.36±54.44	0.002

t-test and Mann-Whitney test were used to compare different markers between the two groups. *P*-value≤0.05 is considered significant. RBG: random blood glucose; CRP: C-reactive protein; INR: international normalized ratio; PT: prothrombin time; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; TP: total protein; T bilirubin: total bilirubin; D bilirubin: direct bilirubin; WBC: white blood cell; SD: standard deviation.

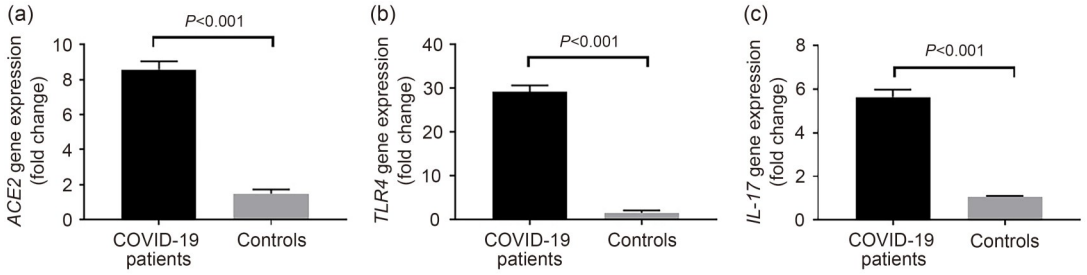

Fig. 1 Fold-change gene expression ($2^{-\Delta\Delta C_T}$) for angiotensin-converting enzyme 2 (*ACE2*) (a), Toll-like receptor 4 (*TLR4*) (b), and interleukin-17 (*IL-17*) (c) in COVID-19 patients and controls. The data are expressed as mean±standard error of the mean (SEM) (*n*=60 for COVID-19 cases and controls).

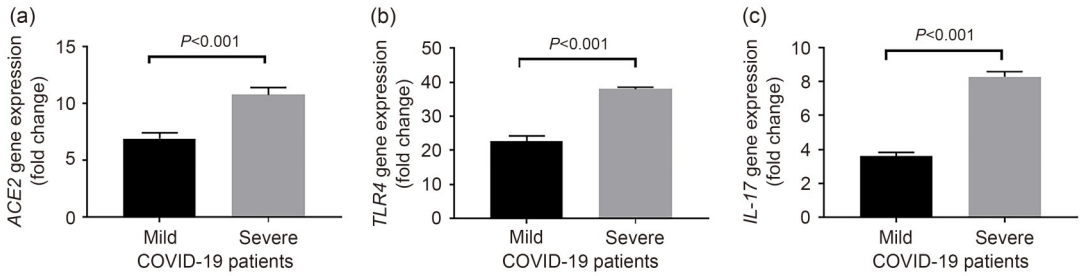

Fig. 2 Fold-change gene expression ($2^{-\Delta\Delta C_T}$) of angiotensin-converting enzyme 2 (*ACE2*) (a), Toll-like receptor 4 (*TLR4*) (b), and interleukin-17 (*IL-17*) (c) in COVID-19 patients subgrouped according to severity of the disease (mild vs. severe). The data are expressed as mean±standard error of the mean (SEM) (*n*=34 for mild/moderate cases and *n*=26 for severe cases).

3.4 Correlation studies

We found significant negative correlations between the expression levels of *ACE2* and *TLR4* and TP, albumin, lymphocyte, platelet, and Hb levels (Table 5). The expression level of *ACE2* showed a significant positive correlation with *IL-17*, *TLR4*, RBG, CRP, ferritin, D-dimer, INR, PT, creatinine, BUN, ALP, ALT, AST, T bilirubin, D bilirubin, WBC, and neutrophil levels in the patient group. More significant positive correlations were found in the more severe groups (Table 5).

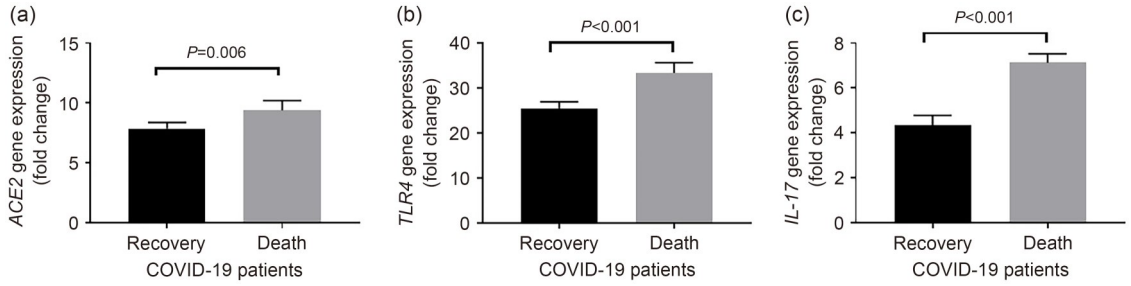

Fig. 3 Fold-change gene expression ($2^{-\Delta\Delta C_T}$) of angiotensin-converting enzyme 2 (*ACE2*) (a), Toll-like receptor 4 (*TLR4*) (b), and interleukin-17 (*IL-17*) (c) in COVID-19 patients subgrouped according to outcomes (recoveries vs. deaths). Data were expressed as mean±standard error of the mean (SEM) (*n*=32 for recovery and *n*=28 for death).

Table 5 Correlation studies between the gene expression of *ACE2*, *TLR4*, and *IL-17* and laboratory data in COVID-19 patients

Parameter	*ACE2*		*TLR4*		*IL-17*	
	r	*P*	*r*	*P*	*r*	*P*
ACE2			0.806**	0.000	0.723**	0.000
TLR4	0.806**	0.000			0.811**	0.000
IL-17	0.723**	0.000	0.811**	0.000		
RBG (mg/dL)	0.252**	0.005	0.261**	0.004	0.361**	0.000
CRP (mg/L)	0.696**	0.000	0.784**	0.000	0.804**	0.000
Ferritin (ng/mL)	0.616**	0.000	0.699**	0.000	0.725**	0.000
D-Dimer (μg/mL)	0.636**	0.000	0.764**	0.000	0.801**	0.000
INR	0.547**	0.000	0.580**	0.000	0.589**	0.000
PT (s)	0.380**	0.000	0.441**	0.000	0.488**	0.000
Creatinine (μmol/L)	0.212*	0.020	0.243**	0.007	0.339**	0.000
BUN (mmol/L)	0.612**	0.000	0.734**	0.000	0.709**	0.000
ALP (IU/L)	0.176	0.055	0.292**	0.001	0.291**	0.001
ALT (IU/L)	0.261**	0.004	0.237**	0.009	0.265**	0.003
AST (IU/L)	0.333**	0.000	0.356**	0.000	0.366**	0.000
TP (g/L)	−0.284**	0.002	−0.241**	0.008	−0.226*	0.013
Albumin (g/L)	−0.287**	0.001	−0.256**	0.005	−0.280**	0.002
T bilirubin (μmol/L)	0.191*	0.037	0.333**	0.000	0.282**	0.002
D bilirubin (μmol/L)	0.600**	0.000	0.731**	0.000	0.706**	0.000
WBC count ($\times10^9$ L^{-1})	0.288**	0.001	0.339**	0.000	0.327**	0.000
Neutrophil ($\times10^9$ L^{-1})	0.390**	0.000	0.385**	0.000	0.458**	0.000
Lymphocyte ($\times10^9$ L^{-1})	−0.282**	0.002	−0.385**	0.000	−0.416**	0.000
Hemoglobin (g/dL)	−0.144	0.116	−0.192*	0.035	−0.227*	0.013
Platelet ($\times10^9$ L^{-1})	−0.293**	0.001	−0.324**	0.000	−0.279**	0.002

Spearman's correlation. The *r* correlation coefficient, *P*-value, and correlation are significant at the 0.05 level (2-tailed). *ACE2*: angiotensin-converting enzyme 2; *TLR4*: Toll-like receptor 4; *IL-17*: interleukin-17; RBG: random blood glucose; CRP: C-reactive protein; INR: international normalized ratio; PT: prothrombin time; BUN: blood urea nitrogen; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; TP: total protein; T bilirubin: total bilirubin; D bilirubin: direct bilirubin; WBC: white blood cell.

3.5 Receiver operating characteristics for the diagnostic performance of ACE2, TLR4, and IL-17 for distinguishing COVID-19 patients from healthy controls

The diagnostic performance of ACE2, TLR4, and IL-17 for distinguishing COVID-19 patients from healthy controls is shown in Table 6 and Fig. 4.

The results showed that at the cutoff value of >3.4850, the serum expression of ACE2 showed 86.7% sensitivity and 96.7% specificity in discriminating between COVID-19 cases and controls. According to the receiver operating characteristic (ROC) curve results for TLR4 and IL-17 at cutoff values of >1.8862 and >1.8021, respectively, the serum expression of the two markers showed 91.7% and 95.0% sensitivity, respectively, and 98.3% specificity for both in discriminating between COVID-19 cases and controls.

4 Discussion

ARDS is now understood to be a multiple-organ dysfunction syndrome. Severe SARS-CoV-2 infection may result in an overactive immunological response that results in a "cytokine storm," a massive synthesis of inflammatory cytokines and chemical mediators. During this systemic inflammatory-response syndrome, cytokine levels increase, and in some people, this can result in multi-organ failure (Sánchez-Díez et al., 2023). The synthesis of pro- and anti-inflammatory cytokines must be carefully balanced to shield the organism from damage (del Carmen Chávez-Ocaña et al., 2023).

Our study showed significant upregulation in the expression of *ACE2*, *TLR4*, and *IL-17* messenger RNAs (mRNAs) in COVID-19 patients; this upregulation persisted with increasing severity of the disease and was also correlated with bad outcomes (death).

Binding of S proteins of the virion with the cellular ACE2 receptor and cleavage of S proteins by host TMPRSS2 are both necessary for SARS-CoV-2 entrance into cells (Hu et al., 2021). The viral genome can enter the host cell in one of two ways: fusion or endocytosis. The S protein, which is present on the surface of SARS-CoV-2, selectively identifies and binds to the ACE2 receptor on the membranes of lung epithelial cells during endocytosis. The virus enters the host cell through the process of endocytosis. Following the S protein's recognition of the ACE2 receptor, TMPRSS2 activates the S protein and breaks the binding site. The virus core enters the cell once the S protein binds with the host-cell membrane. The viral genome is released because of the host cell's lysosomal digestion of the helical N protein. The viral RNA replicates in the host cell after the viral genome enters. After being produced, the new viral protein combines with RNA to make progeny virus particles. A new cycle of infection starts when the newly produced

Table 6 Diagnostic performance of ACE2, TLR4, and IL-17 for distinguishing COVID-19 patients from healthy controls

Parameter	AUC	Cutoff	Accuracy (%)	Sensitivity (%)	Specificity (%)
ACE2	0.908	>3.4850	83.33	86.7	96.7
TLR4	0.968	>1.8862	90.00	91.7	98.3
IL-17	0.973	>1.8021	93.33	95.0	98.3

ACE2: angiotensin-converting enzyme 2; TLR4: Toll-like receptor 4; IL-17: interleukin-17; AUC: area under the curve.

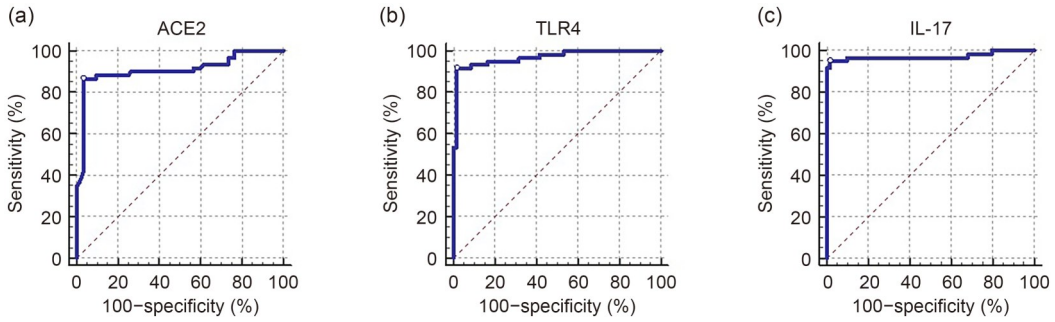

Fig. 4 Receiver operating characteristic (ROC) curves of angiotensin-converting enzyme 2 (ACE2) (a), Toll-like receptor 4 (TLR4) (b), and interleukin-17 (IL-17) (c) in COVID-19 patients vs. controls.

virus particles migrate out of the cell via exocytosis. Alveolar cells are eventually infected by the virus (Xu et al., 2020; Ni et al., 2022).

Human alveolar macrophages can respond to the SARS-CoV-2 S protein by interacting with TLR4, which may play a part in the hyperinflammatory state that COVID-19 patients experience (Corpetti et al., 2021). Given the strong link we observed with IL-17, the binding of the S protein to TLR4 may play a role in SARS-CoV-2 entry into human cells and instigation of the cytokine storm that impacts many organs (Choudhury and Mukherjee, 2020; Gadanec et al., 2021).

TLR4 is one of the most important classes of PRRs and is required for the stimulation of proinflammatory cytokines (Jose et al., 2022). The activation of nuclear factor-κB (NF-κB) leads to the release of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, which are mediated via the TLR4/myeloid differentiation factor 88 (MyD88) pathway. These cytokines cause myeloid dendritic cells to release IL-23, which prompts T cells to produce IL-17 quickly. This has a significant impact on the onset of pulmonary vascular disease and inflammation. Increased TLR4 signaling after viral infection greatly exacerbates the severity of pulmonary illness (Brandão et al., 2021). Furthermore, Luo et al. (2017) found that TLR4 signaling was a critical mechanism of acute lung injury.

Sohn et al. (2020) recently showed that in comparison to peripheral blood mononuclear cells in healthy controls, those in COVID-19 patients exhibited considerably higher expression of TLR4 and its downstream signaling mediators, which is consistent with our findings. In addition, other studies reported that ICU COVID-19 patients had much higher *TLR4* mRNA expression levels than non-ICU COVID-19 patients did (Khanmohammadi and Rezaei, 2021; Alturaiki et al., 2023). Khanmohammadi and Rezaei (2021) documented the fact that TLR4 is known to trigger neutrophil extracellular traps (NETs). The severity of COVID-19 and persistent inflammation have been linked to NET formation. Alturaiki et al. (2023) also came to the conclusion that patients admitted to the ICU had higher levels of TLR4 mRNA expression than patients with non-critical COVID-19, which is consistent with our findings.

Additionally, in the monocytes of severe COVID-19 patients, Dorneles et al. (2023) observed elevated TLR4 expression in conjunction with greater NF-κB p65 phosphorylation. In fact, in vitro tests conducted in the past have shown overactivation of TLR4 due to the simultaneous presence of SARS-CoV-2-related proteins and systemic endotoxemia.

In contrast to our findings, Ghazavi et al. (2021) found that, when comparing the moderate group with the severe group and control group, the mean level of IL-17 in the moderate group was considerably higher. Discrepancies between research populations, study methods, and virus strains could be the cause of the disagreement between this study and ours.

Correlation studies showed that both *ACE2* and *TLR4* expression levels had a significant negative correlation with TP, albumin, lymphocyte, platelet, and Hb levels; and a significant positive correlation with IL-17, RBG, CRP, ferritin, D-dimer, INR, PT, creatinine, BUN, ALP, ALT, AST, T bilirubin, D bilirubin, WBC, and neutrophil levels. Our results confirmed the strong correlation between the *TLR4* expression level and COVID-19 severity, which demonstrates the relation to the pathogenesis of COVID-19 and allows the prediction of disease prognosis and mortality.

The hypothesis that IL17 enhances the host immune response, resulting in severe inflammation and tissue damage, is supported by the decrease in lymphocyte subpopulations and the increase in T helper 17 (Th17) cells and Th17-derived cytokines observed in SARS-CoV patients (Ayhan et al., 2020).

Consequently, it is thought that inhibiting IL-17A may lessen the aberrant immunological response to COVID-19 and reduce the death rate linked to ARDS (Megna et al., 2020).

The strongest protein–protein interaction is thought to exist between the SARS-CoV-2 spike glycoprotein and TLR4. The general view is that the activation of TLR4 after interaction with the spike glycoprotein of SARS-CoV-2 increases the surface expression of ACE2, thus promoting viral entry. Modeling work by Kogan et al. (2022) and Rahman et al. (2021) further demonstrated that in addition to the activation of interferon signalling, antiviral defense, and an anti-inflammatory response, TLR4 activation in alveolar cells may result in excessive inflammatory and fibrotic responses.

In conclusion, our results clarified that the SARS-CoV-2 S protein significantly increases both *TLR4* and *ACE2* gene expression, promoting a marked inflammatory response. These observations were also confirmed by a notable release of pro-inflammatory cytokines IL-17, CRP, ferritin, and D-dimer, which are well-known downstream products of TLR4 activation.

When a viral spike glycoprotein attaches to ACE2 on type II alveolar cells—which are responsible for creating pulmonary surfactant—it exposes the extracellular binding sites of TLR4 on lung epithelial cells. The exposed TLR4 interacts with the viral S protein either directly or indirectly through the rise in ACE2 cell-surface expression caused by interferons upon direct or indirect viral entry into nearby cells. Thus, SARS-CoV-2 can trigger a proliferative antiviral inflammatory state by binding TLR4. If this state is not controlled, it can lead to a major inflammatory response characterized by an increase in cytokines, chemokines, and interferons, a condition known as a cytokine storm. An important aspect of severe COVID-19 pathophysiology is the cytokine storm, which causes epithelial and endothelial apoptosis, as well as vascular leakage, which can ultimately have deadly consequences such as severe lung damage and ARDS (Taha et al., 2021).

Our findings allow us to conclude that increased circulating gene expression of *ACE2*, *TLR4*, and *IL-17* is helpful in assessing the severity of COVID-19. Consequently, targeting these biomarkers may offer additional therapeutic options for COVID-19 patients in the future.

Data availability statement

The data that support the findings of this study are available from the authors, but restrictions apply to the availability of these data, which were used under license from the Faculty of Medicine/Assiut University (Egypt) for the current study and so are not publicly available. Data are, however, available from the corresponding author upon reasonable request and with permission from the Faculty of Medicine/Assiut University.

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Author contributions

Marwa A. DAHPY: conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, validation, writing – original draft, and writing – review & editing. Ragaa H. SALAMA and Abdel-Raheim M. A. MEKI: investigation, methodology, supervision, validation, and writing – review & editing. Ashraf Zein El-ABEDEEN: data curation, supervision, validation, and writing – review & editing. Maiada K. HASHEM and Ebtsam S. ABDULKAREEM: data curation, writing – original draft, and writing – review & editing. Mohamed MOHANY: formal analysis, funding acquisition, investigation, methodology, and writing – review & editing. Sinisa DJURASEVIC: software and writing – review & editing. Amal N. IBRAHIM and Nourhan M. HUSSEIN: data curation, formal analysis, investigation, methodology, project administration, supervision, validation, and writing – original draft. Shima Gafar MANSOR, Mohamed Ramadan IZZALDIN, Suzan Eid Elshishtawy IBRAHIM, Alzahra ABDELBADEA, Islam Khaled Ali HARBY, Marwa A. SABET, and Salwa Seif ElDIN: investigation, methodology, and writing – review & editing. Marwa K. KHAIRALLAH: data curation, methodology, and writing – review & editing. Fatma Y. A. ABBAS and Rasha M. ALI: investigation and writing – review & editing. Abdelraouf M. S. ABDELRAOUF: data curation, investigation, methodology, and writing – review & editing. Amira A. KAMEL: conceptualization, data curation, formal analysis, investigation, methodology, project administration, and writing – review & editing. All authors have read and agreed to the published version of the manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Marwa A. DAHPY, Ragaa H. SALAMA, Abdel-Raheim M. A. MEKI, Ashraf Zein El-ABEDEEN, Maiada K. HASHEM, Ebtsam S. ABDULKAREEM, Mohamed MOHANY, Sinisa DJURASEVIC, Amal N. IBRAHIM, Nourhan M. HUSSEIN, Shima Gafar MANSOR, Mohamed Ramadan IZZALDIN, Marwa K. KHAIRALLAH, Suzan Eid Elshishtawy IBRAHIM, Alzahra ABDELBADEA, Islam Khaled Ali HARBY, Fatma Y. A. ABBAS, Rasha M. ALI, Marwa A. SABET, Salwa Seif ElDIN, Abdelraouf M. S. ABDELRAOUF, and Amira A. KAMEL declare that they have no conflicts of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The ethical committee of Faculty of Medicine, Assiut University has reviewed and approved the study (IRB No. 17101957), with date of original approval 2022. Before participation in the trial, informed consent was acquired from every patient and control subject.

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