Research Article
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Potential relationship between circulating gene expression of ACE2, TLR4, and Il-17 and disease severity and outcome of COVID-19 hospitalized patients

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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 COVID-19. The binding of a SARS-Cov-2 virus spike protein to the cell membrane is mediated through its binding to the 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 relationship between ACE2, TLR4, and Il-17 circulating gene-expression levels and the clinical severity of COVID-19 disease, as well as the associated pathogenic condition of hospitalized patients. Sixty COVID-19 patients (34 mild/moderate and 26 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, TLR-4, and IL-17 levels were assessed by qPCR in addition to all routine baseline laboratory investigations, including CBC with differential analysis, CRP, ferritin, and D-dimer. ACE2, TLR-4, and IL-17 serum expression levels were significantly higher in the COVID-19 group and subgroups and correlated with different laboratory and clinical parameters. The serum expression levels of ACE2, TLR-4, and IL-17 were accurate in differentiating between the patient groups and controls with 86.7%, 91.7%, and 95% sensitivity and 96.7%, 98.3%, and 98.3% specificity, respectively, and correlate to more severe disease courses in COVID-19 patients. Higher levels are associated with overwhelmingly distressing outcomes.

Key words: Acute respiratory distress syndrome (ARDS); COVID-19; Intensive Care Unit; Inter Leukin-17; Polymerase Chain Reaction; Toll Like Receptors-4

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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 acute respiratory distress syndrome (ARDS). Although it was once thought to be a single-organ malfunction, ARDS 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 COVID-19 had been found 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 of the affected cases 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, SARS-CoV-2 is the largest RNA virus. It is an enclosed, positive-sense, single-stranded virus. It has 16 non-structural proteins (nsp1–16) and 4 structural proteins (N, M, S, and E). The nucleocapsid (N) protein forms a helical capsid that contains the genome firmly, and this capsid is encased in a lipid bilayer envelope. The membrane (M), spike (S), and envelope (E) proteins combine to create this envelope. Due to its requirement for virus entry and its role in providing the 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 interaction. 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 TMPRSS or transmembrane protease, or cathepsin proteases found in endolysosomes (Sodhi et al., 2022). Type II transmembrane serine protease TMPRSS2 is abundantly expressed in respiratory epithelial cells, 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 Ang II into angiotensin I–7, is thought to be negatively regulated by ACE2. Nevertheless, ACE1 is a catalyst for biogenesis of Ang II from Ang I (Liu et al., 2022) (13). The degree of epithelial-cell ACE2 expression affects susceptibility to COVID-19. Some have suggested that children have a lower risk of COVID-19 because they have fewer ACE2 receptors than adults do (Bartolák-Suki et al., 2022). Numerous investigations have discovered a link between 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, 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, Tumour necrosis factor (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; Interleukin-17 (IL-17) is active in host defense mechanisms, immune modulation, tissue damage, physiological stress, stimulation of inflammation, and neutrophil activation (Sultan et al., 2022). IL17 increases the inflammatory reactions that initiate cytokine storms by inducing 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 destruction in ARDS (Wu et al., 2022). It also, leads to viral persistence by affecting replication of the virus through inhibition of infected-cell apoptosis (Darif et al., 2021).

Considering the pivotal role of ACE-II, TLR-4, and IL-17 in inflammation, we aimed to assess the potential association of TLR-4 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 in the Assiut University Quarantine Hospital. The ethical committee of Assiut University's Faculty of Medicine reviewed and approved the study (IRB No. 17101957). Before participating in the trial, informed consent was acquired from every patient and control subject.

Participants enrolled in the study were divided into Group 1: sixty positive PCR COVID-19 patients, and Group 2: 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:

I. According to the severity of the disease into:

Group 1A: Thirty-four mild to moderate COVID-19 patients(mild/moderate), and Group 1B: Twenty-six COVID-19 patients with severe ARDS manifestation.

Pandemic severity-assessment scales (PSAs) provide information to estimate the timing, magnitude, and intensity of pandemics. The WHO Pandemic Influenza Severity Assessment (PISA) study characterizes pandemic severity (21) in terms of three indicators: transmissibility, seriousness of disease, and effect. For COVID-19, we modified the PISA approach and classified severity of the disease as critical, severe, or non-severe, our severity evaluation was based on ICU admission and indications of ARDS as follows:

• Critical: people who had ever been hospitalized, admitted to the intensive care unit (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;

• Not severe(mild/moderate): if none of the preconditions listed above were satisfied.

II. Following up on the included patients 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. In addition to positive CT chest features, a confirmed COVID-19 case was classified as positive for the real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of nasal-swab specimens, in accordance with WHO recommendations.

-Exclusion criteria: Patients who were pregnant; under the age of eighteen; diagnosed with cancer; expe-

riencing sepsis, septic shock, or confirmed bacterial infection; on immunosuppressive medication; or who had an autoimmune or chronic inflammatory condition were not eligible for this study.

2.1 Sample collection

Five ml of whole blood samples were drawn under aseptic conditions from all participants. At the time of hospitalization, 2 ml were collected in EDTA tubes and centrifuged at 8000 xg 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

All included participants were subjected to a careful medical history focusing on age, gender, and comorbid conditions, together with the presenting clinical symptoms at the time of hospital admission, during the inpatient period, and at release. Patients also underwent chest computed tomography (CT) examination.

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 was recorded from patient files.

2.3 Evaluation of circulating mRNA expression levels for ACE2, TLR-4, and IL-17 levels

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 cDNA using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit, catalog No. #k1622. Finally, qRT-PCR reaction was performed using Thermo Scientific Maxima SYBR Green qRT-PCR Master Mix, catalog No. #K0251. β-actin and GABDH were used as housekeeping genes for ACE2 and TLR-4, 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.

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

	Forward	Reverse
ACE2 (NM_001389402.1)	CAGGGAACAGGTAGAGGACATT	CAGAGGGTGAACATACAGTTGG
TLR-4 (NM_0032663)	CTTATAAGTGTCTGAACTCCC	TACCAGCACGACTGCTCAG
IL-17	3′-CAAGACTGAACACCGACTAAG-5′	3′-TCTCCAAAGGAAGCCTGA-5′
β-actin	AGGAAGGAAGGCTGGAAGAG	GGAAATCGTGCGTGACATTA
GABDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

3 Results

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

The mean age for all studied participants was 58.97±17.36, and ninety percent of the COVID-19 patient

group showed significant comorbidities such as DM (26.7%), HTN (13.3%), combined DM & HTN (33.3%), and hepatic (16.7%). Regarding the patients presenting symptoms, 53.3 % presented with fever, 66.7 % with cough, 70 % with dyspnea, and 40% with headache and fatigue.

The mean respiratory rate (RR) 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 O_2 saturation mean was 90.40 ± 6.93, while in controls it was 97.18 ± 1.16, which is also statistically significant ($p<0.001$). Assessment of 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% (Table 2).

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

	COVID-19 patients (n=60) Mean ±SD	Controls (n=60) Mean ±SD	P-Value
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	122.0 ± 9.17	0.074
DBP (mmHg)	78.13 ±11.09	76.0 ±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 %)		
Headache& fatigue	24 (40 %)		
Associated comorbidities			
DM	16 (26.7 %)		
HTN	8 (13.3 %)		
DM & HTN	20 (33.3 %)		
Hepatic	10 (16.7 %)		
None	6 (10 %)		
ECG changes			
NSR	40 (66.7 %)		
Sinus tachycardia	14 (23.3 %)		
LBBB	6 (10 %)		
RBG (mg/dl)	200.85 ±87.25	152.49 ±31.89	0.015
CRP (mg/L)	70.440 ± 32.40	9.35 ±3.22	<0.001
Ferritin (ng/ml)	556.834 ±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 (Seconds)	14.29 ± 2.2	12.245 ± .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
WBCS (× 10^9/L)	10.15 ± 4.95	6.89 ± 1.79	0.002
Neutrophils (× 10^9/L)	6.39 ± 2.72	4.55 ± 1.59	< 0.001
Lymphocytes(× 10^9/L)	1.26 ± 0.36	1.73 ± 0.54	< 0.001
Hemoglobin (g/dl)	11.32 ± 2.34	12.21 ±2.02	0.037
Platelets (× 10^9/L)	202.53 ± 69.13	238.12 ± 78.21	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. Systolic blood pressure (SBP),Diastolic blood pressure (DBP), Diabetes mellitus (DM), Hypertension (HTN), Random blood glucose (RBG), C-reactive protein (CRP), International normalized ratio (INR), Prothrombin time (PT), Blood urea nitrogen (BUN), Alkaline phosphatase (ALP),Alanine transaminase (ALT), Aspartate transaminase (AST), Total protein (TP), Total(T) bilirubin, Direct(D) bilirubin, White blood cell count(WBCS).

3. 2 Biochemical parameters of the studied groups

Serum levels of random blood glucose, CRP, ferritin, D-dimer, INR, PT, total bilirubin, direct bilirubin, WBCs, neutrophils, ALT, AST, ALP, BUN, and creatinine were significantly higher among COVID-19 patients in comparison to controls, and among severe COVID-19 cases compared to mild cases. Meanwhile lymphocytes, Hb, platelets, TP, and albumin levels were significantly lower among COVID-19 patients than controls and among severe COVID-19 cases than among mild cases (Tables 2 and 3).

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

	Mild/Moderate COVID-19 patients (n=34) Mean ±SD	Severe COVID-19 patients (n=26) Mean ±SD	P Value
RBG (mg/dl)	179.7 ±82.11	228.5 ± 87.53	0.02
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	1.1± 0.19	1.3 ± 0.21	<0.001
PT (Seconds)	13.76 ± 2.12	14.99 ± 2.14	0.02
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	131.5 ± 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
WBCS ($\times 10^9$/L)	7.76 ± 3.51	13.26 ± 4.86	<0.001
Neutrophils ($\times 10^9$/L)	5.02 ± 1.87	8.19 ± 2.63	< 0.001
Lymphocytes($\times 10^9$/L)	1.41 ± 0.37	1.07 ± 0.24	0.001
Hemoglobin (g/dl)	11.9 ± 1.99	10.56 ± 2.58	0.033
Platelets ($\times 10^9$/L)	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. Systolic blood pressure (SBP),Diastolic blood pressure (DBP), Diabetes mellitus (DM), Hypertension (HTN), Random blood glucose (RBG), C-reactive protein (CRP), International normalized ratio (INR), Prothrombin time (PT), Blood urea nitrogen (BUN), Alkaline phosphatase (ALP),Alanine transaminase (ALT), Aspartate transaminase (AST), Total protein (TP), Total(T) bilirubin, Direct(D) bilirubin, White blood cell count(WBCS).

Results showed exacerbation among all COVID-19 patients who unfortunately did not recover (deaths), whereas all the investigated parameters, including RBG, WBC, and neutrophil counts, 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 lymphocytes, Hb, platelets, TP, and albumin levels showed opposite correlations (Table 4).

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

	Recovery (n=32) Mean ±SD	Death (n=28) Mean ±SD	P Value
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 (Seconds)	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	17.6 ± 7.57	0.025
D bilirubin (μmol/L)	7.31 ± 2.47	8.31 ± 2.55	0.044
WBCS ($\times 10^9$/L)	8.69 ± 3.90	11.81 ± 5.53	0.019
Neutrophils ($\times 10^9$/L)	5.49 ± 1.83	7.43 ± 3.20	0.028
Lymphocytes($\times 10^9$/L)	1.39 ± 0.37	1.11 ± 0.29	0.003
Hemoglobin (g/dl)	11.94 ± 2.34	10.61 ± 2.16	0.39
Platelets ($\times 10^9$/L)	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. Systolic blood pressure (SBP),Diastolic blood pressure (DBP), Diabetes mellitus (DM), Hypertension (HTN), Random blood glucose (RBG), C-reactive protein (CRP), International normalized ratio (INR), Prothrombin time (PT), Blood urea nitrogen (BUN), Alkaline phosphatase (ALP),Alanine transaminase (ALT), Aspartate transaminase (AST), Total protein (TP), Total(T) bilirubin, Direct(D) bilirubin, White blood cell count(WBCS).

3.3 ACE2, TLR-4, and IL-17 fold-change gene expression in the studied groups

The mean fold-change gene-expression levels of ACE2, TLR-4, and IL-17 were 8.58 ± 0.47, 29.26 ± 1.38, and 5.64 ± 0.35, respectively, in COVID-19 patients; and 1.56 ± 0.49, 1.56 ± 0.49, and 1.063 ± 0.044, respectively, in controls (P value < 0.001), as showed shown in Fig 1a-c.

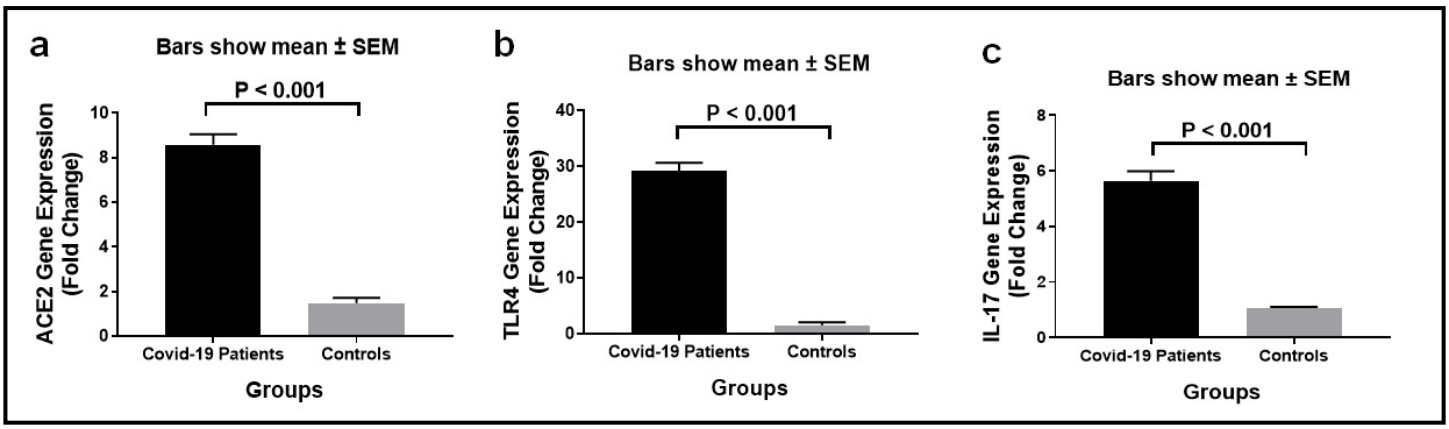

Fig. 1a-c Fold-change gene expression ($2^{-\Delta\Delta Ct}$) For a. ACE2; b. TLR4; c. IL-17 in COVID-19 patients and controls.
Data are expressed as mean ± SEM, (No.of cases=60 and controls=60)

Expression levels of the studied parameters were elevated in severe COVID-19 patients compared to mild cases. Levels were 10.77 ± 0.64 vs 6.91 ± 0.51 for ACE2; 37.98 ± 0.57 vs 22.59 ± 1.65 for TLR-4; and 8.29 ± 0.292 vs 3.62 ± 0.204 for IL-17 (P value < 0.001), as shown in Fig. 2a-c.

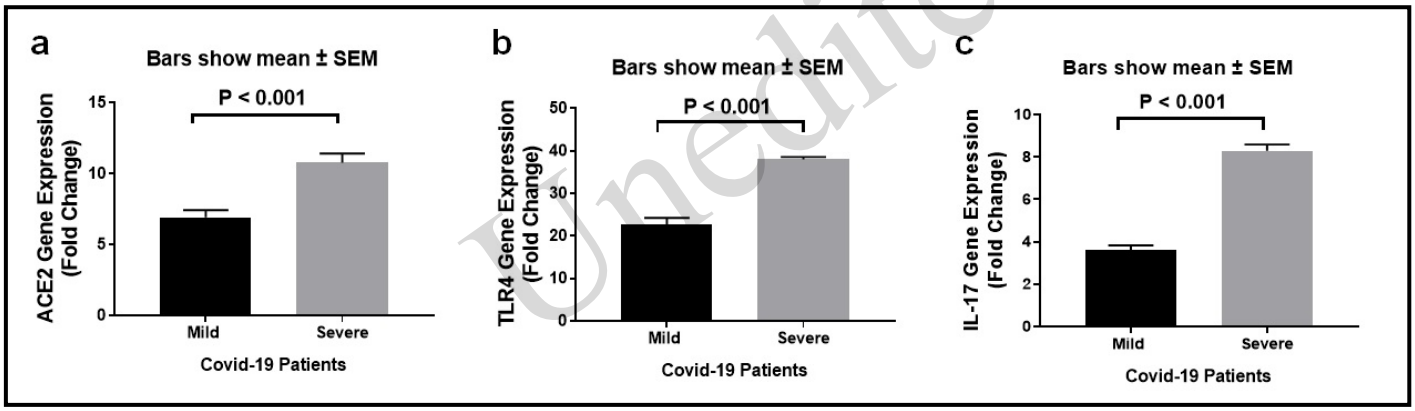

Fig. 2 a-c Fold-change gene expression ($2^{-\Delta\Delta Ct}$) of a. ACE2; b. TLR4; c. IL-17 in COVID19 patients sub grouped according to severity of the disease (Mild vs. Severe).
Data are expressed as mean±SEM. (No.of Mild/Moderate cases =34, Severe cases = 26 patients)

We found increased fold gene-expression levels for ACE2, TLR-4, 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 ± 0.504, 25.58 ± 1.42, and 4.35± 0.43 for recoveries for ACE2, TLR-4, and IL-17, respectively (P value < 0.001 for all; Fig. 3a-c).

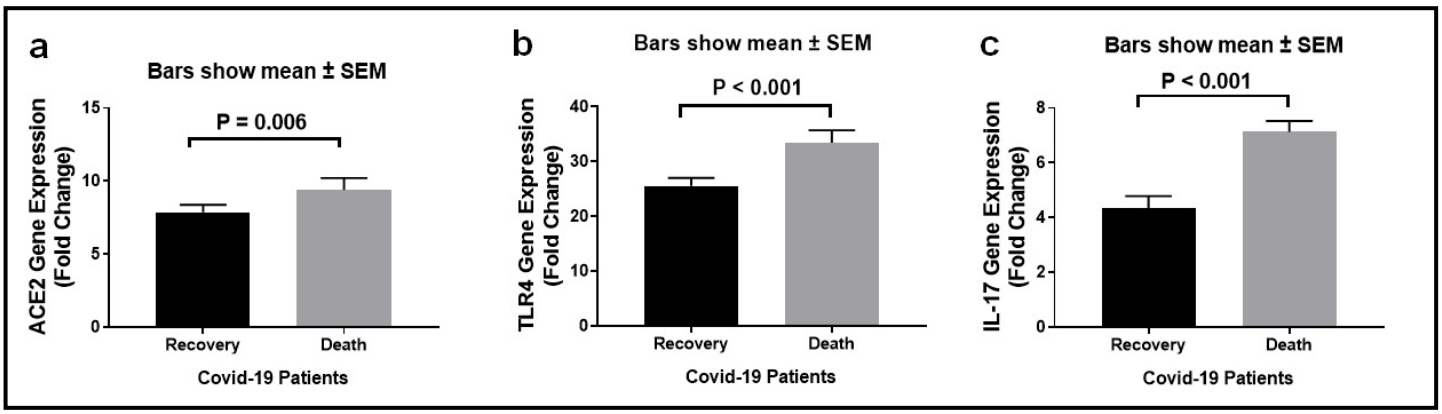

Fig. 3a-c Fold-change gene expression ($2^{-\Delta\Delta Ct}$) of a. ACE2 b. TLR4; c. IL-17 in COVID-19 patients sub grouped according to outcomes (Recoveries vs. deaths). Data were expressed as mean±SE.(No.of Recovery=32 cases,& Deaths =28 patients)

3.4 Correlation studies

We found significant negative correlations between the expression levels of ACE2 and TLR-4 and total protein levels, albumin, lymphocytes, platelets, and HB levels (Table 6). Expression levels of ACE2 showed a significant positive correlation with IL-17, TLR-4, RBG, CRP, ferritin, D dimer, INR, PT, creatinine, BUN, ALP, ALT, AST, T. bilirubin, D. bilirubin, WBCs, and neutrophil levels in the patient group. More significant positive correlations were found in the more severe groups (Table 5).

Table 5 Correlation studies between gene expression of ACE2, TLR-4, IL-17, and laboratory data in COVID-19 patients

	ACE2		TLR4		IL-17	
	R	p	R	P	R	P
ACE2			.806**	0.000	.723**	0.000
TLR-4	.806**	0.000			.811**	0.000
IL17	.723**	0.000	.811**	0.000		
RBG (mg/dl)	.252**	0.005	.261**	0.004	.361**	0.000
CRP (mg/L)	.696**	0.000	.784**	0.000	.804**	0.000
Ferritin (ng/ml)	.616**	0.000	.699**	0.000	.725**	0.000
D-dimer (µg/ml)	.636**	0.000	.764**	0.000	.801**	0.000
INR	.547**	0.000	.580**	0.000	.589**	0.000
PT (Seconds)	.380**	0.000	.441**	0.000	.488**	0.000
Creatinine (µmol/L)	.212*	0.020	.243**	0.007	.339**	0.000
BUN (mmol/L)	.612**	0.000	.734**	0.000	.709**	0.000
ALP (IU/L)	.176	0.055	.292**	0.001	.291**	0.001
ALT (IU/L)	.261**	0.004	.237**	0.009	.265**	0.003
AST (IU/L)	.333**	0.000	.356**	0.000	.366**	0.000
TP (g/L)	-.284**	0.002	-.241**	0.008	-.226*	0.013
Albumin (g/L)	-.287**	0.001	-.256**	0.005	-.280**	0.002
T bilirubin (µmol/L)	.191*	0.037	.333**	0.000	.282**	0.002
D bilirubin (µmol/L)	.600**	0.000	.731**	0.000	.706**	0.000
WBCS ($\times 10^9$/L)	.288**	0.001	.339**	0.000	.327**	0.000
Neutrophils ($\times 10^9$/L)	.390**	0.000	.385**	0.000	.458**	0.000
Lymphocytes($\times 10^9$/L)	-.282**	0.002	-.385**	0.000	-.416**	0.000
Hemoglobin (g/dl)	-.144	0.116	-.192*	0.035	-.227*	0.013

Platelets ($\times 10^9$/L)	-.293**	0.001	-.324**	0.000	-.279**	0.002

Spearman's correlation. r correlation coefficient, p-value, and Correlation are significant at the 0.05 level (2-tailed). Random blood glucose (RBG), C-reactive protein (CRP), International normalized ratio (INR), Prothrombin time (PT), Blood urea nitrogen (BUN), Alkaline phosphatase (ALP),Alanine transaminase (ALT), Aspartate transaminase (AST), Total protein (TP), Total(T) bilirubin, Direct(D) bilirubin, White blood cell count(WBCS).

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. 4a-c.

Results showed that at cutoff values > 3.4856, the serum expression of ACE2 showed 86.75% sensitivity and 96.7 % specificity in discriminating between COVID -19 cases and controls. ROC curve results for TLR4 and IL-17At cutoff values > > 1.8862 and > 1.8021 respectively , the serum expression of TLR-4 and IL-17 showed 91.7% , 95% sensitivity respectively and 98.3 % specificity for both markers in discriminating between COVID -19 cases and controls

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

	AUC	Cutoff	Accuracy%	Sensitivity %	Specificity %
ACE2	0.908	> 3.485	83.33	86. 7	96.7
TLR4	0.968	> 1.8862	90	91.7	98.3
IL-17	0.973	> 1.8021	93.33	95.00	98.3

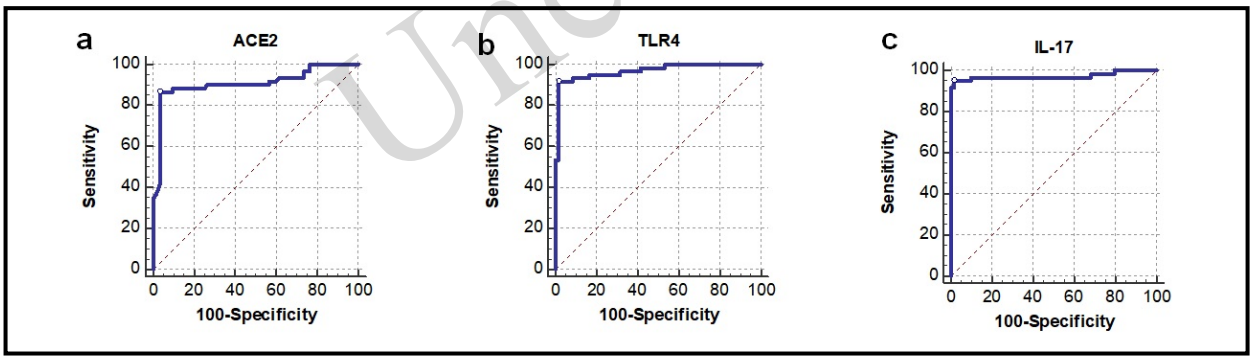

Fig. 4a-c. ROC curve of a. ACE2 b. TLR4; c. IL-17 in COVID-19 patients vs. controls

4 Discussion

Acute respiratory distress syndrome (ARDS) is now understood to be one part of multiple-organ dysfunction syndrome. A severe SARS-CoV-2 infection may result in an overactive immunological response that results in a "cytokines 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 in order to shield the organism from damage (Chávez-Ocaña et al., 2023).

Our study showed significant upregulation in the expression of ACE2, TLR4, and IL-17 mRNA in COVID-19 patients; this upregulation persisted with increasing severity of the disease and was also correlated

with bad outcomes (death).

Binding of Spike proteins of the virion with the cellular ACE2 receptor and cleavage of S proteins by host transmembrane serine proteinase 2 (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 cell membrane 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 lysosome 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 virus particles are migrated out of the cell via exocytosis. Alveolar cells are eventually infected by the virus (Ni et al., 2022, Xu et al., 2020).

Human alveolar macrophages can respond to SARS-CoV-2 spike protein by interacting with TLR-4, 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 spike protein to TLR4 may play a role in SARS-CoV-2 entrance into human cells and instigation of the cytokine storm that impacts many organs (Choudhury and Mukherjee, 2020, Gadanec et al., 2021).

Toll-like receptor-4 is one of the most important classes of PRRS, which are required for the stimulation of proinflammatory cytokines (Jose et al., 2022). Activation of NF-κB leads to the release of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, which are mediated via the TLR4/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 signalling after viral infection greatly exacerbates the severity of pulmonary illness (Brandão et al., 2021). Furthermore, Luo et al. found that TLR4 signalling was a critical mechanism of acute lung injury (Luo et al., 2017).

Sohn et al. (2020) recently showed that in comparison to peripheral blood mononuclear cells in healthy controls, those in COVID-19 exhibited considerably higher expression of TLR4 and its downstream signaling mediators (Sohn et al., 2020), which is consistent with our findings. In addition, Alturaiki et al. (2023) found that ICU COVID-19 patients had much higher TLR-4 mRNA expression levels than did non-ICU COVID-19 patients (Alturaiki et al., 2023; Khanmohammadi and Rezaei, 2021). Khanmohammadi and Rezaei, (2021) documented the fact that TLR-4 is known to trigger neutrophil extracellular traps (NETs). The severity of COVID-19 and persistent inflammation have been linked to NET formation (Khanmohammadi and Rezaei, 2021). Alturaiki and colleagues also came to the conclusion that patients admitted to the ICU had higher levels of TLR-4 mRNA expression than patients with non-critical COVID-19, which is consistent with our findings (Alturaiki et al., 2023).

Additionally, in the monocytes of severe COVID-19 patients, Dorneles et al. (2023) observed elevated TLR-4 expression in conjunction with greater NF-κB p65 phosphorylation. In fact, in vitro tests conducted in the past have shown overactivation of TLR-4 due to the simultaneous presence of SARS-CoV-2-related proteins and systemic endotoxemia (Dorneles et al., 2023).

In contrast to our findings, Ghazavi and colleagues found that, when comparing the moderate group to 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 (Ghazavi et al., 2021).

Correlation studies showed that both ACE2, and TLR4 expression levels had significant negative correlation with total protein, albumin, lymphocyte, platelets, and Hb levels; and significant positive correlation with IL-17, RBG, CRP, ferritin, D dimer, INR, PT, creatinine, BUN, ALP, ALT, AST, T bilirubin, D bilirubin, WBCs, and neutrophil levels. Our results confirmed the strong correlation between TLR4 expression levels and

COVID-19 severity, which demonstrates the relation to the pathogenesis of COVID-19 and allows 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 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 SARS-CoV-2 spike glycoprotein and TLR4. The general view is that activation of TLR4 after interaction with the spike glycoprotein of SARS-CoV-2 increases the surface expression of ACE2, thus promoting viral entry. Modelling work done by Kogan et al and Rahman et al 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 (Kogan et al., 2022; Rahman et al., 2021).

In conclusion, our results clarified that SARS-CoV-2 SP significantly increases both TLR-4 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 each well-known downstream products of TLR-4 activation.

When a viral S glycoprotein attaches to ACE2 on type II alveolar cells—which are responsible for creating pulmonary surfactant—it exposes the extracellular binding sites of TLR-4 on lung epithelial cells. The exposed TLR-4 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 TLR-4. 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-cell 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 ACE-2, TLR4 and IL-17 are helpful in assessing the severity of COVID-19 disease. 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 Faculty of Medicine/Assiut University.

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 conflict 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 Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Approval number by the Institutional Review Board of the Faculty of Medicine/Assiut University (IRB No: (IRB No. 17101957). Assiut University, Date of original approval: 2022.

Author Contributions

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