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Study of ACE2 gene expression and evaluated some cellular and humoral immunity parameters of COVID-19 patients in Ramadi City

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***Abstract*---**The new coronavirus SARS-CoV-2 is responsible for the COVID-19 epidemic. SARS-CoV-2 accesses host cells by ACE2, which is abundantly expressed in the heart, kidneys, and lungs and shed into the plasma. Many SARS-CoV-2 patients' neutralizing antibody titers and memory B cell responses may be transient, exposing them to re-infection. Notably, neutralizing antibody titers and the number of virus-specific T cells had a substantial relationship. Our findings lay the groundwork for additional research into protective immunity against SARS-CoV-2 and the etiology of COVID-19, particularly in severe instances. The most important characteristic of our findings a positive correlation between all parameters (cellular and humoral) and COVID-19 patients compared with healthy group excepting neutrophil cells where no significant difference was observed between the two groups. Lymphopenia and elevated levels of specific cytokines, such as IL-6, IL-10, and TNF-α, have been linked to illness severity in general. T cells are thought to play a key role in the first immune response. In extreme situations, a significant drop in T cell counts is virtually always detected. We observed that lymphocytes were significantly decreased ($p < 0.001$) in COVID-19 patients (630±0.7678) cells/mm^3, as compared to healthy controls (2247±0.1497), Generally COVID-19 patients at severe stage suffering leucopenia and stimulate neutrophils to reduce formazan dye. On the other hand significant increase was recorded in cytokines storm that Hyper-production of

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mostly pro-inflammatory cytokines, such as IL-6, IL-10, and TNF-, which selectively target lung tissue, can significantly impair the prognosis in the most severe cases. Average of IL6, IL-10, TNF- α were significantly increased ($p < 0.001$) in COVID-19 patients (38.2±6.228, 94.87±8.426, 23.61±4.13 pg/ml), as compared to healthy controls (2.66±0.21, 8.06±0.85, 10.53±1.13 pg/ml) respectively. Gene expression of ACE2 significantly decreased at P <0.001 in severe patients to (0.1262±0.0072) whereas reported (1) for healthy controls, thus Fold of expression 7.92 (down regulation). Their impact on results will aid in the development of more effective COVID-19 management strategies.

***Keywords*---**COVID-19, SARS-CoV-2, IL-6, IL-10, TNF-α, NBT, ACE2, RT-PCR.

1. Introduction

Coronavirus disease 2019 (COVID-19) patients were discovered in Wuhan, China, at the end of December 2019 **(1)**, it was first identified as viral pneumonia caused by an unknown infectious agent **(2)**. The virus was described as a novel coronavirus by the Chinese Centre for Disease Control and Prevention on January7, 2020, based on a throat swab examination from an infected patient **(3)**. This disease was first declared a public health emergency of international significance by the World Health Organization (WHO), and then a global pandemic by the WHO **(4), (5)**. SARS-CoV (severe acute respiratory syndrome) in 2002–2004 and Middle East respiratory syndrome (MERS-CoV) in 2012 were also caused by coronaviruses **(6)**. Human endemic infections are caused by four additional coronaviruses (229E, NL63, OC43, and HKU1), which account for 15–30 percent of common cold cases **(7)**. COVID-19 infection, unlike endemic coronaviruses, has a high case mortality rate (CFR 2.2 percent globally) **(8)**, and is characterized by three main symptoms: a fever of more than 38 C°, shortness of breath, and a dry cough **(9)**. Coronaviruses (CoVs) are enveloped, crown-like, positive-stranded RNA viruses with a genome size of 26–32 kilo-bases that belong to the Coronaviridae family **(10)**. SARS-CoV-2 has a genome structure identical to SARS-CoV, with four structural proteins, membrane (M), envelope (E), spike (S), and nucleocapsid (N), as well as 14 open reading frames (ORFs), with ORF1ab encoding 16 non-structural proteins (NSPs) **(11)**. SARS-CoV-2 infection is activated by the spike (S) protein binding to the angiotensin-converting enzyme 2 receptor (ACE2) on host cells figure (1). The S protein is cleaved and activated by the host trans-membrane protease serine 2 (TMPRSS2) after it binds, allowing viral entry **(12)**. N, M, NSP1, NSP3b, NSP4a, NSP4b, and NSP15 are among the other coronaviral proteins implicated in the regulation of the host immune response and immune evasion **(13)**. The expression and distribution of ACE2 may play a key role in the SARS-CoV-2 infection process **(14)**. Recent research has discovered that ACE2 expression is mostly found in the lungs **(15)**, intestine **(16)**, kidneys **(17)**, and heart **(18)**. A group of researchers investigated the RNA expression profile of ACE2 in single cells and discovered that type II alveolar cells (AT2) contain high levels of ACE2 gene expression, as well as other genes that promote viral proliferation and transmission **(19)**. T immune responses and neutralizing antibodies are important

in the clearance of viral infections because specific targeting of pathogen-derived antigens is required for the elimination of infected cells and neutralization of free virions and the adaptive immune system's high specificity allows for a highly regulated and targeted immune response **(20)**. Most viral infections require B cells for clearance, and most COVID-19 patients have a humoral response within two weeks of infection. In certain cases, poor humoral responses are linked to ineffective SARS-CoV-2 clearance, emphasizing the relevance of this response for viral clearance **(21)**, **(22)**. In this study, we aimed to analyze the expression of ACE2 gene and evaluated some cellular and humoral immunity parameters and predict the connection between them and covid-19 disease.

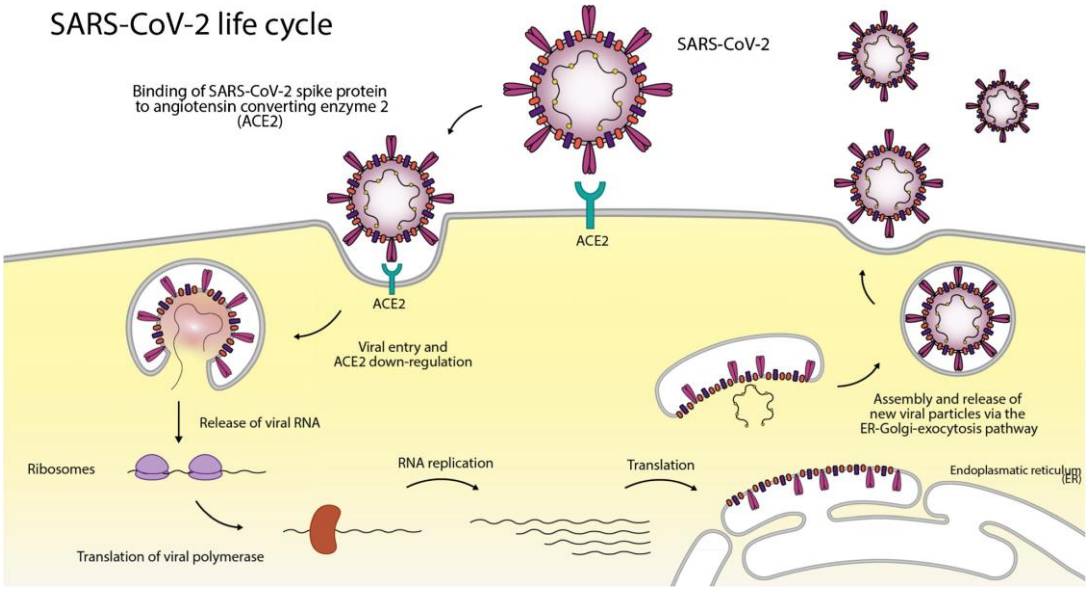

Fig. 1.1 SARS-CoV-2 life cycles: from binding to ACE2 receptor to shedding (23)

2. Materials and methods

2.1 Samples collection

We collected the clinical samples (whole blood and serum) of 100 severe cases who required Quarantine lobbies (need of critical care support, including high-flow oxygen, positive-pressure ventilation or vasoactive drugs) due to COVID-19 (mean age 61.27 years, range 27–77). All the study participants were Iraqi from the region of Anbar (Ramadi, total population 1 million approximaly), and positive for SARS-Cov-2 (PCR test from nasal swabs). We also studied 120 healthy population controls matched with the patients for age (n = 120; mean age 58.32 years, range 30–82).

2.2 Expression of ACE2 gene by RT-PCR

RNA extraction using invitrogen TRIzol Reagent:
RNA was extracted and purified using quantification RNA extraction kit according to the manufacturer's instructions.

Real-Time PCR Amplification (Two-step RT-PCR):

The ACE2 gene was amplified using the sense (5'TCCATTGGTCTTCTGTCACCCG -3') and antisense (5'AGACCATCCACCTCCACTTCTC-3') **(24)** and Homo sapiens Housekeeping β-actin (ACTB) was used for normalization, sense (5'GCGAGAAGATGACCCAGA-3') and anti-sense (5'CAGAGGCGTACAGGGATA-3') **(25)**. qPCR reaction was carried out in a 10ul reaction containing 5ul of qPCR Master Mix (Promega, US), 0.5 ul of 10 pmol/ul of each primer (Macrogen, South Korea), 1.5ul of RNA template, 0.25 of RT (Reverse Transcriptase) enzyme (Promega, US) and the volume was completed to 10ul using nuclease-free water. Thermo cycling conditions were as follows: 35C° for 5min., 42C° for 15min., 95C° for 10 min. (1cycle), followed by 45 cycles of denaturation at 95 C° for 30sec; annealing at 60 C° for 30 sec; extension at 72 C° for 30sec., followed by a melting Stage: Collect data starting at 72 C° to 95 C° at 0.3 C°/s Calculated of Ct value depend on Livak equation as follow:
ΔCt (Patient sample) = Ct*ACE* – Ct B-actin
ΔCt(Healthy control) = Ct*ACE* – Ct B-actin
ΔΔCt = ΔCt(Patient.) - ΔCt(Control).
Folding = $2^{-\Delta\Delta Ct}$ **(26)**

2.3 Cellular immunity parameters
Study of Cellular immunity includes white blood cells count, Neutrophils, lymphocytes, and ability of PMNs for nitroblue tetrazolium (NBT) reduction. Evaluation of WBC, Neutrophils, lymphocytes by Mindray BC-5000 hematology analyzer, while NBT test conducted according to **(27)**

2.4 Humoral immunity parameters
Study of Cellular immunity includes (SARS-CoV-2 (Covid-19) IgG/IgM, Interleukin-6 (IL6), Interleukin-10 (IL-10), Tumor Necrosis Factor alpha (TNF-α). All humoral immunity parameters conducted by Sunlong ELISA kits.

Statistical analysis
The data obtained in the present study were expressed as Mean ± SD and was analyzed using two-tail ANOVA at 1% level of significance using computer software SPSS version 22.

3. Results
Cellular immunity parameters:
3.1 White Blood Cells (WBC)

The results of white blood cells (cells/mm^3) showed significant difference ($p<0.0001$) between **group A (COVID-19 patients)** (3902.857±609.1028) than in **group B (Healthy control)** (6660±976.8351) as shown in Table 3.1 and fig. 3.1

Table 3.1: WBC for Patients compared with HCs

Variable	Groups	Mean ± SD	p-value
WBC (cells/mm^3)	Group A	3902.857±609.1028	<0.0001
	Group B	6660±976.8351	

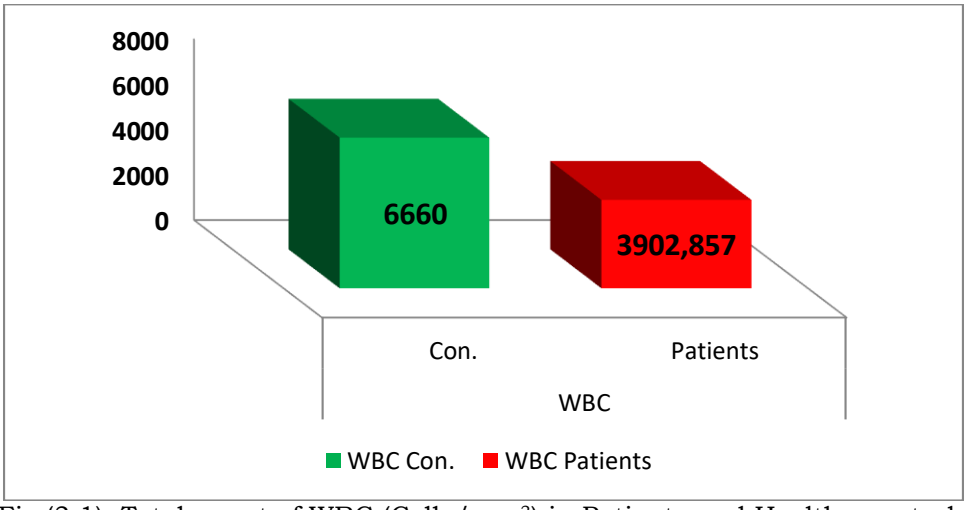

Fig (3.1): Total count of WBC (Cells/mm^3) in Patients and Healthy controls

Until date, the risk variables that determine mortality have remained unknown. Controlling and eliminating CoV infections requires the immune system. Nonetheless, mounting data shows that individuals with severe COVID-19 may be suffering from a cytokine storm (28),(29). Nearly 80% of the patients had normal or lowered white blood cell counts, and 72.3 percent (99/137) exhibited lymphocytopenia, according to Liu *et al.* (30). Zhang *et al.* also reported the results of 9 individuals, all of whom had normal peripheral white blood cell counts and were PCT negative (31). These findings were very comparable to ours. We discovered that the majority of the patients in our research had WBC counts in the lower range. These results are agreement with **(32)** that showed significantly lower counts of leucocytes were observed in the COVID cases than in the controls.

3.2 Neutrophils, Lymphocytes, and NitroBlueTetrazolium (NBT) for Patients compared with HCs

The results of neutrophil cells showed no significant difference ($p<0.793$) between **group A (COVID-19 patients)** and **group B (Healthy control)**. While according to the data obtained from the experiments carried out within the scope of this study, lymphocyte cells in group A was decrease compared with group B, and there was a significant difference ($P< 0.0001$) between the two groups, NBT percentage increased COVID-19 patients compared with healthy controls, it was recorded (72.38) and (55.18) % respectively. As shown in the table 3.2 and fig. 3.2

Table 3.2: Neutrophils, Lymphocytes, and NitroBlueTetrazolium (NBT)

Variable	Groups	Mean ± SD	p-value
Neutrophils (cells/mm^3)	Group A	4880±1.0081	<0.793
	Group B	4937±0.9784	
Lymphocytes (cells/mm^3)	Group A	630±0.7678	<0.0001
	Group B	2247±0.1497	
NBT %	Group A	72.38 %	

	Group B	55.18 %	<0.0001

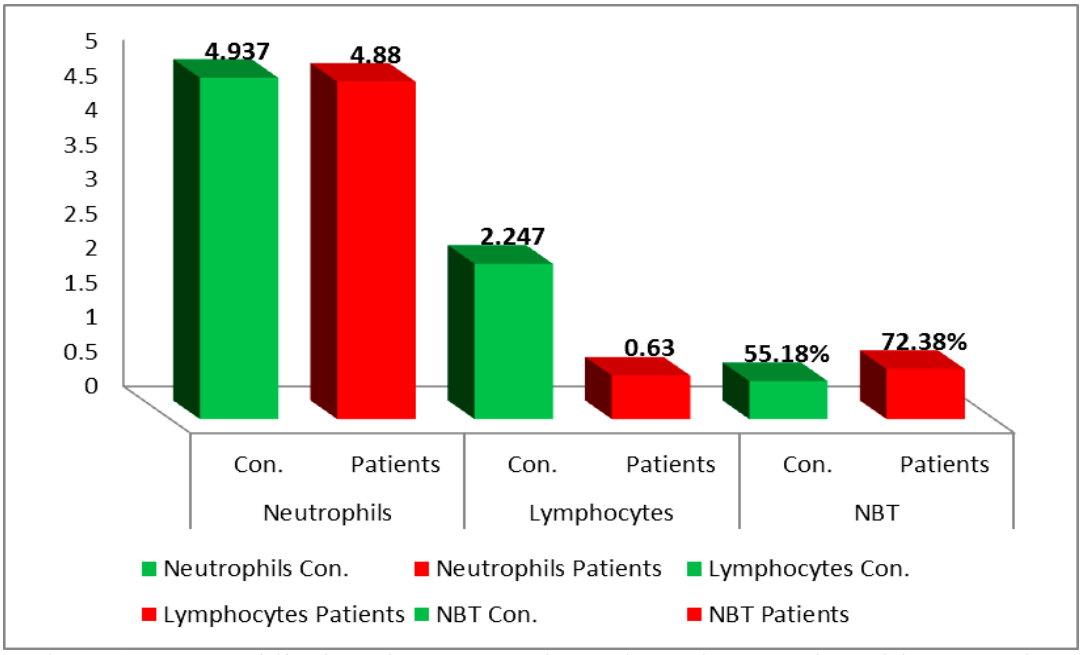

Fig (3.2): Neutrophils, lymphocytes, and NBT in Patients and Healthy controls.

Several investigations found that severe cases of COVID-19 pneumonia included neutrophilia (absolute neutrophil count beyond the normal range; 3–7.5 10^9/L) and/or lymphocytopenia (lymphocyte count 1.5 10^9/L) that were linked with a poor outcome. In the early stages of SARS CoV-2 infection, the ratio of neutrophils to lymphocytes (NLR) has been observed to predict disease severity. Lymphocytopenia is a reliable sign of early SARS CoV-2 infection, and it aids in contact tracing as well as illness development during COVID-19 pneumonia. More research is needed to determine its effect in immune-compromised individuals, particularly those with human immunodeficiency syndrome. Future research is needed to confirm the effect of SARS CoV-2 on diverse subsets of T cells. The results of our current study are agreement with many studies **(33), (34)** that showed decrease levels of lymphocytes in COVID-19 patients related to healthy control group. Cytotoxic lymphocytes, such as cytotoxic T lymphocytes and natural killer cells, are well known for their function in immunological homeostasis and the inflammatory response required to suppress viral infection **(35)**. Apoptosis or functional exhaustion of cytotoxic lymphocytes has been linked to the course of viral infection in previous investigations **(36)**, **(37)**. Although the pathogenic mechanism of lymphopenia on the severe course of COVID-19 is still unknown, we were prompted to hypothesize the production of excessive pro-inflammatory cytokines as a result of the COVID-19 infection, which was expected to progress severely, remain disordered, and induce lymphocyte apoptosis. On the other hand increase of NBT dye in COVID-19 patients compared to healthy controls may be due to the amount of neutrophil NBT is related to the challenging element in the stimulated NBT test **(38)**. However, bacteria, viral, particulate and soluble products, immune complexes, serum complement, and acute phase

proteins, among other things, may produce an increase in dye reduction by neutrophils.

Humoral immunity parameters:

In this context, it is possible to say that there was a high significant difference ($p<0.0001$) between group A and group B for cytokines (Interleukin-6, Interleukin-10, Tumor necrosis factor- alpha) and immunoglobulin's specific COVID-19 (IgM and IgG) recorded (38.2±6.228), (94.87±8.426), (23.61±4.13), (28.54±3.426), (11.62±2.103) for group A (COVID- Patients) and (2.66±0.21), (8.06±0.85), (10.53±1.13), (0.0332±0.0012), (0.143±0.015) for group B (Healthy control) respectively. As shown in table 3.3 and fig. 3.1

Table 3.3: IL-6, IL-10, TNF-α, COVID-IgM, and COVID-IgG parameters assay

Variable	Groups	Mean ± SD	p-value
IL-6 (pg/ml)	Group A	38.2±6.228	<0.0001
	Group B	2.66±0.21	
IL-10 (pg/ml)	Group A	94.87±8.426	<0.0001
	Group B	8.06±0.85	
TNF- α (pg/ml)	Group A	23.61±4.13	<0.0001
	Group B	10.53±1.13	
COVID-IgM (AU/ml)	Group A	28.54±3.426	<0.0001
	Group B	0.0332±0.0012	
COVID-IgG (AU/ml)	Group A	11.62±2.103	<0.0001
	Group B	0.143±0.015	

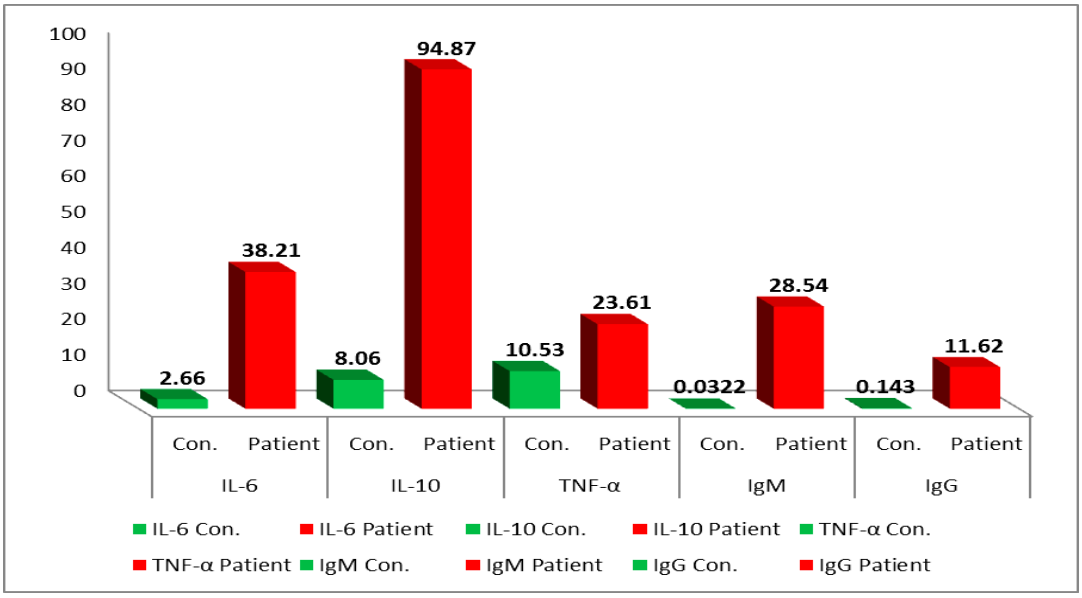

Fig (3.3): Cytokines and immunogloulins parameters assay

Cytokines play a critical role in the regulation of immune and inflammatory responses. Because of its pleiotropic effects, IL-6 is one of the most important among them **(39)**. The evidence that circulating IL-6 levels are closely connected to the severity of COVID-19 infection is described here. In individuals with respiratory dysfunction, an increase in IL-6 levels has previously been found **(40)**, revealing a possible common mechanism of cytokine-mediated lung injury induced by COVID-9 infection. Furthermore, the highly pathogenic SARS-CoV-2 virus appears to be linked to fast virus replication and a proclivity for infecting the lower respiratory tract, leading in an increased sensitivity to IL-6-induced acute respiratory distress. As a result, our findings imply that monitoring circulating IL-6 levels over time might be useful in detecting illness progression in COVID-19-infected individuals. IL-10 blood levels, as well as most other cytokines, have been linked to the severity of COVID-19 in Chinese cohorts from the onset of the pandemic, according to several studies **(41), (42), (43)**. IL-10 was equally elevated in both groups in a recent study of COVID-19 patients hospitalized in Ireland who were stable and required ICU admission 1 week after the onset of symptoms (20 patients per group), while IL-1, IL-6, IL-8, and soluble TNF receptor 1 were all more strongly elevated in the ICU than in the stable group **(44)**. Our results confirm TNF-α as a biomarker of severity in a cohort of covid patients, TNF-α considered one of the more proinflammatory cytokine, thus Several studies are recommend Patients with COVID-19 who are at high risk of developing a life-threatening type may benefit from TNF alpha blocker therapy. Sars-CoV2-induced cytokine release syndrome and immunothrombosis account for the majority of coronavirus illness severity in (COVID-19) **(45)**.

Gene Expression of *ACE2* for COVID-19 patients and Healthy controls

The results showed a significant differences at P <0.001 between group A (COVID-19 patients) and group B (Healthy controls), reduce amount of *ACE2* gene expression in group A to (0.1262±0.0072) whereas reported (1) for group B, thus Fold of expression 7.92 (down regulation) as shown in fig 3.5

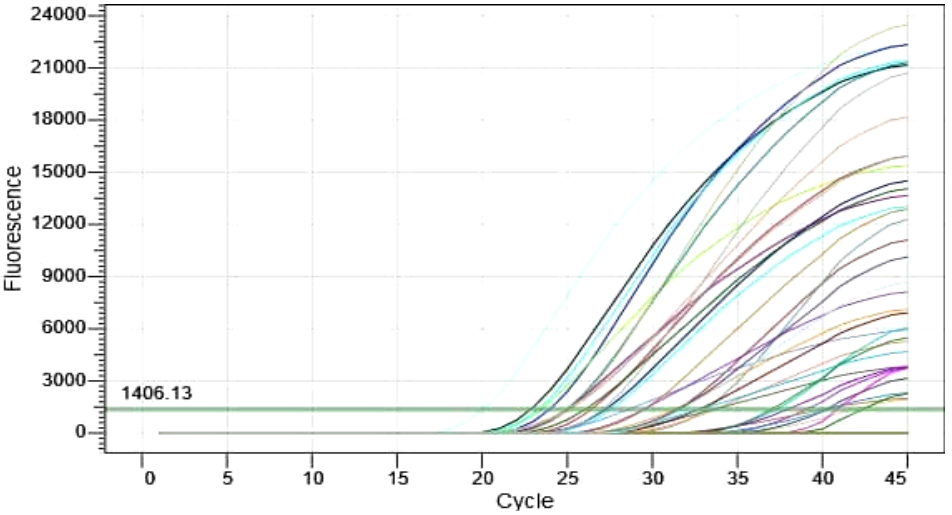

Fig (3.4): Curve of Ct values of Human *ACE2* gene, and β- actin as housekeeping gene.

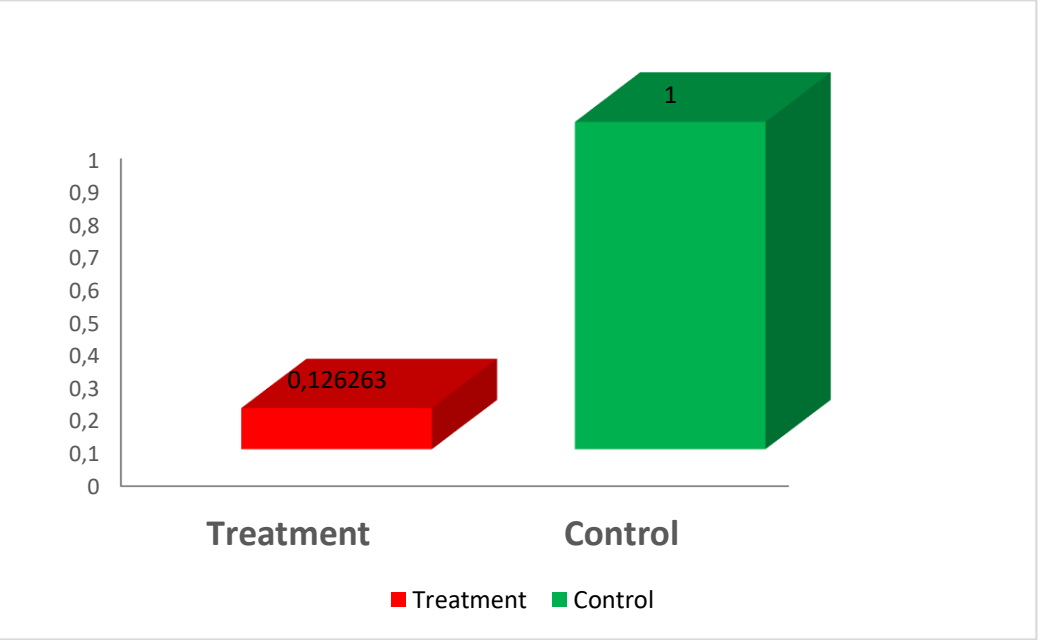

Fig (3.5): Expression folding of Human *ACE2* gene

Ang-II is hydrolyzed by *ACE2* producing Ang-1–7. Ang-II binds to the AT1-receptor, causing vasoconstriction, fibrosis, inflammation, and thrombosis, among other things; Ang-1–7 binds to the AT2-receptor, causing greater vasodilation and decreased fibrosis, inflammation, and thrombosis, among other things. As a result, the *ACE* and *ACE2* are considered as opposing forces in the equation that determines the risk of hypertension and cardiovascular disease. *ACE2* activates a defensive response in the lungs, lowering edema, permeability, and pulmonary injury **(46)**, **(47)**. Lower *ACE2* levels should be harmful for patients with lung disease, so the frequency seen in COVID-19 patients could be a compromise between the negative association with viral infection (lower expression in the airway epithelia) and the positive association with respiratory and cardiovascular disease (lower expression in lung and other organs). The spike protein is an important predictor of the virus's tissue tropism and host range. In terms of internalization, SARS-CoV-2 competes with Ang II for *ACE2*. The binding, on the other hand, inhibits *ACE2* action, lowering enzyme expression in the membrane **(48)**. *ACE2* receptors serve as entrance locations for SARS-CoV-2 to enter the airways and the body in general **(49)**, **(50)**. Spike's glycoprotein (S protein), combined with the type 2 trans-membrane serine protease (TMPRSS2), promotes receptor identification and membrane fusing onto *ACE2* peptidase domain on the virion surface, with a high affinity of KD 15 nM (20– 22). Viral entry increases the activity of the ADAM17 protease, which in turn down-regulates *ACE2* by cleaving the receptor from the cell surface ("shedding"), so moving the protective *ACE2* / angiotensin 1–7/ Mas axis toward the disease state and causing an increase in angiotensin II (ANG II) **(51)**. Some propose that lowering *ACE2* expression reduces vulnerability to the virus by reducing the number of accessible binding sites for SARS-CoV-2 and limiting viral entrance into cells. Others have claimed that after SARS-CoV-2 attaches to *ACE2*, it down-regulates *ACE2* expression, similar to the pathophysiology of other viral

pneumonias, such as those caused by earlier severe acute respiratory syndrome (SARS) viruses. Lack of *ACE2* beneficial effects may aggravate lung damage through a variety of methods. We conducted a literature search and evaluation of important preclinical and clinical papers relating to SARS-CoV-2, COVID-19, and ACE to assist resolve this debate.

Conclusion

The role of Some Cellular and humoral immunity parameters for COVID-19 detection and the *ACE2* gene is critical for COVID-19 prevention, treatment, and medication development. In considerably underlying risk factors for COVID-19, *ACE2* performs comprehensive vascular and organ defense. Patients have low amount of *ACE2* that Complications and death are more likely. Nonetheless, recent research is revealing a link between *ACE2* activity, viral load, and disease severity, with the preliminary consensus being that increased SARS-CoV-2 expression and *ACE2* activity are found in direct viral organ toxicity in severe/critical COVID-19; and increased viral load correlates with increased severity. More clinical research is needed to better understand the role of *ACE2* in illness susceptibility and severity. Novel therapeutics for SARS-CoV-2 will almost certainly investigate the virus's link to *ACE2*.

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