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Immunogenetic Study of IL-12 and ACE-2 Genes Polymorphism among Patients with COVID-19

A Thesis Submitted to Council of College of Science University of Babylon in Partial Fulfiment of the Requirements for the Degree of Master of Sciences in Biology

By

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

(بَدِيعُ السَّمَاوَاتِ وَالْأَرْضِ ۖ وَإِذَا قَضَىٰ أَمْرًا فَإِنَّمَا يَقُولُ لَهُ كُن فَيَكُونُ)

صَدَقَ اللهُ العلي العَظيم

[البقرة: 117]

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Dedication

To the purest heart to my role model, and my ideal in life; He is the one who taught me how to live with dignity and loftiness… My dear father

To the heaven of God on earth, to the bridge that ascends me to heaven, to my ideal… My mother

To my dear companion, my second half, who encourages me to innovate and excel… My husband

To the eyes and heartbeat …to … My brother & sisters

To the endless love and the secret behind my existence… My children

Huda

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Summary

The acute respiratory syndrome caused by the Coronavirus, which is one of the respiratory viruses that spread quickly, swept the world and killed more than six million people. This led to the creation of many vaccines and medicines in record time to lessen the severity of this disease and speed up the healing of patients. This study looks at the genetic heterogeneity of angiotensin-converting enzyme-2 and interleukin-12 in patients with COVID-19 and compares these cytokines and receptors between severe and non-severe cases. This cross-sectional study included 100 blood specimens from COVID-19 patients with ages (15-to 80) years were distributed according to the severity of disease as the following: (46 severe,54 non-severe) who have been hospitalized at the COVID-19 Wards in Merjan Medical City and Imam Sadiq Hospital in Babylon Province for 2 months (November and December 2021). Two parts of the work are the first immunological study for the evaluation of serum IL-12 and ACE-2 and the second work genetic study for the detection of genetic polymorphism of ACE-2, IL-12A, and IL-12B. Results reveal that the serum levels of both angiotensin-converting enzyme-2 (ACE-2) and interleukin-12 (IL-12) were measured in COVID-19 patients and the results were compared using an independent T-test, it was found that their levels for interleukin-12 revealed a significant difference ($P \leq 0.05$) in the serum levels of severe cases when compared with non-severe cases. There was an increase in the serum level of IL-12 in severe cases which was 33.340 ng/L, and in non-severe cases which was 20.913 ng/L. ($P \leq 0.000$), and for angiotensin-converting enzyme-2 this study revealed a significant difference in ACE-2 serum levels in severe cases ($P \leq 0.05$) when compared with the non-severe cases of patients with COVID-19. The serum level of ACE-2 in severe cases was 11.023 ng/ml, and in non-severe cases, it was 5.443ng/ml ($P \leq 0.000$). The result for the IL-12A gene was nine SNPs in the IL-

12A gene, as the following rs1460141649, CG, GG, and CC genotypes the high prevalence rate of genotypes was CC (wild type) in severe COVID-19 patients at a percentage 84.7%, and 92.5% respectively the statistically nonsignificant (P >0.05). rs903858723, TT, TG, TC, CC, GG, and GC genotypes the high prevalence rate of genotypes was (wild type) in severe COVID-19 patients at a percentage of 78.3%, and 85.2% respectively the statistically nonsignificant (P >0.05). and rs772904833 AA, AG, GG, genotypes the high prevalence rate of genotypes was (wild type) in severe COVID-19 patients at a percentage of 78.3%, and 83.4% respectively the statistically nonsignificant (P >0.05).rs1208781994 GG, GC, CC, genotypes the high prevalence rate of genotypes was (wild type) in severe COVID-19 patients at a percentage of 78.3%, and 87% respectively the statistically nonsignificant (P >0.05). rs1720482004 AA, AG, and AC, genotypes the high prevalence rate of genotypes was (wild type) in severe COVID-19 patients at a percentage of 100%, and 89.6% respectively the statistically nonsignificant (P >0.05). and rs550206516 GG, GA, AA, genotypes the high prevalence rate of genotypes was (wild type) in severe COVID-19 patients at a percentage of 100%, and 100% respectively the statistically nonsignificant (P >0.05). and rs4986960 SNP on IL-12A gene show that there was a significant difference between GG and GT genotypes at a significant p-value (0.04) meaning that (IL-12A) rs4986960 SNP contributed to infection and severity in COVID-19. And the result for the IL-12B gene was a novel SNP position on the 159315957 IL-12 B gene showing a significant difference between AA and AG genotypes at a significant p-value (0.01) by mean the novel 159315957 (IL-12 B) SNP was contributed to infection and severity in the COVID-19. While the ACE-2-2 gene, revealed thirteen SNPs on ACE-2-2(GRCh38.p13) as the following rs775089013, rs1180242786, rs760347219, rs768110251,rs776135947,rs761675562,rs199651576,rs1308021517,rs146366965

5, rs746202722, rs201035388 and rs779538833 SNPs there has no significant difference between these SNPs and COVID-19 severity because all patients have the wild genotype and disappear from heterozygote and the homozygote genotype except for rs1482922566 and rs1463669655 where all patients have the heterozygote genotype and disappear from wild type and homozygote. From these findings, this study concluded both ACE-2 and IL-12 contributed to the severity infection of in COVID-19 patients for the immune study but for genetic study the polymorphism in ACE-2-2 gene does not associated with the severity of COVID-19 patients but the polymorphism in IL-12A and IL-12B were associated with the severity of COVID-19 patients.

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List of Abbreviations

Symbol	Description
ACE-2	Angiotensin-converting enzyme- 2
ADAM17	A disintegrin and metalloproteinase 17
AMPs	Antimicrobial peptides
Ang II	Angiotensin II
CD26	Cluster of differentiation
CD4+	A cluster of differentiation 4
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CoVs	Coronaviruses
COVID-19	Coronavirus disease-19
CRP	C- reactive protein

CTLs	Cytotoxic CD8+ T cells
CVD	Cardiovascular disease
DCs	dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ICTV	International committee of Taxonomy virus
IFN	Interferon
Ig	Immunoglobulin
IL-12	Interleukin-12
JAKs	Binding Janus kinases
MERS	Middle- East-respiratory-syndrome
NK	Natural Killer
NLR	Neutrophil-to-lymphocyte ratio
OR	Odds ratio
ORFs	Open reading frames
PCR	Polymerase chain reaction
PLpro	Papain-like protease
PLT	Platelet Count
PP	Polyproteins

RAAS	Renin-angiotensin-aldosterone system
RBD	Receptor-binding domain
RCU	Respiratory care unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
STAT	Signal transducers and activators of transcription
TBE	Tris-Borate EDTA
TMPRSS2	Transmembrane serine protease 2
TNF-α	Tumor necrosis factor-alpha
Vit-D	Vitamin D
WHO	World health organization

Chapter One
Introduction

Introduction

For the past three years, COVID-19 has been a cause of worry for all nations in the globe, including our own, as an economic and commercial movement has been stopped, education in schools has been stopped, and e-learning has evolved. With a fatality rate of 2%, this respiratory virus has become a pandemic that has affected millions of people worldwide. (Wang *et al.*, 2021).

An impressive number of studies have been conducted to understand the pathogenesis of COVID-19, and immunological factors have been revealed to play a key role in the progression of the disease, virus persistence, and risk of severity all we had to do was take a step back and consider studying the human immune system by looking at particular immune receptors and cytokines that play a key role in combating the coronavirus. (Rabaan *et al.*, 2021).

It has been hypothesized that the virus enters cells in two ways: the pH-independent route, in which the virion and host cell membrane fuse and genetic material are delivered to the host cell; and, on the other hand, the pH-dependent route, in which the virion and host cell membrane fuse and genetic material are delivered to the host cell. The second step is the endocytosis route, which is activated when the virus attaches to the ACE2 receptor's cleavage site (Bayati *et al*., 2021).

ACE-2 is a receptor that attaches to the spike glycoprotein ligands in the severe acute respiratory coronavirus envelope and aids the virus's capacity to penetrate the human body (Li *et al*.,2003).

Human host cells have ACE-2 receptors on their membranes. Though to varying degrees, it can be present within every one of the bodily organs. Since the SARS-CoV virus's primary target is the alveolar cells, it is clear that ACE-2 is strongly expressed in alveolar cells, but not in membranes of the nose, mouth, or eye cells.

Both a pH-dependent and a pH-independent pathway have been postulated to be possible entry points for the virus into cells. In the pH-dependent route, genetic material is transmitted to the host cell without the virion or host cell membrane fusing. When the virus binds to the cleavage site of the ACE2 receptor, the endocytosis route is triggered, and this is the final step (Zou *et al*.,2020).

The immunological responses elicited by the novel coronavirus were characterized by the generation of high levels of proinflammatory cytokines (Prompetchara *et al*.,2020). Inflammation of the lungs, significant lung damage and respiratory distress syndrome were all symptoms of both disorders (Harcourt *et al*.,2020). They also have immunological dysregulation and macrophage activation syndrome (Prompetchara *et al*.,2020).

In addition to stimulating the innate immune system, which releases cytokines and chemokines, SARS-CoV-2 also stimulates the adaptive immune system, which causes T and B cells to produce inflammatory cytokines. (Crisci *et al*., 2020).

When comparing critical and non-critical patients, it was revealed that intensive care unit patients' plasma had greater IL-12 concentrations than mild patients' plasma. (Yang *et al*., 2020).

Interleukin-12 (IL-12) is the primary regulator of adaptive type 1 cell-mediated defense (IL-12). It is one of the most important mechanisms in neoplasia and virus defenses. Human clinical research (Leoni *et al*.,2018) examining several studies attributing to improved clinical outcomes substantiates this claim. and IL-12-based therapeutic strategies (Fu *et al*.,2020).

There are two disulfide-linked subunits in the cytokine IL-12 (interleukin-12), and they are known as IL-12p40 and IL-12p35. IL-12 is involved in a wide range of biological processes (Hildenbrand *et al*.,2022). Several additional cytokines,

including interferon, TNF, and granulocyte-macrophage colony-stimulating factors, are synthesized, which aids in the formation and stimulation of CD8+ T helper (CTL) and natural killer (NK) cells (Cox *et al*.,2021). Th1 cell-mediated immunity is dependent on the cytokine IL-12 for induction (Suzuki & Hayashida,2021).

This study aimed to look at the immune and genetic diversity of both angiotensin-converting enzyme-2(ACE-2) and interleukin-12(IL-12) in severe and non-severe patients with COVID-19 and to compare these cases with the following objectives:

1- Detection of ACE-2 gene polymorphism.
2- Evaluation of the IL-12A and IL-12B gene polymorphism.
3- Estimation of the serum levels of ACE-2 and IL-12 by ELISA.
4- Find out the association between IL-12, ACE-2 concentration level, and polymorphism of IL-12, and ACE-2-2 polymorphism respectively in patients with COVID-19.

Chapter Tow

Literature Review

2. Literature Review

2.1 The novel COVID-19 disease

A novel coronavirus causes highly pathogenic disorder 2019 (COVID-19), an invasive severe respiratory disorder. On December 31, 2019, the World Health Organization (WHO) got confirmation of instances of pneumonia with an undetermined microbiological cause in Middletown City, Hubei Region, China. A new flu virus was eventually discovered in clinical specimens from some of these cases, according to the WHO. Ever since, the outbreak has gotten worse and grown rapidly worldwide, prompting the WHO to declare it a health emergency of international concern on Jan 30, 2020, and subsequently an epidemic on Mar 11, 2020. Following that, patients developed symptoms consistent with the SARS outbreak, and the causative culprit was identified as a new coronavirus strain named COVID-19. The international committee on taxonomy virus ICTV updated the designation to R-CoV2 on February 11th, 2020 (Din and Boppana, 2020).In the twenty-first century, have now seen the formation of three main respiratory viruses in human people, one of which, the acute respiratory distress syndrome coronavirus (SARS-CoV), vanished after one period, not ever reappear, and the other, the Mers syndrome coronavirus (MERS-CoV), have not ever fully passed the species border due to it is ineffective transmitted between human people.(Ramadan and Shaib ., 2019; Kaiazek *et al.*,2003).

However, the creation of a new human flu virus during the 2019 virus transmission resulted in the creation of a new human COVID-19, which appears to have an ideal set of traits allowing for rapid dissemination with significant death. (Morens *et al*. 2020). The disease spread rapidly throughout the world, resulting in a pandemic that affected all aspects of life and put strains on all countries as they declared a lockdown and implemented social distancing regulations due to the presence of asymptomatic

carriers, a lack of targeted therapies, and multiple risk factor categories (Yuki *et al*., 2020). Due to their great prevalence and vast distribution, new coronaviruses emerge in humans from time to time, because of genetic diversity, intermittent genome recombination, as well as an increase in human-animal interaction activities, which have all been reported (Cui *et al*., 2019).

2.2 Coronaviruses

2.2.1 Classification of Coronavirus

Coronaviruses are members of Coronaviridae family, and Orthocoronavirinae subfamily of the Nidovirales order. Among RNA viruses, CoVs have the largest genomes, with genome sizes ranging from 26 – to 32 kb (Woo *et al*., 2012; Lu . *et al*., 2020). The COVs are further recognized to have 4 genera: α, β, γ, and δ, and SARS-CoV2 is classified under the genus β-coronavirus (Shchelkanov *et al*., 2020). The taxonomical classification of COVs is illustrated in Table 1-1.

Table 2-1: Taxonomical classification of coronaviruses (Shchelkanov *et al*., 2020).

Kingdom	*Orthornavirae*
Phylum	*Pisuviricota*
Class	*Pisoniviricetes*
Order	*Nidovirales*
Family	*Coronaviridae*
Subfamily	*Orthocoronavirinae*

2.2.2 SARS-COVID-19 variants

1- Alpha (VOC-20DEC-01; B.1.1.7 lineage): first identified in Kent, South East of England in September 2020. There is a piece of evidence that suggests this variant may be more transmissible compared with the wild-type virus. The reported secondary attack rate is 10.2% in household contacts of people with the variant who have not traveled as of 3 August 2021 (Meyer *et al*.,2021; Nyberg *et al*.,2021).

2- Beta (VOC-20DEC-02; B.1.351 lineage): first detected in Nelson Mandela Bay, South Africa in October 2020. The variant has similar spike protein mutations to the Alpha variant. Sequence analysis reveals that the N501Y mutation reported in the UK and South Africa originated independently. The Beta variant is likely to be less transmissible than the Alpha variant. There are limited published data available on whether this variant causes more severe disease (Priesemann *et al*.,2021)

3- Gamma (VOC-21JAN-02; P.1 lineage): a descendant of the B.1.1.28 lineage first detected in Japan in travelers from Brazil. Based on modeling and laboratory data, it is plausible that there is some degree of immune escape, increased transmissibility, or both with this variant. However, the magnitude and clinical significance of these effects are yet to be determined (Priesemann *et al*.,2021).

4- Delta (VOC-21APR-02; B.1.617.2): first identified in India in April 2021. It is now the dominant variant in the UK and has been reported in many countries around the world. There are a small number of cases of the Delta variant with a K417N mutation (AY.1 lineage). Preliminary evidence suggests that this variant is likely to be more transmissible than the Alpha variant based on available data. The secondary attack rate among household contacts of cases that have not traveled is 10.8% (as of 3 August 2021). This is slightly higher compared with the Alpha variant, which is currently 10.2% (Priesemann *et al*.,2021; Twohing *et al*.,2021).

5-It was first discovered in November 2021, declared a VOC by the World Health Organization (WHO), and quickly spread across all of the world's territories. There have been 37 substitutions in the spike protein of the predominant haplotype in the Omicron variant, making it significantly different from any previously described SARS-CoV-2 isolates. These mutations are concentrated in the receptor binding domain RBD, a primary target of neutralizing antibodies after infection or vaccination (Greaney *et al*.,2021). which suggests that Omicron may be able to evade infection- and vaccine-induced antibodies. In the RBD subdomain, nine of these mutations are located in the receptor-binding motif (RBM), which interacts with the host receptor ACE2 directly (Walls *et al*.,2020).

2.2.3 Coronaviruses outer structure

The virions are typically spherical. The crown shape of CoVs is due to the virion–surface spikes that project from the surface of the virus. They are petal-shaped with bulb-like structures on the distal end (Payne, 2017). The virus has three membrane-bound proteins denoted S (spike), M (membrane), and E (envelop), as well as a fourth nucleocapsid (N) protein (Das, 2020) (Figure 1-1).

The S protein, which projects from the viral envelope giving the virion its unique shape, is glycosylated and considered to be responsible for the virus attachment and fusion (Evangelin *et al*., 2020). The M protein is the most predominant and is closely linked to the envelope due to the existence of hydrophobic regions and glycosylated outer domains, and it plays a vital role in increasing membrane curvature (Yao *et al*., 2020). The E protein is a viro- porin that assembles in membranes to establish ion channels that affect the electrochemical balance in subcellular compartments (Sarkar and Saha, 2020). The protein N can bind the genomic RNA and encapsulate it in an extended ductile nucleocapsid envelope. Inside infected cells, it is located in the cytoplasm and plays a role in the assembly and budding of the virus. It can also be

co-localized with replicase–transcriptase which is essential for the synthesis of RNA. Additionally, it has other tasks, such as regulating the cell cycle as it increases cell cycle halting and host translation arrest (Zeng *et al*., 2020).

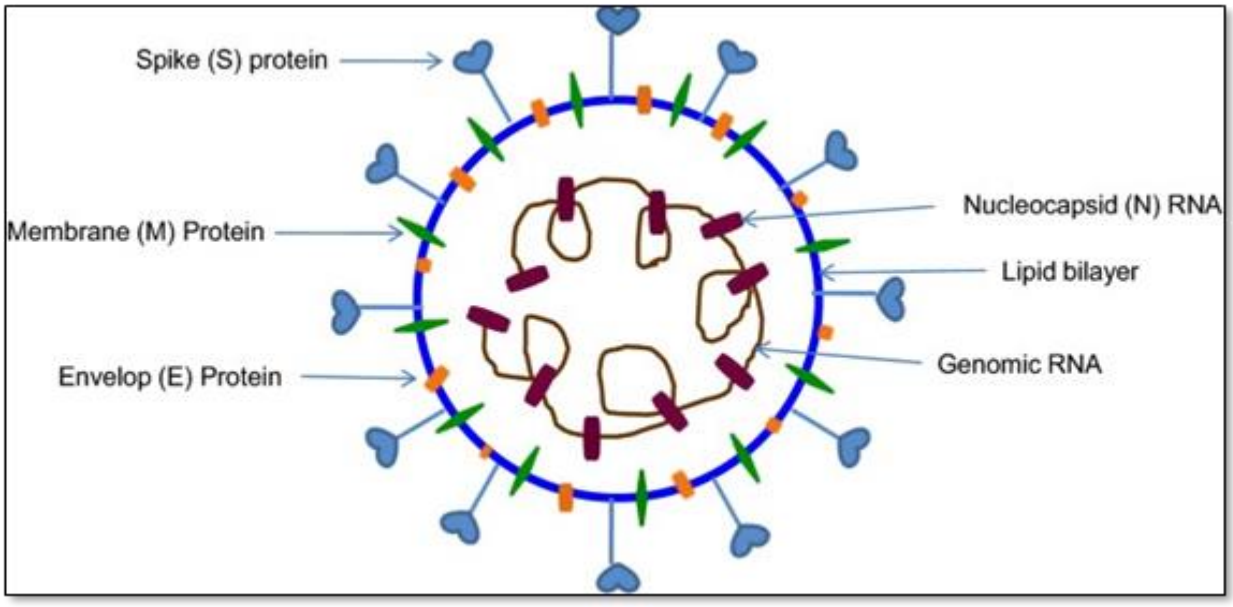

Figure 2-1: Structure of the human coronavirus (Das, 2020).

2.2.4 Coronavirus genomic structure

The genome structure is a polycistronic positive-sense single-stranded RNA with ~30 kb in size and encoding 20 proteins. The 5' end of the genome contains two open reading frames (ORFs), ORF1a and ORF1b, which encode two polyproteins (pp), pp1a, and pp1ab; the production of pp1ab requires a ribosomal frameshift to transcribe the portion encoded by ORF1b. ORF1a encodes viral proteases, main protease (Mopar, also called 3CLpro), and papain-like protease (PLpro), which are responsible for cleavage of the ORF1a and ORF1b. These polyproteins are further cleaved into 16 nonstructural proteins, whereas the 3′ end of the viral genome encodes four structural proteins (Guo *et al.*, 2020; Chan *et al.*, 2020; Yuhang *et al*., 2020). Using all available genetic data, the phylogenetic tree research determined that all current sequenced SARS-CoV-2 genomes had significant sequence

similarity of greater than 99%, with the closest sequence being the bat CoV sequence with approximating sequence identity of 96.2%, validating the proposition of 2019-nCoV as having an animal source (Ceraolo and Giorgi, 2020). Other investigations, such as complete genome sequencing of strains from different countries (China, USA, Japan, Korea, and Finland), reported higher than 99.9% sequence homology (Chitranshi *et al.*, 2020). It was found that the sequence identity of SARS-CoV-2 with SARS-CoV and MERS-CoVexceeded 50% (Kim *et al*., 2020).

2.3 Pathogenesis

2.3.1 Transmission of COVID-19

Both the SARS-CoV in 2002–2003 and the SARS-CoV-2 in 2019 were spread by respiratory droplets and close physical contact (Guo ZD *et al*., 2020). During the SARS outbreak in 2002–2003, SARS-CoV was revealed to be airborne and cutaneous. (Olsen *et al*., 2003). SARS-CoV-2 airborne transmission documentation for 2019 is complete (Lu *et al*., 2020). SARS-CoV and SARS-CoV-2 show that culturable coronavirus can be transmitted from the mouth to the stool (Leung *et al*., 2003; Isakaeva *et al*., 2004). Asymptomatic or presymptomatic SARS-CoV-2 carriers can infect others (Li *et al*.,2020; Bai *et al*.,2020).

2.3.2 Attachment of COVID-19 to a receptor in infected nasal, oral, and conjunctival tissues.

All of the ACE2 receptors in humans are nasal and oral and conjunctival tissues are utilized by both SARS-CoV and its successor SARS-2 during its early stages (Khavandi *et al*.,2020). Conjunctival tissues have been found to contain ACE2 binding sites for SARS-CoV-2 (Wu *et al*.,2020; Khavandi *et al*.,2020), although the eyes are thought to play only a minor role in the spread of SARS-CoV-2 (Liu and Sun,2020). As part of their protective equipment (PPE), healthcare workers who

come into contact with COVID-19 patients should wear eye protection (WHO,2020). Even though they both bind to ACE2 receptors, SARS-CoV, and SARS-CoV-2 coronaviruses have quite distinct binding kinetics (Lu *et al*.,2020). Pathogenicity of SARS-CoV has been found in lung macrophages and type 2 pneumocytes (To and Lo,2004; Sims *et al*.,2005). SARS-CoV-2 also infects pulmonary macrophages and type 2 pneumocytes in addition to ACE2 receptor-expressing heart and kidney cells (Hoffmann *et al*.,2020; Sims *et al*.,2005; Hickmet *et al*.,2020). The SARS-CoV-2 spike protein can be cleaved at the S1S2 cleavage site by the ubiquitous proprotein convertase furin (Izaguirre, 2019). S1 and S2 cleavage sites of coronavirus Spike (S) proteins by host cell proteases are important for the virus's entrance into it cells, allowing the fusion of viral and cellular membranes to take place (Hoffmann *et al*.,2020; Papa *et al*.,2021).

2.3.3 The upper respiratory tract is infected at the outset.

SARS-CoV-2 infection of the upper respiratory tract is the initial occurrence in patients with COVID-19. It has the same symptoms as a cold (Cumming *et al*.,2020). Some people have an acute loss of taste or smell (Xydakis *et al*.,2020).

Many people with an upper respiratory tract infection are asymptomatic, meaning they show no signs of illness. The rapid spread of SARS-CoV-2 has been attributed to the rapid transmission of asymptomatic carriers (Bai *et al*.,2020; Li *et al*.,2020). However, the first binding of SARS-CoV-2 to epithelial cells in the oral and nasal passages is mostly to ACE2 receptors on these cells (Hoffman *et al*.,2020; Sungnak *et al*.,2020).

2.3.4 Lower respiratory tract and other tissues infected by COVID-19

There are two phases to the sickness known as COVID-19. The upper respiratory tract gets infected for around five to seven days in the initial phase. Phase two, which

begins after two weeks of illness, is characterized by pulmonary infection (Cumming *et al*.,2020). Shortness of breath and fever are common symptoms of this phase. If the oxygen saturation goes below 90% on a pulse oximeter, admission to a hospital for oxygen therapy (and other necessary care) is indicated in both asymptomatic and symptomatic patients with silent hypoxemia (Wilkerson *et al*.,2020). Alveolar type II lung cells are infected with SARS-CoV-2. An examination of the pathophysiology of SARS-CoV-2 lung infection can be gained by autopsy research. It is rare to see neutrophils in the early stages of lung infection, but damage to the alveoli, particularly alveolar edema, is present (Tian *et al*.,2020). Tracheobronchitis and diffuse alveolar injury with desquamation of pneumocytes have been seen in other trials. This is consistent with the findings of earlier studies. As a result, the alveolar gaps were filled with a mixture of inflammation and numerous neutrophils. In one of these investigations, a participant was able to see the results of (Martines *et al*.,2020), Colocalized with SARS-CoV-2 antigen, electron-dense projections of spherical virus particles were seen on both ACE2-positive epitheliums and thoracic lymph nodes in electron microscopy pictures. It has been discovered via additional investigations that endothelial dysfunction and hypercoagulability are linked to diffuse alveolar injury and intravascular fibrin thrombi. Fluid leaking into alveolar gaps and decreased air exchange are both caused by the selective death of type II pneumocytes, which appears to be the physiological basis for death in these patients (Hanley *et al*.,2020). When the SARS-CoV-2 virus infects tissue, it decreases the activity of ACE2, which ultimately promotes the development of inflammatory and hypercoagulative conditions (Bryce *et al*.,2020). The alveolar damage caused by an early infection includes alveolar edema. The kallikrein-bradykinin pathway is activated when ACE2 is downregulated, increasing vascular permeability as a result (Li *et al*.,2019). As a result, pulmonary edema is worsened by the increased permeability of the vascular system. This shows that the ratio of membrane-bound

ACE2 to membrane-bound ACE2 may be crucial in the pathogenesis of COVID-19, as evidenced by its ability to reduce SARS-CoV-2 when administered in a soluble form (Monteil *et al*.,2020).

2.3.5 SARS-CoV-2 infections of endothelial cells and their consequences

In COVID-19, endothelial cell infection is the primary pathogenic mechanism (Li *et al*.,2019; Bryce *et al*.,2020; Blanco-Melo *et al*.,2020). When viruses infect a person's endothelium cells, they can cause thrombosis and bleeding (Goeijenbier *et al*.,2012). It is possible that SARS-CoV-2 infection of the endothelial cells in COVID-19 patients is responsible for the observed coagulopathy, including thromboembolic illness (Teuwen *et al*.,2020). Additionally, the presence of SARS-CoV-2 viremia in individuals with severe COVID-19 may be a contributing factor (Li *et al*.,2020). Multi-organ involvement suggests viremia with a live SARS-CoV-2 virus (Bryce *et al*.,2020). Activated platelets may be a factor in the thrombosis experienced by COVID-19 patients. mRNA from the SARS-CoV-2 nucleocapsid gene N1 has been found in the platelets of COVID-19 patients and may be involved in platelet gene expression and function. Faster platelet aggregates and higher collagen and fibrinogen distribution in these COVID-19 individuals (Manne *et al*.,2020).

2.4 Factors associated with COVID-19 pathogenesis

COVID-19 can cause diverse clinical outcomes, and several studies indicated that many risk factors may increase the disease severity and its associated complications. The first of these factors is the patient’s age, as surveys showed that ages ≥ 65 years showed higher rates of mortalities. These patients also showed low lymphocyte count or had a high ratio of neutrophil to lymphocyte (NLR) (Qin *et al*., 2020; Wu *et al*., 2020). Gender is also an important factor, as the prevalence in males showed

to be higher than in females. This can be attributed to differences in steroid hormones between males and females, which influence various aspects of immunity. In addition, the X chromosome harbors some immune regulatory genes (i.e. toll-like receptor 7; TLR7), and their products can lower viral loads and inflammation in females (Conti and Younes, 2020).

Pre-existing morbidities, such as hypertension and cardiovascular disease (CVD), also increase COVID-19 risk, as the disease is linked to myocardial injury due to hypoxia, systemic inflammation, and direct cardiomyocyte injury, as well as increased cytokine response by both type 1 and 2 T-helper cells (Babapoor-Farrokhran *et al*., 2020). Besides, patients with diabetes mellitus (DM) undergo severe complications compared to non-diabetics and this can be attributed to an imbalance in ACE2 activation pathways, resulting in an inflammatory response that can cause pancreatic beta-cell malfunction (Wang *et al*., 2020b). Obesity is also listed as a risk factor and patients with high body mass index (BMI) experience a low prognosis as they have chronic inflammation that leads to an augmented cytokine storm (Yan *et al*., *2021*). Chronic respiratory disorders have been identified as predisposing factors from the start since the patient's lung epithelium is damaged. Typically, patients with chronic obstructive pulmonary disease (COPD) exhibit greater ACE2 receptor levels. This is an ultimate aid in SARS-CoV-2 transmission and infiltration of respiratory epithelium (Hasanagic and Serdarevic, 2020).

Another important factor is Vitamin D (Vit-D), and it has been well established that its deficiency is directly correlated to an elevated risk of viral infections and community-acquired pneumonia (Marik *et al*., 2020). The suggested mechanism for this finding is that Vit-D deficiency increases the expression of CD26, which is accounted as the putative adhesion molecule for host cell invasion. On other hand, the deficiency can elevate the inflammatory responses mediated by interferon-γ

(IFN-γ) and interleukin-6 (IL-6) in COVID-19 patients (McCartney and Byrne, 2020).

2.5.1 Human immune response to COVID-19

It has been illustrated that the immune system is a major player in the pathogenesis of COVID-19 because the causative virus (SARS-CoV-2) can induce dysregulated innate and adaptive immune responses that are ultimately associated with widespread damage to tissues and organs (Thierry and Roch, 2020).

The innate immune response is the first response that is activated immediately upon viral infection to eliminate the pathogen. This system continues to maintain its immune response until the development of adaptive immunity, in which T-cell and B-cell responses are activated within approximately seven days after infection (Lowery *et al.*, 2021). Neutrophils, macrophages, natural killer (NK) cells, and dendritic cells are the most important cells involved in mediating innate immunity (Azkur *et al.*, 2020). Regarding SARS-CoV-2 infection, innate immunity has not been well defined, but the expression of type I interferon (IFN-I) has been proposed to be suppressed in COVID-19 patients (Zanoni, 2021). The IFN-I is usually released from virus-infected cells and can suppress the replication of the virus and limit infected-cell dissemination (Mantlo *et al.*, 2020). However, it has been indicated that the innate immune response and the subsequent adaptive immune responses are efficient in eradicating the virus in most of the infected patients (approximately 80%). In the remaining 20% of patients, the virus replication is sustained and associated with a triggering of inflammatory responses that enhance the recruitment of inflammatory cells, and consequently, moderate and severe pathology can occur in the lungs (Khosroshahi *et al.*, 2021). The most effective factors in recruiting inflammatory cells are cytokines, which are produced by infected alveolar macrophages and airway epithelial cells. Among these cytokines

are interleukin IL-6 and tumor necrosis factor TNF-α, as well as the chemokine CXCL10 (Ni *et al.*, 2020).

Antimicrobial peptides (AMPs) are further important innate immune factors suggested having a role in the resistance to SARS-CoV-2 infection (Ghosh and Weinberg, 2021). They are low molecular weight proteins with a broad range of antimicrobial actions against bacteria, viruses, and fungi (Dijksteel *et al.*, 2021). The AMPs are positively charged with a hydrophobic and a hydrophilic side, allowing them to be soluble in aqueous conditions, and can also enter lipid-rich membranes (Annunziato and Costantino, 2020). These peptides can kill target cells through a variety of processes once they enter a target microbial membrane. The major families of AMPs in mammalian are cathelicidins and defensins (Bahar and Ren, 2013).

In the case of adaptive immunity, it has been shown that both types of T-lymphocytes (T-helper CD4+ and T-cytotoxic CD8+) are markedly involved in COVID-19 pathogenesis (Huang et al., 2020). The available evidence indicates that both cell populations show declined counts and impaired functions during the progression of SARS-CoV-2 infection, particularly in severe cases (Jacques and Apedaile, 2020). Further, it has been reported that virus-specific memory CD8+ T cells were effective in protecting the patients from the lethality of disease due to their role in controlling viral replication (Schulien *et al*., 2021). Similar to CD8+ cells, the counts of CD4+ cells and their function in controlling the replication of SARS-CoV-2 have been indicated to be diminished (Wen *et al*., 2020). In addition to CD4+ and CD8+ cells, B cell populations were also observed to have lower counts in COVID-19 patients with severe disease than in healthy individuals (Zhang *et al*., 2020a). However, despite this numerical decline, the function of these cells is preserved as they continue to produce IgG and IgM antibodies against the virus and

this was correlated with the recovery or persistence of the virus (Sette and Crotty, 2021). The interaction of coronavirus with the immune system is summarized in Figure 2-2.

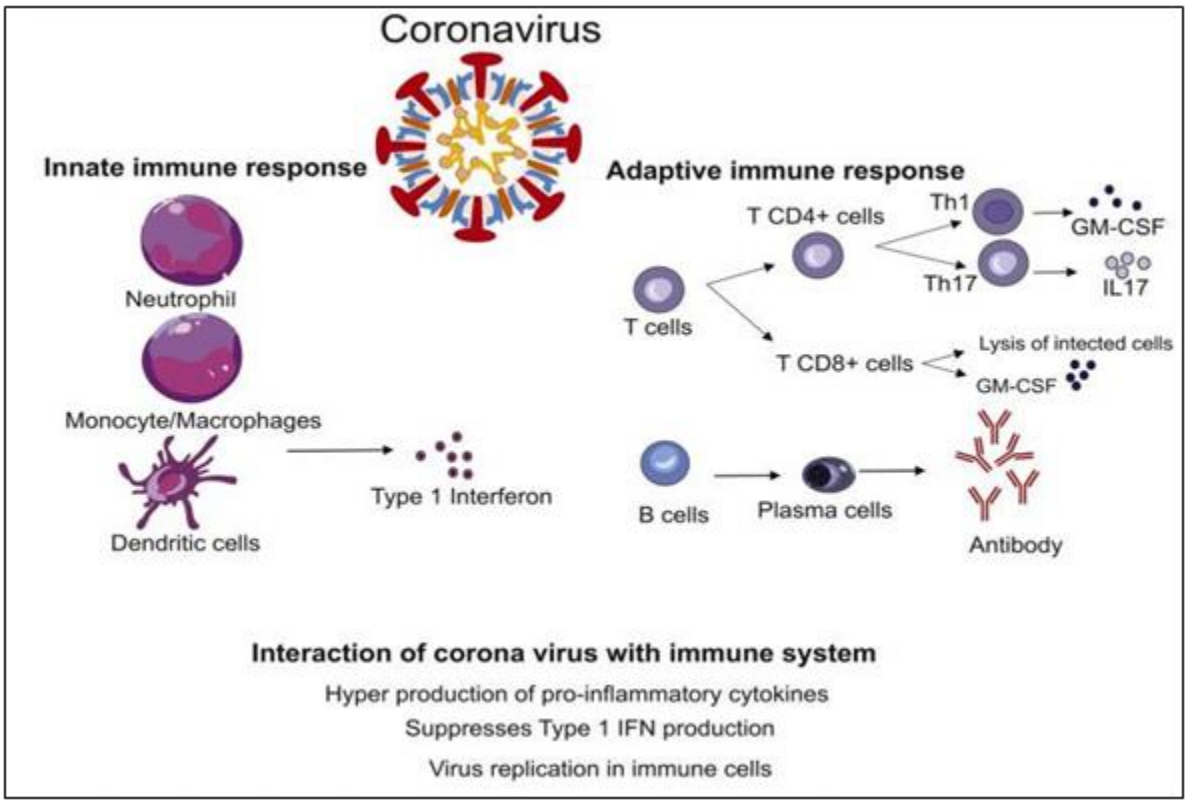

Figure 2-2: The innate and adaptive immune responses against coronavirus (CoV) infection (Hosseini *et al.*, 2020).

Besides these cellular defects in immunity against SARS-CoV-2, the immunopathogenesis of COVID-19 is probably mediated by pro-inflammatory cytokines (Khosroshahi *et al.*, 2021). It has been depicted that the acute respiratory distress in COVID-19 and the development of severe illness were related to up-regulated levels of pro-inflammatory cytokines, and accordingly, this condition was termed cytokine release syndrome or cytokine storm (Pum *et al.*, 2021).

The most important cytokines in this context are IL- 1β, IL-6, IL-18, TNF-α, and IFN-γ, as shown in Figure (2-3) (Olbei *et al.*, 2021). However, IL-6 is the most encountered cytokine, and it has been found that IL-6 is a key cytokine linked with a higher risk of severity and death in COVID-19 patients (Santa Cruz *et al.*, 2021). It is produced by dendritic cells and macrophages in the course of recognizing pathogens via toll-like receptors at infection sites (Ascierto *et al.*, 2021).

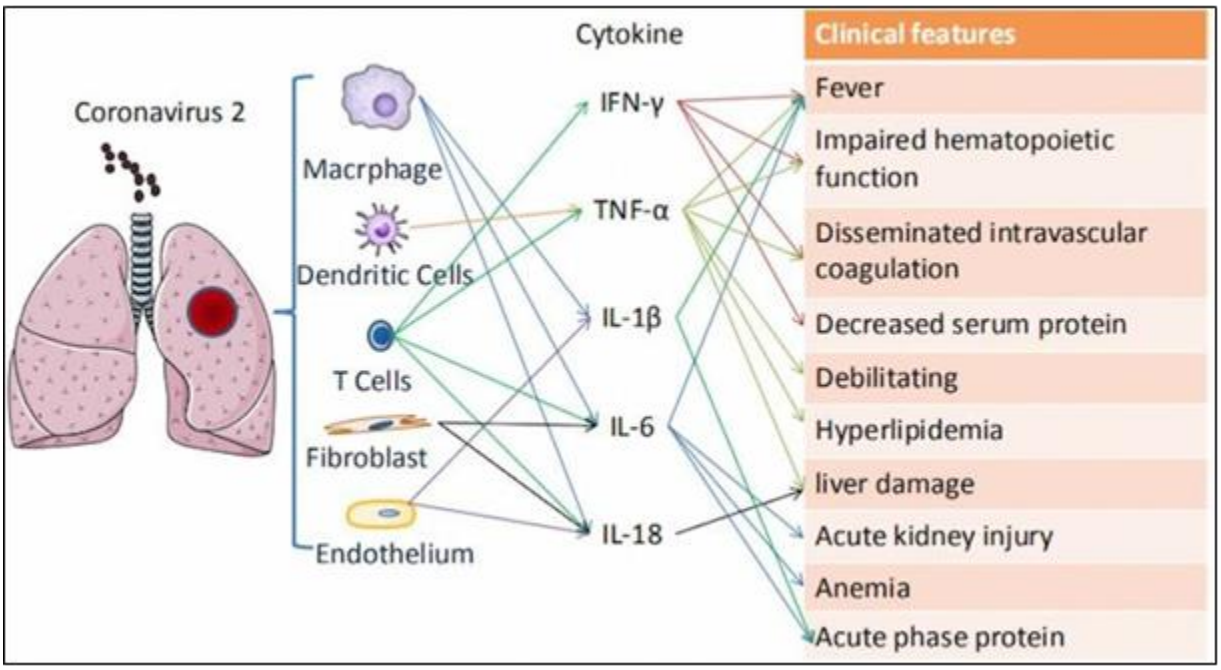

Figure 2-3: Cytokines involved in the pathogenesis of SARS-CoV-2 infection and their cellular source and associated clinical feature (Olbei *et al.*, 2021).

2.5.1 Normal role ACE2 plays in the body

The renin-angiotensin-aldosterone system (RAAS) regulates processes such as blood pressure, wound healing, and inflammation (Sriram *et al.*,2020)

Angiotensin II (ANG II) is a protein that increases blood pressure, inflammation, damage to blood vessel linings, and various types of tissue injury. ACE2 converts ANG II to molecules that neutralize its effects (Sriram *et al.*,2020).

Ang II can cause inflammation and death of cells in the alveoli, which are critical for bringing oxygen into the body; ACE2 reduces these harmful effects of ANG II (Sriram *et al*.,2020).

ACE2 is found in all people, but the amount varies between individuals, tissues, and cells. ACE2 may be elevated in patients with hypertension, diabetes, and coronary heart disease. Lack of ACE2 in mice causes severe tissue injury in the heart, lungs, and other organs (Sriram *et al*.,2020).

2.5.2 Angiotensin-converting enzyme-2 (ACE-2) receptor

ACE2 is a protein found on the outer membranes of a wide variety of cells. It is an enzyme that breaks down angiotensinogen into smaller proteins, which then regulate cell functions (Sriram *et al*.,2020).

Before entering and infecting cells, the SARS-CoV-2 virus binds to ACE2 with the help of a spike-like protein on its surface. As a result, ACE2 serves as a receptor for the virus that causes COVID-19 to enter the body (Sriram *et al*.,2020).

ACE2 is found in the lungs, heart, blood vessels, kidneys, liver, and gastrointestinal tract. It is found in epithelial cells that line tissues and create barriers. Figure 2-4 A(Sriram *et al*.,2020).

This epithelial lining in the lung facilitates oxygen and CO2 exchange between the lungs and blood vessels. ACE2 is found in the nose, mouth, and lungs. ACE2 is highly expressed on type 2 pneumocytes, which are found in the alveoli, where oxygen is absorbed and waste carbon dioxide is expelled (Sriram *et al*.,2020).

An amino acid substitution in SARS-CoV-2 means that five of the six vital amino acids that connect the viral spike (S) protein to human ACE2 are different from the

original version of SARS-CoV. Host tropism is largely determined by viral S-proteins, which are also a major target for the development of antiviral and vaccine therapies. It is also critical for SARS-CoV-2 to enter a cell because both S proteins and ACE2 are modified by host cell proteases during this process. Since the binding affinity of SARS-CoV-2 to the salt bridge protein ACE2 appears to be stronger than that of SARS-CoV, this could explain why COVID-19 has had such a greater global impact than the original SARS virus (Yan *et al*.,2020).

Furthermore, SARS-CoV-2 has evolved to utilize a wide range of host proteases, including cathepsin L, cathepsin B, trypsin, factor X, elastase, furin, and TMPRSS2 (transmembrane protease serine 2), for S-protein priming and facilitating cell entry following receptor binding (Millet *et al*.,2015).

A combination of cathepsin L/B inhibitor camostat mesylate and serine protease inhibitor camostat mesylate E-64d has been shown to block SARS-CoV-2 entry (da Silva *et al*.,2017). The viral S-protein interacts with extracellular domains of transmembrane ACE2 proteins, which facilitates the entry of SARS-CoV and SARS-CoV-2 into cells (Figure2- 5). This is followed by a decrease in surface ACE2 expression (Zhou *et al*.,2020).

Tissue ACE2 downregulation and systemic RAS imbalance have been linked to the development of multiorgan damage from SARS-CoV-2 infections in a cohort of 12 COVID-19 patients, who had significantly higher levels of circulating Ang II than healthy controls (Liu *et al*.,2020).

Potential therapeutic strategies include blocking the viral S protein's receptor-binding domain's (RBD) ability to bind human ACE2 and SARS-CoV-2. ACE2-derived peptides, small molecule inhibitors, ACE2 antibodies, or a single-chain

antibody fragments against ACE2 are all possible treatment options in addition to this receptor-binding domain blocking strategy.

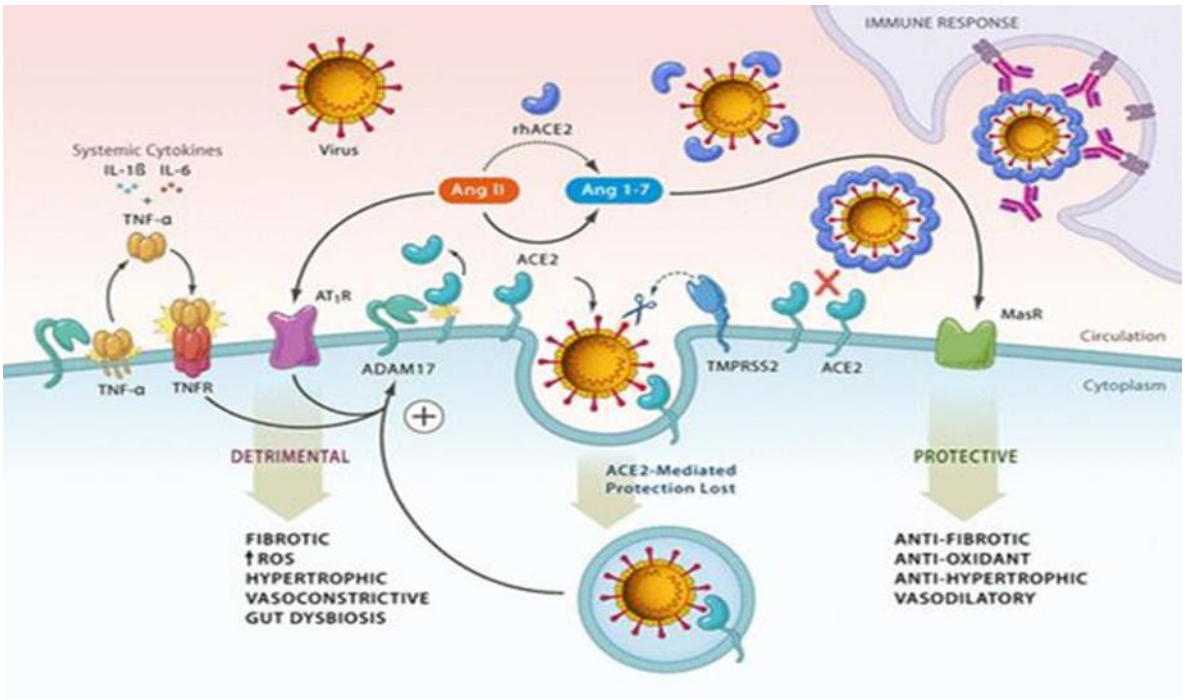

Figure 2-5. ACE2 (angiotensin-converting enzyme 2) and the inflammatory response to coronavirus disease (Zhou *et al*.,2020).

Endocytosis of the enzyme and SARS-CoV-2 viral particles results in the loss of ACE2-mediated cardiovascular protection. Reactive oxygen species (ROS) and vasoconstriction can occur at the expense of ACE2/Ang 1–7 driven pathways, which can lead to fibrotic and hypertrophic changes in the body and gut, as well as the formation of gut dysbiosis. Endocytosed SARS-CoV-2 spike proteins increase ADAM17 (a disintegrin and metalloproteinase)-mediated proteolytic cleavage of ACE2. ADAM17 activity is further increased when the AT1R is activated by elevated Ang II levels. Soluble TNF- α (tumor necrosis factor) is released from

ADAM17 into the extracellular environment, where it has both autocrine and paracrine functions. The TNFR, which is activated by TNF- α, is the third pathway for increasing ADAM17 activity. TNF- α and other systemic cytokines released due to SARS-CoV-2 infection and in conjunction with comorbidities like diabetes and hypertension can lead to a cytokine storm. TMPRSS2 stands for transmembrane serine protease 2. It has been found by (Zhou *et al*., 2020; Li *et al.*, 2003).

2.5.3 The ACE2 Gene and Basic Biochemistry

In contrast to the ACE gene, which is located on human chromosome 17, the 40kb ACE2 gene is located on chromosome Xp22 and contains 18 exons, the majority of which are identical to the ACE gene's exons. Unlike somatic ACE, which contains two active sites, ACE2 contains only one catalytic domain. Both ACE and ACE2 are zinc metallopeptidases, but their substrate specificities are distinct, defining their distinct and complementary roles in the RAS. Whereas ACE hydrolyzes Ang I to form Ang 1–9 (a peptidyl dipeptidase), ACE2 acts as a simple carboxypeptidase capable of hydrolyzing Ang I to form Ang 1–7. (Figure 2-4B)

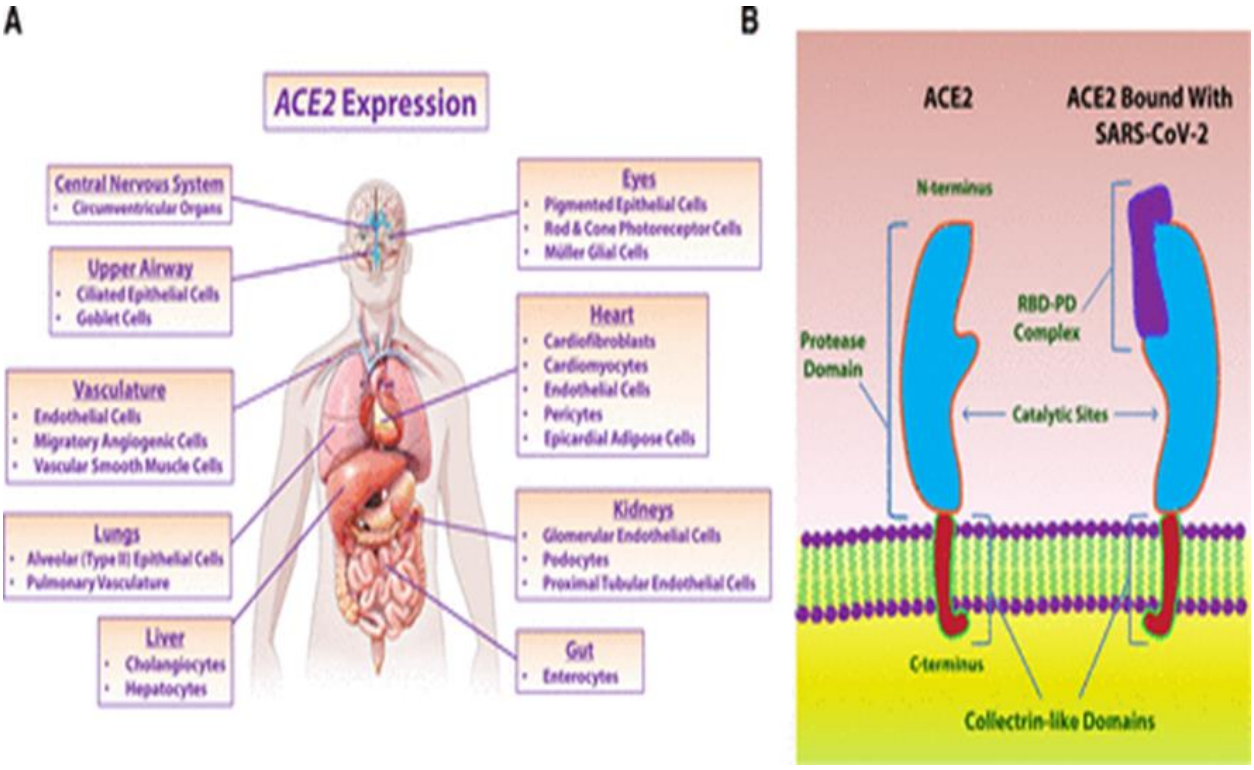

Figure.2-4 expression of ACE2 and a diagram of the primary domains of ACE2 in body A. ACE2 does not cleave bradykinin, further distinguishing its specificity from that of ACE, while conventional ACE inhibitors are also ineffective B (Rice *et al*.,2004).

In contrast to ACE, ACE2's C-terminal domain is a homolog of renal protein collectrin, which controls amino acid transporters' trafficking to the cell surface. This gives ACE2 a wide range of physiological capabilities. SARS-CoV-2 hijacked ACE2 as a receptor because of its many physiological functions, resulting in the COVID-19 pandemic (Yan *et al*.,2020).

Structural studies have revealed the SARS-CoV and, more recently, the SARS-CoV-2 in complex with ACE2 (Figure 2-4B) structures in detail (Wrapp *et al*.,2020). The major spike glycoprotein (S1) of SARS-CoV-2 binds to ACE2 via its N-terminal region. SARS-CoV-2 infection can be better understood and prevented by studying

ACE2's biology and physiology, which has been developed over the past 20 years since its discovery.

ACE2 is expressed in essentially all tissues, with the highest expression in the small intestine, kidneys, testis, thyroid, heart, and adipose tissue followed by the liver, lungs, colon, bladder, and adrenal gland (Li *et al*., 2020). Besides, there is another functional form of ACE2 with 555 amino acids that circulate in small amounts in the blood, named soluble ACE2 (sACE2), which is obtained by shedding the full-length ACE2 through metalloproteinase 17 (ADAM17). It is worth emphasizing that the binding of sACE2 to SARS-CoV-2 blocks the entry of the virus into the target cells and consequently, prevents the progression of COVID-19. Up to now, three major ACE2 functions have been described. In addition to being the viral receptor, ACE2 exhibits a protective role in the cardiovascular system and many other organs and contributes to the absorption of neutral amino acids in the intestine (Kuba *et al*., 2010).

The ACE2 gene and protein show a high degree of genetic polymorphisms including single-nucleotide variation, transcriptional variation, post-transcriptional modifications, and putative protein mutations (Devaux *et al*., 2020a), among which

Single-nucleotide polymorphisms (SNPs) have made their way to the scientific spotlight. Recently, (Suryamohan *et al*.,2021) found 298 unique protein-altering variants across 256 codons distributed throughout the 805 amino acid long human ACE2 (hACE2) from several databases (Suryamohan *et al*., 2021). It is noteworthy that the symptoms and severity of COVID-19 vary greatly, ranging from very mild or no symptoms to pneumonia, acute respiratory distress syndrome (ARDS), and even death (Lopera *et al*., 2020). As SARS-CoV-2 primarily depends on ACE2 for fusion and entry, ACE2 variation is considered to be one of the causes of this difference. Thus, it is important to systematically characterize and evaluate ACE2

polymorphism. Herein, this study provides an update on the possible role of ACE2 variants in people's susceptibility to SARS-CoV-2 infection and the outcome of COVID-19.

2.5.4 Interleukin 12 (IL-12) Cytokines

There are four cytokines in the IL-12 family, each of which has emerged as a key regulator of host immunity: IL-12, IL-33, IL-19, and IL-35 (all of which are derived from the IL-12p35/Ebi3). In each subunit, there are structural similarities to the extracellular regions of type 1 cytokine receptors (e.g., the solubility of the receptor for IL-6), and the β -subunits have helical structures similar to those found in type 1 cytokines (Trinchieri *et al*., 2003; Vignali & Kuchroo,2012).

Both chains are necessary for the bioactive cytokine to be secreted: IL-12p35, IL-23p19, and IL-27p28 are the α- subunits, while IL-12p40 and Ebi3 are β- subunits (Wolf *et al*., 1991).

One theory holds that the expression of the chain limits the secretion of each of the four heterodimeric cytokines, with IL-12 and IL-35 being restricted to tissues or cell types with high levels of IL12p35 or IL23p19, respectively, of the four cytokines (Wolf *et al*., 1991). Many aspects of host immunity are linked to the involvement of IL-12 cytokines because of the unique feature of chain-pairing promiscuity. However, dimerization with Ebi3 produces IL-12 cytokines (e.g., IL-27 or IL-35) that suppress inflammation and mitigate autoimmune diseases, while dimerization with IL-12p40 produces IL-12 cytokines (e.g., IgM or IFN- α) that promote inflammation and the development of chronic inflammation-related disease (Figure 2-6). It is another important feature of IL-12 cytokinesis that they bind Janus kinases (JAKs) and activates JAK-STAT signaling pathways to carry out their biological functions (Trinchieri *et al*., 2003). This is the reason for the unique and overlapping

patterns of gene transcription induced by various IL-12 cytokines, as well as the recruitment and activation of specific members of the STAT (signal transducers and activators of transcription) family of transcription factors (Trinchieri *et al*., 2003).

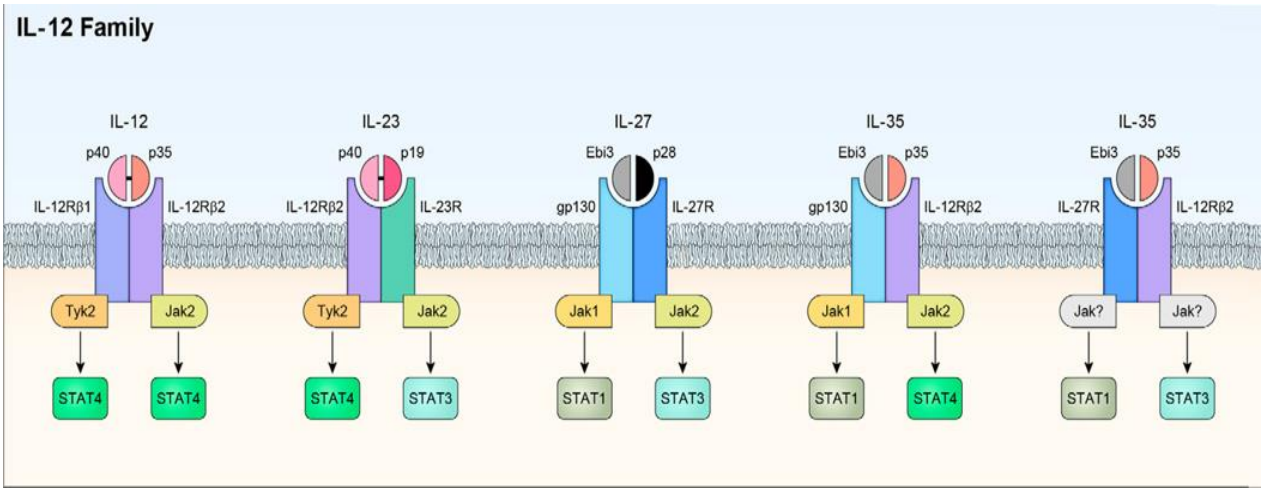

Figure 2-6 IL-12 cytokine family and receptor heterodimers. They share receptor components and activate the overlapping JAK/STAT pathways.

In the IL-6 superfamily, the helical alpha subunit (IL-12p19, IL-12p35, and IL-27p28) shares homology with class I receptor chains. The binding of cognate receptors activates the Jaks and STATs (STAT1, STAT3, STAT4), resulting in the transcription of target genes that mediate biological activities. IL-35 appears to use odd receptors. It can use IL-12R2 or gp130/gp130 to activate STAT1 and STAT4 in T cells. It uses IL-12R/IL-27 to activate STAT1 and STAT3 in B cells. It is unknown whether B cells use Jak in response to IL-35 signaling. With the use of a bicistronic vector, IL27p28/IL12p40 can be produced as a bioengineered heterodimeric IL-12 cytokine. This cytokine inhibits gp130 and IL-27 signaling and does not activate STATs (Sun *et al*.,2015).

2.5.5 IL-12 and TH1 Differentiation

Because of increasing evidence that the T-bet transcription factor is critical to the commitment of the T-cell to TH1, early models of the TH1 differentiation pathway have been progressively revised (Peng ,2006). In 2001, Mullen *et al* reported that T-bet could determine the fate of TH1 effectors in the mouse without the need for IL-12. It was possible to determine the TH1 fate by studying the effects of T-bet on IFN-γ allele chromatin remodeling and inducing the expression of IL-12R2 (Mullen *et al*.,2001). A new TH1 differentiation model now places T-bet before IL-12 as a result. For example, NK cell-derived IFN- γ, which activates STAT1 in T-bet-expressing nave T cells, induced T-bet expression in T-bet-expressing cells, according to this revised model. IL-12, on the other hand, continued to play an important role in the survival of T-bet cells (Murphy and Reiner ,2002). GATA-3, the primary regulator of T cell commitment to the Th2 lineage, is critical to the role of T-bet in determining TH1 fate, rather than solely as a positive regulator of the IFN-γ gene (Usui *et al*.,2006).

2.5.6 Association of IL-12 with COVID-19

Targeting the IL-23/IL-17 axis in COVID-19 may reduce the 'cytokine storm' that can lead to multiple organ failures and death, as evidenced by the current data (Messina and Piaserico , 2020). A real-world database of patients with inflammatory bowel disease affected by COVID-19 found that patients treated with ustekinumab had a lower incidence of adverse outcomes. Similar to the case described by Duret, it appears that tumor necrosis factor inhibitors are linked to lower rates of admission to intensive care units and mortality (Brenner *et al*.,2020). Inhibition of IL-12 may harm SARS-CoV because it induces Th1 polarization, which is necessary for efficient viral clearance. SARS infection has been linked to IL-12 receptor polymorphisms that are less responsive to IL-12 signaling. Although IL-12 may

contribute to the disease's inflammation, it may also have a detrimental effect (Tang *et al*., 2008).

2.5.7 IL-12 gene polymorphism

Cytokine gene polymorphisms warrant consideration as factors explaining variation in the human immune and inflammatory responses and as candidate susceptibility genes for related pathological states. Interleukin 12 (IL-12) is a key regulator of the polarization of immune responses to T helper 1 or 2 categories and plays a role in autoimmune and infectious diseases (Hall *et al*., 2000).

The active cytokine is a 75 kDa heterodimer composed of two covalently linked proteins of 35 kDa (p35) and 40 kDa (p40) (Wolf *et al*.,1991). each of which is encoded on different chromosomes. In humans, the IL-12p35 subunit is encoded on chromosome 3 at 3p12e3q13.2, whereas the gene for the IL-12p40 subunit is located on chromosome 5 at 5q31e33. The p40 subunit is a component of both IL-12 p70 and IL-23. The production of IL-12 heterodimer requires the coordinated expression of both p40 and p35 chains: p35 can only be secreted when associated with p40 (Trinchieri, 2003). Several promoter, intron and 3 untranslated region (UTR) polymorphisms were identified at IL-12 p40 gene, IL12B (Huang *et al*., 2000). The presence of a single nucleotide polymorphism (SNP) at 3 UTR (Taq I) has been correlated with increased in vitro IL-12 secretion recently (Seegers *et al*.,2002). The same single base change showed strong linkage disequilibrium with a type 1 diabetes (T1D) susceptibility locus and IL12B 3UTR alleles showed different levels of expression in cell lines (Morahan *et al*.,2001). However, this polymorphism was not associated with T1D and no correlation was observed between genotype and IL-12 expression in other studies (Bergholdt *et al*.,2004).

Chapter Three
Materials and Methods

3. Materials and methods

3.1: Materials

3.1.1: Laboratory equipment and instruments

The laboratory types of equipment and instruments used in the present study were listed in table (3.1).

Table (3.1): Laboratory Equipment and Instruments

Instruments and Equipment	Origin	Company
Incubator	Germany	Memmert
Incubator	USA	Fisher Scientific
Vortex	Holland	LABINCO
Micropipette 20-200 µL	Japan	GHADEER. LAB
Micropipette 100-1000 µL	Japan	DRAGON LAB
Microcentrifuge	Germany	BECKMAN COULTER
Centrifuge	Germany	Eppendorf
Centrifuge	Germany	ROTOFIX32A
Autoclave	Hirayama	(Japan)
Distillator	GFL	(Germany)
Electric sensitive balance	Denver	(USA)
ELISA reader, ELISA washer	BioTeck	(USA)
Gel documentation system	Vilber	(France)
Gel electrophoresis system	Cleaver Scientific	(UK)

PCR Thermal cycler	Techne	(UK)
Refrigerator	Concord	(Lebanon)
Nanodrop 2000c spectrophotometer	Fisher Scientific	(Canada)

3.1.2: Kits, biological and chemical materials

The kits, biological and chemical materials used in this study are listed in the table (3-2)

Table (3-2): Kits, biological and chemical materials

Type of Kit	Company (Origin)
DNA extraction Kit	Favorgen(Taiwan)
DNA ladder	Bioner(Korea)
Green master mix	Bioner(Korea)
primers	Macrogen (Korea)
ACE-2 ELISA Kit	Biotech(China)
IL-12 ELISA Kit	Biotech(China)
Nuclease Free water	Bioner(Korea)
Agarose, TBE buffer	Condalab (Spain)
Simply Safe	Eurx (Poland)

3.1.3. Primer Pairs

The Primer pair (Macrogen/Korea) (Table 3-3)

Table (3-3) Primer Sequencing and PCR Conditions

Primer for characterization							
Primer	**Sequences 5 to 3**	**product**		**Conditions Time**		**NO. CYCLE**	**Reference**
ACE2-2-F	TGGAGGCAAACATCCAATCTCA	500	Step 1	2 min	95ºC	1	This study
			Step 2	30 sec	95ºC	35	
ACE2-2-R	CTGTCCTCTCCAGGATGAACTT		Step 3	30 sec	59 ºC		
			Step 4	1 min	72 ºC		
			Step 6	5 min	72 ºC	1	
			Step 7	forever	4 ºC		
IL-12A-F	AGACACAACTCCCACTGATGAT	650	Step 1	2 min	95ºC	1	This study
			Step 2	30 sec	95ºC	35	
IL-12A-R	TGAAAAACACCTTCAGGATGGAT		Step 3	30 sec	57.4 ºC		
			Step 4	70 sec	72 ºC		
			Step 6	5 min	72 ºC	1	
			Step 7	forever	4 ºC		
IL-12B-F	CTGATGTACTTGCAGCCTTGC	473	Step 1	2 min	95ºC	1	This study
			Step 2	30 sec	95ºC	35	
IL-12B-R	GCTGTTACAATGTCACCCCAC		Step 3	30 sec	59 ºC		
			Step 4	70 sec	72 ºC		
			Step 6	5 min	72 ºC	1	
			Step 7	forever	4 ºC		

3.2: Methods

3.2.1. Patients

This cross-sectional study includes 100 blood specimens from COVID-19 patients aged (15-to 80) years distributed according to the severity of disease as the

following: (46 severe who have needed ventilators and lying in the respiratory care unit (RCU) with severe respiratory distress, respiratory rate ≥30 breaths/minute and pulse oxygen saturation (SpO2) ≤ 93% on resting state, the case was considered to be in severe illness with PCR positive, 54 non-severe with positive PCR result those who did not need respirators with an oxygen rate higher than 93% (SpO2) ≥93% on resting state who have been hospitalized at the COVID-19 Wards in Merjan Medical City and Al-Imam Al-Sadiq Hospital in Babylon Province during 2 months (November and December 2021). All patients were diagnosed based on previous clinical reports, clinical examinations, and PCR tests, and the result of the plate late count was taken from the CBC medical reports. These cases were compared with each other, all of them were asked to fill out a questionnaire and all had no family history of any disease. All patients suffering from Covid-19 pneumonia were included and excluded from other types of respiratory diseases.

3.2.2 Ethical considerations

The approvals were obtained from all the participants and also agreed to study scientifically and morally in Merjan Medical City and Imam Sadiq Hospital in Babylon. The following information is recorded (patient name, age, sex, date of infection, and chronic disease).

3.2.3 Collection of specimens

All subjects had venous blood specimens of five milliliters obtained. The blood specimens are then separated into two groups. For DNA extraction, the initial part (2.5 ml) is transferred to the anticoagulant tube (EDTA) tube. The remaining fraction (2.5ml) is transferred to a Gel tube for serum separation, the blood is allowed to coagulate for about 30 minutes at room air and then centrifuged for 5 minutes at

3000 rpm. The serum was then collected in four repeaters in a sterile Eppendorf tube and kept frozen at -20.

3.2.4 Study design

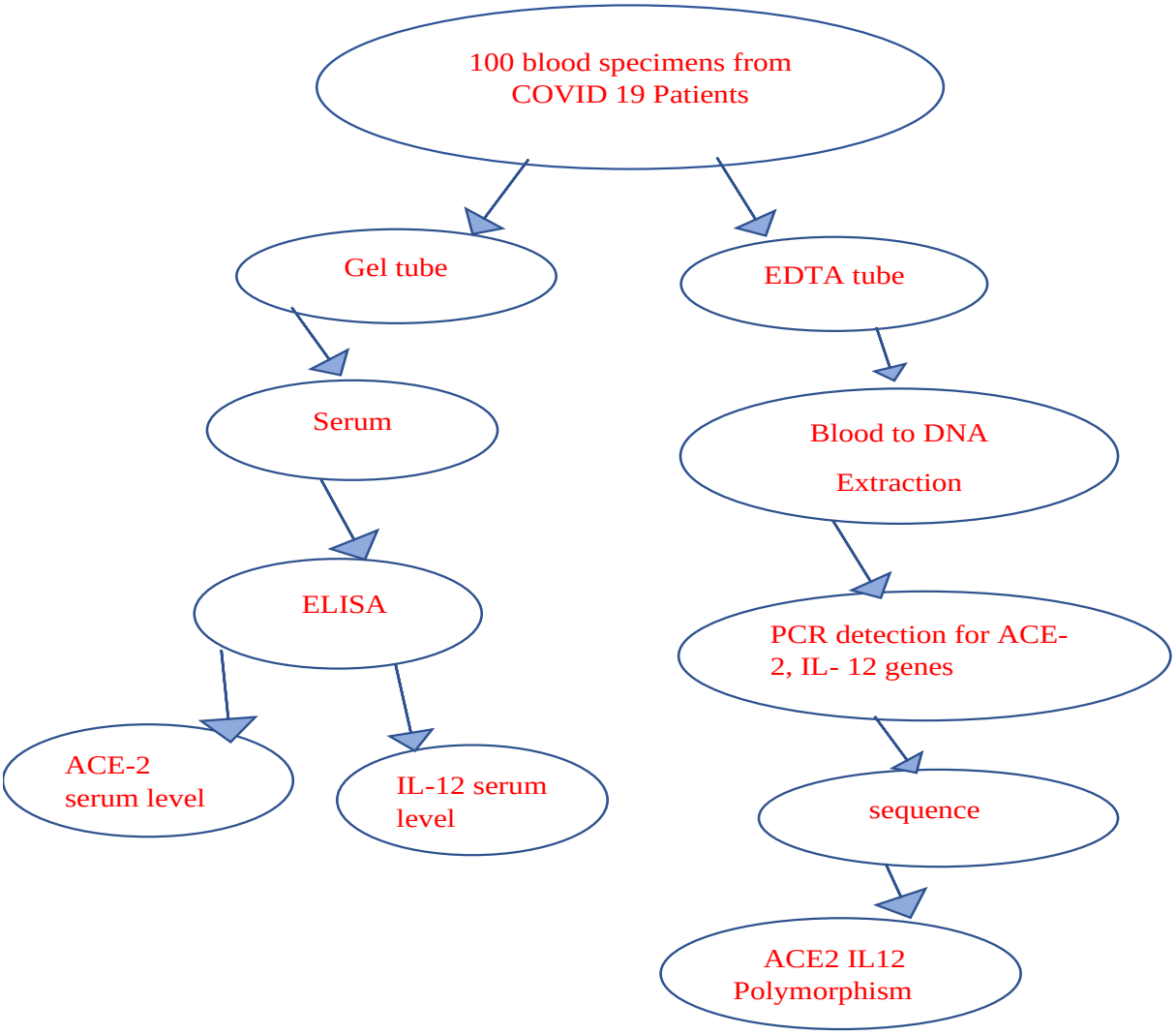

Figure (3.1): Study design

3.2.5: Genetic Study

3.2.5.1: Genomic DNA extraction from frozen human blood

Favor the PrepTM Genomic Mini Kit obtained genomic DNA from frozen human blood following the manufacturer's protocol.

1- A 1.5 mL microcentrifuge tube was used to transfer approximately 200 µL of thawed frozen blood from an EDTA tube. Following this step, the sample was incubated in the incubator for 15 minutes at 60 ° C with 30 µL of proteinase K (10mg/ml, not provided).

2- As soon as the blood turned greenish-black from the addition of 200 µL of FABG Buffer for cell lysis, the sample was incubated for 15 minutes at 70 ° C. and vortexed or shaken to ensure that the cells were fully lysed before being placed back into the assay. Every three minutes, the sample was inverted to create a new one. For DNA elution, the Elution Buffer was placed in an incubator set to 70°C.

3- In order to ensure that no precipitation formed during DNA binding was added 200 µL of ethanol (96-100%) was to the sample and vortexed for 10 seconds. This was followed by one minute of 1400-rpm centrifugation on the FABG column after it was carefully transferred to the FABG column. Discarded the Flow-Through Two milliliters of new collection tubes were used to hold the FABG column in place.

4-After adding 400 µL of W1 Buffer to the FABG tube, the tube was centrifuged at 1400 rpm for 30 seconds. After discarding the flow-through, the FABG column was reinserted into the collection tube. well and toward the positive (red) electrodes. Add power by turning on the switch.

5-To dry the column, 600 µL of wash Buffer was added to the FABG tube and centrifuged at 1400 rpm for 30 seconds. The flow-through was then discarded and

the FABG tube was placed in the collection tube and centrifuged for an additional 3 minutes.

6- After that, the dry FABG column was transferred to a new 1.5 ml microcentrifuge tube, and 100 µL of preheated elution buffer or TE was added directly to the FABG column membrane. After 10 minutes of incubation at 37°C, the DNA was extracted by centrifugation at 1400 rpm for 1 minute. DNA was kept at 4°C until it was needed.

3.2.5.2: Primer preparation

TB Buffer (8.0) was used to dissolve the primer pairs used in this study, which contained Tris-HCL (10 Mm) and EDTA-Na2. Initially, the primer stock tube is prepared, and then the working solution is prepared from the primer stock tube

The nuclease-free water was added in accordance with the manufacturer's instructions (macrogen/Korea) to obtain a (300 picomole/microliter) concentration of primer stock solution. By diluting the stock solution with nuclease-free water, the working solution was obtained at a concentration of 10 picomole/microliter (Green and Sambrook,2012).

3.2.5.3: Reaction mixture

Amplification of DNA was carried out in a final volume of 50 µL reaction mixture as mentioned in Table (3-4).

Table 3-4: contents of the Reaction Mixture

No.	Contents of the reaction mixture	Volume
1.	Green master mix	25 µL
2.	Upstream primer (10pmol/ µL)	3 µL

3.	Downstream primer (10pmol/ µL)	3 µL
4.	Nuclease free water	14 µL
5.	DNA from sample	5 µL
Total volume		50 µL

3.2.5.4: Polymerase chain reaction (PCR)

The target DNA was amplified using specific primer pairs in a conventional PCR as previously described (Table 3-3). PCR product (amplicon) is obtained by repeating three consecutive steps for a specific number of cycles, which can then be visualized after agarose gel electrophoresis, as shown in Table (3-3) which contains information on the thermal cycling conditions.

3.2.5.5: TBE Buffer (Tris-Borate-EDTA)

DNA and RNA gel electrophoresis was commonly performed using TBE running buffer. The stock solution of TBE was prepared and stored at a concentration of 10x working solution. Dissolving 108 grams of Tris, 55 grams of boric acid, and 40 ml of 0.5 M EDTA in 1000 ml of D.W. yielded the 1x solution. When preparing gel electrophoresis gel, the final concentration of the TBE solution was prepared by adding 100ml of 10x TBE buffer to the final volume of D.W of 900 ml and using a concentration of 1x TBE solution prepared by adding 100ml of 10x TBE buffer to the final volume 950ml (Sambrook and Russel,2001).

3.2.5.6: Agarose Gel Electrophoresis

Agarose gel electrophoresis is a good way to separate DNA fragments. The amount of agarose in a gel depends on the size of the DNA fragments that need to be separated. It ranges from 0.5% to 2%. (Lee *et al*.,2012). A 1.5 gel was used to get a good picture of small parts of the PCR product (100-700 bp). However, the specific weight of agarose was added to 100 ml of 1x TBE buffer and then melted in the microwave until the solution was clear. This is how the solution was made. Afterward, the agarose gel cooled down to 50-55°C. 5 ml of a dye called "simply safe" (10 mg/ml) was added to 100 ml of melting agarose gel to get a concentration of 0.5%. (Sambrook and Russel,2006). The gel tray was filled with agarose, the comb was put in the right place, and then it was left to dry. They were put in a separate well of the gel, and markers were put in another well. Using the gel's percentage and size, a run was done in the correct way for the electrodes to connect (The time of agarose gel electrophoresis is 70 minutes for PCR product).

1-When using a plastic tray, make sure that the comb's teeth are at least 0.5mm higher than the tray's bottom.

2-1 mL TBE (10X) stock solution + 1000 mL deionized water = 1 mL TBE (10X) solution (1X).

3-Mix the buffer and 1.5g agarose in a 500ml flask. Microwave the solution for 90 seconds on high power to dissolve the agarose. Make sure the agarose solution is completely dissolved and has no visible agarose particles.

4-Use 50°C agarose solution with 5 µL Simple safe stock solution. Pour the agarose into the gel-casting tray in a steady stream. Remove any air bubbles with a yellow tip.

5-When applying the gel, keep the comb 1.5cm away from the gel's edge. Wait 20–30 minutes before using the agarose solution. Taking the comb out of the agarose gel after it has solidified requires extreme caution to avoid tearing it. Set the gel-casting tray on the gel box's central platform.

6-After removing the gel tray from the gel box, place it on the central platform.

7-The electrophoresis buffer should be raised 0.5–1cm above the gel surface by adding a buffer to the buffer chamber.

8-Place the samples in the yellow-tipped wells. Make sure that the syringe tip is well above the electrophoresis buffer if injecting. Wait a few moments before slowly ejecting the sample. Avoid contaminating nearby water sources. Sequentially fill each well with a sample. One gel well should be sufficient if you have enough samples to fill the other gel wells.

9- First load 5µl of ladder molecular weight marker to each side of the gel(flanking the sample line) and 5 µl of DNA specimen in the other well.

10-The electrodes can be connected to the gel box lid. The positive electrode can be used to draw DNA from a well (red). The machine needs the power supply turned on to work properly.

11-No electrophoresis should be done until the tracking dye has traveled at least 10 cm across the gel.

3.2.5.7: Sequencing of PCR product

Forty microliters of IL-12A, IL-12B, and ACE-2-2 products were sent to Macrogene/ Korea for Sanger sequencing. After trimming each sequence, the result of the trimmed sequence was blasted in NCBI to check the similarities and differences with the database. Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA,

USA; (http://www.geospiza.com) was used to check the polymorphism of the genes above.

3.2.6: Immunological study

3.2.6.1 Estimation of serum Human ACE-2 and IL-12

ELISA kit was applied to the in vitro quantitative determination of Human ACE-2 and IL-12.

1. Test principle

In the ELISA kit, the Sandwich-ELISA method is used to detect ACE-2 and IL-12, respectively. A Human IL-12 antibody is pre-coated on a 96-well microtiter plate included in this kit. Samples or standards were mixed with the appropriate antibody in the appropriate wells. A biotinylated detection antibody for ACE-2 was then added. To each well, we added an avidin-horseradish peroxidase conjugate (HRP). Washing was performed after the chromatogenic step to remove any remaining free components. Each well was incubated with a different substrate. Only the wells containing ACE-2 and IL-12 contain biotinylated detection antibodies and Avidin HRP conjugates. The enzyme-substrate reaction turns yellow after the stop solution is added. The optical density (OD) at an excitation wavelength was measured using a spectrophotometric technique (450 nm 2 nm). The OD value and Perforin concentration were found to be highly correlated. The concentration of ACE-2 and IL-12 was determined by comparing the OD of the samples to the standard curve.

2. Reagent preparation

1- Before using any of the samples or kit components, they were all removed from the refrigerator.

2-Standard solution preparation: reconstitute the lyophilized recombinant protein to make (80ng/l) of IL-12 and (24ng/ml) of ACE-2 solutions by adding 1ml standard and sample diluents buffer to a tube of lyophilized protein and mixing thoroughly. To avoid foam formation, the tube was gently shaken with a vortex before being left at room temperature for 10 minutes and then thoroughly mixed.

Dilution method:

Seven EP tubes were taken, and 500 µl of reference standard and sample diluent was added to each tube. 120 µl from 80,24 ng/ml IL-12, ACE-2 respectively. The working solution was pipetted to the first tube and mixed up to produce a 40,12 ng/ml working solution. Pipetted 120 µl of the solution from the former tube into the latter one according to this step. Then 120 µl was moved from each tube to another to prepare a series to dilute. Each tube has been thoroughly before the next transfer.

3. Wash Buffer – 20 ml of concentrated (Wash Buffer) was added to 25x of deionized H2O to yield 500 ml of Wash Buffer.

4. Biotinylated detection Ab working solution: The stock tube was centrifuged before use, then diluted by Biotinylated Detection Ab Diluent (1:100). The required amount was calculated before the experiment (100µL/well).

5- concentrated HRP Conjugate was less concentrated, by adding HRP Conjugate Diluent to the mix (1:100).

6- sterilized tips were used, and got the amount of reagent you need. did not put the leftover reagent back into the vial because it is sensitive to light and other things.

3. Assay procedure

Each well gets 100 mL standard, blank, or sample. A sample is drawn first. After 60 minutes of incubation at 37°C, the plate was sealed.

2. No washing was required for each well. Each well got 100 mL of biotinylated detection Ab working solution. A plate sealer seals the plate. To ensure complete mixing, it was incubated at 37°C for 1 hour.

3. Rinse: Aspirate and wash each one three times. Fill each well with wash buffer to wash. At each step, drain the system. Any remaining wash buffer was aspirated or decanted after the final wash. flipped over and placed on clean absorbent paper.

Workup solution: 100 mL per well. Then 30 minutes at 37°C with the plate sealer.

5. Wash: As in step three, wash five times.

6. Substrate: 90 mL substrate solution per well, sealed and incubated for 15 minutes at 37°C. Because the plate was not exposed to light, the reaction time can vary but should not exceed 15 minutes.

7. Stop: 50µLof stop solution is added to each well, then the color turns yellow immediately. The order to add the stop solution should be the same as the substrate solution.

8. Optical density (OD): The optical density of each well was determined at once, using a microplate reader set to 450 nm.

3.2.7: Biosafety and Hazard Material Disposing

Biosafety aspects were followed during the work including wearing all personal safety equipment such as masks, paws, gloves, and glasses when taking samples

from patients sleeping in the epidemiological corridor. The samples were stored after sterilization with ethanol in freezers designated for storing covid-19 samples. Then all contaminated syringes and supplies were disposed of supplies by autoclaving and then incineration. All benches were cleaned with alcohol (70%) before and after work. Simply safe was used instead of ethidium bromide to reduce biohazard. (Bergen, and Shelhamer, 1996).

3.2.8: Statistical analysis

Results were expressed as a mean ± standard deviation (SD)and performed using an independent T-test. Correlations between variables were assessed by correlation analysis. The association between the polymorphism and susceptibility to COVID-19 was expressed as OR and 96% CI. A probability (p) value ≤ 0.05 was considered significant. The statistical analysis was performed using IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 8.0.0 (San Diego, California USA).

Chapter Four

Result and Discussion

4. Result and Discussion

4.1. Clinical characteristics of patients

Clinical characteristics for 100 COVID-19 patients who have been hospitalized at the COVID-19 Wards in Merjan Medical City and Al-Imam Al-Sadiq Hospital in Babylon Province for two months (November and December 2021). By cross-sectional study, aims to reveal the following results.

4.1.1. Study subjects' demographics

During this study ACE-2, and IL-12 were studied from an immunological study, which was done for 90 specimens including 46 severe COVID-19 patients and 44 patients with non-severe COVID-19 patients the second part of the current study deal with polymorphism of immune genes (ACE-2 and IL-12) which carried out to 100 specimens includes 46 patients with severe COVID-19 and 54 patients with non-severe COVID-19. The study revealed that all patients have positive PCR tests and C-reactive protein (CRP) tests with low platelet counts in complete blood count (CBC) in severe patients.

According to gender distribution, the immunological study revealed that severe COVID-19 patients consisted of (23 (50%) male&23 (50%) female) cases and non-severe COVID-19 patients consisted of (22 (50%) male and 22 (50%)female) cases this means the number of patients equal in the immunological study of patients

And the gender distribution for genetic study the result revealed that severe COVID-19 patients consisted of 23 (44.3%) males in severe patients and 29 (55.7%) in non-severe patients and 22 (47.9%) females in severe patients and 25 (52.1%) in n0n-severe COVID-19 patients with A slight increase in the number of males in non-severe patients as shown in table (4-1).

Table (4-1). Gender distribution in COVID-19 patients

Subject character	Immunological study		Genetic study	
	severe	Non-severe	Severe	Non-severe
Male	23(50%)	22 (50%)	23 (44.3%)	29(55.7%)
Female	23 (50%)	22 (50%)	23(47.9%)	25(52.1%)
Total	90		100	

Individual studies, including the current study, have reported different incidences of COVID-19 in males and females but seemed to be that the disease shows a preponderance in males. It has also been observed that males tend to have a more severe illness than females within a worse prognosis (Chang, 2020; Ortolan *et al*., 2020).

It has been argued that there are some differences between males and females that may explain why COVID-19 was more preponderant in males than females in some studies, and why females perform better outcomes in terms of disease prevalence and mortality. In this context, three relevant determinants were addressed and included X-chromosome-related immune functions, the possible influence of sex hormones, and some behavioral patterns related to gender (Lakbar *et al.*, 2020).

Age median range in severe patients is between (15-80) years and in non-severe patients (10-75) years, although the mutant viruses that followed the emerging coronavirus, delta, and omicron, can severely infect younger ages, unlike the original virus, the elderly are still the largest percentage of exposure to acute infection, lung

damage, and admission to intensive care. The study showed that there are significant differences in p-value (0.05) between severe and non-severe patients.

chronic diseases associated with age progression including diabetes mellitus, cardiac diseases, and hypertension may be related to scoring of lung damage and oxygen consumption. It seems that the elderly are more susceptible to COVID-19 and this hypothesis can be interpreted due to the reduction in the number and size of ciliated cells in the airway and decreased nasal resistance in the elderly. Additionally the elderly was significantly associated with more extensive lung involvement, lower oxygen saturation levels, and a higher need for oxygen supplementation. Consequently, these patients had more severe COVID-19 and required mechanical ventilation and ICU admission more often than younger patients (Lian *et al*., 2020).

Regarding immune changes in the elderly, a disruption of the innate and adaptive immune system was observed (Xu *et al*., 2019), resulting in an extensive production of cytokines and inflammatory mediators in the so-called inflammation process as well as a more profound depletion of CD4+ cells that consequently lead to a disproportionate cytokine storm and a reduced virus clearance (Aw *et al*., 2007; Napoli *et al*., 2020).

According to this study, which was corroborated by others, elderly individuals were more sensitive to COVID-19 than younger adults (Yang *et al*., 2020). And (Jingchun *et al*., 2020) concur with the study's findings, noting that the median age of COVID-19 patients was 62 years, with no significant variation in median age between male and female groups.

According to studies, chronic diseases associated with aging are the primary reason for the hospitalization of approximately 80% of elderly people infected with

the virus, lung damage, and the need for artificial respiration devices compared to younger patients ((Mueller *et al*., 2020).

This study revealed the mean platelet level was significantly lower in severe COVID-19 patients (207.76±71.01) while in non-severe COVID-19 patients was (276.95±56.78) p-value<0.000.

This study found very decreased platelet counts when examining complete blood count (CBC) in severe patients. In some cases, the number of platelets dropped to 26×10^9/L at the start of the infection and entry to the hospital. With the appropriate treatment, the number of platelets rose to the normal range of (150-450) $\times10^9$/L. In comparison to non-severe patients, the number of platelets did not collapse below the normal limit. The average platelet count was between (190 -330) $\times10^9$/L.

Liu *et al*.,(2020) agree with our findings, a study conducted in Wuhan, China discovered that a significant decrease in platelet count was associated with an increase in the number of deaths caused by an exacerbation of thrombotic state. The platelet count was (0-50) $\times10^9$/L. in deceased patients, (50-100) $\times10^9$/L in severe cases, and (≥150) $\times10^9$/L in healthy individuals. On the contrary, the observed increase in platelet count in COVID-19 patients may indicate that clinical improvement is imminent. The study's findings showed that patients with thrombocytopenia in COVID-19 had a considerably bigger mean platelet volume (MPV, median 10.3 fL) than COVID-19 patients with retained platelet counts (median 9.9 fL). In the absence of congenital platelet abnormalities, an increase in mean platelet volume indicates an increase in the number of circulating young platelets, and this is the body's response to thrombocytopenia (low platelet counts).

Chabert *et al*.,(2015) observed that viral infection can be associated with thrombocytopenia for a variety of causes. The majority of patients who experience

hypoproliferative thrombocytopenia are in the later stages of a viral infection. The rapid development of thrombocytopenia in response to viral infections is generally mediated by increased platelet clearance/destruction platelets which can be activated by viral antigen-antibody complexes or inflammatory responses in the host; activated platelets are more easily removed from circulation by the reticuloendothelial system. according to a study by (Assinger ,2014).

A study conducted by (Weyrich & Zimmerman ,2013) observed that platelets help keep the alveolar capillaries' basal barrier intact, but where can also cause lung damage in a wide range of pulmonary diseases and syndromes.

4.2 Immunological study

4.2.1 Estimation of interleukin-12 (IL-12) serum level in severe and non-severe COVID-19 patients

The results revealed a significant difference ($P \leq 0.05$) in the serum levels of IL-12 in severe cases when compared with non-severe cases this study showed an increase in the serum level of IL-12 in severe cases which was (33.340) ng/L, in the serum levels of non-severe cases which were (20.913 ng/L, $P \leq 0.000$) as shown in Table (4-2).

Table. (4-2) Estimation of IL-12 level between severe and non-severe COVID-19 patients

patients	No. of patients	IL-12 ng/L Mean± S.D	P-value
Severe	45	33.340±11.987	0.000
Non-severe	45	20.913±4.361	

*Significant ($P \leq 0.05$).

This study found an increase in serum IL-12 levels in a respiratory care unit (RCU) patients as they approach the normal limit of non-severe patients resulting in the formation of an immune storm that contributed to the damage to the alveoli in severely COVID-19 patients.

Long *et al*. (2020) agreed with the present study and confirmed that IL-12 serum level was increased in the symptomatic group of COVID-19 patients when compared with the asymptomatic group was, observing a reduction of IL-12 serum level and other inflammatory cytokines and chemokines

The results of the Tjan *et al*. (2021) study, which contradict the current findings, showed that serum levels of IL-12 were increased in asymptomatic patients and moderate patients, while low serum levels of IL-12 were found in severely symptomatic COVID-19 patients. The discrepancy between the two studies may be attributable to the time of specimen collection after infection and the number of samples collected for severe cases. From these findings, the increase in serum IL-12 levels in respiratory care unit (RCU) patients as they approach the normal limit in non-severe patients resulted in the formation of an immune storm that contributed to the damage to the alveoli in severely COVID-19 patients.

Wahyuningtyas *et al.*(2021) observed that Cell-mediated immunity and Th1-type immune responses can be boosted by IL-12, which plays an important role in this process. Immunomodulatory factor 12 (IL-12) has many biological functions; it promotes the proliferation of activated NK and T cells, enhances their cytolytic activity, and induces IFN-γ production by both cells (T and NK). An important link between innate and adaptive immunity is the production of interferon-γ, which is induced by IL-12.

Floss *et al*.(2020) reasoned that IL-12 might also be involved in SARS-CoV resistance, as it directly binds to its receptor (IL- 12R). IL-12R complexes expressed on activated T-cells and NK-cells have been shown to contain at least two distinct subunits, B1 and B2.

Tang *et al*.(2008) showed that IL-12 contributes to the inflammatory manifestations of the disease, thereby playing a negative role in the progression of the disease.

4.2.2 Estimation of angiotensin-converting enzyme (ACE-2) serum level in severe and non-severe COVID-19 patients

The present study measured the serum levels of ACE-2 for all cases of patients with COVID-19, the findings revealed a significant difference in ACE-2 serum levels in severe cases (P ≤0.05). Compared with the non-severe case, the serum level of ACE-2 in severe cases was (11.023) ng/ml and in non-severe cases, (5.443) ng/ml. as shown in Table (4-3).

Table (4-3) Estimation ofACE-2 level between severe and non-severe COVID-19 patients

patients	No. of patients	ACE-2 ng/ml Mean± S.D	P-value
severe	45	11.023 ±5.731	0.000
Non-severe	45	5.443±1.211	

*Significant (P≤0.05).

Sriram *et al*.(2020) support this finding they said that ACE2 is found in all people, but the amount varies between individuals, tissues, and cells. ACE2 may be elevated in patients with hypertension, diabetes, and coronary heart disease.

Liu *et al*.(2020) consistent with study findings, when they examined a cohort of 12 COVID-19 patients, they discovered that circulating Ang II levels were significantly higher than in healthy controls (and were linearly related to viral load), indicating a direct link between tissue ACE2 downregulation and systemic RAS imbalance, thereby facilitating the development of multiorgan damage from SARS-CoV-2 infections.

Another study found that COVID-19 participants had an imbalance in the renin-angiotensin-aldosterone system, with an increased expression of the ACE2, renin, and kallikrein enzymes in their lavage fluids (Garvin *et al*.,2020).

Xu *et al*.(2020) observed that the high prevalence of pneumonia and bronchitis in patients with severe COVID-19 infection can be attributed to the increased expression of ACE2 in the lung, intestine, kidney, and blood vessel epithelial cells.

Linsky *et al*.(2020) showed that extreme COVID-19 patients have elevated circulating ACE2 and soluble E-selectin levels. Since viral titers were still high when ACE2 activity peaked at about 40fold above average, it's interesting to speculate that increased serum ACE2 could provide endogenous defense against SARS-CoV- 2 by blocking the virus binding sites.

Kaseb *et al*.(2021) who revealed that differences in incidence and severity of COVID-19 infection might be directly related to the degree of ACE2 expression and blood levels of ACE2 may provide a risk-stratification opportunity to identify individuals at greater risk of infection and severe illness.

Ahmadpoor *et al*.(2020) who mentioned that coronavirus enters the cell via angiotensin-converting enzyme-2 (ACE-2), mainly through the TLR-7 present in endosomes that requires the production of TNF-α, and IL-6 to enable the generation

of specific cytotoxic CD8+ T cells. This involves the formation of antigen-specific B cells and antibody production through CD4+ helper T cells.

The present study compared (ACE-2 and IL-12) between males and females in both severe and non-severe patients. It was found no significant differences between males and females in these two parameters in both severe and non-severe patients as shown in Table (4-4).

Table (4-4) Estimation of ACE-2, and IL-12 between male &female severe and non-severe COVID-19 patients

Parameters		Males Mean± S. D	Females Mean± S. D	P-value
IL-12 ng/L	Severe	35.044±12.948	31.636±10.962	0.341
	Non-severe	21.164± 4.838	20.722±3.873	0.372
ACE-2 ng/ml	Severe	12.128±7.330	9.917±3.303	0.194
	Non-severe	5.411±0.996	5.403±1.409	0.166

*Significant (P≤0.05).

Inferring from this study can conclude that these two variables are not associated with an increase in symptoms of infection in males when compared to females and that the cause of male infection was stronger than that of female infection because females have a strong immune response; they are less susceptible to viral infections; because they have a high level of the protective hormone estrogen, progesterone (Hussain *et al*.,2020; Valencia, 2020).

4.3 Correlation among the immunological parameter

This study correlated ACE-2 and IL-12 serum levels in COVID-19 patients and found that there is a direct correlation between the cytokine and this immune

receptor, and the results were as follows: Correlation is significant at the 0.01 level (2-tailed). As shown in table (4-5).

Table (4-5) correlations between ACE-2 and IL-12 serum levels in COVID-19 patients.

correlation	r-value	P-value
IL-12&ACE-2	0.586**	0.000

** Significant correlation at the level of significance (0.01)

From this study, can infer that the new Coronavirus, when it enters the human body, triggers a storm of cytokines, which is associated with an increase in the severity of infection and death among COVID-19 patients.

Yao *et al*.(2020) support this study that clarified that antigen presentation stimulates the body's humoral and cellular immunity, mediated by virus-specific immune cells. One of the main causes of death from coronavirus is cytokine storm, which is an uncontrolled systemic inflammatory response. COVID-19 induced a strong immune response by releasing pro-inflammatory cytokines and chemokines, similar to SARS-CoV and MERS-CoV infections.

4.4 Genetic study

Humane DNA extraction for detecting and identifying specific PCR amplified fragments (ACE-2-2, IL-12 A, and IL-12 B) are shown in Figures (4-1,2,3).

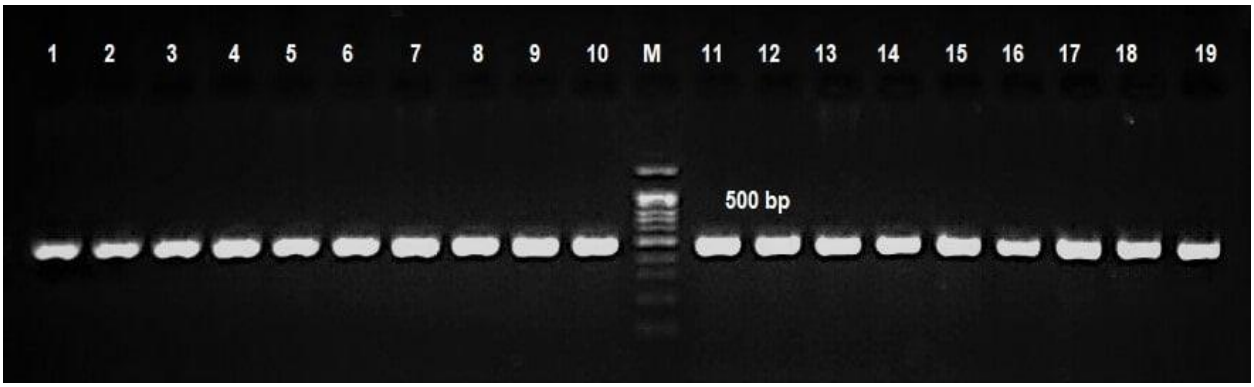

Figure 4-1 : (1.5 %) Agarose gel electrophoresis at 72 volts for 80 minutes of PCR product of ACE2-2 amplicon (500bp) , 1-19 represented sample , M (DNA marker size (100bp).

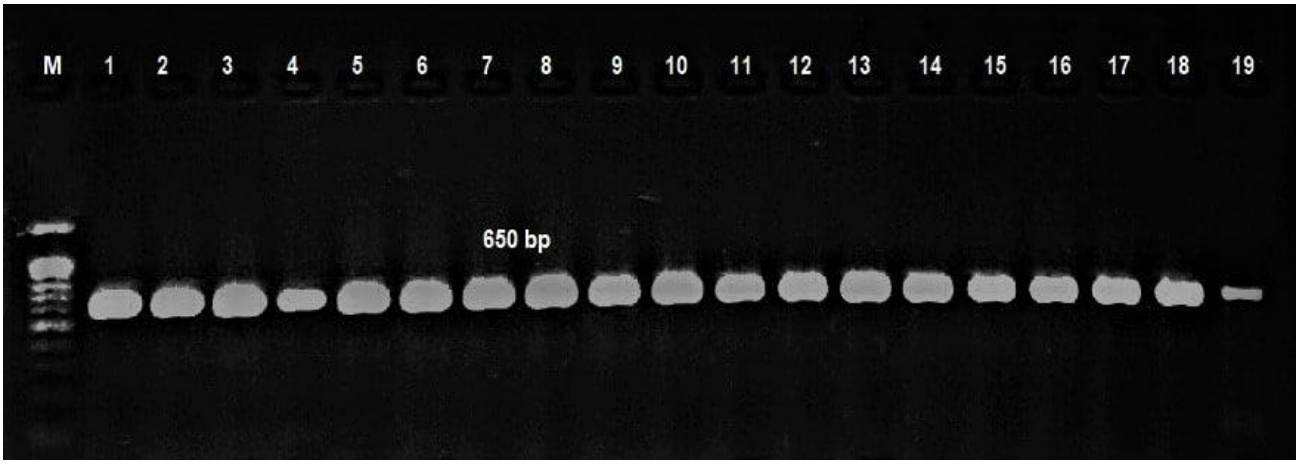

Figure 4-2: (1.5 %) Agarose gel electrophoresis at 72 volts for 80 minutes of PCR product of IL-12 A amplicon (650bp), 1-19 represented samples, M (DNA marker size (100bp).

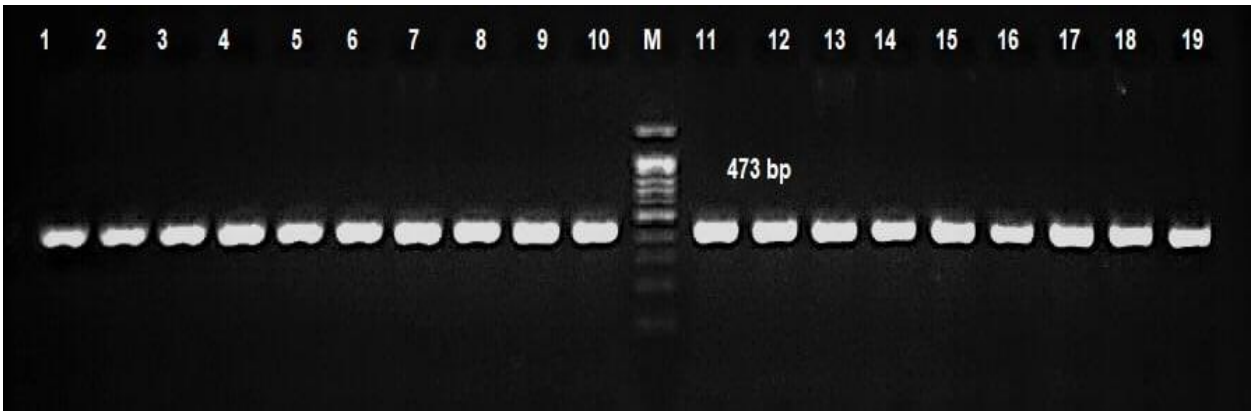

Figure 4-3: (1.5 %) Agarose gel electrophoresis at 72 volts for 80 minutes of PCR product of IL-12 B amplicon (473bp), 1-19 represented samples, M (DNA marker size (100bp).

4.4.1 Association of IL-12 A single nucleotide polymorphisms (SNPs) and risk of COVID-19

Trimming of Sanger sequencing results for IL-12 A PCR product. Multiple alignments for each were prepared using Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; (http://www.geospiza.com) to know the genotype differences between severe and non-severe COVID-19 patients and compared with the NCBI database. Figures (4-4) and Figure (4-5) show SNP distributions on the IL-12 A gene.

Figure 4-4: Distributions of rs1460141649, rs903858723, rs102618861, rs906249297, rs772904833 SNPs on IL-12A gene

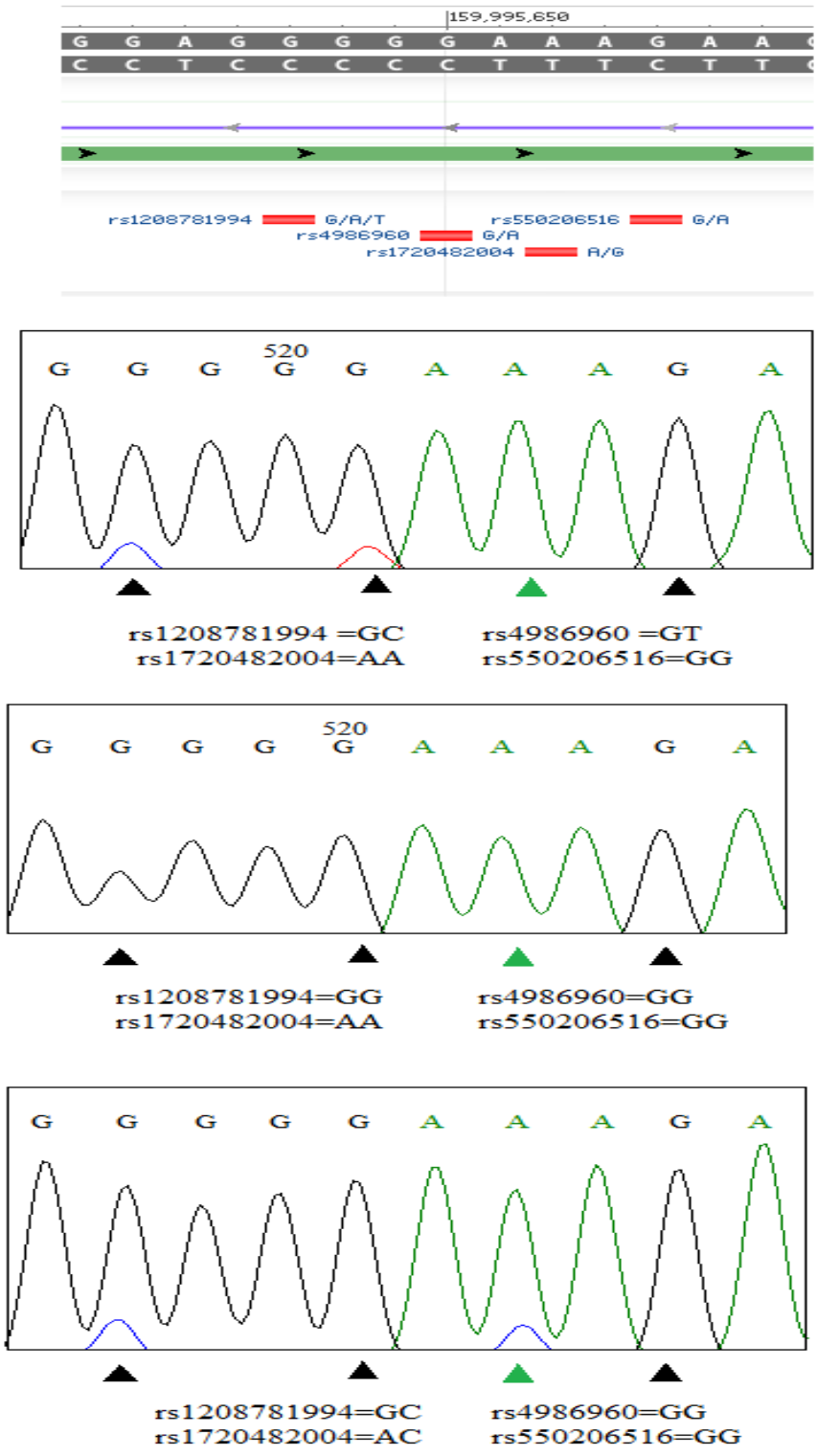

Figure 4-5: Distributions rs1208781994, rs4986960, rs1720482004, rs550206516 SNPs on IL-12A gene

A. IL-12A rs1460141649

IL-12A rs1460141649, this first SNP conducted by the current study found 84.7 % CC,15.3 % CG, and 0 % GG genotype carries in severe COVID-19 patients, with an allele frequency of 0.43 % for C-allele and 0.03% for G-allele. The genotype distribution in non-severe patients is as the following 92.5 % CC,7.5 % CG, 0% GG. The C-allele and G-allele frequencies are 0.52% and 0.02 % in non-severe cases respectively 0.43% and 0.03 for severe cases. According to these findings, there were statistically no significant differences between the C and G alleles with little increase of the G allele in severe patients, but the C allele remains more prominent in each of the severe and non-severe cases. And when comparing genotype distribution in IL-12A rs1460141649 CG (OR: 0.445, Cl 95%:0.12- 1.63, p=0.222) and the CC, GG genotypes in severe and non-severe patients there were no statically significant differences at p-value (0.222)for both CC, CG genotypes and (0.937)for GG genotype these results indicate that there is no relationship between rs1460141649 (IL-12A) SNPs and infection severity in COVID-19 as shown in (Table 4-6).

Table (4-6). Distributions of genotypes and allele frequencies in IL-12A rs1460141649 SNP in severe and non-severe COVID-19 patients

IL-12A rs1460141649 Genotype	Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC	39 (84.7)	50 (92.5)	0.222	0.445	0.12-1.63
Heterozygote CG	7 (15.3)	4 (7.5)	0.222	2.243	0.61- 8.21

Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	85(0.43)	104(0.52)	0.236	0.467	0.13- 1.64
	G	7 (0.03)	4 (0.02)	0.236	2.141	0.60- 7.55

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

B. IL-12 A rs903858723

IL-12 A rs903858723, is the second SNP conducted by the current study found 78.3% TT, 21.7 % TG, and 0% TC as the genotype distribution in severe cases, and the genotype distribution in non-severe cases as the following 85.2 % TT, 11.1 %TG, 3.7 % TC. The T-allele, G-allele, and C-allele frequencies are (T/82 (0.41), G/10 (0.05), and C/0 (0)) % in severe patients, and T/100 [(0.5), G/6 (0.03), C/2 (0.01)] % for non-severe cases. According to these findings, there were noticed statistically non-significant differences between the T, G, and C alleles with little increase of the G allele in severe patients with little appearance of the C-allele in non-severe patients, but the T-allele remains more prominent in each of the severe and non-severe cases. The explanation for the appearance of three different alleles was either because of the presence of two heterozygotes genotypes at the SNP site or because of proofreading during the annealing step of PCR amplification. From these findings can be said that there was no relationship between (IL-12A) rs903858723 SNPs and infection severity in COVID-19 as shown in (Table 4-7).

Table (4-7). Distributions of genotype and allele frequencies in IL-12A rs903858723 SNP in severe and non-severe COVID-19 patients.

IL12Ars903858723 Genotype		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type TT		36 (78.3)	46(85.2)	0.371	0.626	0.22- 1.74
Heterozygote TG		10 (21.7)	6 (11.1)	0.155	2.222	0.73- 6.67
Heterozygote TC		0 (0)	2 (3.7)	0.340	0.225	0.01- 4.82
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Heterozygote GC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	T	82 (0.41)	100 (0.5)	0.396	0.656	0.24- 1.73
	G	10 (0.05)	6 (0.03)	0.174	2.073	0.72- 5.94
	C	0 (0)	2 (0.01)	0.345	0.230	0.01- 4.85

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

C. IL-12 A rs102618861

IL-12 A rs102618861, is the third SNP conducted by the current study found 100 % TT, 0 % TC, and 0% CC as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % TT, 0 %TC, and 0 % CC, with T-allele frequency 92(0.47)%, 108 (0.54) % in both severe and non-severe cases respectively and disappeared of C-allele in both two cases and when compared the genotype distribution for IL-12 A rs102618861 (TT/P=0.973, OR=0.853 ,Cl=0.01-

43.8) and TC, CC the same result with (p=0.937,OR=1.172,Cl=0.02-69.2) this finding shows that there were no significant differences between IL-12A rs102618861 SNPs and the COVID-19 disease as shown in (Table 4-8).

Table (4-8). Distributions of genotype and allele frequencies in IL-12A rs102618861 SNP in severe and non-severe COVID-19 patients.

Genotype IL-12 A rs102618861		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type TT		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote TC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	T	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	C	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

D. IL-12 rs906249297

IL-12 rs906249297 is the third SNP conducted by the current study noticed that 100 % TT, 0 % TC, and 0% CC was the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % TT, 0 %TC, and 0 % CC, with T-allele frequency 92(0.46)%, 108 (0.54) % in both severe and non-severe cases and disappeared of C-allele in both two cases and when compared the genotype distribution for IL-12 A rs906249297 (TT/P=0.937, OR=0.853, Cl=0.01- 43.8) and TC, CC the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between IL-12A rs906249297 SNPs and the COVID-19 disease because all severe and non-severe patients contain the wild

genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-9).

Table (4-9). Distributions of genotype and allele frequencies in IL-12A rs906249297 SNP in severe and non-severe COVID-19 patients.

Genotype IL-12 rs906249297		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type TT		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote TC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	T	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	C	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles

E. IL-12 A rs772904833

And their IL-12 A rs772904833 SNP gene polymorphism was detected by this study which has been shown that 78.3 % AA, 21.7 % AG, and 0 % GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases as the following 83.4 % AA, 16.6 % AG, and 0% GG with A-allele frequency (82 (0.41) %, 99 (0.495) % and G-allele frequency 10 (0.05) %,9 (0.45) in severe and non-severe cases respectively this founding showed that there were no significant differences between the A and G alleles with almost the same ratio of G-allele between severe and non-severe cases and remained A-allele more prominent in each of the severe , and non-severe cases., the results indicate that there were no

relationship between (IL-12A) rs772904833 SNPs and infection severity in COV-19 (Table 4-10).

Table (4-10). Distributions of genotype and allele frequencies in IL-12A rs772904833 SNP in severe and non-severe COVID-19 patients

Genotype rs772904833(IL-12A)		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		36 (78.3)	45 (83.4)	0.520	0.720	0.26- 1.96
Heterozygote AG		10 (21.7)	9 (16.6)	0.520	1.388	0.51- 3.78
Homozygote GG		0 (0)	0(0)	0.937	1.172	0.02-60.2
Alleles (%)	A	82 (0.41)	99 (0.495)	0.543	0.745	0.28- 1.92
	G	10 (0.05)	9 (0.45)	0.543	1.341	0.52- 3.45

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles

F. IL-12 A rs1208781994 SNP

IL-12 A rs1208781994 SNP is the fifth SNP conducted by the current study noticed that 78.3 % GG, 21.7 % GC, and 0 % CC as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was the following 87 % GG, 13 % GC, and 0 % CC with G-allele frequencies 82 (0.41) %,101 (0.505) % and C- allele frequencies 10 (0.05) %, 7 (0.035) % in severe and non-severe patients respectively this founding shows the C-allele approximated for both severe and non-severe cases with remains G-allele more prominent in each of the severe and non-severe cases. And when comparing the genotype distribution of three genotypes with severe and non-severe patients, IL-12 A rs1208781994 GG (P=0248, OR=0.536,

Cl=0.18- 1.54), G C(p=0.248, 0R=1.865 Cl=0.64-5.37), CC (p=0.937, OR=1.172, Cl=0.02- 60.2) were noticed from these that there were no significant differences between the three genotypes in severe and non-severe patients, these results indicate that there is no relationship between (IL-12A) rs1208781994 SNP and infection severity in COVID-19 as shown in (Table 4-11).

Table (4-11). Distributions of genotype and allele frequencies in IL-12A rs1208781994 SNP in severe and non-severe COVID-19 patients

Genotype IL-12A rs1208781994		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		36(78.3)	47 (87)	0.248	0.536	0.18- 1.54
Heterozygote GC		10 (21.7)	7 (13)	0.248	1.865	0.64- 5.37
Homozygote CC		0 (0) %	0 (0) %	0.937	1.172	0.02-60.2
Alleles (%)	G	82 (0.41)	101 (0.505)	0.272	0.568	0.20- 1.55
	C	10 (0.05)	7 (0.035)	0.272	1.759	0.64- 4.82

OR=Odd ratio, Cl (95%) =confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles

G. IL-12 A rs4986960

For more insurance, this study took another IL-12 A rs4986960, and the result is the following 78.3 % GG, 21.7 % GT, and 0% TT as the genotype distribution in severe patients and non-severe patients as the following 93 % GG, 7 % GT, and 0 %TT, with G-allele frequencies 82 (0.41) %,104 (0.52) % and T- allele frequencies 10 (0.05) %, 4 (0.02) % in severe and non-severe patients respectively this founding shows the T-allele increase in severe patients when compared with T-allele in non-

severe patients with remaining G-allele more prominent in both cases. And when comparing the genotype distribution of three genotypes with severe and non-severe patients, IL-12A rs4986960 GG (P=0.04, OR=0.288, Cl=0.08- 0.99), GT(p=0.04, 0R=3.472 Cl=1.00 -11.95), CC (p=0.937, OR=1.172, Cl=0.02- 60.2) from this founding, it can be shown that there was significant difference between GG and GT genotypes at significant p-value (0.04) by means that (IL-12A) rs4986960 SNP was contributed with infection and severity in COVID-19 as shown in (Table 4-12).

Table (4-12). Distributions of genotype and allele frequencies in IL-12A rs4986960 SNP in severe and non-severe COVID-19 patients

Genotype IL-12 A rs4986960		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		36 (78.3)	50(92.6)	0.04	0.288	0.08- 0.99
Heterozygote GT		10(21.7)	4 (7.4)	0.04	3.472	1.00- 11.95
Homozygote TT		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	G	82 (0.41)	104 (0.52)	0.058	0.315	0.09- 1.04
	T	10 (0.05)	4 (0.02)		3.170	0.95- 10.47

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

H. IL-12 A rs1720482004 SNP

IL-12 A rs1720482004 SNP is the seventh SNP conducted by the current study the results were as follows 100 % AA, 0 % AG, and 0 % AC as the genotype

distribution in severe patients, and the genotype distribution in non-severe patients as the following 89.6 %AA,7.4 % AG, and 3 % AC. The A-allele, G-allele, and C-allele frequencies are (A/92 (0.46), G/0 (0), and C/0 (0)) % in severe patients, and A/102 [(0.51), G/4 (0.02), C/2 (0.01)] % for non-severe cases. According to these findings, there were noticed no statistically significant differences between the A, G, and C alleles with little increase of the G allele in non-severe patients with little appearance of the C-allele in non-severe patients, but the A-allele remains more prominent in each of the severe and non-severe cases. And when comparing the genotype distribution of three genotypes in severe and non-severe patients, IL-12 A rs1720482004 AA (P=0.088, OR=12.463, Cl=0.682-227), AG (p=0.159, 0R=0.120 Cl=0.006-2.3), AC (p=0.340, OR=0.225, Cl=0.01-4.82) were noticed from these results that the AG genotype has appeared only in non-severe patients and disappeared from severe patients but the number of non-severe patients was with AG genotype very little to said that this SNP has positivity effect on patients because of that there was no significant difference between AA and AG genotype. The AC genotype appeared only in two of the non-severe patients this result is not sufficient to occur as a significant difference between AC and AA, AG genotypes. The explanation for the appearance of three different alleles was either because of the presence of two heterozygotes genotypes at the SNP site or because of proofreading during the annealing step of PCR amplification. From these findings can be said that there was no relationship between (IL-12A) rs1720482004 SNPs and infection severity in COVID-19 as shown in (Table 4-13).

Table (4-13). Distributions of genotype and allele frequencies in IL-12A rs1720482004 SNP in severe and non-severe COVID-19 patients

Genotype IL12A rs1720482004		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		46 (100)	48 (89.6)	0.088	12.463	0.682-227
Heterozygote AG		0 (0)	4 (7.4)	0.159	0.120	0.006- 2.3
Heterozygote AC		0 (0)	2 (3)	0.340	0.225	0.01- 4.82
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Heterozygote GC		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	A	92 (0.46)	102 (0.51)	0.095	11.73	0.65- 211
	G	0 (0)	4(0.02)	0.165	0.125	0.00- 2.36
	C		2 (0.01)	0.345	0.230	0.01- 4.85

OR=Odd ratio, Cl=(95%) confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

I. IL-12 A rs550206516

IL-12 A rs550206516 SNP is the eighth SNP conducted by the current study results were as follows 100 % GG, 0 % GA, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % GG, 0 %GA, and 0 % AA, with G-allele frequency 92 (0.46)%, 104 (0.54) % in both severe and non-severe cases and disappeared of A-allele in both two cases and when compared the genotype distribution for IL-12 A rs550206516 (GG/P=0.937,

OR=0.853, Cl=0.01- 43.8) this finding shows that there were no significant differences between IL-12A rs550206516 SNPs and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-16).

Table (4-14). Distributions of genotype and allele frequencies in IL-12A SNP in rs550206516severe and non-severe COVID-19 patients

Genotype IL-12 A rs550206516		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote GA		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	G	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	A	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

4.4.2 Association of IL-12 B single nucleotide polymorphisms (SNPs) and risk of COVID-19

Trimming of Sanger sequencing results for IL-12 B PCR product. Multiple alignments for each were prepared using Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; (http://www.geospiza.com) to know the genotype differences

between severe and non-severe COVID-19 patients and compared with the NCBI database. Figures (4-6) show SNP distributions on the IL-12 B gene.

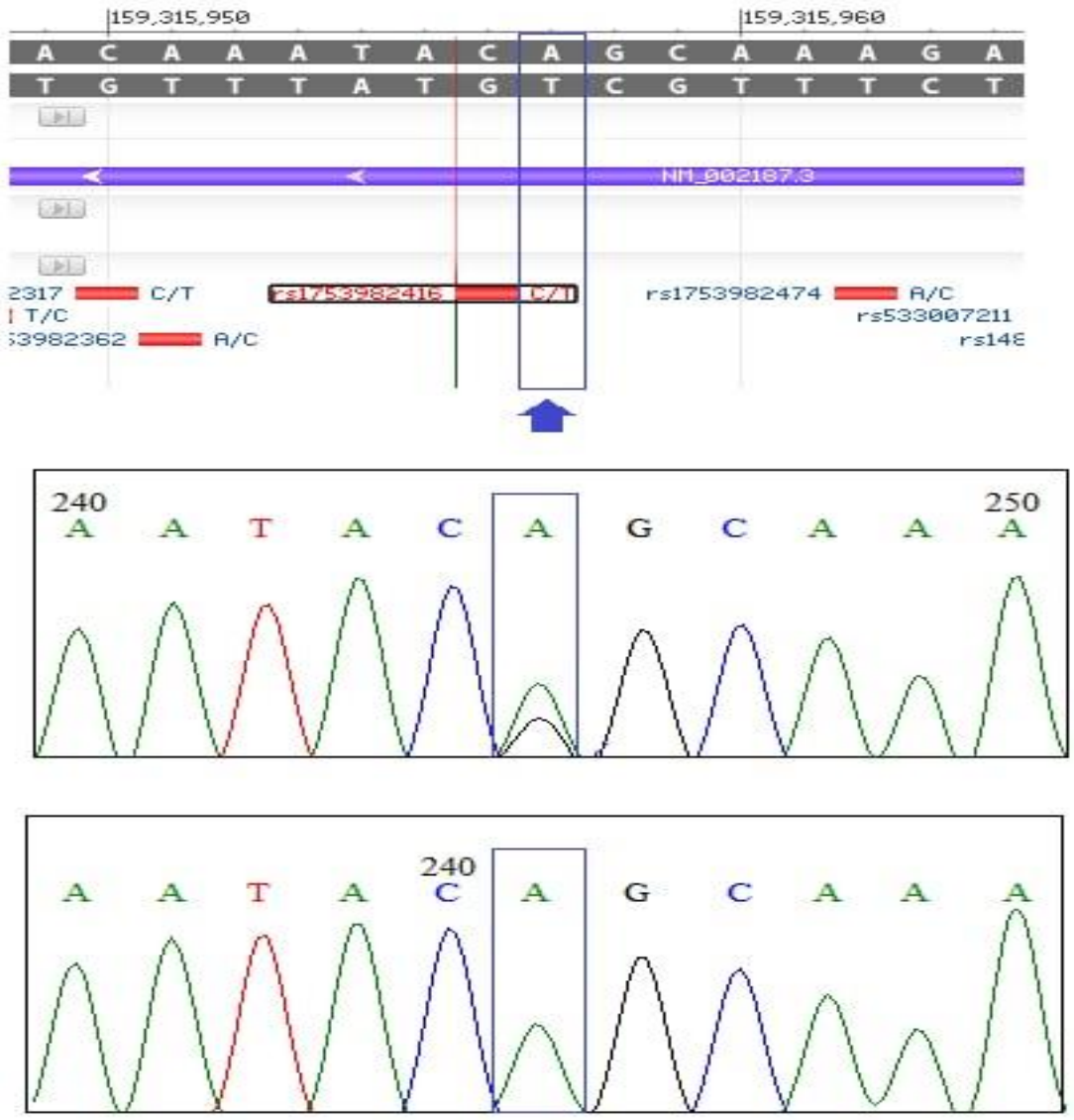

Figure 4-6: Distributions of novel SNP position on the 159315957 IL-12B gene

A. Novel SNP position on the 159315957 IL-12B gene.

This study showed that there was a novel SNP position on the 159315957 IL-12B gene and the result is the following AA 74 %, AG 26 %, and GG 0 % as a genotype distribution in severe cases, and the genotype distribution in non-severe cases was AA 100 %, AG 0 %, and GG 0 % with A allele frequency 80 (0.4) %,108 (0.54)% and G allele frequency 12 (0.06), 0 (0) in severe and non-severe cases respectively this finding shows the G-allele increase in severe patients when compared with G-allele in non-severe patients with remains A-allele more prominent in cases. And when comparing the genotype distribution of three genotypes in severe and non-severe patients from this finding, it can be shown that there was a significant difference between AA and AG genotypes at a significant p-value (0.01) meaning this novel SNP position on the 159315957 IL-12B gene contributed to infection and severity in COVID-19 as shown in (Table 4-15).

Table (4-15). Distributions of genotype and allele frequencies in novel SNP positions on 159315957 IL-12B gene in severe and non-severe COVID-19 patients

Genotype 159315957 (IL-12B)		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		34 (74)	54 (100)	0.01	0.025	0.00-0.44
Heterozygote AG		12 (26)	0 (0)	0.01	39.49	2.26-688
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles	A	80 (0.4)	108 (0.54)	0.01	0.029	0.001-0.50

(%)	G	12 (0.06)	0 (0)	0.01	33.69	1.96- 577

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

IL-12R has previously been reported by (Mohite *et al*.,2021) to be associated with infectious diseases such as tuberculosis, hepatitis B virus infection, and parvovirus infection revealing its potential role of function in host defense against microbial infections.

Kazemi *et al*.(2019) studied the relationship between IL-12 polymorphism and serum levels with the susceptibility to brucellosis and observed that patients the serum level of IL-12 were significantly more frequent in patients than in the control groups, and there was no difference in allele and genotype frequencies of 238(G/A), IL-12 between patients and controls.

Tang *et al*.(2008) show there was a relationship between SARS infection severity and IL-12 RB1 polymorphism. They observed that these genetic variations might predispose individuals to SARS infection by diminishing receptor responsiveness to IL-12, leading to partial dysfunction of interferon-γ-mediated immunity.

Karimi *et al*.(2019) observed that there were no significant differences in the distribution of IL-12 (rs3212227 +1188 A/C) genotypes and alleles were not risk factors for the development of Graft-Versus-Host Disease GVHD.

Another study by Yılmaz *et al*.(2005) observed that IL-12 production varied between genotype groups with PPD and LPS stimulations. Genetic variations of cytokines conferring susceptibility to diseases may still be functionally relevant, as polymorphisms are distributed not homogenously among different populations and seem to be influenced by various stimulations differently in different settings.

A systematic review Dos *et al.*(2021) reviewed another study about L-12 and emphasized the association of polymorphisms with susceptibility or protection to SARS-COV infection The study of genetic polymorphisms related to SARS-COV has been explored in the literature. Therefore, new studies with this proposal may contribute to a better understanding of the disease susceptibility, mainly of COVID-19 (SARS-COV-2) which has been a current focus on global health, considering clinical syndromes associated with mild disease, uncomplicated pneumonia, severe pneumonia, SARS, sepsis, and septic shock.

4.4.3 Association of ACE-2-2(GRCh38.p13) single nucleotide polymorphisms (SNPs) and potential risk of COVID-19

Trimming of Sanger sequencing results for ACA-2-2 PCR product. Multiple alignments for each were prepared using Finch TV version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; (http://www.geospiza.com) to know the genotype differences between severe and non-severe COVID-19 patients and compared with the NCBI database. Figures (4-7), (4-8) and (4-9) show SNPs distributions on the ACE-2-2(GRCh38.p13) gene.

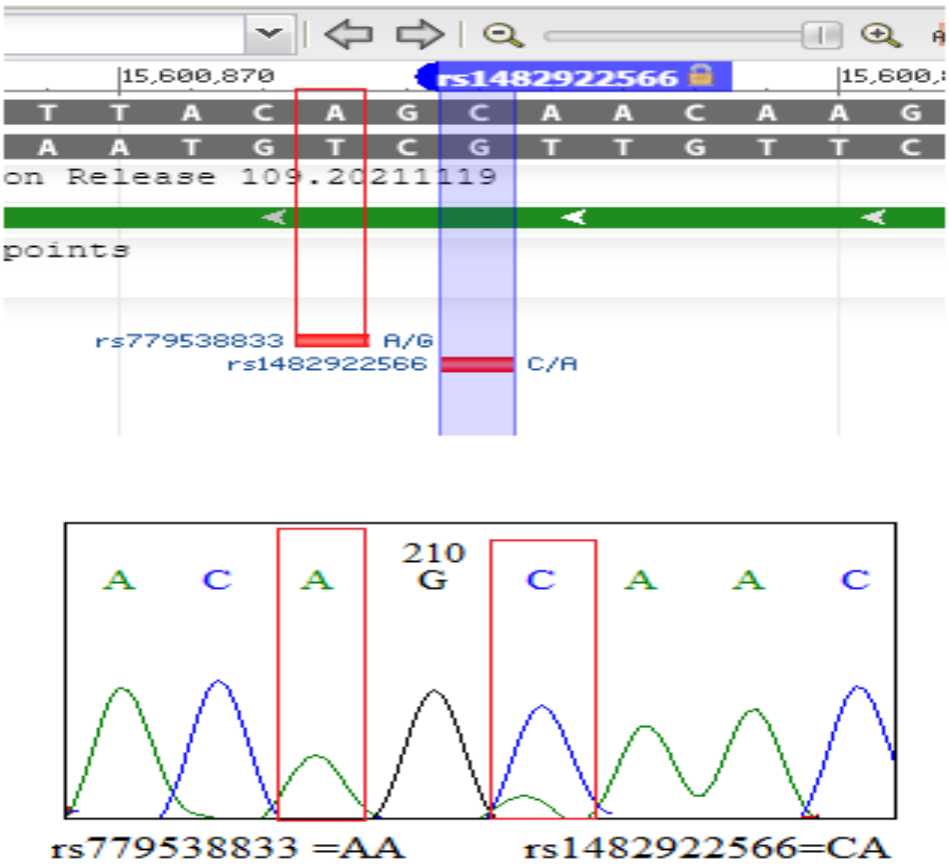

Figure 4-7: Distributions of rs779538833, rs1482922566 SNPs on ACE-2-2(GRCh38.p13) gene

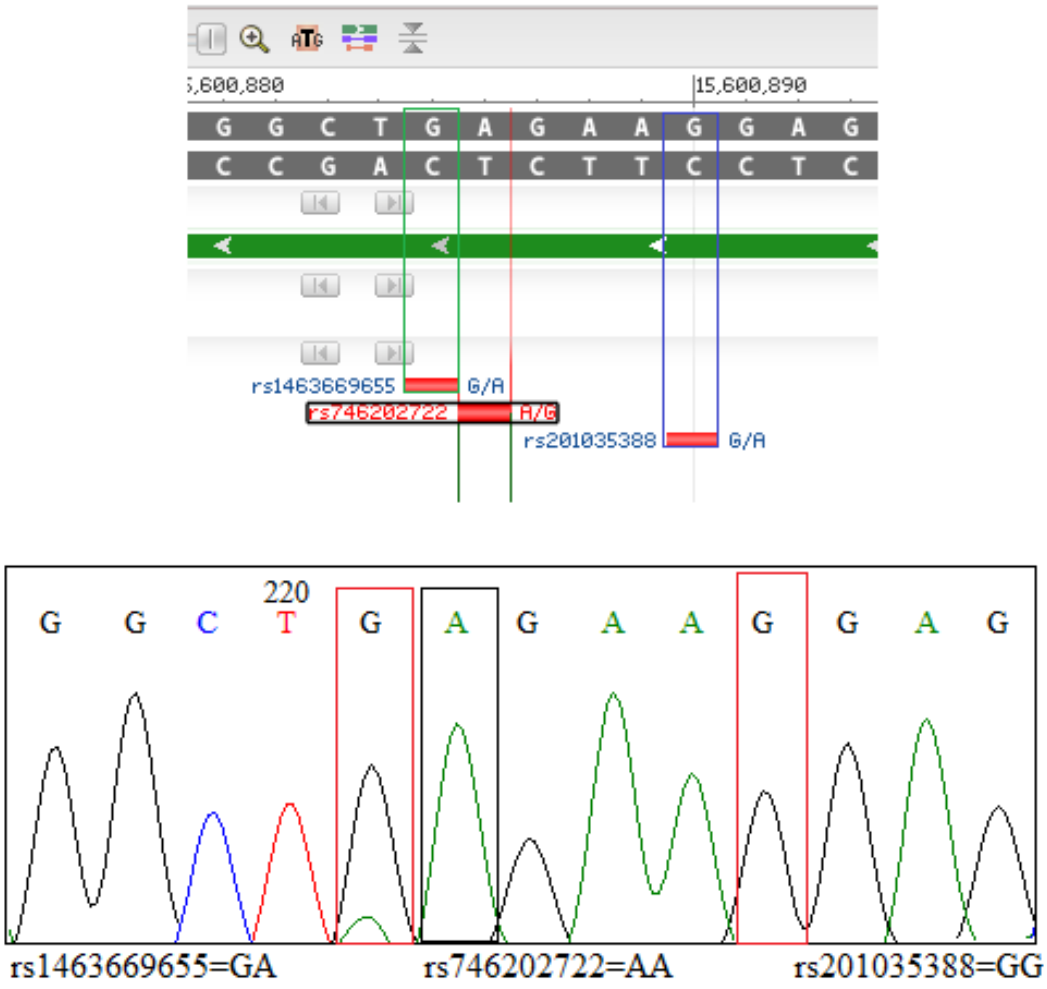

Figure 4-8: Distributions of rs1463669655, rs746202722, rs201035388 SNPs on ACE-2-2(GRCh38.p13) gene

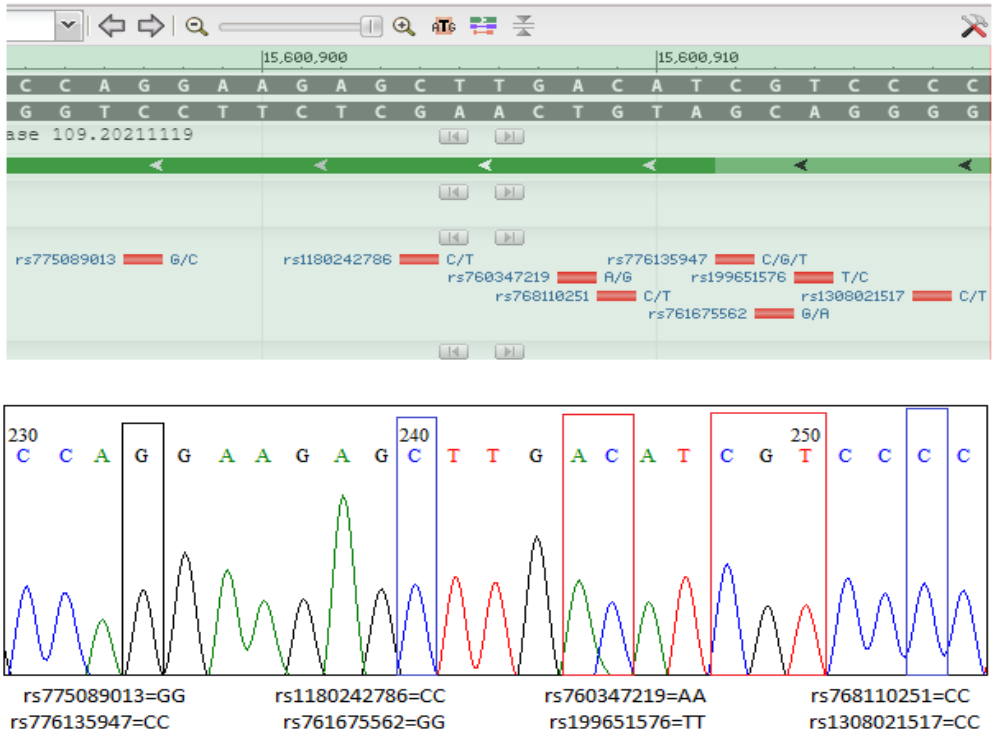

Figure 4-9: Distributions of rs775089013, rs1180242786, rs760347219, rs768110251, rs776135947, rs761675562, rs199651576, rs1308021517 SNPs on ACE-2-2(GRCh38.p13) gene

A. ACE-2-2 rs779538833

The first SNP on ACE-2-2 rs779538833 and the result following 100 % AA, 0 % AG, and 0% GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % AA, 0 %AG, and 0 % GG, with A-allele frequency 92(0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of C-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs779538833 (AA/P=0.937, OR=0.853, Cl=0.01- 43.8) and AG, GG the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs779538833

SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-16).

Table (4-16). Distributions of genotype and allele frequencies in ACE-2-2 rs779538833 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs779538833		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote AG		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02-60.2
Alleles (%)	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

B. ACE-2-2 rs1482922566 SNP

The second ACE-2-2 rs1482922566 SNP with the results 0 % CC, 100 % CA, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 0 % CC, 100 %CA, and 0 % AA, with C-allele and A-allele frequency 46 (0.23) %, 46(0.23) % in severe cases and non-severe were 54(0.27)% for C-allele frequency and 54(0.27)% for A- allele frequencies and when compared the genotype distribution for ACE-2-2 rs1482922566 CC and AA the same result

with (P=0.937, OR=1.172, Cl=0.02- 60.2) and CA result was (p=0.937, OR=0.853, Cl=0.01-43.8) this finding shows that there were no significant differences between ACE-2-2 rs1482922566 SNP and the COVID-19 disease because all severe and non-severe patients contain the heterozygous genotype and disappearance of the wild genotype and homozygous genotype in all patients as shown in (Table 4-17).

Table (4-17). Distributions of genotype and allele frequencies in ACE-2-2 SNP in rs1482922566 severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs1482922566		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Heterozygote CA		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	46 (0.23)	54(0.27)	1.000	1.000	0.57- 1.74
	A	46(0.23)	54(0.27)	1.000	1.000	0.57- 1.74

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

C. ACE-2-2 rs1463669655 SNP

The third ACE-2-2 rs1463669655 SNP and the results were 0 % GG, 100 % AG, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 0 % GG, 100 %AG, and 0 % AA, with G-allele

and A-allele frequency 46 (0.23) %, 46(0.23) % in severe cases and non-severe were 54(0.27)% for G-allele frequency and 54(0.27)% for A- allele frequencies and when compared the genotype distribution for ACE-2-2 rs1463669655 GG and AA the same result with (P=0.937, OR=1.172, Cl=0.02- 60.2) and GA result was (p=0.937, OR=0.853, Cl=0.01-43.8) this finding shows that there were no significant differences between ACE-2-2 rs1463669655 SNP and the COVID-19 disease because all severe and non-severe patients contain the heterozygous genotype and disappearance of the wild genotype and homozygous genotype in all patients as shown in (Table 4-18).

Table (4-18). Distributions of genotype and allele frequencies in ACE-2-2 rs1463669655 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs1463669655		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Heterozygote GA		52 (100)	58 (100)	0.937	0.853	0.01- 43.8
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	46 (0.23)	54(0.27)	1.000	1.000	0.57- 1.74
	A	46(0.23)	54(0.27)	1.000	1.000	0.57- 1.74

OR=Odd ratio, Cl (95%)=confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

D. ACE-2-2 rs746202722 SNP

The fourth ACE-2-2 rs746202722 SNP and the results of the following 100 % AA, 0 % AG, and 0% GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % AA, 0 %AG, and 0 % GG, with A-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of G-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs746202722 (AA/P=0.937, OR=0.853, Cl=0.01- 43.8) and AG, GG the same result with (P=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs746202722 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-19).

Table (4-19). Distributions of genotype and allele frequencies in ACE-2-2 rs746202722 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs746202722		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote AG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3

Alleles (%)	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

E. ACE-2-2 rs201035388

The fifth ACE-2-2 rs201035388 SNP with the results 100 % GG, 0 % GA, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % GG, 0 %GA, and 0 % AA, with G-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of A-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs201035388 (GG/P=0.937, OR=0.853, Cl=0.01- 43.8) and GA, AA the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs201035388 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-20).

Table (4-20). Distributions of genotype and allele frequencies in ACE-2-2 rs201035388 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs201035388	Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG	46(100)	54 (100)	0.937	0.853	0.01- 43.8

Heterozygote GA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

F. ACE-2-2 rs775089013SNP

The sixth ACE-2-2 rs775089013SNP and the result were 100 % GG, 0 % GC, and 0% CC as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % GG, 0 %GC, and 0 % CC, with G-allele frequency 92 (0.46)%, 108(0.54) % in severe and non-severe cases respectively and disappeared of C-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs775089013(GG/P=0.937, OR=0.853, Cl=0.01- 43.8) and GC, CC the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows there were no significant differences between ACE-2-2 rs775089013 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (table 4-21).

Table (4-21). Distributions of genotype and allele frequencies in ACE-2-2 rs775089013 SNP in severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs775089013		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG		46 (100)	54 (100)	0.937	0.897	0.01- 43.8
Heterozygote GC		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	G	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	C	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

G. ACE-2-2 rs1180242786

The seventh ACE-2-2 rs1180242786 SNP with the results 100 % CC, 0 % CT, and 0% TT as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % CC, 0 %CT, and 0 % TT, with C-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of T-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs1180242786 (CC/P=0.937, OR=0.853, Cl=0.01- 43.8) and CT, TT the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs1180242786 SNP and the COVID-

19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-22).

Table (4-22). Distributions of genotype and allele frequencies in ACE-2-2 rs1180242786 SNP in severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs1180242786		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote CT		0 (0)	0 (0)	0.957	1.172	0.02-60 .2
Homozygote TT		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	T	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

H. ACE-2-2 rs760347219 SNP

The results of the eighth ACE-2-2 rs760347219 SNP were 100 % AA, 0 % AG, and 0% GG as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % AA, 0 %AG, and 0 % GG, with A-allele frequency 92 (0.46)%, 108(0.54) % in severe and non-severe cases respectively and disappeared of G-allele in both two cases and when compared the genotype

distribution for ACE-2-2 rs760347219 (AA/P=0.937, OR=0.853, Cl=0.01- 43.8) and AG, GG the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs760347219 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-23).

Table (4-23). Distributions of genotype and allele frequencies in ACE-2-2 rs760347219 SNP in severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs760347219		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type AA		46 (100)	54(100)	0.937	0.853	0.01- 43.8
Heterozygote AG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote GG		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	A	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

I. ACE-2-2 rs768110251 SNP

The results of the ninth ACE-2-2 rs768110251 SNP were 100 % CC, 0 % CT, and 0% TT as the genotype distribution in severe cases, and the genotype distribution in

non-severe cases was 100 % CC, 0 %CT, and 0 % TT, with C-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of T-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs768110251 (CC/P=0.937, OR=0.853, Cl=0.0172- 43.8) and CT, TT the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs768110251 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-24).

Table (4-24). Distributions of genotype and allele frequencies in ACE-2-2 SNP in rs768110251severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs768110251		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC		46 (100)	54 (100)	0.937	0.897	0.017- 43.8
Heterozygote CT		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote TT		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	92 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	T	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

J. ACE-2-2 rs776135947

The results of the tenth ACE-2-2 rs776135947 SNP were 100 % CC, 0 % CT, and 0% TT as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % CC, 0 %CT, and 0 % TT, with C-allele frequency 92 (0.46)%, 108(0.53) % in severe and non-severe cases respectively and disappeared of T-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs768110251 (CC/P=0.937, OR=0.853, Cl=0.01- 43.8) and CT, TT the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs776135947 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-25).

Table (4-25). Distributions of genotype and allele frequencies in ACE-2-2 SNP in rs776135947 severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs776135947	Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC	46(100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote CT	0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote TT	0 (0)	0 (0)	0.937	1.172	0.02- 60.2
C	92 (0.47)	108 (0.54)	0.936	0.852	0.01- 43.3

Alleles (%)	T	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

K. ACE-2-2 rs761675562 SNP

The results of the eleventh ACE-2-2 rs761675562 SNP were 100 % GG, 0 % GA, and 0% AA as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % GG, 0 %GA, and 0 % AA, with G-allele frequency 92 (0.46)%, 108 (0.54) % in severe and non-severe cases respectively and disappeared of A-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs761675562 (GG/P=0.937, OR=0.853, Cl=0.01- 43.8) and GA, AA the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs761675562 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-26).

Table (4-26). Distributions of genotype and allele frequencies in ACE-2-2 rs761675562 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs761675562	Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type GG	46 (100)	54(100)	0.937	0.853	0.01- 43.8
Heterozygote GA	0 (0)	0 (0)	0.937	1.172	0.02- 60.2

Homozygote AA		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	A	92 (0.46)	108(0.54)	0.936	0.852	0.01- 43.3
	G	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

L. ACE-2-2 rs199651576

The results of the twelfth ACE-2-2 rs199651576 SNP were 100 % TT, 0 % TC, and 0% CC as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % CC, 0 %TC, and 0 % CC, with T-allele frequency 92 (0.46)%, 108(0.54) % in severe and non-severe cases respectively and disappeared of C-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs199651576(TT/P=0.937, OR=0.853, Cl=0.01- 43.8) and TC, CC the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs199651576 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-27).

Table (4-27). Distributions of genotype and allele frequencies in ACE-2-2 rs199651576 SNP in severe and non-severe COVID-19 patients

Genotype ACE-2-2 rs199651576	Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%

Wild type TT		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote TC		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Homozygote CC		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	T	92(0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	C	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%)confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

M. ACE-2-2 rs1308021517

The results of the thirteenth ACE-2-2 rs1308021517 SNP were 100 % CC, 0 % CT, and 0% TT as the genotype distribution in severe cases, and the genotype distribution in non-severe cases was 100 % CC, 0 %CT, and 0 % TT, with C-allele frequency 92 (0.46)%, 108(0.54) % in severe and non-severe cases respectively and disappeared of T-allele in both two cases and when compared the genotype distribution for ACE-2-2 rs768110251 (CC/P=0.937, OR=0.853, Cl=0.01- 43.8) and CT, TT the same result with (p=0.937, OR=1.172, Cl=0.02-60.2) this finding shows that there were no significant differences between ACE-2-2 rs1308021517 SNP and the COVID-19 disease because all severe and non-severe patients contain the wild genotype and disappearance of the heterozygous genotype and homozygous genotype in all patients as shown in (Table 4-28).

Table (4-28). Distributions of genotype and allele frequencies in ACE-2-2 SNP in rs1308021517 severe and non-severe COVID-19 patients

GenotypeACE-2-2 rs1308021517		Severe N=46(%)	Non-severe N=54(%)	P-Value	OR	CL=95%
Wild type CC		46 (100)	54 (100)	0.937	0.853	0.01- 43.8
Heterozygote CT		0 (0)	0 (0)	0.937	1.172	0.02- 57.2
Homozygote TT		0 (0)	0 (0)	0.937	1.172	0.02- 60.2
Alleles (%)	C	46 (0.46)	108 (0.54)	0.936	0.852	0.01- 43.3
	T	0 (0)	0 (0)	0.936	1.173	0.02- 59.7

OR=Odd ratio, Cl=(95%) confidence interval, p-value < 0.05 calculated for estimation significant difference of the patients genotypes and alleles.

All these single nucleotide polymorphisms (SNPs) are positioned in ACEII (NCBI Reference Sequence: NC_000023.11) as shown in (table 4-29).

Table (4-29) position of SNPs on ACE-2-2 gene

SNPs	Position (GRCh38.p13)
rs779538833	15600873
rs1482922566	15600875
rs1463669655	15600885
rs746202722	15600886
rs201035388	15600890
rs775089013	15600897

rs1180242786	15600904
rs760347219	15600908

A review study by Chen *et al*. (2021) on 23 SNPs present in ACE-2 in different populations showed that they could partially account for the differences in COVID-19 prevalence and mortality rates. And when comparing the affinity of the ACE2 variant to the S1 protein, their findings were controversial among studies, and the results lack validation by systems biology studies even though some variants have been believed to enhance the affinity in several reports. Thus, there is an urgent need for in vitro validation studies to assess the involvement of population-specific SNPs of ACE2 and other host factors in susceptibility toward SARS-CoV-2 infection.

Another study by Möhlendick *et al*. (2021) shows that there was a relationship between ACE2 rs2285666 and COVID-19 severity if A-allele carriers actually produce more ACE2 than those with GG genotypes, A-allele carriers could be protected by at least partially against the ACE/ACE2. This indicates that the ACE-2 gene polymorphisms may affect the severity of the infection, but the difference in the results of these studies was the difference in the type of SNPs between this study and other studies.

In addition, the study by Cao *et al*. (2020) by analyzing 1700 ACE2 variants from China MAP and 1000 Genomes Project databases and comparing the allele frequency differences between different populations identified a truncating mutation and seven hotspot variants potentially related to differently SARS-CoV-2 infection.

Finally, a study by Cafiero *et al*. (2021) on the ACE-2 gene has identified rs2074192 (ACE2), rs1799752 (ACE1), and rs699 (AGT) SNPs could potentially be valuable tools for predicting the clinical outcome of SARS-CoV-2 infected patients. And suggested furthering molecular–epidemiological studies are required to

understand the exact mechanisms underlying the clinical variability of COVID-19 disease, even in populations from different ethnic groups, and predict the most severe clinical manifestations, to develop personalized approaches or alternative strategies.

Conclusions and Recommendations

Conclusions

Depending on the current study results, the works conclude the following.

1. IL-12 and ACE-2 play a role in pathogenesis.
2. Cytokine storm causes increased IL-12 and ACE-2 serum levels and contributes to severity and death in COVID-19 patients.
3. rs1460141649, rs903858723, rs102618861, rs906249297, rs772904833, rs1208781994, rs1720482004, rs550206516 eight SNPs on the IL-12A gene did not considered a risk factor for COVID-19 patients.
4. rs4986960 SNP on the IL-12A gene and considered as a risk factor for COVID-19 patients.
5. rs775089013, rs1180242786, rs760347219, rs768110251, rs776135947, rs761675562, rs199651576, rs1308021517, rs1463669655, rs746202722, rs201035388, rs779538833 SNPs on the ACE-2-2(GRCh38.p13) gene did not considered a risk factor for COVID-19 patients.
6. A novel SNP position on the 159315957 IL-12 B gene is considered a risk factor for COVID-19 patients.
7. Polymorphism in IL-12 A and IL-12 B may contribute to severe infection.

Recommendations

The results of this study encouraged the investigator to propose the following recommendations:

1. Detection of other cytokines such as interferon-α and interferon-γ and determining their specificity and their polymorphism to detect if they contribute to the severity of COVID-19. And further studies are required to detect IL-6 serum levels and determine their specificity and their polymorphism to detect if they contribute to the severity of COVID-19 and especially can be served as an early detection marker.
2. Further studies are required to detect HLA-DRB1 and determine its specificity and its polymorphism to detect if it contributes to the severity of COVID-19.
3. The use of ACE-2 and IL-12 in the serum for early detection of infection with COVID-19.

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Appendices

Appendix: Questionnaire of information

Sample number		
Patient's name:		**Age:**
Weight	**Height**	**BMI**
Diabetes ○ **Other**		

Diagnostic method: ○ **RT-PCR** ○ **CT scan** ○ **Rapid COVID-19Ab**

Date of admission	**Case severity** ○ **non-severe** ○ **sever** ○ **critical**

Date of sample collection

Laboratory tests

HEMATOLOGY

WBC	
Hb	
platelet count	
ESR	

RANDOM BLOOD SUGAR

LIVER FUNCTIONS TESTS

GPT	
GPT	
Alkaline Phosphatase	

RENAL FUNCTIONS TESTS

S. creatinine	
Urea	
Uric acid	

CRP

POSITIVE NEGATIVE

LIPID PROFILE

cholesterol	
Triglycerides	

ELECTROLYTES LEVELS

Ca	
Na	
K	
Cl	

Patient's consent

Vaccination

Yes **No**

الخلاصة

متلازمة الجهاز التنفسي الحادة الناجمة عن فيروس كورونا ، وهو أحد فيروسات الجهاز التنفسي التي انتشرت بسرعة ، اجتاحت العالم وأودت بحياة أكثر من ستة ملايين شخص. وأدى ذلك إلى ابتكار العديد من اللقاحات والأدوية في وقت قياسي للتخفيف من حدة هذا المرض وتسريع شفاء المرضى. تبحث هذه الدراسة في التباين الجيني بين الإنزيم 2 المحول للأنجيوتنسين والإنترلوكين 12 في مرضى COVID-19 وتقارن هذه السيتوكينات والمستقبلات بين الحالات الشديدة وغير الشديدة. تضمنت هذه الدراسة المقطعية المستعرضة 100 عينة دم من مرضى كوفيد -19 الذين تتراوح أعمارهم (15 إلى 80) سنة وزعت حسب شدة المرض على النحو التالي: (46 حالة شديدة ، 54 غير شديدة) تم نقلهم إلى المستشفى في الردهة الوبائية ب كوفيد -19 في مدينة مرجان الطبية ومستشفى الإمام صادق بمحافظة بابل لمدة شهرين (نوفمبر وديسمبر 2021). جزءان من العمل هما الدراسة الجزء الاول دراسة المناعية لتقييم مصل IL-12 و ACE-2 و الجزء الثاني الدراسة الجينية للكشف عن تعدد الأشكال الوراثي لـ ACE-2 و IL-12A و IL-12. كشفت النتائج أن مستويات المصل لكل من الإنزيم المحول للأنجيوتنسين -2 (ACE-2) والإنترلوكين -12 (IL-12) تم قياسها في مرضى COVID-19 وتمت مقارنة النتائج باستخدام اختبار T مستقل ، وتم العثور عليه أن مستويات الإنترلوكين - 12 الخاصة بهم كشفت عن فرق معنوي ($P \leq 0.05$) في مستويات المصل للحالات الشديدة عند مقارنتها بالحالات غير الشديدة. كانت هناك زيادة في مستوى المصل من IL-12 في الحالات الشديدة حيث بلغ 33.340 نانوغرام / لتر ، وفي الحالات غير الشديدة كان 20.913 نانوغرام / لتر. ($P \leq 0.000$) ، وبالنسبة للإنزيم المحول للأنجيوتنسين -2 ، كشفت هذه الدراسة عن اختلاف كبير في مستويات مصل ACE-2 في الحالات الشديدة ($P \leq 0.05$) عند مقارنتها بالحالات غير الشديدة للمرضى المصابين بـ COVID-19. . كان مستوى الإنزيم المحول للأنجيوتنسين 2 في المصل في الحالات الشديدة 11.023 نانوغرام / مل ، وفي الحالات غير الشديدة كان 5.443 نانوغرام / مل ($P \leq 0.000$). كانت نتيجة جين IL-12A تسعة SNPs في جين IL-12A ، مثل الأنماط الجينية التالية rs1460141649 و CG و GG و CC ، كان معدل الانتشار المرتفع للأنماط الجينية CC (النوع البري) في مرضى COVID-19 الحاد بنسبة 84.7٪ ، و 92.5٪ على التوالي غير ذات دلالة إحصائية (ف> 0.05). rs903858723، TT، TG، TC، CC، GG ، و GC للأنماط الجينية كان معدل الانتشار المرتفع للأنماط الجينية (النوع البري) في مرضى COVID-19 الوخيمين بنسبة 78.3٪ ، و 85.2٪ على التوالي غير ذات دلالة إحصائية (P> 0.05). و rs772904833 AAو AG و GG ، كان معدل الانتشار المرتفع للأنماط الجينية (النوع البري) في مرضى COVID-19 الوخيمين بنسبة

78.3٪ ، و 83.4٪ على التوالي غير ذات دلالة إحصائية (P> 0.05). rs1208781994 GG ، GC، CC ، الأنماط الجينية كان معدل الانتشار المرتفع للأنماط الجينية (النوع البري) في مرضى COVID-19 الوخيمين بنسبة 78.3٪ ، و 87٪ على التوالي غير ذات دلالة إحصائية (P> 0.05). rs1720482004 AA, AG, AC ، الأنماط الجينية كان معدل الانتشار المرتفع للأنماط الجينية (النوع البري) في مرضى COVID-19 الوخيم بنسبة 100٪ ، و 89.6٪ على التوالي غير معنوي إحصائياً (P> 0.05). rs550206516 GG, GA, AA, الأنماط الجينية كان معدل الانتشار المرتفع للأنماط الجينية (النوع البري) في مرضى COVID-19 الوخيم بنسبة 100٪ ، و 100٪ على التوالي غير ذات دلالة إحصائية (P> 0.05).

و rs4986960 SNP على جين IL-12A يظهران أن هناك فرقًا كبيرًا بين الأنماط الجينية GG و GT عند قيمة p كبيرة (0.04) مما يعني أن rs4986960 SNP (IL-12A) ساهم في العدوى وشدة في COVID-19. وكانت نتيجة جين IL-12B وضع SNP جديد على الجين 159315957 IL-12 B يظهر فرقًا كبيرًا بين الأنماط الجينية AA و AG بقيمة p معنوية (0.01) عن طريق الرواية 159315957 (IL-12 B).) ساهم SNP في العدوى والشدة في COVID-19. بينما كشف جين ACE-2-2 عن ثلاثة عشر SNPs على ACE-2-2 (GRCh38.p13) على النحو التالي rs760347219, rs1180242786, rs775089013,

rs768110251,rs776135947,rs761675562,rs199651576,rs1308021517,rs146366965 5 rs779538833 rs201035388 rs746202722, لا توجد فروق ذات دلالة إحصائية بين SNPs و COVID-19 لأن جميع المرضى لديهم النمط الجيني البري ولا يملكون الزيجوت المتغاير والنمط الجيني متماثل الزيجوت باستثناء rs1482922566 و rs1463669655 حيث يكون لدى جميع المرضى النمط الوراثي متغاير الزيجوت ولا يملكون النوع البري ومتجانسة الزيجوت. من هذه النتائج ، خلصت هذه الدراسة إلى أن كلاً من ACE-2 و IL-12 يساهمان في الإصابة الشديدة لمرضى COVID-19 للدراسة المناعية ولكن بالنسبة للدراسة الجينية ، فإن تعدد الأشكال في جين ACE-2-2 لا يرتبط بشدة الإصابة. ارتبط مرضى COVID-19 ولكن تعدد الأشكال في IL-12A و IL-12B بخطورة مرضى COVID-19.

وزارة التعليم والعالي والبحث العلمي
جامعة بابل
كلية العلوم
قسم علوم الحياة

دراسة مناعية جزيئية للتغيرات الوراثية لجينات ACE-٢ و IL-١٢بين مرضى COVID-١٩

رسالة مقدمة لمجلس كلية العلوم جامعة بابل استيفاء جزئي لمتطلبات درجة ماجستيرفي علوم الحياة

من قبل

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