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Study of Genetics Polymorphism of Some Human Genes Associated with the Severity and Progression of COVID-19 Infections

A Dissertation

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By

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿قُلْ هَلْ يَسْتَوِي الَّذِينَ يَعْلَمُونَ وَالَّذِينَ لَا يَعْلَمُونَ إِنَّمَا يَتَذَكَّرُ أُولُو الْأَلْبَابِ﴾

صدق الله العظيم

سورة الزمر، الآية (٩)

Supervisor Certification

I certify that this thesis entitled **(Study of Genetics Polymorphism of some Human Genes Associated with the Severity and Progression of COVID-19 Infections)** has been conducted under my supervision by (**Ghazwan Faisal Hussein**) and submitted in partial fulfillment of the requirements for Degree of **Doctor of Philosophy in Biology** at the Department of Biology, College of Science, University of Babylon.

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Dedication

To:

My family (mother, father, brother, and sisters)

My friends

To all COVID-19 Patients, those who are fighting for life, and those who have left us.

Ghazwan, 2023

Acknowledgments

I would like to thank the **University of Babylon**, **College of Science-Department of Biology** for giving me this opportunity to undertake my Ph.D. degree. Babylon is the name capital city of the ancient Babylonian Empire.

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Summary

A global health epidemic emerged due to the Coronavirus disease 2019 outbreak (COVID-19), caused by the novel severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2). A high mortality rate and poor prognosis are associated with severe COVID-19 cases due to hypercoagulability and progressive hyperinflammation.

This study investigates genetic markers and protein levels as potential COVID-19 severity indicators in Iraqi patients. It involves 102 patients (aged 52.66±18.82 years) and 92 healthy controls (ages 37.88±14.19 years). Patients are categorized as non-severe (45) or severe (57) based on hospital/ICU admission for respiratory failure or not.

In the course of the study, specimens were gathered from several Iraqi healthcare facilities, specifically Al-Diwaniyah Teaching Hospital, Marjan Medical City, Al-Hussein Teaching Hospital, and Al-Hamza Hospital during (Dec 2021- May 2022) including Covid-19 positive patients and a healthy control group. The identified polymorphisms in four single nucleotide polymorphisms (SNPs) are as follows: Methylenetetrahydrofolate reductase gene (MTHFR) rs1801133 C677T (p. Ala222Val), Angiotensinogen gene (AGT) rs699 T>C (p. Met268Thr), angiotensin-converting enzyme 2 gene (ACE2) rs2106809 T>C, and serpin family E member 1 (SERPINE1) rs1799889 4G/5G polymorphism.

The polymorphisms are detected using the ARMS analysis (AS-PCR) and tetra amplification refractory mutation system (T-ARMS-PCR) methods, along with sequencing techniques, the genotyping involved using three primers for AS-PCR and four primers for T-ARMS-PCR to detect the polymorphisms. Subsequently, Sanger sequencing is conducted on the target DNA. Changes in serum concentrations of four biomarkers (homocysteine, angiotensinogen, angiotensin 1-7, and plasminogen activator inhibitor-1) are determined using ELISA analysis to detect specific antibodies in human serum. In silico tools were employed to examine the impact of SNPs on protein structure and function.

Additionally, it is noted that SNPs located in introns may influence splicing, gene regulation, and promoter activity.

The study results indicate that the mean age of COVID-19 cases is significantly increased compared to HC (52.66±18.84 vs. 37.88±14.19, $p < 0.05$), female and male distribution in patients and healthy groups did not differ significantly ($p=0.99$). The patient data comprises 58.6% males and 47.1% females, while healthy individuals included 52.9% males and 41.3% females. Eight laboratory parameters, including hemoglobin level, platelets, white blood cell (WBC) count, neutrophils, lymphocytes, D-dimer test, neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR), were also determined for Covid-19 cases. Among the parameters compared with the reference ranges, five exceeded them; WBC count (13.5×109/L ±5.5), D.D (1.37±1), NUE (10.88±4.35), NLR (11±9), and PLR (307.96±271.76). Lymphocytes were also measured; they were significantly decreased (1.55±0.96). Based on allelic discrimination, the CT and TT genotypes of the MTHFR gene (rs1801133) are associated with an increased risk of Covid-19 infection and higher severity. In the AGT gene (rs699), the TC genotype is a statistically significant risk factor for Covid-19 infection, but no impact on disease severity is observed. The genotype variation of the ACE2 gene (rs2106809) on the X-chromosome does not affect the risk or severity of infection in females. However, in males, the C allele is associated with an increased risk and severity of Covid-19. The 4G/5G genotype of the SERPINE1 gene (rs1799889) is linked to a higher risk and worsening severity of infection. Significantly higher levels of homocysteine in patients (16.17 nmol/l) than in HC (4.46 nmol/l). Similarly, homocysteine levels are significantly higher in death and severe cases compared to non-severe cases (12.5 and 10.4 *vs.* 3.2, respectively). Patients exhibited a notable disparity in serum angiotensinogen levels compared to the HC group (69.9 vs. 86.07 ng/L). However, no significant differences are observed between severe and non-severe patients. The average Ang 1-7 level in Covid-19 patients

was notably lower compared to the control subjects (70.77 vs. 91.38 ng/L). Furthermore, serum Ang 1-7 levels in deceased and severe cases were significantly lower than those observed in non-severe cases (60.6 and 75.2 vs. 109.44 ng/L, respectively). The PAI-1 levels in patients were significantly higher than in healthy controls (6.5 *vs.* 3.56 ng/ml). Additionally, both dead and severe cases had significantly higher PAI-1 levels than non-severe cases (13.2 and 8.36 *vs.* 3.43 ng/ml), respectively. Significant associations were observed between biomarkers levels (Hcy, Ang 1-7, and PAI-1) and genotypes/alleles of three polymorphisms (rs1801133, rs2106809, and 1799889). However, AGT levels did not show significance in genotypes related to rs699.

Multiple web servers are utilized to verify the polymorphisms in the study and display their in-silico results. The rs1801133 p.A222V poly-morphism of MTHFR shows decreased protein stability and potential disease implications. Conversely, the AGT rs699 p.M268T polymorphism demonstrated a benign decrease in protein stability without causing disease. Additionally, the rs2106809 in ACE2-intron and rs1799889 in SERPINE1 promoter were found to modulate transcriptional activity by affecting the presence or absence of transcription factors.

In conclusion, the polymorphisms in MTHFR, ACE2, and SERPINE-1, along with the biomarker levels of Hcy, Ang 1-7, and PAI-1, show promise as potential biomarkers that warranted further validation in studies investigating their association with Covid-19 severity.

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List of Abbreviations

Abbreviations	Meaning
2019-nCoV	2019-Noval CoV
ARDS	Acute-respiratory-distress-syndrome
Ang 1-7	Angiotensin (1-7)
Ang II	Angiotensin II
ACE2	Angiotensin-converting-enzyme-2
AGT	Angiotensinogen
Ag	Antigen
AUC	Area under the curve
BALF	Bronchoalveolar lavage fluid
CVD	Cardiovascular diseases
CDC	Centre for Disease Control and Prevention
COPD	Chronic obstructive pulmonary disease
CD	Cluster of differentiation
CT	Computerized tomography
CI	Confidence interval
COVID-19	Coronavirus disease-19
CoVs	Coronaviruses
CRP	C-reactive protein
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DM	Diabetes mellitus
DIC	Disseminated intravascular coagulation
E	Envelope
ELISA	Enzyme-linked immunosorbent assays
EDTA	Ethylenediamine tetra acetic acid
D-dimer	Fibrin detected in blood
GWAS	Genome-wide association study
HWE	Hardy-Weinberg equilibrium
HC	Healthy control
Hb	Hemoglobin
HSV	herpes simplex virus
Hcy	Homocysteine
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HPIVs	human parainfluenza viruses
Ig	Immunoglobulin
IAV	Influenza A virus
INS/DEL	Insertion/deletion
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin

ICTV	International Committee on Taxonomy of Viruses
IQR	Interquartile range
LDH	Lactate dehydrogenase
LYM	Lymphocytes
M	Membrane
MTHFR	Methylenetetrahydrofolate reductase
MERS	Middle- East-respiratory-syndrome
MAF	Minor allele frequency
NCBI	National Center for Biotechnology Information
NK	Natural killer
NEUT	Neutrophils
NLR	Neutrophil-to-lymphocyte ratio
NAD	Nicotinamide adenine dinucleotide
nsp	Non-structural proteins
N	Nucleocapsid
OR	Odds ratio
ORFs	Open reading frames
PTT	Partial thromboplastin time
PAI-1	Plasminogen activator inhibitor-1
PLR	Platelet to lymphocyte ratio.
PLT	Platelets
PCR	Polymerase Chain Reaction
Ph	Potential of hydrogen
p	Probability
ROC	Receiver operating characteristic
rs	Reference sequence
RAS	Renin-angiotensin system
RSV	Respiratory *syncytial virus*
RPM	Revolutions per minute of rotor (rpm)
RNA	Ribonucleic acid
SERPINE1	Serine Protease Inhibitor Family E member 1
SARS-COV-2	Severe acute respiratory syndrome coronavirus 2
SARS	Severe-acute-respiratory-syndrome
SNP	Single nucleotide polymorphism
S	Spike
SD	Standard deviation
s-HRP	Streptavidin-horse radish peroxidase
TGF-β	Transforming growth factor-β
TMPRSS2	Trans-membrane protease serine2
TBE	Tris-Borate EDTA
TNF-α	Tumor necrosis factor-alpha
UTR	Untranslated region
Vit-D	Vitamin D
WBC	White blood cell

Chapter One

Introduction

1. Introduction

Since December 2019, in Wuhan, Hubei Province, China, a noticeable increase is observed in the number of pneumonia patients and has rapidly spread across China, which has drawn international attention(Wang C. *et al*., 2020). After the pathogen for this pneumonia was identified and isolated, it was originally referred to as the 2019 novel coronavirus (2019-nCoV), The World Health Organization (WHO) subsequently dubbed it severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the coronavirus has caused a pandemic of an acute respiratory disease called "coronavirus disease 2019" (COVID-19)(Zheng Y-Y. *et al*., 2020).

A virus known as COVID-19 was originally classified as part of the same virus family as the severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) and the Middle East respiratory coronavirus syndrome (MERS-CoVs), these two coronavirus-based respiratory syndromes have affected over 10,000 people in the last two decades around the world, with mortality rates of 11% and 35%, respectively (Organization 2003; Leung *et al*., 2004; De Wit *et al*., 2016).

The main symptoms of COVID-19 at the time of illness onset are fever, fatigue, coughing, myalgia, and dyspnea(Wang D. *et al*., 2020), and according to the WHO, COVID-19 has an incubation period of (1-14) days(WHO 2020). On February 24, 2020, Iraq reported its first COVID-19 patient, an Iranian student in Al-Najaf Al-Ashraf province who had traveled from Iran, on February 25, there were four cases reported from the same family in the province of Kirkuk, the number of confirmed COVID-19 infections and deaths increased to 4,469 by May 24, 2020, among those affected by the virus, 160 people died, and 2,738 recovered(Dawood and Dawood, 2021). As an RNA virus, SARS-CoV-2 can infect both animals and humans, coronavirus infection causes respiratory, gastrointestinal, and neurological symptoms, COVID-19 has the potential to cause pulmonary and systemic inflammation, potentially

leading to multi-organ dysfunction may be associated with direct virus invasion via angiotensin-converting enzyme 2 (*ACE2*) binding(Zhou P. *et al*., 2020a), Cardio-renal inflammation, endothelial dysfunction (Guzik *et al.*, 2020), and thrombotic microvascular angiopathy (Varga *et al.*, 2020).

The COVID-19 causes excessive inflammation, endothelial cell activation and injury, platelet activation, and hypercoagulability, which predisposes patients to thrombotic and thromboembolic events, COVID-19 patients are prothrombotic or thrombophilic, with elevated levels of several thrombotic biomarkers related to disease severity and prognosis(Gorog *et al.*, 2022). The presence of thrombosis and elevated levels of coagulation markers are important prognostic factors in COVID-19 patients(Tang *et al.*, 2020).

Early in the pandemic, hospitalized COVID-19 patients were frequently found to have abnormal levels of coagulant biomarkers activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen are some examples, and suggested that these biomarkers be routinely measured(Bonaventura *et al.*, 2021). A high homocysteine level (Hcy), an amino acid that plays a significant role in coagulation, appears to be related to COVID-19's severity(Abu-Farha *et al.*, 2020). The Hcy has previously been shown to have a role in numerous metabolic and inflammatory processes, and different groups with distinct ethnic predominances have varying prevalence of *MTHFR* gene variants and *MTHFR* activity which is clearly connected to prothrombotic events as a result of increased homocysteine metabolism(Liew and Gupta, 2015).

As genetic predisposition is identified as a factor in the progression and infection of SARS-CoV-2, polymorphisms in renin-angiotensin-aldosterone system (RAAS)-related genes have been proposed to be involved with risk factors for COVID-19(Devaux *et al*., 2020). The *ACE2* a transmembrane glycoprotein, is a key renin-angiotensin system (RAAS) component. The new SARS coronavirus 2 uses *ACE2* as its entrance receptor SARS-CoV-2. Gue and Gorog propose that the decrease in angiotensin-converting enzyme 2 (*ACE2*)

activity seen in COVID-19 could potentially result in heightened vascular permeability, elevated tissue factor expression, activation of the extrinsic coagulation pathway, as well as an upsurge in plasminogen activator inhibitor 1 levels and a reduction in fibrinolysis. Importantly, prior research had already hinted at a connection between the ACE2 pathway and thrombotic events(Gue and Gorog, 2020; Zhang *et al.*, 2020). The *ACE2* is downregulated, resulting in a rise in angiotensin-II levels and a decrease in 1-7 angiotensin, which are connected to pro-apoptotic characteristics, inflammation, and fibrosis, all of which are the core of COVID-19 pathobiology(Sriram *et al*., 2020).

Angiotensinogen (*AGT*) protein is a vital part of the RAAS system that efficiently controls blood pressure and other cardiovascular functions. High blood pressure (BP) is well acknowledged as a substantial risk factor for arterial stroke (Wajngarten and Silva, 2019). Studies data suggests that COVID-19 may be an important modifier of the onset, characteristics and outcome of acute ischemic stroke(Perry *et al.*, 2021). Ischemic stroke occurs due to the blockage of blood vessels, often caused by thrombosis, subsequently resulting in tissue ischemia, inflammation, and cell death.

The COVID-19 patients are found to have higher plasma D-dimer levels in many studies, which is linked with severity, thrombosis, and mortality, such as high amounts of active plasminogen activator inhibitor 1 (PAI-1) in COVID-19 patients may inhibit plasmin breakdown of crosslinked fibrin and D-dimer release(Campbell *et al.*, 2021). The PAI-1 regulates fibrinolytic action, impaired fibrinolysis is suggested in COVID-19 patients, which could increase thrombotic risk (Zuo *et al.*, 2021). High levels of active PAI-1 may induce thrombosis in COVID-19 patients due to simultaneous stimulation of coagulation and inhibition of fibrinolysis (Campbell *et al.*, 2021). Endothelial dysfunction, RAAS activation through the production of procoagulant plasminogen activator inhibitor (PAI-1), and hyperimmune response via activated platelets seem to be important factors to thrombogenesis in COVID-

19(Ahmed *et al.*, 2020). Thrombotic consequences are common in COVID-19, and they contribute considerably to death and morbidity(Hanff *et al.*, 2020). Genetic variations can influence individual differences in traits, including disease susceptibility and response to medications, and understanding how these variations affect gene expression and protein function can help pinpoint causal factors(Jin Yu. *et al.*, 2018). Modern genetics research included as one of their primary objectives the discovery of SNPs that are linked to an increased risk of developing chronic diseases. It is hypothesized that genetic variants in various populations may alter the course of COVID-19 reassortment and classify the levels of severity vulnerability of patients to the virus(Kwok A. *et al.*, 2021).

1.1. Aim of the Study

This study aims to detect of some genetic polymorphisms, correlate of each SNP with biomarker serum level, and determine the effect of each SNP on structure and function of protein by in silico analysis to identify which could increase susceptibility or have associated with severity and mortality of Covid-19 infection. The specific objectives were as follows:

1) The study explored the relationship between laboratory features and the severity of COVID-19.
2) The study involved examining the following genetic polymorphisms: rs2106809 *ACE2*, rs699 *AGT*, rs1801133 *MTHFR*, and rs1799889 *SERPINE-1*, to estimate their relationships with interindividual variations in severity and mortality rates of COVID-19 among samples of Iraqi patients, employing both in vitro and in silico analyses.
3) The study assessed the concentrations of biomarkers (Angiotensinogen, homocysteine, Angiotensin-(1-7), and Plasminogen activator inhibitor-1) associated with the identified genetic variants in patients and compared them with the control group using ELISA kits.

Chapter Two

Literature Review

2. Review of Literature

2.1. The family of Coronaviridae

The family Coronaviridae is defined a monogeneric family of RNA-containing pathogens that has been associated to a variety of illnesses in both laboratory and domestic animals as well as respiratory disorders in humans. The family was given its name to describe the unique fringe of crownlike projections that can be seen around the viruses under an electron microscope; these extensions are club-shaped rather than sharp or pointed, as is the case with myxoviruses. Coronaviruses have a diameter ranging from 80 to 160 nm and contain vital lipids(Monto, 1989; Liu *et al.*, 2021). Animals from all over the world can be infected by members of this family, which was previously known as the myxovirus group despite the fact that it had several anomalous traits(McIntosh, 1974; Lehtinen *et al.*, 2022).

2.2. Taxonomy

In 1968, electron microscopy revealed the coronavirus to have a crown-like shape, for this reason, it was renamed to the coronavirus(Cunningham *et al.*, 1968; Chinedu *et al.*, 2022). Coronaviridae viruses have large enveloped single-stranded RNA genomes ranging from 25 to 32 kb. This family was classified by the International Committee on Virus Taxonomy(Adams *et al.*, 2017).

The subfamilies Letovirinae and Orthocoronavirinae are identified, orthocoronaviridae is further subdivided into four genera based on phylogenetic studies and comparative genome studies: Alphacoronavirus (CoV), Betacoronavirus (CoV), Gammacoronavirus (CoV), and Deltacoronavirus (CoV), Currently, there are 17 α-CoV species, 12 β-CoV species, 2 γ-CoV species, and 7 δ-CoV species(Chen B. *et al*., 2020). While Letovirinae includes the Alphaletovirus genus(Kielian *et al.*, 2018). Alpha- and Beta-coronaviruses impact mammals, while Gamma-coronaviruses lead to

avian diseases. In contrast, Delta-coronaviruses, on the other hand, have limited mammalian infection and primarily target birds(Li F. 2016; Cui J. *et al.* , 2019).

2.3. Human Coronaviruses

There are numerous human Coronaviruses identified to date, including HCoVs, including HCoV-NL63 and HCoV-229E, as well as SARS-CoVs and MERS-CoVss. The SARS-CoV and MERS-CoVs viruses are the most frequent human infections that cause pneumonia in humans that may be deadly(Drosten *et al*., 2003; Yin and Wunderink, 2018). There are seven human-infecting CoVs, including SARS-CoV-2. More than 50 years after the first isolation of a prototype murine coronavirus, numerous animal species, including humans, have been infected by coronaviruses, with only the common cold as its only known human disease, coronaviruses were thought to be nearly extinct, though it was not widely known until 2003, this virus eventually came to be identified as a cause of severe acute respiratory syndrome (SARS)(Weiss and Navas-Martin, 2005).

The SARS-CoV infections can cause a wide range of illnesses in humans, mostly in the respiratory and digestive tracts. Diseases like the common cold can be mild or very bad. They range in severity from mild to very bad, from pneumonia to renal disease, to bronchitis to the central nervous systems of mammals(Wevers and van der Hoek, 2009).

Coronaviruses are a kind of enveloped positive-strand RNA virus that may infect humans and other animals(Masters, 2006; Frye *et al.*, 2022). In the past, it was believed that these viruses were the primary cause of respiratory and gastrointestinal diseases in domestic animals and that they were also responsible for around 15% of all cases of the common cold(Perlman and Netland, 2009) then, a few years later, with Middle East respiratory disease (MERS) in Saudi Arabia in June 2012, more than 2260 human cases of confirmed MERS-CoV infection(Zumla *et al.*, 2015; Bleibtreu *et al.*, 2020).

Each time, the virus was classified using information gleaned from a sequence-based family classification(Van Boheemen *et al.*, 2012). SARS-CoV-2 has currently undergone genome sequencing analysis, and the results showed 96.2% overall genome sequence identity with Bat CoV RaTG13, indicating that Bat CoV and human SARS-CoV-2 may have a common ancestor(Zhou P. *et al.*, 2020a).

2.4. Transmission Mode of SARS-CoV-2

A virus that originated in animals has evolved into primarily from one human to another human transmission mechanism(Ye Z. *et al*. 2020). It is possible to spread coronavirus by inhaling aerosols from virus-infected surfaces, as well as by sneezing, coughing, or talking(Zingade *et al.*, 2021). It is possible to become infected with SARS-CoV-2 by touching an infected surface, inhaling a droplet, or by infecting the nose, mouth, or eye with the virus (Khatod *et al.*, 2020). The COVID-19 infection and transmission to infants is considered to be low at this time , due to the fact that women who are pregnant with SARS-CoV-2 infections are not at a greater risk of contracting COVID-19 and transmitting it to their children, which typically occurs in the third trimester of pregnancy(Dashraath *et al.*, 2020). Most reported series have had difficulty determining the virus particle dose required to cause a true infection with COVID-19 in the patient(Wiersinga *et al.*, 2020).

There are two types of COVID-19 transmission: direct transmission and indirect transmission. As part of the direct transmission mode, (1) aerosols are produced during surgical and dental procedures and/or in the form of respiratory droplets; (2) feces, saliva, urine, semen, and tears serve as transmission vehicles; and (3) mothers and children are carriers. There are several indirect transmission mechanisms that can occur at work, including fomites (e.g., furniture, fixtures) and objects used on an infected person (e.g., stethoscopes, thermometers)(Karia *et al.*, 2020).

2.5. Genome Structure

Around 79 % of the "SARS-COV-2" genome sequence is shared with the SARS-CoV and approximately 50 % with MERS-CoVs(Li X. *et al*., 2020; Lu R. *et al*., 2020; Sheikhshahrokh *et al*., 2020) as in figure (2-1).

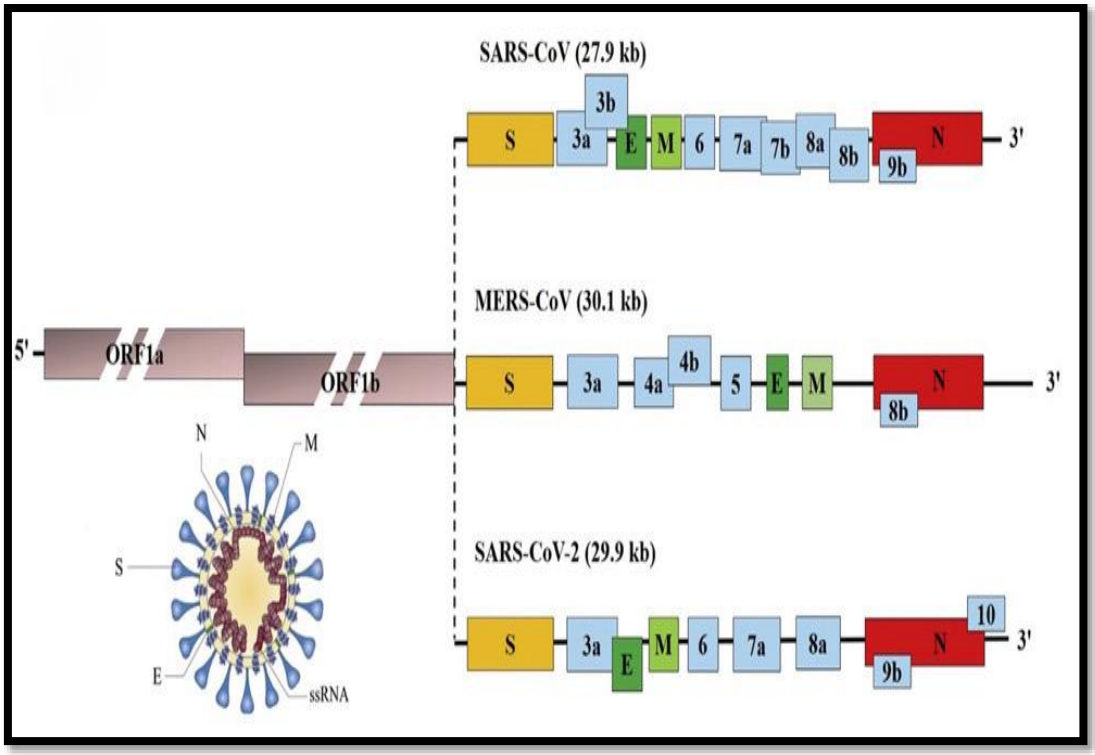


Figure (2-1)Genomes structures of SARS-CoV, MERS-CoVs and SARS-CoV-2(Li X. *et al*., 2020).

The genome is composed of the following elements: 5′ UTR, replicase (ORF1a/ORF1b), four structural genes, 3′ UTR, and a poly (A) tail. The ecological diversity of coronaviruses is characterized by their high rates of genetic recombination and mutation(Cui J. *et al*., 2019). The SARS-CoV-2 is characterized by a single-stranded, positive-sense RNA structure. Its genome consists of 14 distinct open reading frames (ORFs), which collectively code for 27 proteins. Among these proteins, there are four major structural ones: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N)(Nelson *et al*., 2020; Wu A. *et al*., 2020) as in figure (2-2). Interspersed between these structural genes are ORFs responsible for encoding non-structural proteins, such as papain-like protease, 3-chymotrypsin-like protease, RNA-dependent RNA

polymerase, and helicase. Additionally, the SARS-CoV-2 genome contains accessory proteins, the functions of which during infection are still not fully understood(Chan, J.F. *et al*., 2020).

When a cell is infected ORFs 1a and 1b are translated into two large polyproteins, pp1a and pp1ab, of approximately 490 and 790 kDa, respectively. The pp1a and pp1ab polyproteins are found in a viral genomic region and both encoded by the ORF1a and ORF1ab genes, respectively. pp1a occupied by about two-thirds of the virus first 20 kilobases to its 5' end, and they encode non-structural proteins (nsps), such as NSP1 to NSP11, while pp1ab polyproteins correspond to NSP12 to NSP16, while encode the remaining 10 kb of structural proteins, ORF3a, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14, and ORF10 genes were added to the structural genes.

In coronaviruses the S protein enables the virus's recognition of the receptor on the surface of the host cell and the subsequent fusion of the membrane(Li, 2016; Zhang Q. *et al.*, 2021). The E and M proteins played a key role in the development of the viral envelope, virus packaging, and viral particle budding(Neuman *et al*., 2011; Schoeman and Fielding, 2019). The N protein is a multifunctional protein with a variety of functions in the viral life cycle(Chang *et al.*, 2014; McBride *et al.*, 2014).

The RNA translation is aided by a 5' cap and a 3' poly (A) at both ends of the genome(Fehr and Perlman, 2015). Through a leader sequence containing numerous loop structures, the genome virus 5' terminus aids in RNA replication and transcription. The 3'-end is necessary for viral RNA replication and expression, whereas accessory proteins contribute to viral pathogenesis but are not required for replication mechanisms(Zhao *et al.*, 2012). When a genome is replicated, it produces multiple copies of itself; when it is transcribed, it produces the mRNA that encodes the viral structural and accessory proteins(Enjuanes *et al.*, 2006). Genome replication in coronaviruses is a continuous synthesis process that employs a full-length complementary

negative-strand RNA as the template for the formation of progeny virus genomes, similar to that of other positive-strand RNA viruses(Sola *et al.*, 2011).

According to bioinformatics analysis, the SARS-CoV genome contains 11 genes encoding 14 proteins located in (29.757 kb). The SARS-CoV-2 genome, which was published in March 2020 on the Gene Bank database, is approximately 29.903 kb in length and encodes for ten proteins organized similarly to the MERS genome, while the MERS genome contains eleven proteins organized similarly to the SARS genome (29.903 kb)(Hassanin *et al.*, 2021).

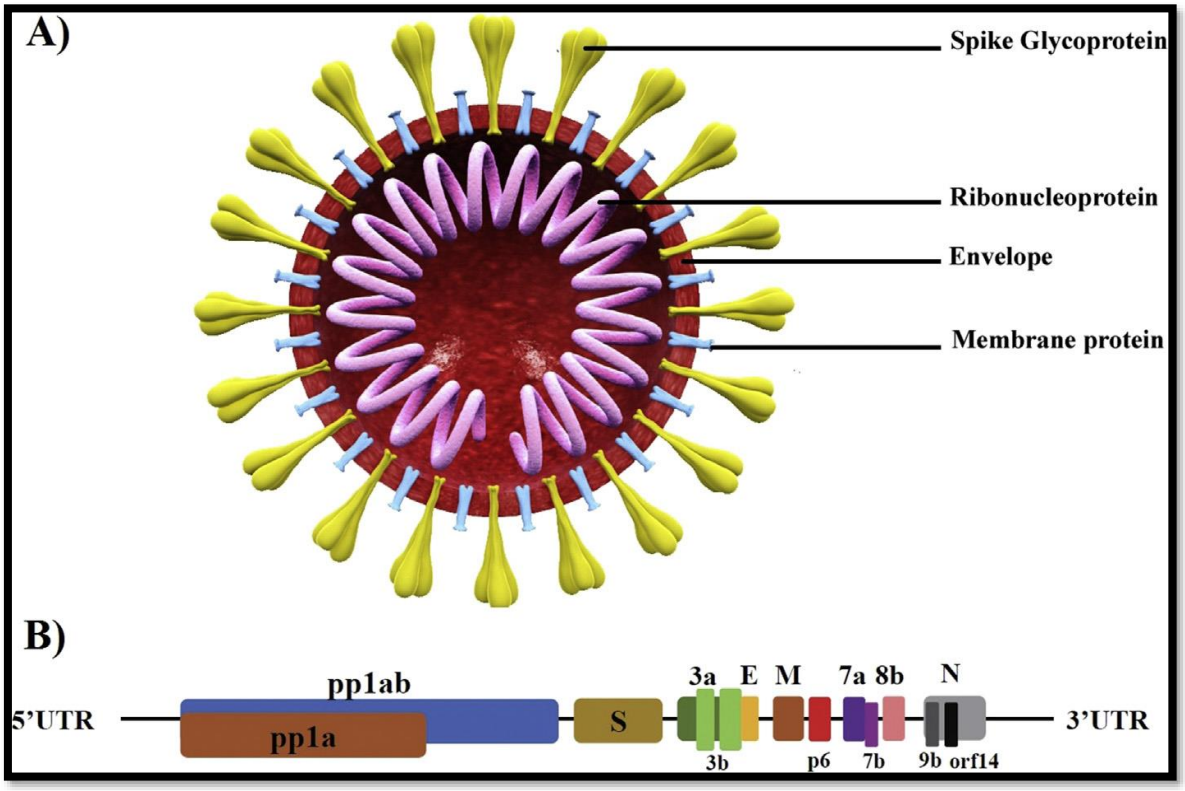


Figure (2-2) the SARS-CoV-2 genome. Figures A and B showed the SARS-CoV-2 genome organization (Wuhan from NCBI, accession number NC 045512.2), as well as the virus's structural proteins ,components that include open-reading frames (ORFs)(Wu A. *et al*., 2020).

2.5.1. Spike Protein

In SARS-CoV-2, the S protein is a transmembrane fusion protein composed of two subunits called S1 and S2, S protein is a structural

glycoprotein(Mittal *et al.*, 2020). It is between 180 and 200 kDa in size and has a homotrimer structure(Huang Y. *et al*., 2020), and contains between 1273 and 1800 amino acids in total(Yesudhas *et al*., 2021). Like SARS-CoV, SARS-CoV-2 has an N-terminal and a C-terminal domain, as well as two major subunits (S1 and S2), a viral entry process is mediated by this protein(Li F. , 2016 ; Huang Y. *et al*., 2020).

The N-terminus contains the signal peptide(1-13), while the S1 and S2 subunits are responsible for membrane binding and fusion, with the S1 subunit (14–685 residues) and the S2 subunit (686–1273 residues) respectively, the virus infects the host and is recognized by the ACE-2 receptor(Huang Y. *et al*., 2020), by cleaving the protein into S1 and S2 subunits and fusing it to the membrane target cell, assisting the proteases in activating the S protein(Bertram *et al.*, 2013; Hoffmann *et al.*, 2020). When the virus enters the target cell, the S1 subunit binds to the sugar and *ACE2* receptors(Huang X. *et al*., 2015), while the S2 subunit undergoes conformational and fusion state changes(Millet and Whittaker, 2015). The SARS-CoV-2 S protein of C-terminal subunit called S2 that facilitates fusion between the virus and host, and an N-terminal subunit called S1 that interacts with receptor-binding domain (RBD) of Angiotensin converting enzyme2(Walls *et al.*, 2020; Wrapp *et al.*, 2020). The spike protein may be involved in pathogenesis via the endoplasmic reticulum's stress response(Liu D. *et al*. 2021).

2.5.2. Membrane Protein

Among all of the proteins in coronaviruses, the M protein is the most prevalent(Alsaadi and Jones, 2019). The M protein, which is 25-30 kDa in size, is abundant in the virion. The N-terminus of the M protein contains an ectodomain, while the C-terminus contains an endo-domain, it determines the virion's appearance(Nal *et al*., 2005; Arndt *et al*., 2010).

The M protein is present in the virion as a dimer and is involved in maintaining the curvature of the membrane and nucleocapsid binding. The coronavirus M protein is a critical component of virus assembly, converting cellular membranes into workshops where viral and host components work together to create new virus particles(Neuman *et al.*, 2011). The M protein is the virus's most abundant structural protein and is thought to be the core coordinator of coronavirus assembly because it interacts with all of the other key coronaviral structural proteins(Schoeman and Fielding, 2019).

In addition to stabilizing the nucleocapsid (the N protein-RNA complex), binding of M to N aids in the completion of viral assembly(Escors *et al.*, 2001). As a result, the SARS-CoV-2 M protein is critical for maintaining the virus's morphology and for stabilizing the positions of other structural proteins and the coronavirus's size(Cao Y. *et al*., 2022). The M proteins of the coronavirus envelope are the most important. Structure proteins interact with each other and played a critical role during viral assembly. In the endoplasmic reticulum–Golgi intercellular compartment (ERGIC) Coronaviruses mature, but the mechanisms by which M proteins are transported from the ER to the budding site remain a mystery(Perrier *et al.*, 2019).

2.5.3. Envelope Protein

Virion maturation, assembly, and egress are all aided by the multifunctional E protein(Sarkar and Saha, 2020). The E protein is the smallest and has the fewest copies of all the membrane proteins identified in the mature virus's lipid envelope(Ruch and Machamer, 2012; Schoeman and Fielding, 2019). It is, nonetheless, necessary for the pathogenesis of other human coronaviruses(Almazán *et al.*, 2013). Despite the fact that the E protein encoded by sgRNA has a low copy number in mature viruses, it is one of the most widely produced transcripts(Brant *et al.*, 2021). Despite its short size (between 76 and 109 amino acids depending on the CoV), E protein contains

multiple active motifs. The addition or deletion of E protein in different CoVs has resulted in viruses with distinct behaviors and altered virus–host interactions, such as the activation of stress and unfolded protein responses, or changes in cellular ion concentrations caused by E protein's ion channel activity. All of these actions have a significant impact on the pathogenesis of CoV(Nieto-Torres *et al.*, 2014). The E protein also played a role in causing membrane curvature and preventing M protein aggregation. Virions are assembled and transported to the cell surface in vesicles, where they are discharged by exocytosis(Malik, 2020).

2.5.4. Nucleocapsid Protein

Nucleocapsid protein (N protein) is the primary antigen of the virus for development of sensitive diagnostic assays of COVID-19(Khan W.H. *et al*., 2022). The coronavirus nucleocapsid (N) is a structural protein that forms complexes with genomic RNA, interacts with the viral membrane protein during virion formation, and aids virus transcription and assembly efficiency(McBride *et al*., 2014). The nucleocapsid (N) protein of the severe acute respiratory syndrome coronavirus (SARS-CoV) is the most abundant protein in virus-infected cells and It's a protein with a lot of different functions. The N protein of SARS-CoV-2 is a 45.6 kDa phosphoprotein that consists of an N-terminal domain (NTD) and a C-terminal domain (CTD)(Chang *et al.*, 2014; Ya Peng *et al.*, 2020).

Two RNA-binding domains are joined by a central spacer that contains a serine- and arginine-rich (SR) region in the coronavirus nucleocapsid (N) protein(Koetzner *et al.*, 2022). Unmodified N protein forms a structured oligomer suited for nucleocapsid assembly, whereas phosphorylated N protein creates a liquid-like compartment suitable for viral genome processing, the coronavirus nucleocapsid (N) protein has two main functions: it compacts the RNA genome in the virion and regulates viral gene transcription(Carlson *et al*.,

2020). One study's findings demonstrated that the viral proteins of SARS-CoV-2, including ORF6, ORF8, and nucleocapsid proteins, have the potential to act as inhibitors of the type I interferon signaling pathway, which is a crucial component of the host's innate immune system's antiviral response(Li, J.-Y. *et al.*, 2020).

2.6. Life Cycle of SARS-CoV2

2.6.1. Coronavirus's Primary Reservoirs and Hosts

It is critical to understand the source of infection and its spread in order to design preventive methods for infection containment. In the case of SARS-CoV, researchers first concentrated on raccoon dogs and palm civets as potential infection reservoirs. However, only samples isolated from civets in the food market were positive for viral RNA, implying that the civet palm may serve as a secondary host(Kan *et al.*, 2005). A molecular analysis of samples obtained from healthy Hongkong residents in 2001 revealed a 2.5% prevalence of antibodies against the SARS Coronavirus. According to these findings, the SARS-coronavirus circulated in humans before the 2003 outbreak(Zheng B.J. *et al*., 2004).

Later on, anti-SARS-CoV antibodies are discovered in Rhinolophus bats, implicating the bats as a source of viral replication(Shi and Hu, 2008). Coronavirus, which causes Middle East respiratory syndrome (MERS), was detected the Kingdom of Saudi Arabia for the first time in 2012(Memish *et al.*, 2013).

The MERS-coronavirus is also associated with beta-coronavirus and camels as a source of infection or primary host(Paden *et al.*, 2018). However, numerous observations, combined with the virus's sequence identity, have called attention to the virus's potential zoonotic origin, after SARS-CoV, MERS-CoVs, and Ebola virus, the SARS-CoV-2 infection may be another significant example of the One Health idea, as there is excellent overlap in

human, animal, and environmental health (Hemida, 2019). Numerous investigations indicated that bats are a common reservoir for SARS-CoV-2 since viruses obtained from Chinese horseshoe bats exhibited close similarities to SARS-CoV sequences(Fan Y. *et al*., 2019). There are around 200 coronaviruses found in diverse bat species (Chen L. *et al*., 2014).

In 2003, the SARS-primary CoV's bat reservoirs were identified (Guan Y. *et al*. 2003). Meanwhile, numerous bat species, including *Taphozous perforatus, Rhinopoma hardwickii, and Pipistrellus kuhlii*, were thought to represent the MERS-forebears (Lau *et al.*, 2018). The spread of SARS-CoV-2 may have started in what is known as Huanan Seafood Market located in Wuhan, South China, a live-animal market(Tiwari *et al.*, 2020).

2.6.2. Entry of Virus

The coronaviruses employ the spike (S) protein to help them enter target cells, the surface unit of the S protein, S1, must bind to a cellular receptor in order for the virus to attach to the surface of target cells(Hoffmann *et al.*, 2020). The receptor-binding domain (RBD) in the S1 subunit of spike protein binds to *ACE2*, which is expressed on the surface of epithelial cells in the respiratory and gastrointestinal systems, to start the SARS-CoV-2 life cycle(Zhou P. *et al*., 2020a). This virus is infecting the host when it fuses directly with the membrane of the cell, causing it to become infected and/or endocytosis using the spike protein's S2 subunit(Bestle *et al.*, 2020; Hoffmann *et al.*, 2020).

The heptad repeats parts of the transmembrane S2 domain, as well as the fusion peptide, improve the fusing of viral and cellular membranes after considerable conformational rearrangements(Tortorici and Veesler 2019; Letko *et al.*, 2020). Shortly after the 2002–2003 SARS-CoV outbreak, *ACE2* was discovered to be the functional receptor that permits SARS- CoV infection(Li W. *et al*. 2003). The *ACE2*, a cell surface receptor for SARS-CoV-2, is genetically and structurally similar to SARS-CoV's S proteins (76% amino

acid identity)(Walls *et al.*, 2020). The SARS- CoV-2 RBD was found to have a similar or greater affinity for *ACE2* as the SARS- CoV RBD(Lan *et al.*, 2020; Shang J. *et al.*, 2020). A transmembrane protease, serine 2 (TMPRSS2) is required by the virus at the time of entry into the host in order to separate the virus's S-spike and attach to the cell membrane efficiently, S1 and S2 of the Coronavirus S-spike are separated by these serine proteases(Wrapp *et al.*, 2020).

A common misunderstanding is that SARS-CoV-2 only attaches to *ACE2*, however, attachment to only this receptor would prevent the virion from delivering the RNA into the cell, as viral entry requires attachment to both the *ACE2* receptor (S1-part of spike) and the membrane (S2-part of spike), thus human serine proteases are critical in altering the configuration of the S-spike in order for the virus to attach successfully(Mönkemüller *et al*., 2020).

The spike protein is generated as an inactive precursor, and repeated cleavages by cellular proteases cause conformational changes in the S2 subunit, allowing the spike protein to become functional and ready for membrane fusion(Chakraborty and Bhattacharjya, 2020).

The TMPRSS2 and lysosomal proteases have been determined to be required for virus entry (Ou *et al.*, 2020). SARS-CoV-2 to enter host cells, viral spike proteins must attach to the host receptor protein *ACE2* and be primed by the host serine protease, cell surface transmembrane protease serine 2 (TMPRSS2)(Shang J. *et al*. 2020; Hoffmann *et al*., 2020). In both pulmonary and non-pulmonary organs, SARS-CoV-2 viral RNA and viral replication events were found. Look for changes in viral load and cytopathic consequences across organ systems, between people, and within the same patient to do this. The presence of host factors like *ACE2*, TMPRSS2, and AR has also been linked to infection sites (Wang *et al*., 2021).

Transcribing the genome upon entry, the coronavirus genome is released into the host cell cytoplasm, initiating a complicated program of viral gene

expression that is highly controlled in space and time. ORF1a and ORF1b are translated from genomic RNA to generate two polyproteins, pp1a and pp1ab. The latter occurs as a result of a designed –1 ribosomal frameshift at the ORF1a–ORF1b overlap(Perlman and Netland, 2009). According to ribosome profiling, SARS-CoV-2 is capable of frameshifting between ORF1a and ORF1b with a 45-70% efficiency(Finkel *et al.*, 2021), which is comparable to the efficiency found for mouse hepatitis virus (MHV)(Irigoyen *et al.*, 2016).

Assembly and translation occur following viral RNA synthesis, which generates both genomic and sub-genomic RNAs. Sub-genomic RNAs function as messenger RNAs for accessory and structural genes located downstream of replicase polyproteins. Negative strand intermediates are used to synthesize both genomic and sub-genomic RNAs. Finally, coronaviruses are unique in their ability to recombine via homologous and nonhomologous recombination(Malik, 2020).

2.7. Immune Response of the Host to COVID 19

Once the virus gains entry into the target cell, the host immune system recognizes the entire virus or specific epitopes on its surface, eliciting an innate or adaptive immune response. The virus is initially recognized by pathogen recognition receptors (PRRs) on immune cells, primarily Toll-like receptors 3, 7, and 8, which results in increased interferon (IFN) production. The non-structural proteins of SARS-CoV and MERS-CoVs impair the function of host innate immune cells, thereby impairing overall cytokine production(Le Bon and Tough 2002; Hu W. *et al.*, 2012). Pathogenesis of COVID-19 is complex, and virulence and pathogenicity are also related to the activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome by the virus. A pro-inflammatory cytokine examples include interleukin (IL-1 and IL-18) is released by macrophages, epithelial cells, and possibly endothelial cells when the inflammasome is activated. They are responsible for the pathogenic

inflammation that leads to COVID-19 symptoms(Deftereos *et al.*, 2020; Shneider *et al*., 2020).

Dendritic cells, as part of their normal patrolling function, have a strong ability to phagocytose dying and apoptotic cells, such as those infected with SARS-CoV-2. They also enhance their migration to draining lymph nodes by upregulating chemokine receptors. In these lymph nodes, they activate naive B and T cells, leading to the production of antiviral antibodies and effector T cells. These effector T cells, both CD4+ and CD8+, can then return to inflamed tissues to inhibit virus replication by releasing antiviral cytokines like IFN-γ and inducing antigen-specific cell death(Piconese *et al.*, 2014).

The SARS-CoV-2 virus proteins specifically target several innate immunes signaling proteins. Nsp13, Nsp15, and open reading frame (ORF)9b all target the interferon (IFN) pathway, whereas Nsp13 and Orf9c all target the NF-B pathway. SARS-CoV-2 Orf6 impedes the nuclear export of NUP98-RAE1, an interferon-inducible mRNA complex. The SARS-CoV-2 Orf3b and Orf9c are replication-canonical. As previously stated, these findings will aid in the discovery of antivirals against SARS-CoV-2. Remdesivir is one of the antiviral medications available. It is a nucleoside analog RNA-dependent RNA polymerase (RdRP) inhibitor indicated to treat COVID-19 infections(Gordon *et al.*, 2020).

The COVID-19 is frequently associated with lymphopenia, particularly in more severe cases, characterized by a decrease in both CD8+ and CD4+ T cells (Liu J. *et al*., 2020; Oja *et al*., 2020). The CD4+ and CD8+ T cells played a critical role, CD4+ T cells activate B cells to promote the production of virus-specific antibody, while CD8+ T cells can kill viral infected cells(Yuki *et al.*, 2020).

It remains unknown whether the T-cell response played a role in disease pathogenesis, infection recovery, or both. Additionally, studies have focused on various aspects of the T-cell response or on patients at various stages of

infection, resulting in a lack of clarity regarding the big picture. T-cell responses in critically ill patients are frequently robust and comparable to or superior to those in non-critically ill patients(Thieme *et al.*, 2020). In severe cases, when compared to moderate cases, more CD4+ and CD8+ T-cell responses were found, both in breadth and magnitude, CD8+ T cells, however, accounted for a bigger proportion of T-cell responses to SARS-CoV-2 structural proteins in mild cases than in severe cases, implying that the CD8+ T-cell response may be beneficial, whereas the CD4+ T-cell response may contribute to pathogenesis(Yanchun Peng *et al.*, 2020). The SARS-CoV-2 infects human T-cell lines via a new pathway via the CD147 spike protein found on the surface of T cells ,the transmembrane protein, as a novel pathway for SARS-CoV-2 entrance(Wang X. *et al*., 2020).

The CD147 (a.k.a. basigin or EMMPRIN) is expressed in a wide variety of organs and cells and is involved in cell proliferation, apoptosis, tumor cell migration, metastasis, and differentiation, particularly under hypoxic settings. A second CD147 isoform, designated CD147 Ig0-Ig1–Ig2, has also been identified. Preventing SARS-CoV-2 spike binding and infection with meplazumab, a CD147 protein blocker, may be helpful in the treatment of COVID-19(Ulrich and Pillat, 2020). Notably, multiple investigations have showed that patients who are more critically ill create larger amounts of antibody, particularly IgG, to S or N than do individuals with milder infections(Hashem *et al.*, 2020; Rijkers *et al.*, 2020). Antibody levels and severity of infection have been found to be related in a similar manner for infections caused by other viruses, including SARS-CoV-1 and MERS-CoVs(Lee *et al*., 2006; Choe *et al*., 2017). This is most likely because increased antigen exposure, in combination with larger or more persistent viral loads, generates a stronger antibody response. However, antibody reactions may potentially contribute to the etiology of COVID-19. Despite the fact that neutralizing antibodies are likely adequate to prevent infection or influence

disease course if administered early after infection, the significance of neutralizing antibodies in pathogenesis remains unclear(Moss, 2022).

Infections caused by SARS-CoV-2 may or may not be prevented by SARS-CoV-2-specific T cells. In light of the fact that viruses change under immunological pressure, it is crucial to remain vigilant for strains of SARS-CoV-2 that are able to evade infection-induced or vaccine-induced humoral or cellular immunity. It remains unclear whether immunity to other Coronaviruses had an effect on COVID-19 results and whether exposure to SARS-CoV-2 could elicit an immune response when the virus wasn't reproduced clearly. Due to the brief period of contact with the pathogen, the duration of both humoral and T-cell immunity after infection is difficult to predict(Forthal, 2021).

2.8. Clinical Picture

2.8.1. Signs and Symptoms

The SARS-CoV-2 infection can have a wide range of clinical consequences, some persons infected with SARS-CoV2 remain asymptomatic, while others develop mild to moderate COVID-19 pneumonia, this can result in some individuals requiring extensive care and, in severe situations, death, particularly in elderly persons(Struyf *et al.*, 2021). COVID-19 is most frequently accompanied with a sudden onset of fever, coughing, and dyspnea(Jiang *et al.*, 2020). One or more of the following can occur as complications: acute respiratory distress syndrome (ARDS), pneumonia, renal failure with infection opportunistic problems such as bacterial superinfections and major hazard such as coagulation abnormalities and thromboembolic events, sepsis, or death(Llitjos *et al.*, 2020; Yang X. *et al.*, 2020).

Long COVID-19 is a term that refers to persons who have recovered from the acute phase of COVID-19 but continue to experience enduring consequences of the infection, have had the predicted clinical picture for a much longer period of time than expected, or have developed new symptoms

and signs. Two distinct manifestations of this illness have been observed: (a) a severe form associated with thromboembolic consequences, such as: and (b) a nonspecific type, which is frequently accentuated by weariness and dyspnea(Greenhalgh *et al.*, 2020).

When it comes to cardiovascular disease risk factors, arterial hypertension (50 %), high cholesterol (28 %), and type 2 diabetes mellitus were the most common conditions (22%). Other Asthma (14.4%), sleep apnoea/hypopnea (8.5%), and other comorbidities were found. Chronic obstructive pulmonary disease (COPD) syndrome (6%), heart failure (6%), and chronic kidney disease (6%)(Rosales-Castillo *et al*., 2021).

In the cohorts studied, the prevalence of protracted COVID-19 varied between 4.7 and 80 %, and the most common clinical symptoms were chest pain (reported by up to 89% of patients), fatigue, dyspnea, coughing, and sputum production. Temporal criteria for defining protracted COVID-19 differed significantly between three and twenty-four weeks following the acute phase and/or hospital discharge. Advanced age, female sex, a large number of comorbidities and poor clinical condition, hospitalization, and oxygen supplementation during the acute phase were all considered to be associated with prolonged COVID-19 survival Nevertheless, none of the research examined the duration of persistent signs/symptoms(Cabrera Martimbianco *et al*., 2021).

2.8.2. Epidemiology

The COVID-19 virus is isolated in Wuhan, China, when the first cases were discovered, towards the end of 2019, the infection spread to the majority of countries. Positive infections have been reported in excess of 300 million people. On the World Health Organization and European Centre for Disease Prevention and Control websites, you can access the most recent cases(McIntosh *et al*., 2021).

The SARS-CoV-2 spread to other provinces of China in mid-January 2020, owing to the Spring Festival travel season. SARS-CoV-2 was spread by international travelers from China to other countries. It was transmitted from the source of infection to other countries via travel; the first case was confirmed in Thailand on 13 January 2020, followed by another in Japan; by the end of January, there were 2106 infected cases, with infection present in more than 20 countries(World Health Organization, 2020).

Coronaviruses appear to have a significant role in the history of the twenty-first century. This century has seen the isolation of five of the seven human coronaviruses. Regrettably, the last three of them entered lives amid fears of pandemic, breakout, or death. SARS CoV-2, the most recent human coronavirus to originate from Wuhan, China, and its clinical manifestation, Coronavirus disease (COVID-19), have lately gained a considerable foothold in daily practice. Initially, reports indicated that it was caused by bats, in epidemiology, an epidemic curve of infection is a statistical chart used to depict the commencement of a coronavirus outbreak. There are three phases in an epidemic curve: rising, plateauing, and falling. Phase of growth This period is influenced by a variety of factors, including country demographics, age distribution, health system preparedness for an epidemic, implementation of some preventive measures, country response time to a pandemic, and society's reaction to new implementing policies. Distinct countries can exhibit markedly different curve patterns, complicating any attempt to predict a country's pandemic trend. However, it appears that this period is normally between three and four weeks for COVID-19, during Plateau phase: Disease incidence is steady during this phase. COVID-19, according to daily nation reports, takes two to three weeks to complete while Decreasing phase: which indicates that disease activity could be observed at extremely low levels two or three weeks later(Bulut and Kato 2020; Tashiro and Shaw 2020;Guthrie *et al*., 2021;)

Symptoms typically disappear within 1–2 weeks in most COVID-19 cases, SARS-CoV-2 infection can result in five distinct outcomes: asymptomatic infection (1.2%), mild to moderate illness (80.9%), severe infection (13.8%), critical infection (4.7%), and death (2.3 % in all reported cases)(Team E., 2020). Human CoVs continue to pose a serious threat to global health. However, humans have lacked sufficient experience in previous battles against SARS and MERS, following the outbreak of COVID-19 in China, SARS-CoV-2 gained global attention as a significant pathogen associated with respiratory tract infection(Jin Y. *et al*., 2020).

2.9. Markers of COVID-19 Infection and Severe Progression

Many of biomarkers identify abnormalities between the severe cases and compared to mild/moderate such as hematologic, biochemical, inflammatory, coagulation, and immune biomarker, furthermore, homocysteine and Angiotensin II levels are significantly elevated in COVID-19 patients who had severe disease according to a recent study(Ponti and Ruini 2020).

2.9.1. Hematologic Biomarkers

Patients with COVID-19 are stratified based on a number of hematologic biomarkers, including a count of white blood cells, lymphocytes, neutrophils, neutrophil to lymphocyte ratio (NLR), platelets, eosinophils, and hemoglobin. Patients with severe COVID-19 infections, as in studies Yang Y. *et al*. (2020) and Chen N. *et al*. (2020), had lymphopenia rates of 80% and 25%, respectively. These findings suggest a correlation between lymphopenia and infection severity.

According to Qin C. *et al*. (2020) an analysis of 450 patients infected with COVID-19 and markers associated with immune dysregulation revealed that severe cases had lower lymphocyte counts, higher leukocyte counts, higher NLR counts, as well as lower monocyte, eosinophil, and basophil percentages

in comparison with mild cases. Henry B. *et al.* (2020) conducted a meta-analysis of 21 studies that included 3377 COVID-19 positive patients. The study found that patients with severe or fatal diseases had significantly higher WBC and lower lymphocyte and platelet counts than those with mild or non-severe diseases.

The COVID-19 patients with mild-moderate disease had both low levels of helper T cells and suppressor T cells, with higher levels of helper T cells being associated with severe disease. A higher percentage of naive helper T cells was observed in severe cases, while a lower percentage of memory helper T cells was observed. In addition, those with COVID-19 have a lower level of regulatory T cells, which are more clearly damaged when the disease is severe(Cossarizza *et al.*, 2020).

It is essential that cytotoxic lymphocytes, such as cytotoxic T lymphocytes (CTLs) and natural killer cells (NKs), are present in order to control viral infection, and its functional exhaustion correlates with an increase in disease severity(Zhang C. *et al.*, 2019). In confirmed cases of COVID-19, laboratory examinations revealed abnormally low mean lymphocyte counts(Luo *et al.*, 2020). In study showed 66 % of 32 COVID-19 patients studied, the eosinophil count decreased, positive correlation exists between eosinophil and lymphocyte counts ($r = 0.305$, p .001)(Yun *et al.*, 2020). Among 140 COVID-19 patients, 52.9% had eosinopenia (0.02 109/L) and in both mild ($r = 0.449$, p.001) and severe ($r = 0.486$, p.001) cases of COVID-19, eosinophil count was positively correlated with lymphocyte count(Zhang J. *et al.*, 2020). In order to determine the sensitivity and specificity of the eosinophil count in COVID-19, further research is required, including larger patient cohorts(Li Q. *et al.*, 2020).

While platelets count Numerous studies have demonstrated a correlation between low PLT levels and the severity of COVID-19, making the PLT count a valuable prognostic indicator for COVID-19 patients. In a recent meta-

analysis, Bashash *et al.* (2020)found that thrombocytopenia significantly increases the risk of disease progression and suggested that daily monitoring of platelet count can accurately reflect the outcome of COVID-19 patients.

Lippi (2020) found in a meta-analysis on 1779 patients noticed that a decrease in the PLT count is linked to an increased mortality rate. Platelets in the lungs may be activated by damaged lung tissue or pulmonary endothelial cells, causing them to aggregate and form microthrombi, thereby increasing platelet consumption(Xu P. *et al.,* 2020).

Both CD4+,CD8+, and WBCs are affected by lymphocytopenia in patients with severe disease(Tan M. *et al*., 2020). There is also a marked decrease in monocytes, and an apparent increase in neutrophils and NLR. It is possible to diagnose and identify critically ill patients using these simple parameters(Henry B. *et al.,* 2020).

2.9.2. Biochemical Biomarkers

Three large studies evaluating the primary laboratory changes in patients suffering from severe or fatal COVID-19 examined the differences between survivors and non-survivors. A significant difference was observed between survivors and non-survivors in terms of total bilirubin levels, CK, serum ferritin, white blood cell count, and interleukin-6, the presence of creatinine (CREA), cystatin C (cysC), Direct bilirubin (DBIL), cholinesterase (CHE) and lactate dehydrogenase (LDH), alonag with serum urea could be used to distinguish severe COVID-19 cases from mild COVID-19 cases. There may be some significance in the early diagnosis of severe COVID-19 and the differentiation of it from mild COVID-19 based on serum biomarkers including urea, CREA, and CysC, all of which reflect glomerular filtration function(Ruan *et al*., 2020; Xiang *et al*., 2020; Yang X. *et al*., 2020; Zhou F. *et al*., 2020).

In the majority of severe cases, infection-related biomarkers (procalcitonin, serum ferritin, and CRP) and inflammatory cytokines (IL-2R, IL-6, IL-8, IL-10, and TNF) were elevated(Chuan Qin *et al.*, 2020).

According to study found elevated LDH, a marker of lung tissue damage, is one of the most common biochemical abnormalities in COVID-19 patients admitted to the hospital(Lippi and Plebani, 2020). Numerous studies have demonstrated that patients with mild infection have LDH levels within the reference ranges, in contrast to patients in critical condition(Fan Z. *et al*., 2020).

Also identified as a significant predictor of COVID-19 patient mortality is liver function. Recent research found significant elevations in liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are associated with significant alterations in renal function parameters (blood urea nitrogen, creatinine) and coagulation markers associated with worse outcomes in COVID-19 patients(Krishnan *et al.*, 2022).

2.9.3. Immunity and Inflammatory Biomarkers

Infection with SARS-CoV-2 can cause a "cytokine storm," which is the massive release of pro-inflammatory cytokines that contribute to acute lung injury and poor prognosis(Rokni *et al*., 2020; Cao X. *et al.*, 2020). The majority of parenchymal lesions in vital organs are caused by an increase in inflammatory markers and defects in coagulation processes. When severe COVID-19 patients were first infected, the CRP marker was significantly elevated, even before CT scans revealed critical findings. Importantly, CRP is linked to the development of disease and is a predictor of severe COVID-19(Tan C. *et al*., 2020). A rapid increase in C-reactive protein (CRP) levels precedes respiratory deterioration and intubation, whereas CRP levels plateau in stable patients. Increasing CRP levels during the first 48 hours of admission to hospital are a more sensitive predictor of respiratory decline than initial CRP levels(Mueller *et al.*, 2020).

Interleukin-six (IL-6) and serum ferritin concentrations were significantly higher in non-survivors compared to survivors (WMD: 4.6 pg/ml and 760.2 ng/ml, respectively) and in those with severe disease compared to those without severe disease (WMD: 1.7 pg/ml and 408 ng/ml respectively)(Henry B. *et al.*, 2020). IL-6 levels were elevated in both mild and severe patients, irrespective of comorbidities. Regardless of disease severity or the presence of co-morbidities, the Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) levels were increased. Diabetic patients and patients who developed ARDS possessed elevated levels of D-dimer and LDH. The procalcitonin levels of severely and critically ill patients were elevated to varying degrees(Iwamura *et al.*, 2021). Some of the previously mentioned parameters appear to be associated not only with disease severity but also with mortality(Ruan *et al.*, 2020).

2.9.4. Coagulation Biomarkers

Coagulation abnormalities are associated with a poor prognosis. COVID-19 non-survivor patients have significantly elevated D-dimer and FDP levels(Tang *et al.*, 2020). D-dimer levels appear to be frequently elevated in COVID-19 patients (36–43 %)(Lippi and Favaloro, 2020). It is reported that coagulation abnormalities like disseminated intravascular coagulation (DIC), as well as elevated D-dimer and FDP, are common in COVID-19, but these are rarely seen in other coronavirus infection(Cao and Li, 2020).

Patients with COVID-19 have higher rates of systemic thrombotic complications. It's becoming clear that SARS-CoV-2 immuno-thrombotic responses in the lung microvasculature may played a role in advancing the illness, COVID-19 pneumonia is characterized by fibrin deposition in the lungs, elevated D-dimer levels, and thrombosis rates ranging from 5 % to 30 % despite thromboprophylaxis(Cui *et al*., 2020; Rali *et al*., 2021).

Prothrombin time is linked to disease severity in some large-scale studies, however. In a retrospective analysis of 296 COVID-19 patients (of whom 17 died), the non-survivors' D-dimer and thrombin time were higher and their (aPTT) was lower than those of the survivors(Wang *et al*., 2020).

Tang (2020) examined 207 non-survivor COVID-19 patients and found that non-survivors had significantly higher D-dimer and FDP levels and longer PT at admission than survivors. The COVID-19 patients have a rapid progression to death and a limited ability to benefit from mechanical ventilation, according to this evidence. In some cases, mechanical ventilation protocols for patients with ARDS may actually worsen their condition. On the other hand, full anti coagulation therapy may explain the quick and positive response of some patients(Ponti and Tomasi 2020).

As early as the pandemic began, coagulant biomarkers such as fibrinogen, D-dimer, and aPTT were frequently observed in COVID-19 patients hospitalized for treatment, and these biomarkers are recommended to be routinely measured because in large scale studies D-dimer and PT have been linked to severe disease and mortality(Bonaventura *et al.*, 2021).

2.10. Clinical and Pathological Investigation of COVID-19 Patients

The COVID-19 is a respiratory illness that can result in mild to moderate disease in 80% of cases, severe disease in 15% of cases, and critical illness in 5% of cases. The overall case fatality rate is 0.5–2.8 %, but rates in octogenarians are much higher (3.7–14.8 %)(Team E.E. , 2020). SARS-CoV-2 infection symptoms can range from asymptomatic and mild to pneumonia and life-threatening complications such as acute respiratory distress syndrome (ARDS), septic shock, and multiorgan failure, with the final outcome being death for those who contract the disease(Zhu *et al.,* 2020;Wang *et al*., 2020).

Notably, approximately 11% of individuals with modest symptoms experience a rapid deterioration, leading to severe presentations such as respiratory failure, multiorgan failure, or death. Patients with COVID-19 have been found to have host risk factors that are linked to critical illness and mortality, although early symptoms of disease progression have been documented infrequently(Chen *et al*., 2020). In COVID-19, there is still a lot don't know about how cytokines, pulmonary inflammation, and the links between an abnormal immune response and immunopathology in the lung work. This is important for how treat COVID-19 in the clinic(Li S. *et al*., 2020).

Pulmonary bilateral widespread alveolar destruction with cellular fibro myxoid exudates were found in the first report of pathology results from a severe COVID-19(Xu X. *et al*., 2020). According to Carsana *et al*. (2020) and colleagues, who studied the lungs of 38 COVID-19-deceased patients, diffuse alveolar destruction in the early or middle phases, together with fibrin thrombi in tiny artery arteries, are the most common pathological findings. Other autoptic studies with less instances also demonstrate thrombotic events occurred in association with COVID-19(Fox *et al.*, 2020; Wichmann *et al.*, 2020). Reported in individuals with COVID-19 may enhance the risk of venous thrombosis and pulmonary embolism in critically sick COVID-19 patients receiving deep sedation and neuromuscular blockers for Acute Respiratory Distress Syndrome (ARDS)(Helms *et al.*, 2020; Klok *et al.*, 2020).

Additionally, only a few mononuclear interstitial inflammatory infiltrates were found in cardiac tissue, indicating that SARS-CoV-2 may have no direct effect on the heart(Xu Z. *et al*., 2020). Massive mucus discharge was found in both lungs in COVID-19 fatality cases, which was not reported in SARS or MERS cases(Liu X. *et al.*, 2020).

2.10.1. Direct Effect of SARS-CoV-2 on the Cerebrovascular Events

A hospital network in New York City, the rate of ischemic stroke admission increased sevenfold when compared to a typical flu season, implying a fundamental link between SARS-CoV-2 and cerebrovascular events(Merkler *et al.*, 2020).

The COVID–19 virus is associated with a higher frequency of neurological manifestations, cerebrovascular events have been reported to occur in approximately 3% of patients in the majority of studies conducted worldwide(Mishra *et al.*, 2020). The CoroNerve Study Group in the United Kingdom reported a staggering 62% rate of cerebrovascular events in hospitalized COVID-19 patients in the largest collection of neurologic complications(Varatharaj *et al.*, 2020). Strokes that occur in the context of COVID-19 may be more common in some people than others, based on these findings. Ischemic stroke was observed in 2.3% of 214 patients hospitalized with COVID-19 in a preliminary study from Wuhan, China(Mao *et al.*, 2020). Ischemic stroke was found in 0.9% of 3556 patients with COVID-19 who were hospitalized in New York City(Yaghi *et al.*, 2020).

An increased risk of thrombotic events, including severe cerebrovascular events, has been found in young patients who have COVID-19(Oxley *et al.*, 2020). Studies describing neurological involvement have reported headaches, dizziness, impaired vision, decreased consciousness, agitation, ataxia, and seizures in patients with COVID-19(Kalinsky *et al.*, 2020).

Patients with "COVID-19" diagnoses often suffer a lobar stroke, were more likely to have a cryptogenic cause, and had poorer outcomes(Dhamoon *et al.*, 2021). Cerebrovascular disease risk factors other than the usual suspects have been identified in the context of COVID-19 infection. These include hypercoagulability as a result of infection-induced systemic inflammation or a cytokine storm, as well as endothelial inflammation specific to COVID-19 infection(Benussi *et al.*, 2020).

2.10.2 Direct Effect of SARS-CoV-2 on the Liver

The virus may directly infect liver cells in COVID-19 patients, resulting in liver damage. The research previously conducted on SARS-CoV, which causes SARS, and MERS-CoVs, which causes Middle East respiratory syndrome, revealed that these viruses also affect the liver(Guan W. *et al*., 2020).

The SARS-CoV patients showed 70% decreased lymphocytes, 25% diarrhea, and 66% elevated liver enzymes in their plasma, according to Greenough *et al*. (2005) . In these patients, special attention should be paid to liver enzyme elevations and liver lesions. In deceased SARS patients, liver cells expressing the SARS-CoV protein were discovered. This suggested that liver cells could be infected directly by viruses. Additionally, autopsy findings in SARS patients revealed a high number of mitotic liver cells, balloon degeneration of hepatocytes, mild inflammation, moderate lymphocyte infiltration, steatosis, and central lobular necrosis, all of which were associated with obvious apoptosis(Guo *et al*., 2008).

It is found that approximately 2% – 10% of COVID-19 patients had positive SARS-CoV-2 RNA in feces and blood, which was associated with diarrhea, abdominal pain, nausea, and vomiting, suggesting the virus may infect liver cells during early phases of infection(Yeo *et al*., 2020).The coronavirus that causes SARS uses *ACE2* as a receptor on host cells. Numerous studies one of it (Wang D. *et al*., 2020), have demonstrated that SARS-CoV-2 can also bind to the *ACE2* receptor, allowing it to replicate in cells. Additionally, while *ACE2* expression was low in liver cells (2.6% of total cells), it was highly specific in bile duct cells (59.7%), which is comparable to the expression level of SARS-CoV and SARS-CoV-2 in the lung's major targeted cells (type II alveolar cells)(Zhang H. *et al*., 2020).

As a result, the novel coronavirus doesn't necessarily infect liver cells, but rather, it causes bile-duct dysfunction by binding to bile duct cells, which

played a critical role in liver regeneration and immune response, as a result, surmise that COVID-19-induced bile duct cell damage could lead to liver injury(Chenlu Huang *et al.*, 2020).

A cytokine storm caused by the virus's excessive immune response may also be one of the ways in which liver damage is caused(Cao X. *et al.*, 2020). Patients with a more severe course of disease may be more at risk for COVID-19-associated liver injury than those with a milder injury. COVID-19 multisystem manifestations like ARDS, coagulopathy, and multiorgan failure may have an impact on the liver's ability to produce albumin, acute phase reactants, and coagulation factors(Varga *et al*., 2020; Xie and Chen, 2020). Numerous studies published in the last few months have revealed a possible role for the liver in COVID-19 infection and pathology(Nardo *et al.*, 2021).

2.10.3. Direct Effect of SARS-CoV-2 on Coagulation Disorder and Thromboembolic Disease

Thrombogenic events, which include ischemic stroke, pulmonary embolism, deep vein thrombosis, mesenteric ischemia, and acute coronary syndrome, are significant complications of certain pathological conditions, including hypertension, atherosclerosis, and diabetes mellitus(Willoughby *et al*., 2002). In SARS-CoV-2 patients, coagulopathy has been observed. Coagulation abnormalities, including disseminated intravascular coagulopathy (DIC) and sepsis-induced coagulopathy, have been observed in patients with COVID-19 (SIC)(Connors and Levy, 2020). Coagulation disorders are common in COVID-19, and the severity of these disorders is inversely proportional to the severity of the disease. It is believed that endothelial dysfunction, specifically pulmonary fibrinous microthrombi in alveolar capillaries, is responsible for the imbalance between pro- and anticoagulant mechanisms that occurs when a viral disease is triggered by inflammatory responses(Subramaniam and Scharrer, 2018; Jun Zhang *et al*., 2020). Patients

with a history of coagulation issues are more concerned than other patients about the potential risks of COVID-19(Kirchberger *et al.*, 2021).

In COVID-19 patients with vascular damage are more likely to develop thrombosis, microvascular thrombosis, and hemorrhage(McGonagle *et al.*, 2020), because coagulopathy was common in COVID-19 patients, these systems have values. In COVID-19 patients, dynamic monitoring of coagulation system parameters may be essential for preventing the development of thrombus or death due to DIC. COVID-19 patients with thrombotic disease showed higher coagulation parameters than non-thrombotic patients, thrombotic disease was more common in "COVID-19" patients(Bikdeli *et al.*, 2020; Jin X. *et al.*, 2020) as in figure (2-3).

Increased fibrinogen and D-dimer levels are frequently observed. Anticoagulation at a prophylactic dose does not prevent venous thrombosis, intensive care unit (ICU) patients in particular. While bleeding is less common, it is possible, and pulmonary microvascular thrombosis has been documented, the risk of developing arterial thrombotic diseases appears to be increased. Vein embolism can be challenging to diagnose due to the fact that many of the symptoms of pulmonary embolism are similar to those of COVID-19(Zanza *et al.*, 2021).

Researchers are now attempting to mitigate the disease's consequences on a global scale. In general, abnormal blood coagulation can be regarded as a prognostic factor in patients with COVID-19. Due to the severity of this disease, coagulation indicators such as fibrinogen degradation products, lymphocytes, d-dimer, and accumulated platelets may fluctuate in patients; as a result, examining these factors can be used to initiate treatment promptly(Liu Y. *et al.*, 2020). Currently, most studies focus on use D-dimers solely as a marker of disease progression(Pryzdial *et al.*, 2020; Tang *et al.*, 2020).The D-dimer is a byproduct of the clotting and breakdown of blood that can be quantified through blood sample analysis. When a blood clot starts to dissolve,

D-dimer is released. However, D-dimers have a high sensitivity but a low specificity for pulmonary embolism and deep vein thrombosis detection in low-risk populations(Ryu *et al.*, 2019).

D-dimer levels above 1000 ng/ml are associated with a 20 higher mortality risk than those at lower D-dimer levels(Zhou *et al.*, 2020a). Additionally, as numerous studies have demonstrated, severe SARS-CoV-2 infection is frequently complicated by coagulopathy, and thromboembolic events are observed in several patients (Huang C. *et al*., 2020).

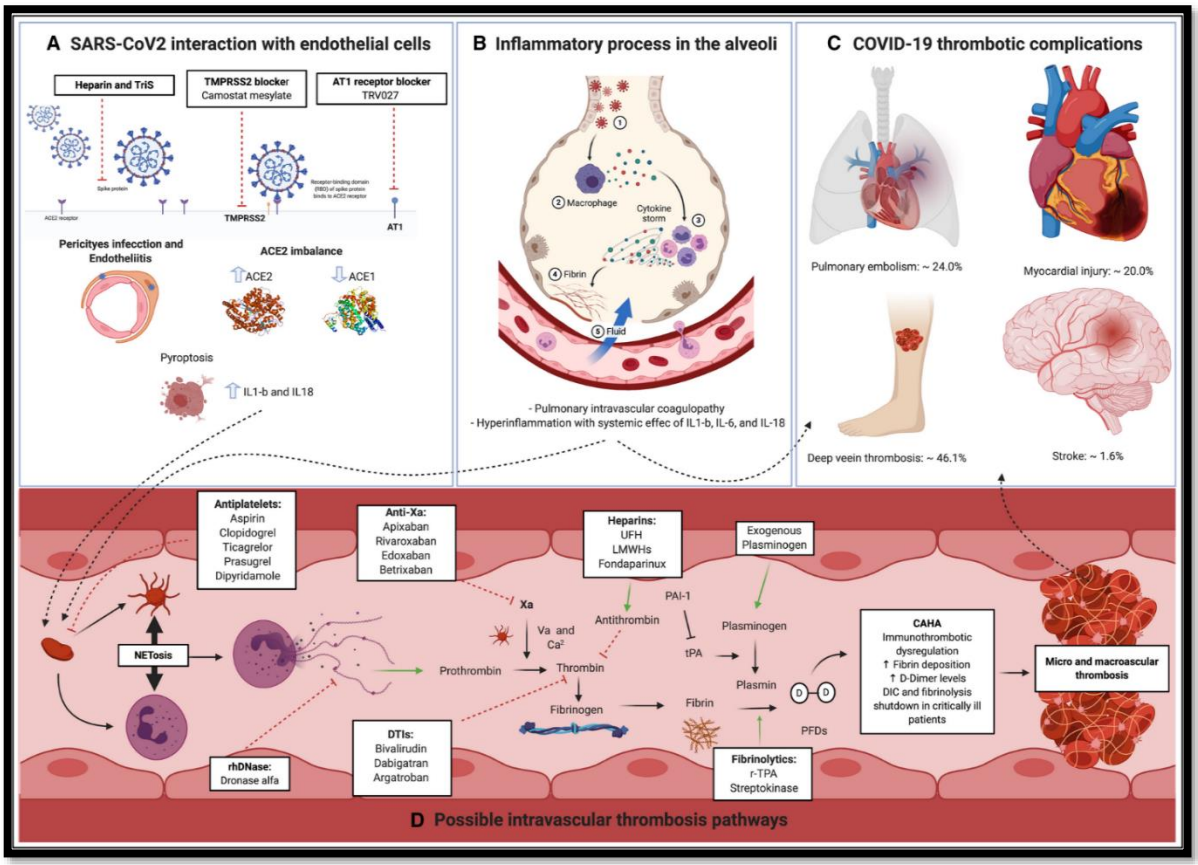


Figure (2-3) Pathophysiological mechanism related to "coronavirus disease 2019" (COVID-19)–associated with thrombosis and coagulopathy(Bikdeli *et al.*, 2020).

2.11. The Role of Host Genetics in the Severity and Progression of COVID-19

The genetic makeup of an individual has an impact on his or her susceptibility to and response to a virus. The risk of SARS-CoV-2 infection and the severity of COVID-19 are both influenced by environmental, clinical, and

social factors, in addition, the genetic makeup of the host may be important. Identifying host-specific genetic factors may shed light on therapeutically relevant biological mechanisms, as well as on the causal relationships between modifiable environmental risk factors for SARS-CoV-2 infection and outcome(COVID-19 Host Genetics Initiative 2021).

A large proportion of COVID-19 cases, its severity, and its mortality can be explained by genetic variations. In addition, there have been regional differences in the frequency of some clinical manifestations of COVID-19. Wuhan patients had higher rates of fever and dyspnea than those from other parts of China (91.7 % and 21.1 %,) (78.1% and 3.80 %) respectively(Park *et al.*, 2020).

In 2020, the COVID-19 Host Genetics Initiative conduct the largest genome-wide association study (GWAS) for COVID-19 to date. The primary objective of this GWAS is to gain a better understanding of influence of host genetics in pandemic susceptibility and severity. The summary dataset for the COVID-19 GWAS to statistical and counted (14,134 cases and 1284,876 controls with European ancestry), these findings were validated by the COVID-19 Host Genetics Initiative (COVID-19 Host Genetics Initiative 2020).

Wang and Fang's (2020) study of Chinese patients provides genetic insight into the phenotypic differences between COVID-19 patient groups and identifies genes and variants that may aid in directing targeted efforts to contain the outbreak. There was a wide range of disease severity and symptom presentation among patients during the outbreak of COVID-19. One in five laboratory-confirmed cases was mild (non-pneumonia or mild pneumonia), while one in five went on to a severe or critical stage with a high risk of respiratory failure(Fu *et al.*, 2020).

Notably, prior research has established the critical role of genetic background in determining host responses in a variety of common viruses as

examples, HIV (Fellay *et al.*, 2009), HBV(An *et al.*, 2018), HCV(Rau *et al.*, 2012), influenza (Schulert *et al.*, 2016), SARS-CoV(Lin M. *et al.*, 2003 ; Ching *et al.*, 2010).These studies shed light on several genes involved in interferon production, the viral replication pathway, and numerous postconditions, indicating that genetic factors may also contribute significantly to the interindividual clinical variability observed among SARS-CoV-2 infection patients.

An initial genetic study of COVID-19 disease severity in China, by analyzing the association between genetic variants present in patients' genomes and disease progression, they registered 332 hospitalized patients from Shenzhen City's designated infectious disease hospital(Cai *et al.*, 2020).

The patients exhibit a variety of clinical and laboratory characteristics and were classified as asymptomatic, mild, moderate, severe, or critical cases using Chinese Center for Disease Control and Prevention criteria(Wu and McGoogan 2020). Currently, there is a worldwide outbreak of COVID-19 that poses a significant threat to all researchers investigating the role of human genetics in the face of a novel infectious disease will identify rare pathogenic mutations that result in SARS-CoV-2 infection and a severe clinical outcome. This will provide unprecedented insights into disease pathogenesis in nature, undoubtedly opening up new therapeutic options(Casanova *et al.*, 2020). The Coronaviruses occur on a regular basis, and understand the genetic mechanisms underlying the development of COVID-19 is critical not only for containing the current pandemic, but also for preventing future outbreaks(Carter-Timofte *et al.*, 2020).

The identification of genetic variants associated with individuals' variable susceptibility to COVID-19 infection and the severity of adverse complications may eventually help open new avenues, including innovative personalized treatments, risk stratification, and protection prioritization, assisting current biomedical research efforts to combat the virus, and also

guiding current genetics and genomics research toward a cure, thus, additional experiments are being conducted in several laboratories to examine the effect of polymorphisms in different genes on COVID-19 severity in a larger patient population(Abdollahzadeh *et al.*, 2021).

2.11.1. The *ACE2* Gene and COVID-19 Disease

The *ACE2* acts as a negative regulator of the human body's Renin Angiotensin System (RAS). Angiotensin converting enzyme 2 (*ACE2*) converts Angiotensin (Ang) I to Angiotensin (Ang) (1–9) and disrupts Angiotensin (Ang) II to Angiotensin (Ang) (1–7) by *ACE2* as in figure (2-4). Ang II induces inflammation, promotes fibrosis, and causes vasoconstriction, whereas Ang (1–7) is a vasodilator peptide with anti-proliferative and apoptotic properties(Hamming *et al.*, 2007), and because of the importance of *ACE2* in cardiovascular disease and the use of ACE inhibitors to treat hypertension and heart failure, there has been a surge of interest in the function and expression of *ACE2* in various human organs(Singh *et al.*, 2021). The *ACE2* protein is found in the mucosa of the oral and nasal passages, the nasopharynx, the lungs, the skin, the lymphatic system, the liver, the stomach, the small intestine, the colon, the kidney, the brain, and the heart, as well as in vascular endothelial and smooth muscle cells(Harmer *et al.*, 2002; Hamming *et al.*, 2004).

Angiotensin II stimulates the synthesis of thrombin and inhibits fibrinolysis, the level of COVID-19 in the cases of severe COVID-19 was strongly correlated with the viral load and lung dysfunction in these patients(Miesbach, 2020). For the proper functioning of the heart, *ACE2* is required, angiotensin II (Ang II) increases blood pressure, myocardial hypertrophy, and fibrosis in mice, but recombinant human *ACE2* reverses these effects(Zhong *et al.*, 2010). Angiotensin converting enzyme 2 in the human

genome, *ACE2* occupies 39.98 kilobases (kb) of space on chromosome Xp22. There are a total of 18 exons and 20 introns(Vickers *et al.*, 2002).

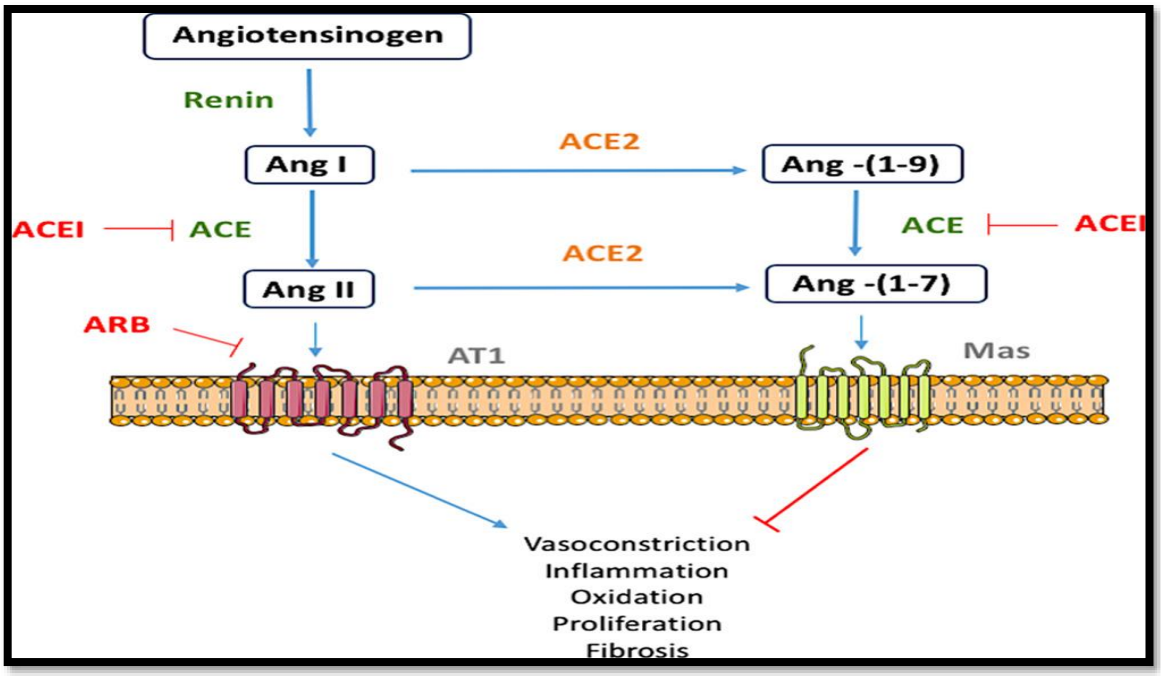


Figure (2-4) A schematic diagram of the major metabolic pathways that regulate the levels of Ang II and Ang 1–7 in the renin–angiotensin system that are controlled by ACE and *ACE2* enzymes(Peiró and Moncada, 2020).

It is a membrane-bound glycoprotein of type I with an 805 amino acid catalytic domain(Tipnis *et al.*, 2000). Similar to the 2002 pandemic-causing severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1), An infection with SARS-CoV-2 begins when the virus's S-protein binds to the *ACE2* receptor, allowing it to enter the host's cells(Kuba *et al.*, 2005; Walls *et al.*, 2020). SARS-CoV-2 has a 10 times greater affinity for the *ACE2* receptor than SARS-CoV-1(Wrapp *et al.*, 2020). According to studies, the cell-virus interaction is mediated by the transmembrane glycoprotein spike (S), which is present on the viral surface in the form of trimers (*ACE2*, also called h*ACE2*)(Yan *et al.*, 2020). This receptor is predominantly expressed in the lungs, but also in other organs such as the heart, kidneys, and intestines to a lesser extent(Bavishi *et al.*, 2020). Low levels of *ACE2* in the lungs have been found in children, and this group is described as having a low death rate as well (Bunyavanich *et al.*, 2020).

In terms of *ACE2* expression, human beings are not equal(Bosso *et al.*, 2020). At the moment, researchers are examining potential genetic variants that could facilitate or complicate virus-host interactions(Othman *et al.*, 2020). Experimental evidence showed that changing the high number of codons in this gene alters how well the virus interacts with cells (Chan K.K. *et al.*, 2020). There are over 2000 polymorphisms in the region 5' upstream of *ACE2*, as well as in exonic regions and introns regions within the *ACE2* locus, based on many analysis of the polymorphisms present (Khayat *et al.*, 2020). As a result, *ACE2* has a dual function in COVID-19: Initially, it acts as a receptor for SARS-CoV-2 entry; however, when SARS-CoV-2 infection occurs, *ACE2* expression is decreased, resulting in an increase in Ang II and decrease Ang 1-7, to enter host cells, SARS-CoV-2 utilizes *ACE2* as a receptor(González-Rayas *et al.*, 2020) as in figure (2-5).

Increased *ACE2* expression due to upregulation enhances SARS-CoV-2 binding sites, thereby increasing susceptibility to COVID-19 infection. Additionally, it is demonstrated that Ang II infusion induces aberrant inflammatory and thrombotic responses in the microcirculation. Ang II accelerates carotid artery thrombosis in hypertensive rats via the AT1 receptor (Kaminska *et al.*, 2005). Prothrombogenic activity is not limited to large arteries. As well, microvascular thrombosis is accelerated in arterioles and less so venules following Ang II infusions(Senchenkova *et al.*, 2019). Numerous results showed that single-nucleotide polymorphisms (SNPs) in the *ACE2* (Xp22.2) gene could influence its expression and also the high affinity of SARS-CoV-2, affecting susceptibility to infection with SARS-CoV-2 and COVID-19 severity(Hou *et al.*, 2020).

According to a study, eighty-one thousand genomes were analyzed to determine major mutations in *ACE2*, possibly linked to pulmonary and cardiovascular injury (Hou *et al.*, 2020a). Thus, those *ACE2* variants are promising candidates for genetic association studies aimed at elucidating the

genetic basis of susceptibility to SARS-CoV-2 risk and the severity of COVID-19 (Möhlendick *et al.*, 2021).

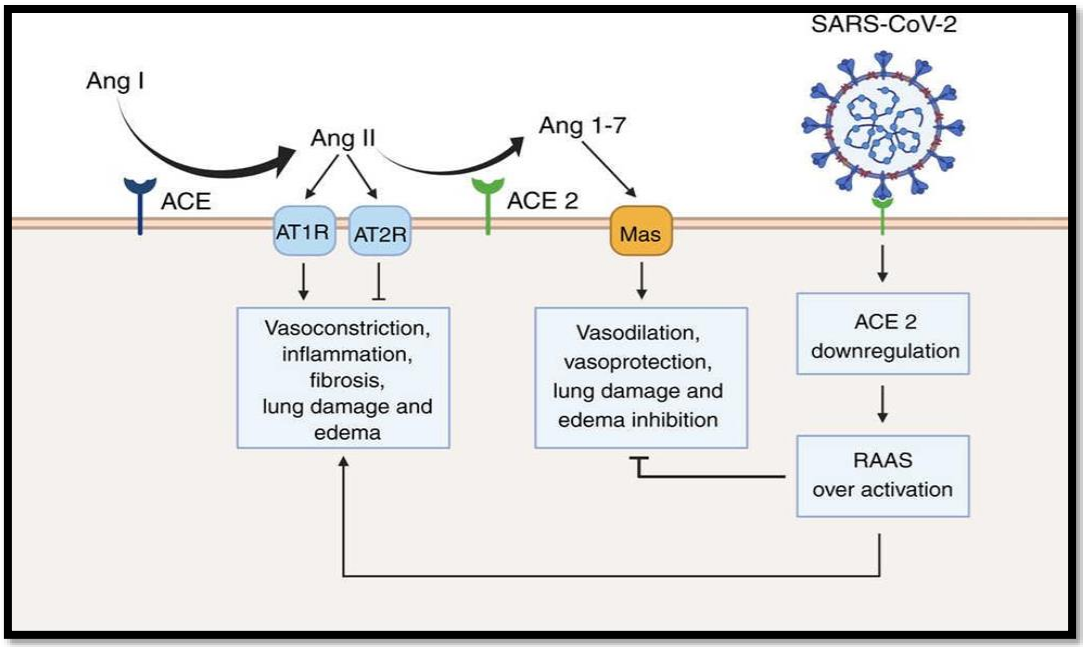


Figure (2-5) illustrates how SARS-CoV-2 infection leads to RAAS overactivation. The angiotensin converting enzyme (ACE) triggers the conversion of angiotensin I (Ang I) into angiotensin II (Ang II), causing increased vasoconstriction, inflammation, fibrosis, and lung injury. Conversely, angiotensin converting enzyme 2 (ACE 2) deactivates Ang I by generating angiotensin 1-7 (Ang 1-7), which binds to the Mas G-protein-coupled receptor, countering the effects of Ang I. However, SARS-CoV-2 reduces *ACE2* expression, intensifying RAAS activity and leading to increased lung injury and edema (González-Rayas et al., 2020).

2.11.2. The *AGT* Gene and COVID-19 Disease

The *AGT* gene on chromosome 1q42.2 encodes angiotensinogen, a peptide hormone(Corvol and Jeunemaitre, 1997). *AGT* in humans contains 485 amino acids, including a signal peptide of 33 amino acids(Wu C. *et al.*, 2011). The renin-angiotensin system (RAS) is critical for blood pressure regulation(Kumar *et al.*, 2005). Renin is primarily released by juxtaglomerular cells in response to a decrease in blood pressure. It acts on angiotensinogen (*AGT*), cleaving off the decapeptide angiotensin-I (Ang I). Angiotensin-converting enzyme (ACE) is then used to convert the latter to angiotensin-II (Ang II), an octapeptide. Ang II is a potent vasopressor that constricts the smooth muscle of the arteries(Skeggs *et al.*, 1976).

D-dimer levels may play a role in the assessment of Primary Pulmonary hypertension patients(Shitrit *et al.*, 2002). The renin–angiotensin–aldosterone (RAAS) system, a metabolic cascade that regulates blood pressure and circulating blood volume, has been implicated in the pathogenesis of severe lung injury and organ failure in COVID-19 patients. The angiotensin I-converting enzyme (ACE1), Angiotensin converting enzyme 2 (*ACE2*), and angiotensinogen (*AGT*) during SARS-CoV-2 entry, these factors are vital, sodium and water retention with an increase in blood pressure, and promotion of fibrotic and inflammatory phenomena, culminating in a cytokine storm(Cafiero *et al.*, 2021).

Angiotensinogen (*AGT*) is the major precursor responsible for hypertension and hypertension-related conditions. Angiotensin-converting enzyme converts angiotensin I into angiotensin II (ACE). Angiotensin II played a critical role in the development of hypertension and hypertension-related conditions via multiple pathways(Lu H. *et al*., 2016). Along with its direct effects on blood pressure, Ang II stimulates the adrenal cortex, resulting in the release of the sodium-retaining hormone aldosterone. With increasing disease COVID-19 severity the serum angiotensinogen concentration increased. In spite of the fact that the RAS is well established as being a critical component of the response to COVID-19 infection, other pathways such as the kallikrein-kinin system (KKS), plasminogen activation, and complement activation all influence the systemic response to the infection(Tepasse *et al.*, 2022).

It is possible for anyone to become infected with SARS-CoV-2, the disease's outcome and severity vary by individual and country and are determined by a dual interaction between the virus and the infected host, the importance of host genetic polymorphisms (particularly those of the RAS system) and other confounding factors such as age, gender, lifestyle and habits have been demonstrated in numerous studies. Those with underlying pathologies or comorbidities (diabetes, hypertension, and cardiovascular

diseases) may be more susceptible to infection and pathogenicity(Braga *et al.*, 2020; El-Arif *et al.*, 2021). Variations in the expression and function of RAS elements, such as those caused by genetic variants, can affect susceptibility to COVID-19 pathogenesis, because of this, the COVID-19 virus may be associated with a gene variant(Kouhpayeh *et al.*, 2021).

2.11.3. The *MTHFR* Gene and COVID-19 Disease

In the blood, homocysteine is produced by the breakdown of the amino acid methionine (a building block of protein). High homocysteine levels (also referred to as hyper-homocysteinemia) may irritate the blood vessels. Homocysteine levels that are too high indicate an increased risk for (1) atherosclerosis, which could lead to a heart attack and/or stroke, and (2) venous thrombosis, also known as blood clots in the veins. Elevated homocysteine levels are associated with blood clots in the arteries and veins; common genetic variants in *MTHFR* may be responsible for elevated homocysteine levels(Moll and Varga, 2015).

Methylene Tetrahydrofolate Reductase (*MTHFR*; OMIM:607093) is a regulatory enzyme of folate and homocysteine metabolism that has been identified as a disease progression predictor(Ponti and Pastorino, *et al.*, 2021). The human *MTHFR* gene is 20 kb in length, located at 1p36.3 (OMIM 607093), and has 11 exons(Rai *et al.*, 2012). As 5,10-methylenetetrahydrofolate reductase (*MTHFR*) plays an important role in the one-carbon-methionine pathway, which regulates DNA repair, neurotransmitter function, and membrane transport in the absence of enough folate intake, MTHFR activity is reduced, which reduces 5-methyltetrahydrofolate (5-MTHF) levels and consequently methionine synthase activity, leading to toxic amounts of homocysteine(Yafei *et al.*, 2012).

Serum levels of H-Hcy may serve as a biomarker for adverse outcomes and higher mortality in COVID-19 disease instead of genetic testing for

MTHFR polymorphism. The antiviral properties of omega-3 polyunsaturated fatty acids, selenium, zinc, and iron, as well as B vitamins A, C, D, and E, have been demonstrated(Zhang and Liu 2020).

Acute respiratory distress syndrome (ARDS) and multiple organ failure can result from SARS-CoV-2. Venous thromboembolism has been observed in severe patients, most likely as a result of a combination of endothelial injury, stasis, and hypercoagulability in an inflammatory state(Bilaloglu *et al.*, 2020). Homocysteine accumulates systemically in genetic variants that significantly impair *MTHFR* function, this can result in abnormal clotting(Undas *et al.*, 2005). In order to understand mortality, clinical, epidemiological, and laboratory markers must be identified, particularly those related to the potentially modifiable damage to the microvasculature. A severe clinical course of COVID-19 infection is associated with serological and clinical biomarkers(Van Der Made *et al.*, 2020).

In recent years, the level of homocysteine has been hypothesized to be an important prognostic indicator(Ponti G. *et al.*, 2020). Homocysteine played an important role in metabolic and inflammatory processes, and the frequency of *MTHFR* gene mutations and *MTHFR* activity varies across populations based on racial predominance(Liew and Gupta, 2015). Homocysteine has been identified as a significant predictor of disease progression in 273 mild cases of COVID-19 in Shanghai; lung CT scans revealed disease progression in 72 of these patients(Ponti and Pastorino, *et al.*, 2021).

The 273 patients at admission were measured for more than 40 parameters, but the monocyte-to-lymphocyte ratio (MLR), age, and homocysteine were only significant predictors of disease progression, according to chest CT changes from COVID-19 patients at the first week for severe pneumonia(Yang Z. *et al.*, 2020).

The pathogenic relationship between COVID-19 infection and Hcy metabolism has been elucidated by a number of studies. Recent reports indicate

that regulatory pathways directly associated with Hcy activate the angiotensin II type I receptor(Li T. *et al*., 2018). The findings by Ponti *et al*. (2021) indicate that Hcy is a marker for predicting the outcome of hospitalized COVID-19 patients. Plasma Hcy levels correlate significantly both as a continuous value and as a dichotomous value, with the optimal cutoff being 16 mol/L. A correlation between MTHFR genetic polymorphisms and an increase in COVID-19 infection incidence and severity will aid in predicting population-based risk factors and developing diagnostic and therapeutic interventions for patients who may benefit from vitamins B and Folic acid supplements(Ponti G. *et al.*, 2020). In large studies, plasma Hcy levels, and *MTHFR* gene sequencing may be established as standard indicators of COVID-19 infection(Fouda *et al.*, 2022).

2.11.4. The *SERPINE1* Gene and COVID-19 Disease

Serpin family E member 1 (*SERPINE1*), which encodes plasminogen activator inhibitor 1 (PAI-1), is the most potent inhibitor of uridylyl phosphate adenosine (uPA) and tissue plasminogen activator (tPA)(Huang J. *et al*., 2012). Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor (*SERPINE1*), is involved in blood clotting. It is linked to cardiovascular disease and resides on seventh chromosome 7q21.3–q22(Hayashida *et al.*, 2010). One of the main functions of PAI-1, a serine protease inhibitor, is to regulate clot decomposition in the body by inhibiting the activity of tissue plasminogen activator (tPA) and the enzyme urokinase (uPA)(Rockway *et al.*, 2002; Kruithof, 2008) The PAI-1 presents its binding loop as a "pseudo-substrate" to the protease; the loop is subsequently cleaved and forms a covalent complex with the protease(Antalis *et al*., 2011).

Fibrinolysis is a tightly controlled process in which the protease plasmin degrades and remodels a fibrin-rich thrombus(Longstaff and Kolev, 2015). Numerous biological processes, including atherosclerosis, thrombosis, fibrosis,

cancer, inflammation, ageing, apoptosis, and infection, involve the fibrinolytic system. Prior to COVID-19, the role of fibrinolytic system components has been extensively studied in a number of other pathological conditions(Flevaris and Vaughan, 2017).

This process is regulated by plasminogen activators and inhibitors, with the end result being the conversion of plasminogen to plasmin, which supports fibrinolysis. The interaction of plasminogen activators (tPA and uPA) and their main inhibitor, plasminogen activator inhibitor-1 (PAI-1), was critical in regulating fibrinolytic activity(Longstaff and Kolev, 2015). However, the primary An important fibrinolytic inhibitor in ARDS pathogenesis is plasminogen activator inhibitor 1 (PAI-1), whose levels are elevated in the SARS-CoV strain epidemic in 2002 and 2003 and acute injury lungs (ALI)(Gralinski *et al.*, 2013). Pro-coagulation factors, such as von Willebrand factor and plasminogen activator inhibitor-1(PAI-1), played crucial roles in the inflammation and coagulation dysfunction associated with COVID-19, indicating that these soluble mediators may serve as biomarkers of endothelial activation and endotheliosis(Zhang Jun *et al*., 2020).

Plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA) levels in plasma concentrations are essential for the regulation of the fibrinolytic system. Plasmin is produced by the activation of its inactive precursor, plasminogen, and is the primary defense against fibrosis in the body. As previously discussed, COVID-19a is associated with poor outcomes due to elevated d-dimer levels, which are fibrin breakdown products(Hayıroğlu *et al*., 2020). Patients with critical illnesses tend to have elevated levels of PAI-1 and t-PA, according to prior research(Zuo *et al.*, 2021). In COVID-19 associated coagulopathy, fibrinolytic parameters, including PAI-1 and t-PA, are also significantly dysregulated. Increased PAI-1 activity and inhibition of endogenous fibrinolysis may be responsible for the increase in thrombosis in COVID-19(Agirbasli, 2022). In the case of D-dimer formation in COVID-19,

the question is how much plasmin-generating capacity each form of uPA possesses in the affected organs. Obviously, this depends on their respective sensitivity to PAI-1's inhibitory effect. PAI-1 levels are elevated in COVID-19(Zuo *et al.*, 2021). A recent study predicted mortality with 100 percent accuracy by plasma PAI-1 levels above 640 ng/mL in patients who progressed from Acute lung injury (ALI) to acute respiratory distress syndrome(ARDS)(Prabhakaran *et al.*, 2003). Similar pathology of fibrin depositions in the lungs has been identified in COVID-19(Yao *et al.*, 2020), ARDS patients who need intensive care and ventilation may benefit from PAI-1 values as a prognostic indicator(Whyte *et al.*, 2020).

Chapter Three

Subjects, Materials and Methods

3. Subjects, Materials, and Methods

3.1. Subjects

In this part of the study, the subjects were divided into two categories:

1) Control Group

This group consisted of (92) apparently healthy individuals, (54) males and (38) females between the ages of 18 and 65, None of them had contracted coronavirus disease at the time when the samples were collected. During direct communications with patients and control persons, all information about both groups of patients and the control were recorded in a questionnaire form, which included name, age, sex, employment type, linked disease, and symptoms, (Appendix 1).

2) Patients Group

This group consisted of (102), physician-diagnosed patients who experienced various symptoms, including fever, dry cough, headache, loss of smell and taste, and occasionally vomiting and/or diarrhea, between December 2021 and May 2022.

They are (48) males and (54) females between the ages of (18-80), with the diagnosis confirmed by a nasopharyngeal or oropharyngeal swab for PCR or a blood sample for biomarker and CT scan in some cases, as per the WHO-established protocol for the detection of coronavirus using different tests.

all samples were collected from different places and hospitals in Iraq (Al-Diwaniyah Teaching Hospital, Marjan Medical City, Al-Hussein Teaching Hospital, Al-Hamza Teaching Hospital, and many special laboratories) while the control (92 blood samples) was collected from people with negative COVID-19 tests.

The patient groups were categorized into two distinct subgroups: severe (consisting of 57 cases) and non-severe (comprising 45 cases), using specific criteria based on hospitalization duration and the worsening of symptoms.

Unfortunately, within these groups, there were instances of patient mortality observed in the severe category, while the non-severe category had fewer cases exhibiting severe symptoms. The classification of COVID-19 severity was defined as follows:

- **Non-severe:** Non-severe: Patients with mild symptoms, no pneumonia on lung CT.
- **Severe:** Patients with severe symptoms including respiratory distress, O2Sat at/below 90%, and the need for mechanical ventilation, often leading to ICU admission(WHO, 2020a).

3.2. Materials

This part includes the materials, equipment, chemicals, and kits, and also the companies and countries involved in the study.

3.2.1. Equipment

An overview of the equipment used and its sources in table (3-1).

Table (3-1) Includes all study equipment required for the study

No.	Name	Company	Origin
1.	**Centrifuge**	Hattich	Germany
2.	**Cool box**	Eskimo	India
3.	**EDTA tube (2 ml)**	Shanghai Ningbo	China
4.	**Electronic Balance**	Huma Scale huma	Germany
5.	**ELISA reader**	PKL/PARAMEDICAL	Italia
6.	**Gel electrophoresis apparatus and power supply**	Bio-Rad	USA
7.	**Gel tube (6 ml)**	V-TUBE	Korea
8.	**Incubator**	Hettich	Germany
9.	**Micropipette (different sizes)**	Slamed	Japan
10.	**Microwave oven**	SAMSUNG	Korea
11.	**Nanodrop**	Thermos fisher	USA
12.	**Refrigerator**	Beko	Japan
13.	**Thermal cycler (PCR)**	Bio-Rad	USA
14.	**UV Transilluminator**	Major science	Taiwan
15.	**Vortex**	Huma Twist	Germany
16.	**Water bath**	Memmert	Germany

3.2.2. Kits

Listed below is a list of the kits used in this study in Table (3-2).

Table (3-2) kits used in the study

No.	Kits	Company	Origin
1.	**Kit DNA extraction**	Geneaid	Taiwan
2.	**Kit ELISA assays (N=4)**	BT-LAB	China
3.	**Master mix**	Promega	USA

3.2.3. Reagents and Chemicals

This study used the chemicals and reagents presented in table (3-3) and their sources.

Table (3-3): Chemicals list used in this study.

No.	Chemical	Company	Origin
1.	**Absolute ethanol**	Fluka	Germany
2.	**Agarose**	Pronadisa Conda	Spain
3.	**DNA ladder marker**	Bioneer	South Korea
4.	**Ethidium Bromide**	Biobasic	Canada
5.	**Nuclease Free Water**	Bioneer	South Korea
6.	**Isopropanol**	Fluka	Germany
7.	**Loading dye**	Geneaid	Taiwan
8.	**Primers**	Bioneer	South Korea
9.	**TBE buffer (10x)**	Transgen biotech	China
10.	**Proteinase K**	Transgen biotech	China

3.3. Collection Samples and Types

3.3.1. EDTA Tube

Blood samples (5ml) were obtained from 105 patients with positive COVID-19 results.

3.3.2. Sodium Citrate Tube

Collect from each patient 2ml of blood put into sodium citrate tube to obtain plasma that used for D-Dimer test.

3.3.3. Serum Sampling

Each patient with a positive COVID-19 test was provided a serum sample along with a blood sample, in addition to each COVID-19 negative test with no symptoms, serum and blood samples were provided.

3.3.4. Study Design

The study utilized a case-control design to compare patients with a specific disease or outcome of interest (cases) to those without the disease or outcome (controls). The final results were obtained through a series of steps outlined in Figure (3-1).

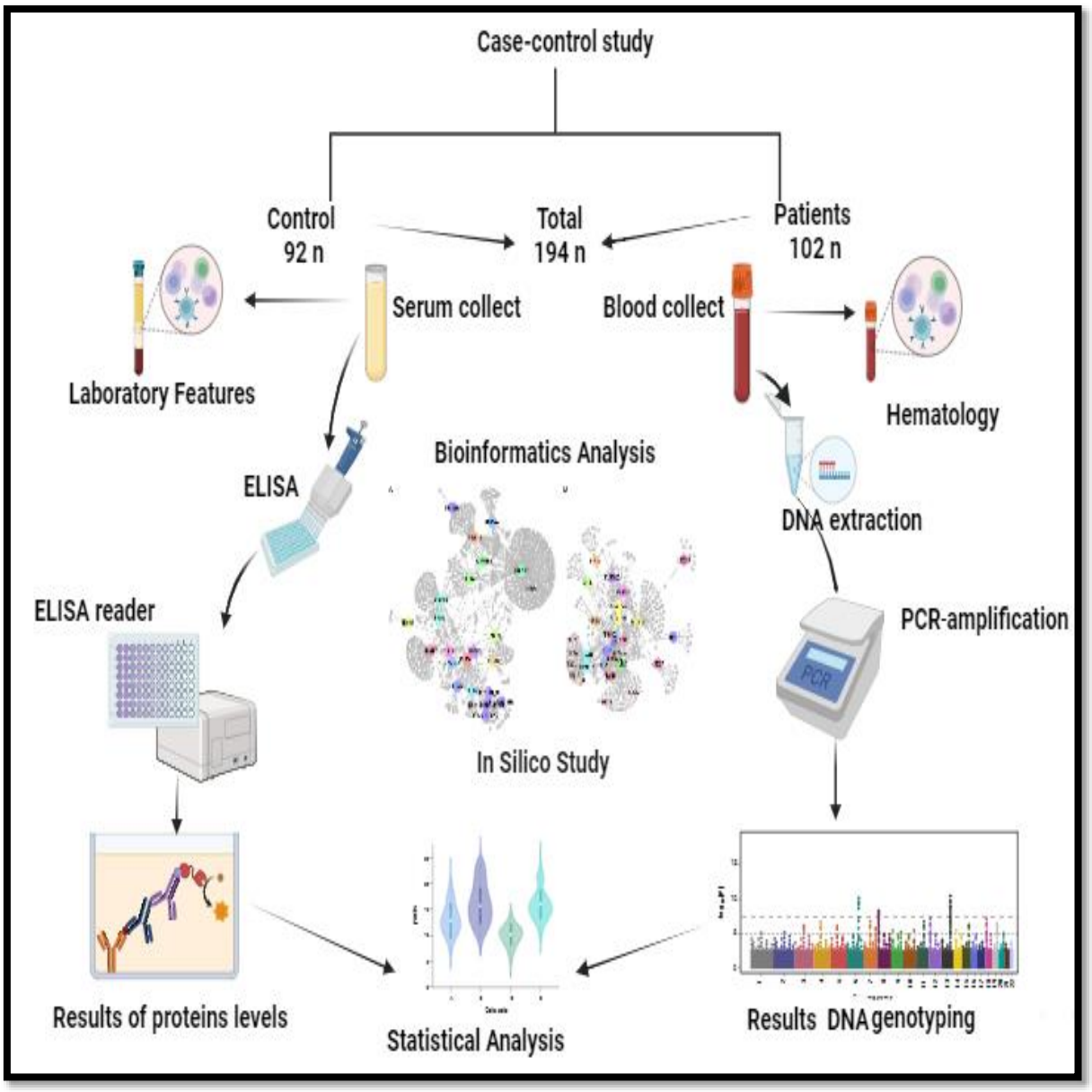


Figure (3-1) depicted an experimental study design involving genotyping and ELISA measurements of blood and serum samples from both healthy individuals and patients.

3.3.5. Ethical Approval and Participants' Consent

This study was approved by the Council of the Department of Biology/ College of the Science/ University of Babylon under number (B220102, date Jan 17, 2022), each individual participating in the study was taken consented him/her or the consent of his family for severe cases.

3.4. Methods

Blood samples and serum samples were collected in EDTA and GEL tubes respectively, from 194 participants; 102 positive COVID-19 test patients and 92 negative COVID-19 tests.

3.4.1. Genomic DNA Extraction

Using DNA Extraction Mini Kit supplied by Geneaid/Taiwan, the extracted DNA from whole blood in accordance with the manufacturer's instructions:

1. **Sample Preparation;** A clean 1.5-milliliter tube was filled with 20 microliters of Proteinase K, after which the Proteinase K was mixed with 200 microliters of whole blood.
2. **Cell Lysis;** Introduce 200 µl of lysis buffer (GSB Buffer) and vigorously shake to mix. Incubate at 60ºC for 5 minutes, gently inverting the tube every 2 minutes.
3. **DNA Binding;** Added 200 µl of absolute ethanol to the sample lysate and shake vigorously for 10 seconds. If there's any precipitate, disperse it with a pipette. Placed a GS Column into a 2 ml Collection Tube. Transfer the entire mixture (including precipitate) to the GS Column. Centrifuge at 12,000 rpm for 1 minute. If the mixture doesn't pass through the GS Column membrane, extend centrifugation. Dispose of the 2 ml Collection Tube with flow-through, then move the GS Column to a new 2 ml Collection Tube.

4. **Washing;** Added 400 µl of W1 Buffer onto the GS Column. Centrifuge at 12,000 rpm for 1 minute, and then remove the flow-through. Return the GS Column to the 2 ml Collection Tube. Added 600 µl of Wash Buffer (W2) to the GS Column. Centrifuge at 12,000 rpm for 1 minute, and again remove the flow-through. Placed the GS Column back in the 2 ml Collection Tube. Centrifuge for 3 minutes at 12,000 rpm to desiccate the column matrix.
5. **Elution;** The binding column tube was transferred to a fresh 1.5 ml tube for elution. Following this, 200 µl of pre-heated Elution buffer (EL) was added, and a minimum of 5 minutes was allowed for full absorption of the EL at room temperature (15-25°C). For elution, centrifugation at 10,000 rpm for 1 minute was performed, yielding an approximate eluent volume of (180-200) µl when using 200 µl of Elution buffer. Subsequently, the collection tube was discarded, and the Eppendorf tube containing eluted DNA was securely capped and stored at -20°C.

3.4.2. DNA Quantity and Purity

The efficiency of subsequent reactions depends critically on the nucleic acid concentration of the specimen. nucleic acid quantification was done with using a nanodrop device that only requires 1 µl of sample to quantify and measure optical density (OD) at 260 nm and 280 nm. The Nanodrop is a reliable machine that can calculate g/ml and purity from OD measurements as in Appendix (2). In general, a ratio of A260/A280 between 1.7 and 1.9 indicates pure DNA(Lucena-Aguilar *et al.*, 2016).

3.4.3. Primer Preparations

3.4.3.1. Primer Design

Several genotyping methods are used for the molecular diagnosis of polymorphisms in this study, two genotyping methods using in this study and the both methods were faster and less expensive technologies such Allele

specific-PCR (AS-PCR) and Tetra-ARMS-PCR (T-ARMS-PCR). In design allele specific primers in present study used two websites Web-based Allele-Specific Primer (WASP) and BatchPrimer3, WASP is a tool for designing allele-specific (AS) primers that were applied in this work, in order to enhance the resultant AS primers, as a result, WASP introduces one deliberate "mismatch" at the penultimate base of AS primers (second-to-last) to create various destabilizing effects(Wangkumhang *et al.*, 2007), while BatchPrimer3 is a comprehensive web-based primer design application that uses the Primer3 core software as its primary primer design engine to build various PCR primers included (including allele-specific primers, tetra-primers for tetra-primer ARMS PCR and single-base extension primers)(You F. *et al*. 2008) .

The AS-PCR is easy to use, quick, affordable, and trustworthy in addition does not require advanced equipment or technology(Liu *et al*. 2012; Yang *et al*. 2013). Hence, various natural populations were examined for the preferred SNP genotype. This method relies on three primers (mutant, wild, common) and their binding, determined by the base in the targeted region as in figure (3-2). Details of primer sequences and fragment sizes are outlined in Table 3-4.

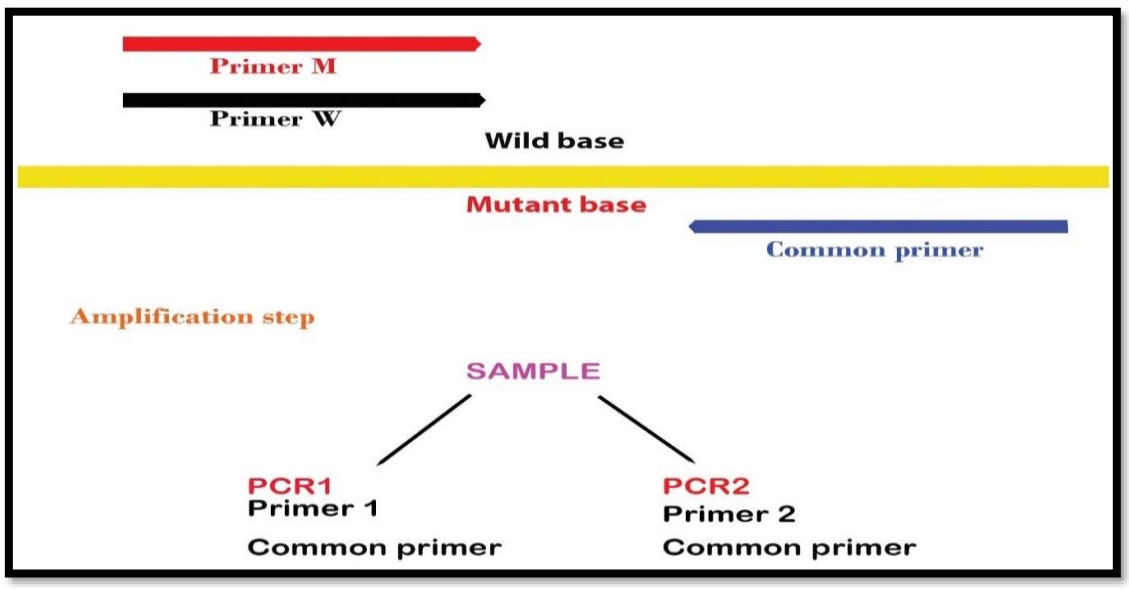


Figure (3-2) Schematic summary of Allele Specific-PCR when designing primers according to parameters by classified into two reactions, primer M= mutant, primer W= wild and common primer.

Table (3-4) The primers sequences of allele-specific PCR were design by WASP website for the genes in study (*AGT*, SERPIN1, *MTHFR*, and *ACE2*).

No.	Gene/rs	Common primer (5' - 3')	Wild type primer	Mutant type primer	Size of product
1.	*AGT*/rs699	Forward CTGGCTGATCTCAGCTACAC	Reverse AAGACTGGCTGCTCCCTGAT (T allele)	Reverse AAGACTGGCTGCTCCCTGAC (C allele)	142 bp
2.	*SERPINE1* / rs1799889	Forward GTCTGTGTCTGGAGGAAGAG	Reverse TCCGATGATACACGGCTGAAT (4G allele)	Reverse CCGATGATACACGGCTGAAC (5G allele)	255 bp
3.	*MTHFR*/ rs1801133	Forward AGTTCTGGACCTGAGAGGAG	Reverse GAAGGTGTCTGCGGGAGC (C allele)	Reverse GAAGGTGTCTGCGGGAGT (T allele)	266 bp
4.	*ACE2*/ rs2106809	Forward GCTACAACGTGTCCTTGATT	Reverse TGATGTAGA*AGT*GTGGAGAACT (T allele)	Reverse TGATGTAGA*AGT*GTGGAGAACC (C allele)	196 bp

The T-ARMS-PCR is an acronym that stands for tetra primer-amplification refractory mutation system-based polymerase chain reaction. This method is used for genotyping as well as identifying any mutation that involves single base changes or small deletions. Compared to genotyping techniques such as allele-specific PCR (AS-PCR), T-ARMS PCR offers a flexible, fast, and cost-effective method for SNP detection(Gaudet *et al.*, 2009), However, it does require significant optimization during the initial phases of the experiment, as noted by Alyethodi *et al*. (2018). The optimization stage might be laborious and time-consuming in this method(Zabala *et al.*, 2017).

Tetra-primer PCR was initially introduced by Ye *et al*. (2001), a method of amplification in which allele-specific amplification may be done in a single reaction by Primer1 design web service to creates four primers, in addition BatchPrimer3 was mentioned in AS-PCR. The T-ARMS- PCR based method involved two SNP-specific inner primers and two outer primers are used in a single reaction as seen in figure (3-3), with one of the inner primers being intentionally mismatched at position -2 from the 3' end. In study summarizes sequences primers designed for profiling of DNA polymorphism in table (3-5).

Table (3-5) the primers were designed for T-ARAMS-PCR to detected variants in this study (*T*/rs699, *SERPIN1*/rs1799889, *MTHFR*/rs1801133, and *ACE2*/2106809).

No.	Gene/rs	Outer primers (5' - 3')	Size of product	Inner primers (5' - 3')	Size of product
1.	*AGT*/rs699	**Forward** CTGAAGCAGCCGTTTGTGCA **Reverse** CACCAGGTATGTCCGCAGGG	319 bp	**Forward inner (T allele):** TGGAAGACTGGCTGCTCCCTTAT **Reverse inner (C allele):** GCTGTCCACACTGGCTCACG	194 bp (T allele) 168 bp (C allele)
2.	*SERPINE1*/ rs1799889	**Forward** CCATGGTAACCCCTGGTCCC **Reverse** GCTCTGGACCACCTCCAGGA	315 bp	**Reverse inner (5G allele):** GAGAGAGTCTGGACACGTGGTGG **Forward inner (4G alleles):** CCGATGATACACGGCTGCCT	145 bp (4G) 213 bp (5G)
3.	*MTHFR*/ rs1801133	**Forward** GAGGCCAGCCTCTCCTGACT **Reverse** AGTGGGGTGGAGGGAGCTTA	273 bp	**Reverse inner (T allele):** GAAGGTGTCTGCGGGCGT **Forward inner (C allele):** AAAGCTGCGTGATGATGAAATAGG	176 bp (T allele) 139 bp (C allele)
4.	*ACE2*/ rs2106809	**Forward** TCACAGATCCCAAAACAGTA **Reverse** TAGCTACAACGTGTCCTTGA	312 bp	**Reverse inner (T allele):** TTTTCCATATCTCTATCTGATTGA **Forward inner (C allele):** TGTAGAAGTGTGGAGACGC	159 bp (T allele) 196 bp (C allele)

3.4.3.2. Reconstitute PCR Primers

Centrifuge primers to force all of the lyophilized powder to the bottom of the tube. A 100pmol/µl concentration of lyophilized primer was reconstituted in free DNase and RNase water, and stored at -20°C until use. The amount of nuclease water to add is determined by the number of nanomoles (nmoles) in the oligo primer. This is normally located on the tube itself or on the priming sheet that comes with the order, to prepare a 10pmol working primer solution, The sample was resuspended 10µl in 90µl of deionized water to a final concentration of 10 pmol/µl and vortexed.

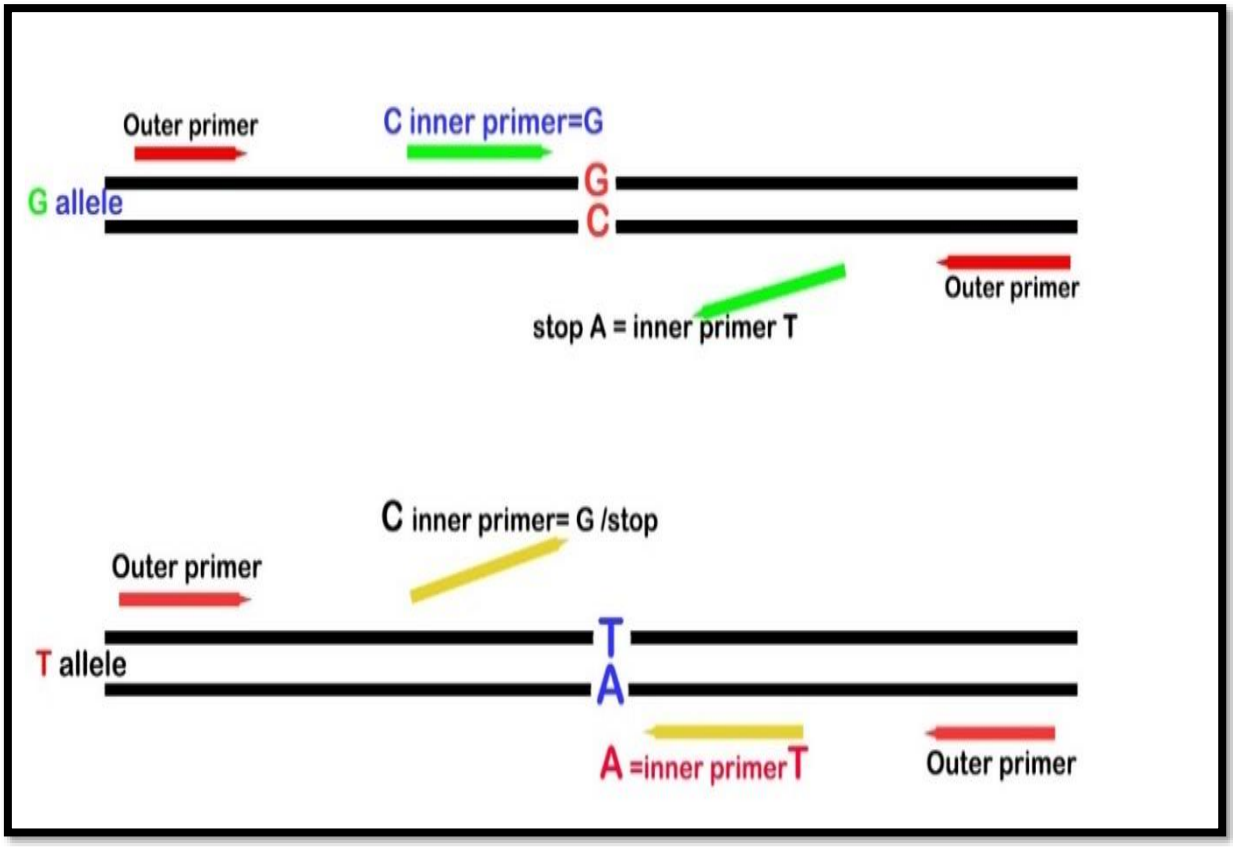


Figure (3-3) A schematic overview of the ARMS-PCR primer design. Red: exterior primers; green: inner C-allele-specific primer and T-allele primer in G allele; yellow: inner T-allele- and C allele in T allele specific primer; stop: inner primer with a second mismatch.

3.4.4. Amplification of DNA

The target region was amplified using PCR conventional using a specific primer. The amount of the reaction mixture was completed to 25µl, and this reaction carried out under sterile conditions.

Two PCR reactions were performed for each sample, including two allele-specific forward primers for the mutant with a common primer and wild with a common primer in the second reaction, in order to determine the wild-type and mutant alleles of this study polymorphisms as in table (3-6). All the reactions under optimal conditions of PCR by calculation the temperature to each cycle to get of results as in table (3-7).

Table (3-6) PCR reaction components for amplification of *ACE2, AGT, SERPINE1,* and *MTHFR* by allele specific-PCR.

Component reaction 1	Quantity(µl)/ concentration	Component reaction 2	Quantity(µl)/ concentration
Master mix	12.5 µl	**Master mix**	12.5 µl
Common primer	2 µl / (10pmol)	**Common primer**	2 µl / (10pmol)
Wild primer	2 µl/ (10pmol)	**Mutant primer**	2 µl/ (10pmol)
DNA template	5 µl	**DNA template**	5 µl
Nuclease free water	3.5 µl	**Nuclease free water**	3.5 µl
Total volume	25 µl	**Total volume**	25 µl

Table (3-7) PCR amplification program of allele specific-PCR to *ACE2, AGT, SERPINE1,* and *MTHFR.*

Steps	*ACE2*	*AGT*	*SERPINE1*	*MTHFR*
Initial denaturation Tm	95°C/3 min	95°C/3 min	95°C/3 min	95°C/3 min
Denaturation Tm	95°C/30 sec	95°C/30 sec	95°C/30 sec	95°C/3sec
Annealing	Touch down PCR (54-59) °C /45 sec -1°C/ cycle	57°C/45 sec	59°C/45 sec	60 °C/45 sec
Extension	72°C/45sec	72°C/30sec	72°C/30sec	72°C/45sec
Final extension	72°C/5 min	72°C/5 min	72°C/5 min	72°C/5 min
No. of cycles	33X	34X	34X	34X

X: PCR yield for number cycles.

While T-ARMS-PCR the method were in single reaction by using four primers with all the components of reaction to amplify the target region contain the polymorphism as in table (3-88), Optimizing the optimum PCR conditions for the T-ARMS PCR was a challenging process(Medrano and De Oliveira, 2014), the list conditions of protocol T-ARMS PCR in this study as in table (3-9).

PCR Master Mix Composition Taq DNA polymerase is provided at a concentration of 50 units per milliliter, and it comes supplied with a proprietary reaction buffer at a pH of 8.5. Additionally, the reaction mixture contains 400 micromolar (µM) of each of the four deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), as well as 3 millimolar (mM) of MgCl2.

Table (3-8) PCR reaction components for amplification of *ACE2, AGT, SERPINE1,* and *MTHFR* by T-ARMS PCR.

Component single reaction	Quantity(µl)/ Concentration
Master mix	12.5 µl
Outer primers **1-Forward** **2-Reverse**	1 µl each primer/ (20pmol) =2 20stock primer+80 free nuclease
Inner primers **1-Wild primer** **2-Mutant primer**	1 µl each primer/ (20pmol) =2 20stock primer+80 free nuclease
DNA template	5 µl
Nuclease free water	3.5 µl
Total volume	25 µl

Table (3-9) PCR amplification program of T-ARMS PCR to *ACE2, AGT, SERPINE1,* and *MTHFR.*

Steps	*ACE2*	*AGT*	*SERPINE1*	*MTHFR*
Initial denaturation Tm	95°C/3 min	95°C/3 min	95°C/3 min	95°C/3 min
Denaturation Tm	95°C/30 sec	95°C/30 sec	95°C/30 sec	95°C/30 sec
Annealing	Touch down PCR (55-60) °C /1 min -1°C/ cycle	62°C/1 min	59°C/1 min	Touch down PCR (56-62) °C /1 min -1°C/ cycle
Extension	72°C/30 sec	72°C/30 sec	72°C/30 sec	72°C/45 sec
Final extension	72°C/5min	72°C/5min	72°C/5min	72°C/5min
No. of cycles	33X	34X	34X	33X

3.4.5. Agarose Gel Electrophoresis

When extraction of genomic DNA was done, Agarose gel electrophoresis is the most efficient method for separating DNA fragments and confirming the presence and integrity of the extracted DNA(Russell and Sambrook, 2001). The Solutions used with Agarose gel electrophoresis were as follows:

- 1 X TBE buffer
- Loading dye
- DNA ladder marker
- Gel stain

A- Preparation of 1X TBE Buffer

To make 1X TBE buffer, add 100 ml of 10X TBE stock solution to 900 ml distilled water.

B- Preparation of Agarose Gel

1. One hundred ml of 1X TBE buffer was placed in a beaker.
2. To the buffer, 1 gm of agarose gel (1% for DNA extraction) or 2 gm of agarose gel (2% for PCR amplification) was added.
3. In order to prevent evaporation, the solution was heated in a microwave until all the gel particles were dissolved.
4. To make the DNA visible, 3-5 µl of gel stain (Ethidium Bromide) was added to the agarose.
5. The agarose was thoroughly mixed by stirring.
6. The solution was then cooled to a temperature of 50-60°C.
7. The gel tray was prepared by placing the comb inside it, and the agarose solution was poured into the tray. The agarose was allowed to solidify at room temperature for 10-20 minutes.

8. The comb was carefully removed, and the tray was filled with 1X TBE-electrophoresis buffer until it reached a level 3-5 mm above the gel surface.

C- DNA Loading

The following DNA samples were loaded into the agarose gel's wells:

1. Seven µl of DNA was added to each well in the gel box.
2. At 75v and 20A, the electricity was turned on for one hour. DNA moves from the negative cathode to the positive anode (anode).
3. A source of ultraviolet light with a wavelength of 350 nm was used to examine the gel's bands.
4. Gel imaging was used to visualize stained bands in gel.
5. Analyzing the gel band to find fit size by using the DNA ladder in the first lane.

3.4.6. Direct DNA Sequencing

The PCR product (20 µl) sent to Macrogen Company, South Korea to perform PCR product sequencing based on primers in table (3-10). Sequencing was carried on the 250 samples to confirm the accuracy and correctness of the genotyping data from the AS-PCR and T-ARMS-PCR methods, the products were forwarded for Sanger sequencing. The shipped samples for sequencing were categorized into groups, with 60 samples assigned to each polymorphism. Within these, 20 samples were designated as controls, another 20 as severe patients, and the remaining 20 as non-severe patients. The results of sequencing came as three files included (chromatogram, text file sequence, and PDF file of picture chromatograms), the technique employed for sequencing, known as Sanger sequencing, was carried out using an ABI3730XL automated DNA sequencer, The results were subsequently received via email, followed by data

analysis using specialized software programs like MEQA11 and Geneious Prime software to perform alignment analysis and identify genetic variations.

Table (3-10) the primers were designed for sequencing variants included in study (*AGT*/rs699, *SERPIN1*/rs1799889, *MTHFR*/rs1801133, and *ACE2*/2106809).

NO.	Gene/rs	Forward primer (5' - 3')	Reverse primer (5' - 3')	Size of product
1.	*AGT*/rs699	Forward AGCAGCCACTTCCCCACT	Reverse CACAGCTGACAGGCTACAGG	545 bp
2.	*SERPINE1*/ rs1799889	Forward TCTGTGTCTGGAGGAAGAGGA	Reverse CAGCCACGTGATTGTCTAGG	350 bp
3.	*MTHFR*/ rs1801133	Forward TGGGAAGAACTCAGCGAACT	Reverse CAGCCTCTCCTGACTGTCATC	336 bp
4.	*ACE2*/ rs2106809	Forward TATGGGCCTCATGCTCTCTC	Reverse TTCTCTCACAGATCCCAAAACA	366 bp

3.4.7. Laboratory Methods

3.4.7.1. Hematological Parameters and D-Dimer

Level of Hb, platelet count, neutrophils and total WBC count were determined using a fully automated hematology analyzer.

3.4.7.2 Homocysteine, ANG 1-7, PAI-1, and Angiotensinogen A-Kit Content

Using commercially available ELISA kits, the levels of homocysteine, ANG 1-7, PAI-1, and Angiotensinogen were determined in the serum of COVID-19 patients and healthy controls. The same company all of the kits, which were based on the same principles; As a result, the kit components, assay process, and calculations are all presented together as in table (3-11).

Table (3-11) Kit content of ELISA.

Components	Quantity (96T)
Biotinylated Human Hcy, ANG 1-7, *AGT*, and PAI-1 antibody	1ml x1
Plate sealer	2 pics
Pre-coated ELISA plate	12 * 8 well strips x1
Standard diluent	3ml x1
Standard solution (24ng/ml)	0.5ml x1
Stop solution	6ml x1
Streptavidin-HRP	6ml x1
Substrate solution A	6ml x1
Substrate solution B	6ml x1
User instruction	1
Wash buffer Concentrate (25x)	20ml x1

B-Standard Preparations

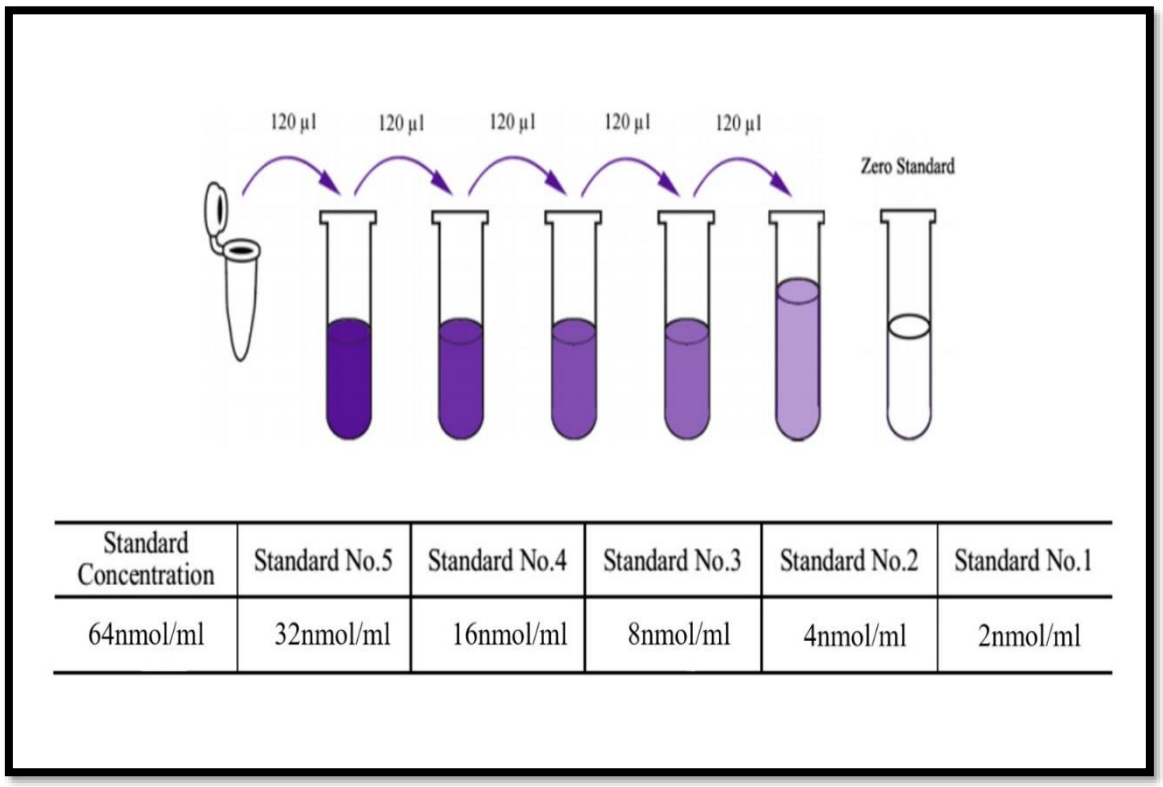
Reagent preparation: the following manufacturer's recommendations were followed while preparing standard solutions: It is recommended that all reagents be stored at room temperature and all concentrations in table (3-12) below:

Table (3-12) the standard of concentration to each ELISA used in study based on the kits.

NO.	ELISA name	Standard Concentration	Standard No.1	Standard No.2	Standard No.3	Standard No.4	Standard No.5
1.	**Human *AGT***	1440ng/L	720ng/L	360ng/L	180ng/L	90ng/L	45ng/L
2.	**Human Hcy**	64nmol/ml	32nmol/ml	16nmol/ml	8nmol/ml	4nmol/ml	2nmol/ml
3.	**Human Ang 1-7**	960ng/L	480ng/L	240ng/L	120ng/L	60ng/L	30ng/L
4.	**Human PAI-1**	24ng/ml	12ng/ml	6ng/ml	3ng/ml	1.5ng/ml	0.75ng/ml

A similar method was used for creating criteria for the four markers evaluated in this study. As an example, here is a typical Human Hcy procedure: Standard to create a 32nmol/ml standard stock solution, reconstitute 120ul of the standard (64nmol/ml) with 120ul of the standard diluent. Ensure that the standard is moderately agitated for 15 minutes before preparing dilutions.

The standard stock solution (32nmol/ml) should be serially diluted (1:2) with the standard diluent to create 16nmol/ml, 8nmol/ml, 4nmol/ml, and 2nmol/ml solutions. It is recommended to dilute standard solutions as follows figure (3-4). Dilute 20ml of wash buffer to 500ml of 1x wash buffer. If crystals develop in the concentrate, gently mix until the crystals dissolve entirely.



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
64nmol/ml	32nmol/ml	16nmol/ml	8nmol/ml	4nmol/ml	2nmol/ml

Figure (3-4): Schematic presentation of standard preparation of hHCY.

C-Assay Technique

1. All reagents, standard solutions, and samples were prepared. It was ensured that all reagents were at room temperature before use. The experiment was conducted at room temperature.

2. To perform the test, the required size of the strips was determined. The strips were placed into the frames and kept between 2 and 8 °C.

3. The standard wells were filled with 50 µl of standard solution. Biotinylated antibody was avoided in the standard solution as it was already present.

4. The sample wells received both the specimen and 10 µl of Human antibody, selected based on the specific kit used for (AGT, Ang 1-7, Hcy, and PAI-1). Additionally, 50 µl of streptavidin-HRP was inserted into the standard wells (absolute blanks could not be used). The contents were mixed well and incubated for 60 minutes at 37 °C after applying a sealer to the plate.

5. The sealant was removed from the plate, and it was washed five times with a wash buffer.

6. Substrate solution A (50 µl) was introduced into each well, followed by the addition of 50 µl of substrate solution B. Subsequently, the plate was incubated in darkness at 37 °C for 10 minutes.

7. Stop solution was added to each well, causing the blue color to quickly turn yellow.

8. The optical density (OD value) was calculated by using a 450 nm microplate reader within 10 minutes after applying the stop solution.

D- Calculation of the Concentration

The absorbance of the standards for each serum marker was recorded in an EXCEL sheet and then plotted against the concentrations of the standard. Then, the level of the unknown samples was identified by generating an equation that fits best the curve as in figure (3-5).

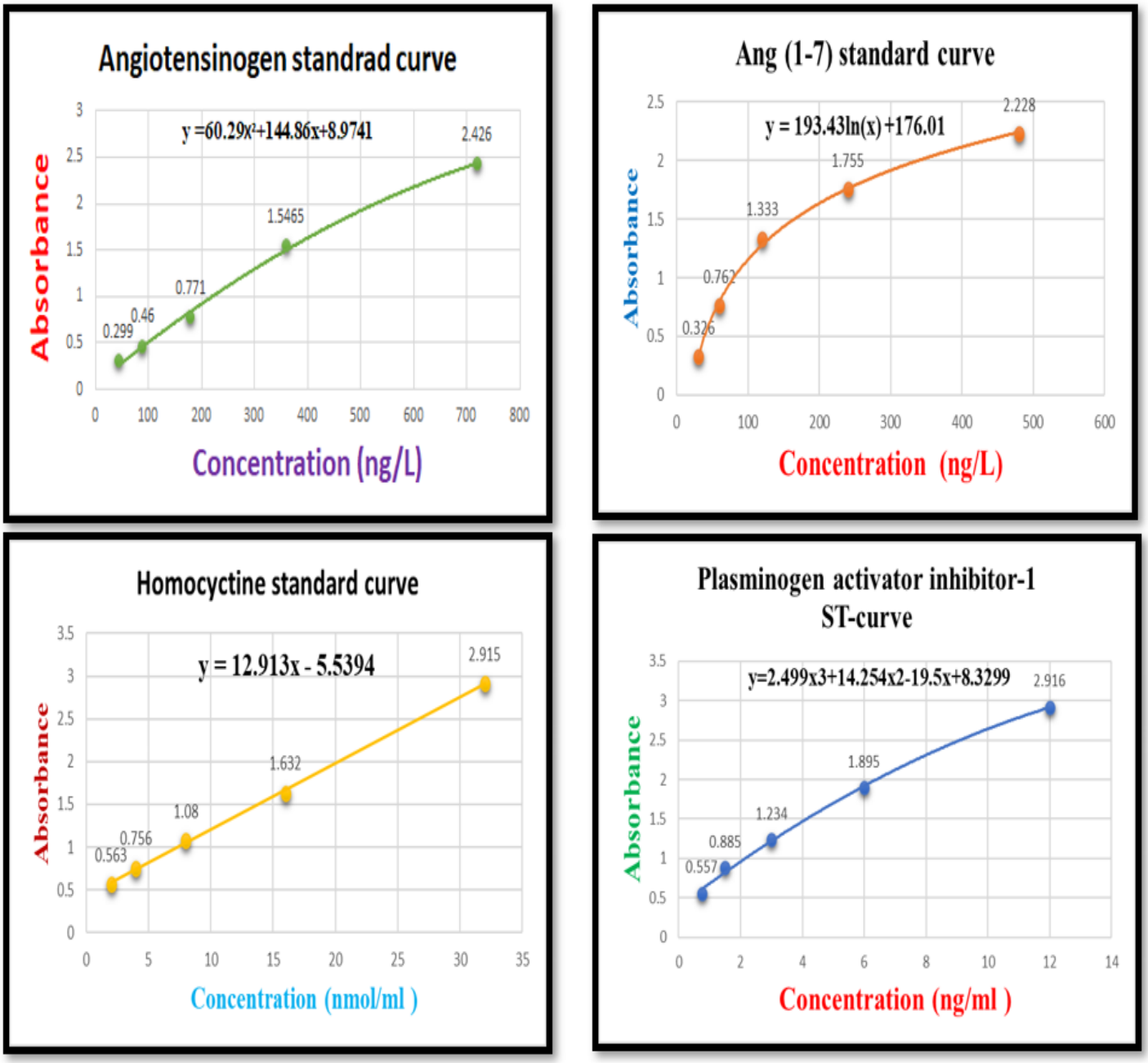


Figure (3-5): Standard curves of AGT, Ang 1-7, Hcy, and PAI-1.

3.5. In Silico Tools Study

Several web servers and software were used to primers design and predict impact SNPs in the genes of this study based on present polymorphisms in exon sites of *AGT* and *MTHFR* and polymorphisms in non-coding sites of *SERPINE1* and *ACE2*, in addition to analysis of the sequencing results and calculate the curve of ELISA concentration as in table (3-12).

Table (3-13) list of web servers and software used in results of the study.

Servers name	Types of analysis
SIFT prediction	Coding variants
PROVEAN tool	Coding variants
PhD-SNP tool	Coding variants
MutPred2 tool	Coding variants
I-mutant2.0 tool	Coding variants
PolyPhen-2 software	Coding variants
TMHMM tool	Coding variants
SpliceAid	Non-coding variants
WASP tool	Allele specific primers
BatchPrimer3	Allele specific primers +ARMS PCR primers
PRIMER1	ARMS-PCR primers
Primer3Plus	Sequencing primers
Curve finder	ELISA concentration curve
MEQA11	Sequencing analysis
Geneoius prime and Blast alignment	Sequencing alignment
BioEdit	Data sequencing analysis
CurveExpert Professional	Curve fitting and data analysis

3.6. Statistical Analysis

The current study data was analyzed using the statistical packages for Social Sciences-version 27 (SPSS-27) with GraphPad prism software, was used to determine the influence of various factors on study parameters. The T-test was used to compare means in a statistically significant manner. The Chi-square test was used to compare percentages in a significant manner (0.05 and 0.01 probability). The odds ratios (ORs) and 95% confidence intervals (95 % CIs) were utilized to evaluate the potential associations between genetic variants of the (*ACE2, AGT, SERPIN-1, and MTHFR*) SNPs genes and severity COVID-19 disease in addition, levels of proteins (Hcy, AGT, Ang 1-7, and PAI-1) were measured. If a test's P value was less than 0.05, it was deemed significant. The Hardy-Weinberg equilibrium (HWE) was used to examine the allele and genotype frequency distributions.

In addition to the area under the curve (AUC), 95 % confidence interval (CI), cut-off value, sensitivity, and specificity were calculated using ROC analysis. The ROC curve for calculated ages, hematological markers, and

ELISA concentrations. The ROC curve was used to plot sensitivity against 1-specificity (false-positive rate) for various cut-off values measured in the diagnostic test. The black curved line in the ROC curve represents the specific diagnostic test and illustrates the trade-off between sensitivity and specificity at different cut-off values. For instance, point A on the curve indicates a stricter cut-off with high specificity (low false-positive rate) but poor sensitivity (around 40%). On the other hand, point B represents a more lenient cut-off with better sensitivity (>90%) but lower specificity (approximately 30%, corresponding to a false-positive rate of 70%).

The AUC (Area Under the Curve) of the ROC curve reflects the overall accuracy of the test. A test that performs no better than chance would be indicated by a straight line (shown as a dashed red line) with an AUC of 0.5. Conversely, a nearly perfect test would have a rectangular configuration (shown as a dashed blue line) with an AUC approaching 1.0.

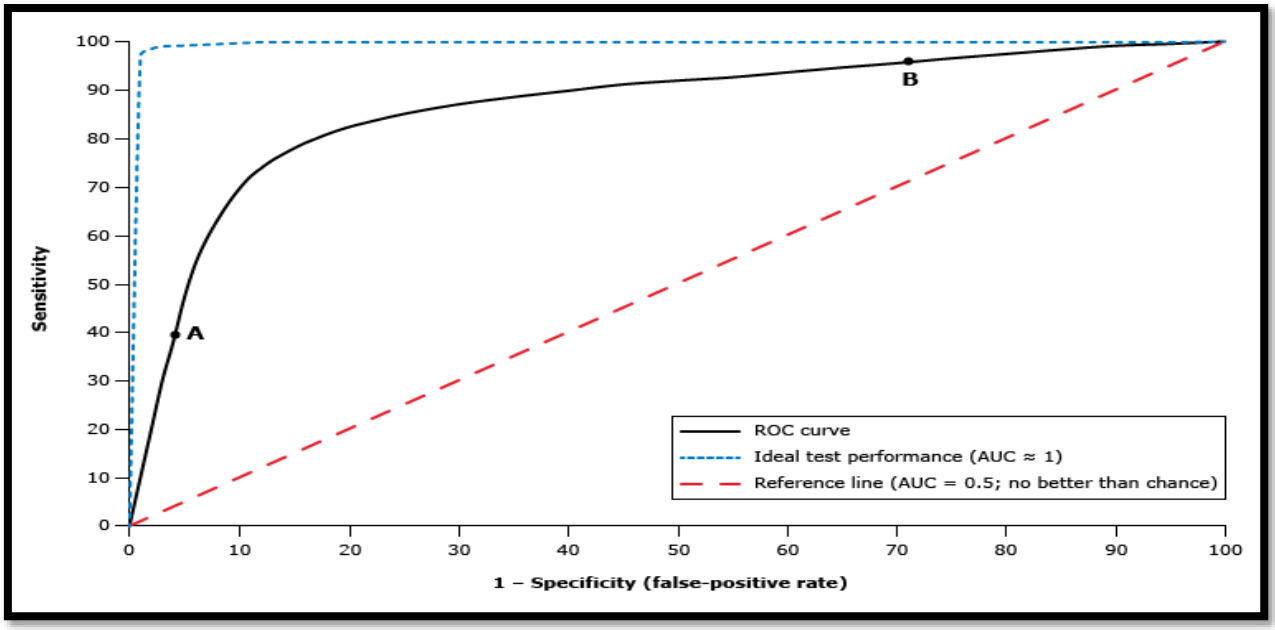


Figure (3-6): The Receiver Operating Characteristic (ROC) curve is an example of a graphical representation used to evaluate the performance of a diagnostic test.

Chapter Four

Results and Discussion

Results and Discussion

4. Subject

The participants in this study were one hundred ninety-four individuals from patients and healthy, one hundred two COVID-19 patients, and ninety-two apparently healthy individuals as a control group figure (4-1).

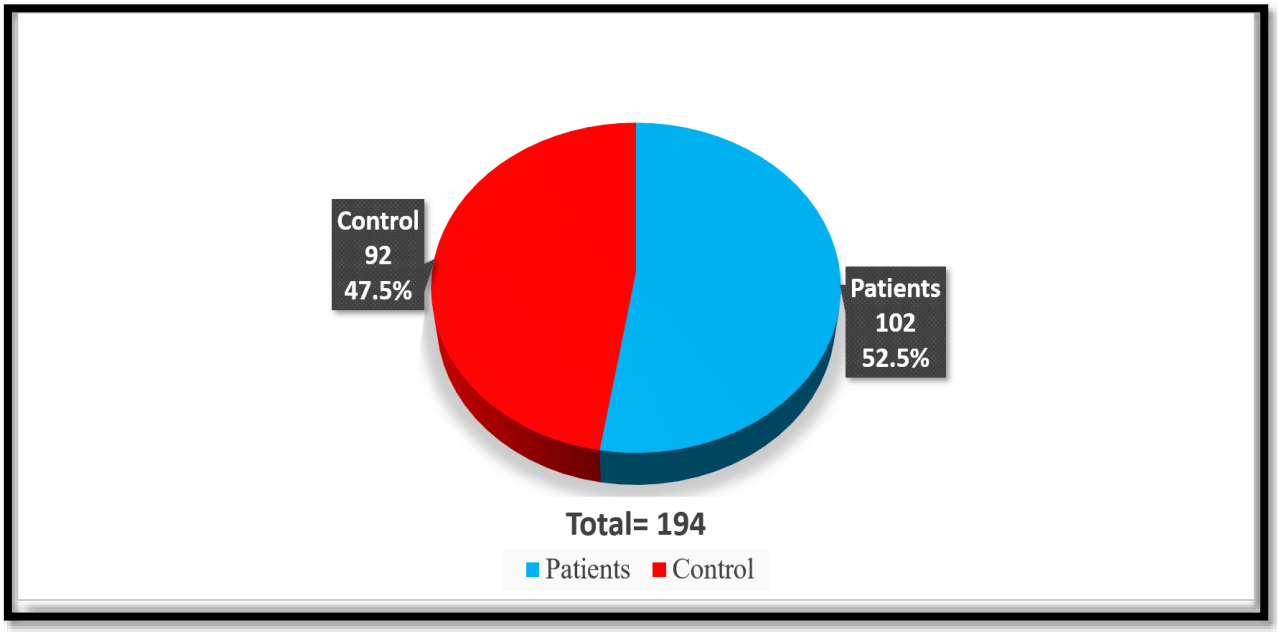


Figure (4-1): Pie Chart showed COVID-19 cases and healthy group distributed according to the number of participants.

4.1. Age and gender distribution

According to the demographic survey, the age of patients ranges from (18-85) years with a high number of older patients. According to the data, older people were more likely to suffer a severe infection. Previous studies also predicted these results, COVID-19 symptoms and non-recovery were more common in older patients, and they were more likely to die in hospital(Zhang J. *et al.*, 2020; Speretta and Leite 2020). The mean±SD age of COVID-19 patients and healthy group (52.66±18.84 *vs.* 37.88±14.19) respectively, as in figure (4-2), with a median (IQR) was [53(36-69) *vs.* 31(24-43)] respectively. In the sense that most COVID-19 cases were 45 years and older in comparison

to the healthy group most of them ≤40 years. That is why predicted a significant level at a (*p*-value ≤0.05).

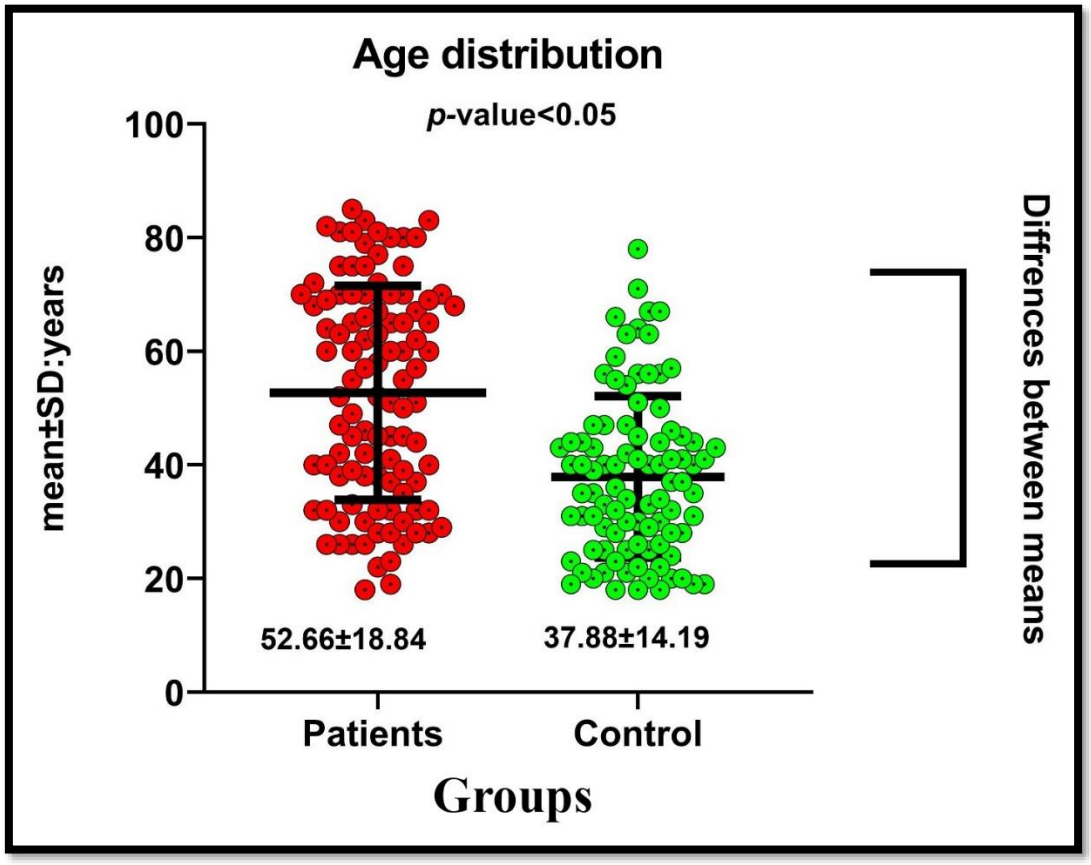


Figure (4-2): COVID-19 cases and control distributed according to mean±SD age.

Figure (4-3) showed the distribution of the males and females between patient group and healthy group did not a significant variation (p=0.99), where collected as patients (54 *vs.*58.6% and 48 *vs.*47.1%) of both male and female are respectively, in addition to heathy male (54 *vs.* 52.9%) and female (38 *vs.* 41.3%). It means that men and women had both susceptibility of COVID-19 infection according to the early clinical reports from China, a recent study showed that both men and women were infected with COVID-19, and further investigation revealed sex differences in mortality and susceptibility to SARS-CoV-2 infection(Kopel *et al.*, 2020).

A study by Levkovich *et al*. (2021) there seems to be gender variations in mortality and susceptibility to the illness, despite the fact that gender-disaggregated data for COVID-19 so far showed an equal number of cases for men and women. Men may be higher mortality, probably because of immunological variations between the genders or because of the higher incidence of smoking among men(Chen *et al*., 2020).

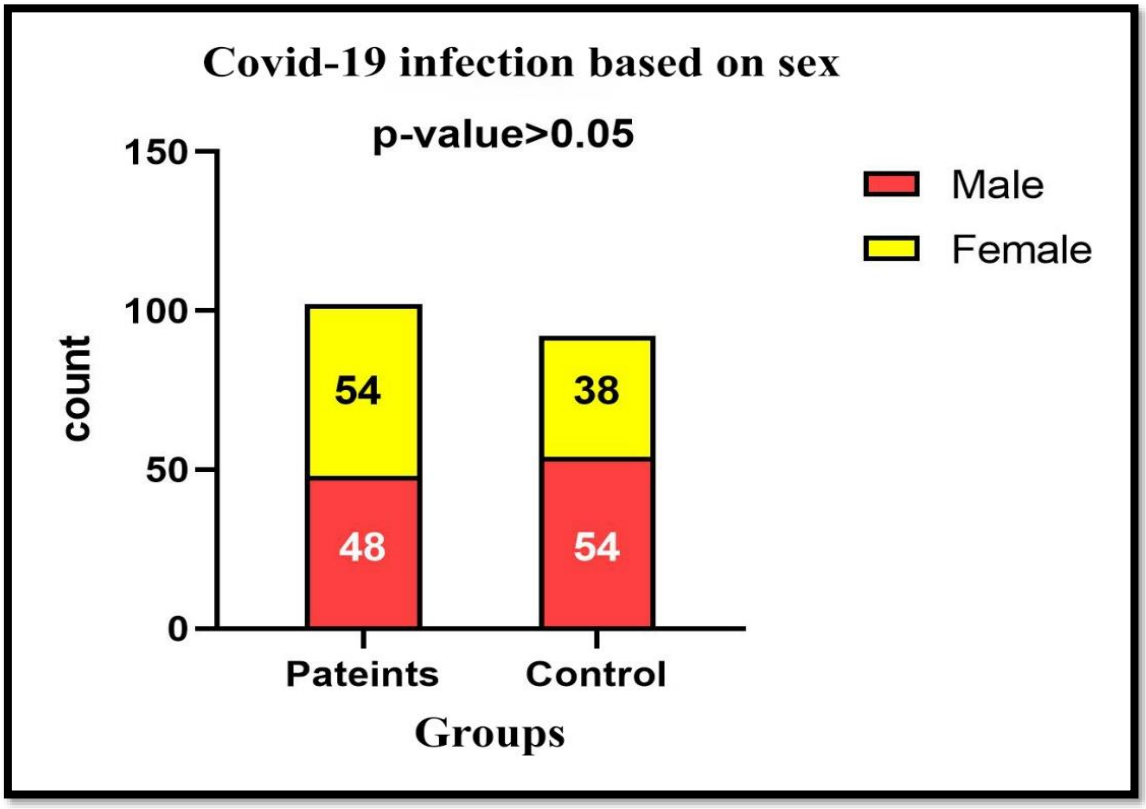


Figure (4-3): Covid-19 cases and control distributed according to gender.

The ROC curve was drawn for predicted age which is to increase the risk of infection COVID-19 between patients compared to control in this study by calculating the AUC, sensitivity, and specificity to collected data, and the results found cut off value when age > 44 years old while the AUC was 0.76 at sensitivity 63% and specificity 89% as in shown in figure (4-4).

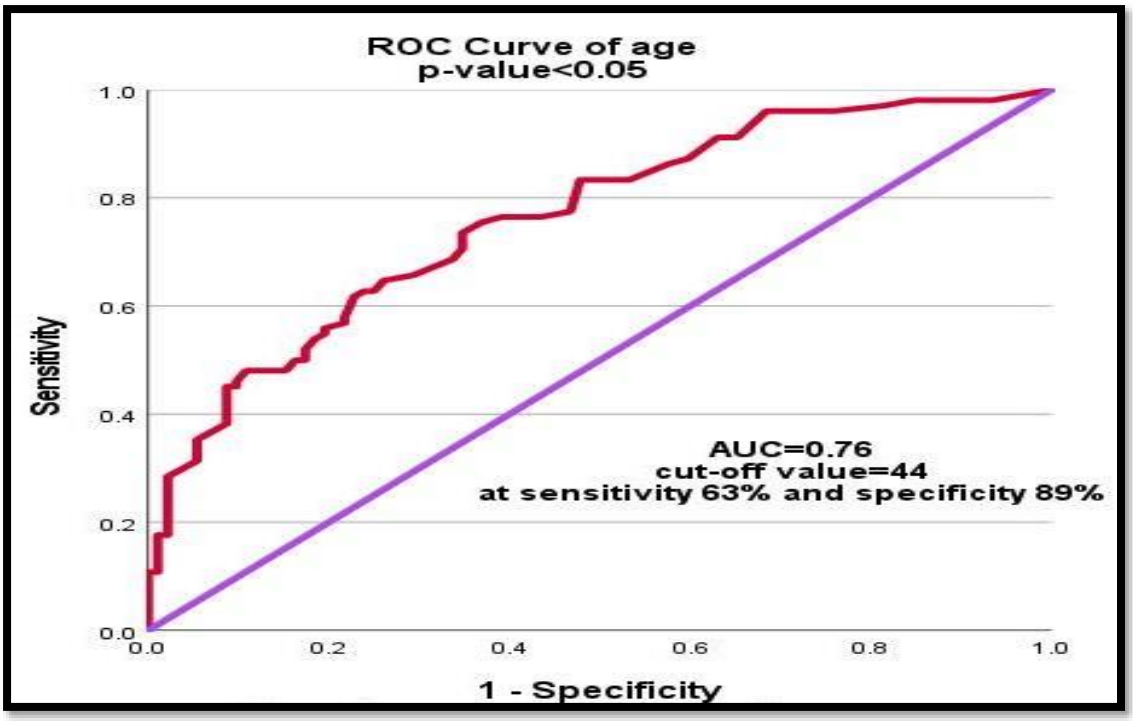


Figure (4-4): ROC curve analysis of age in COVID-19 patients versus control.

4.2. Severity classification among of COVID-19 patients

The study included 102 COVID-19 patients; SARS-CoV-2 positive for all of the COVID-19 study participants was determined by RT-PCR. The participants were categorized into two subgroups, namely severe and non-severe, based on the severity of their condition as illustrated in Figure (4-5) of the present study. In many studies categories of COVID-19 cases depends on who needs ICU and who needs hospitalization as a severe case while non-severe from mild to moderate who was symptoms not observed deeply or there were symptoms needed to see the physician but not hospitals admission(Al Mutair *et al.*, 2022; Flikweert *et al.*, 2022). There were 57(55.9%) severe/critical cases (33 *vs.* 57.9% - 24 *vs.* 42.1%) as male and female respectively, while 45(44.1%) (male 15 *vs.* 33.3% and female 30 *vs.* 66.7%) as non-severe cases with significant difference (*p*-value≤0.01).

In figure (4-6) the mean±SD of age non-severe cases 39.24±10.4 and median (IQR) 38(32-45) compares with severe cases the mean±SD were

65.72±12.83 and median (IQR) 69(59-76), therefore; the result of this study was significance difference (*p*-value≤0.05) between severe and non-severe by the age distribution. One of the important risk factors to predicted severity of COVID-19 in this study was older people more likely to severe infection.

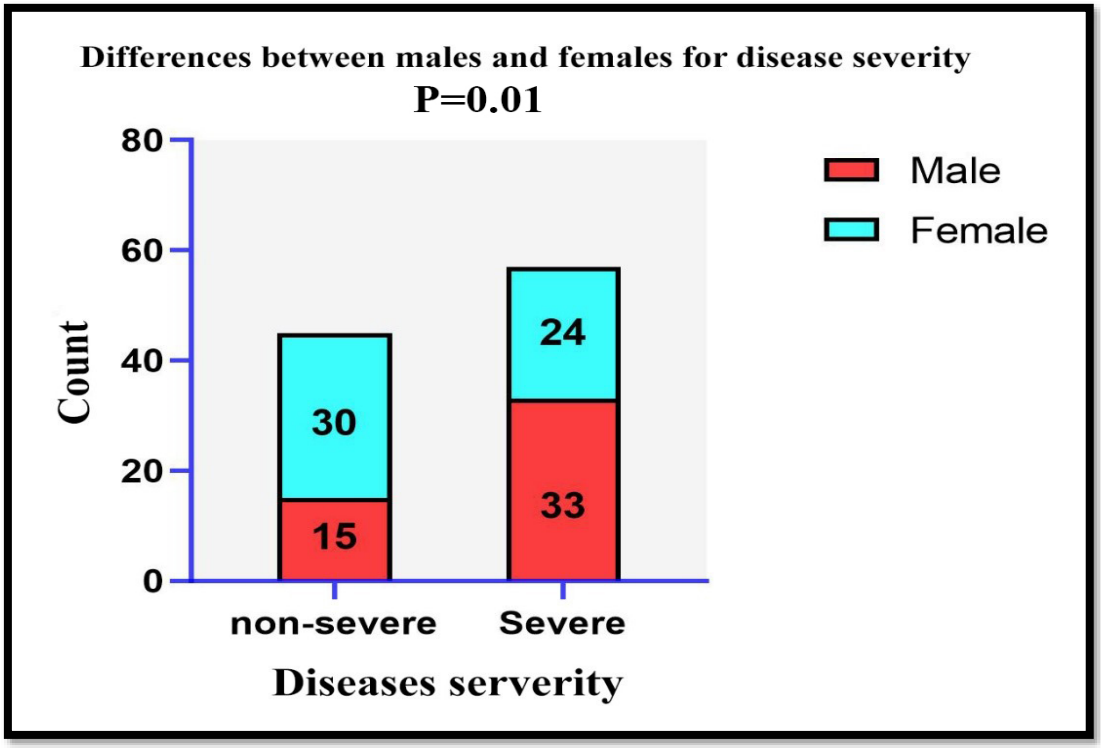


Figure (4-5): COVID-19 severe and non-severe distributed according to gender.

A study from Spain revealed that older age was associated with a greater death rate in those data and in all major series(Sisó-Almirall *et al.*, 2020). Another finding revealed three risk variables related to COVID-19 patient survival: age, gender, and comorbidities(Mi *et al.*, 2020). Recent studies found that age was significantly related to increase the severity of the disease (p≤0.05)(Fang X. *et al.*, 2020; Kose *et al.*, 2023). According to several studies, the majority of COVID-19 patients tend to exhibit mild to moderate symptoms, also severe cases may manifest fewer initial symptoms before progressing to organ failure(Verity *et al.*, 2020; Blair *et al.*, 2021).

A recent study from China indicated that none of the mild patients needed intensive care unit (ICU) care, but 23 (or 77%) of the 30 severe cases did, cases of severe COVID-19 frequently have high viral loads and prolonged viral shedding. These findings suggest the possibility that SARS-CoV-2 viral load might serve as a marker for determining the severity and prognosis of the disease(Liu Y.Y. *et al*. 2020).

Among severe patients in present study, 5 died (8.7%) of severe group, the median age (min-max) was 70(51-83) years. A receiver operating characteristic (ROC curve) analysis was performed in order to investigate the role of age in infection, as well as the prognostic ability of clinical characteristics, as well as some related factors in order to categorize continuous variables, cut-off values must be determined, followed by plotting the resulting sensitivities against specificities. In figure (4-7) predicted cut-off value 48 years to measure effect of the age to increase severity of COVID-19 at higher the specificity 93% and sensitivity 94%.

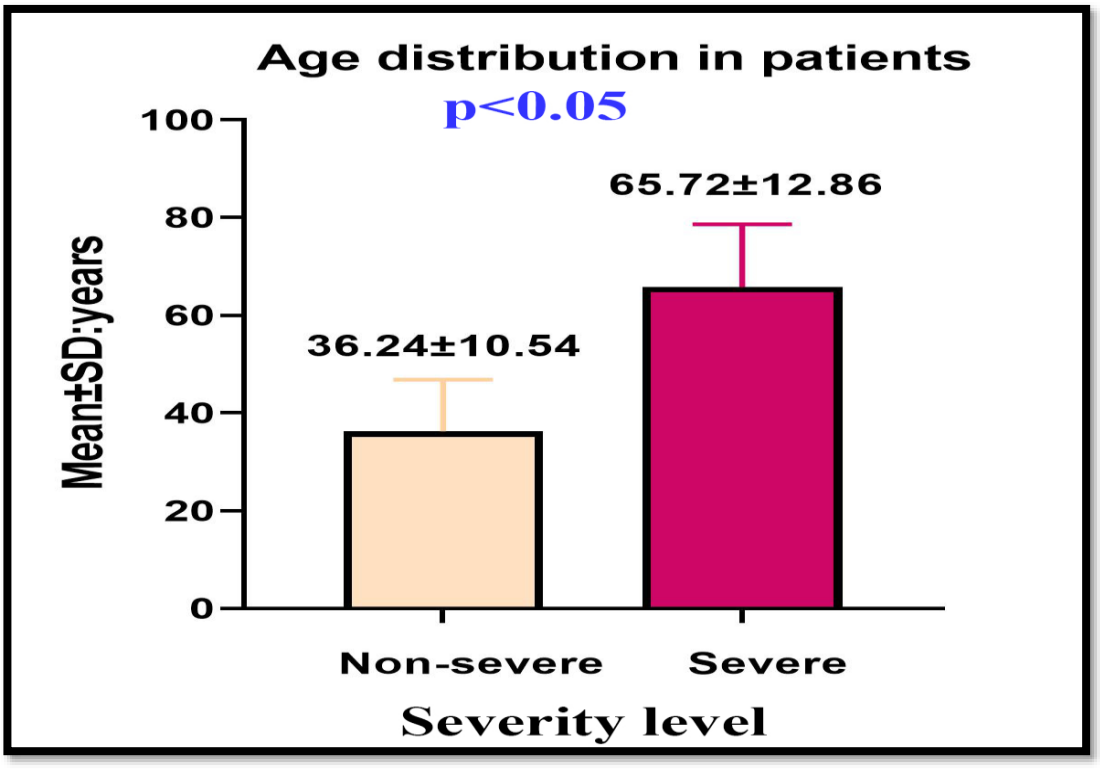


Figure (4-6): COVID-19 patients (non-severe vs severe) distributed according to mean±SD age.

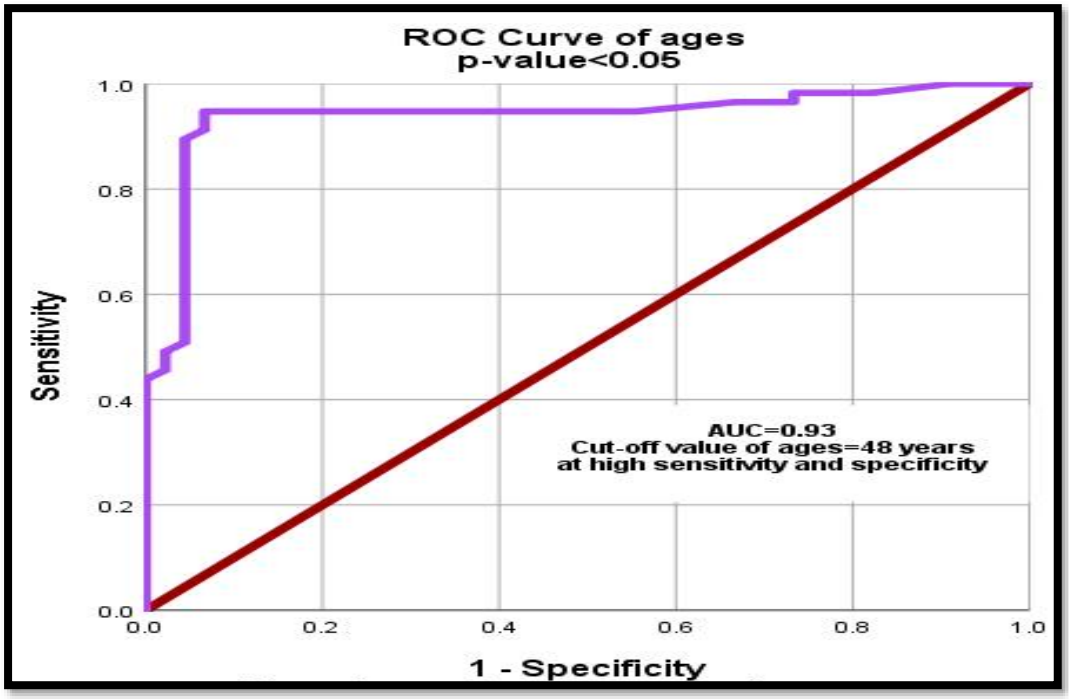


Figure (4-7): ROC curve analysis of age in COVID-19 severe versus non-severe.

4.3. Comorbidities diseases among COVID-19 patients

According to results of present study, there is a relationship between preexisting chronic conditions and clinical outcomes and severity of COVID-19, such as diabetes, hypertension, cardiovascular disease (CVD), malignancy, and chronic kidney diseases (CKD), figure (4-8) showed 14(13.7%) had hypertension (HTN), 15(14.76%) of diabetes mellitus (DM), 9(8.82%) of CVD, 6(5.8%) of CKD, 3(2.4%) of malignancy, 2(1.9%), and 53 (51.9%) of without chronic diseases. The prevalence of chronic diseases between men and women of patients was not similar as in figure (4-9), HTN and DM in male less than female while CVD and CKD in male greater than female. Figure (4-9) exhibited 14 patients had two chronic diseases and 6 patients with three diseases. In the study, the hypertension status, vaccination status of the cases, and the level of oxygen support provided to the patients emerged as noteworthy factors when compared to other variables. The table (4-1) contains all the data of the subjects that were collected during the study.

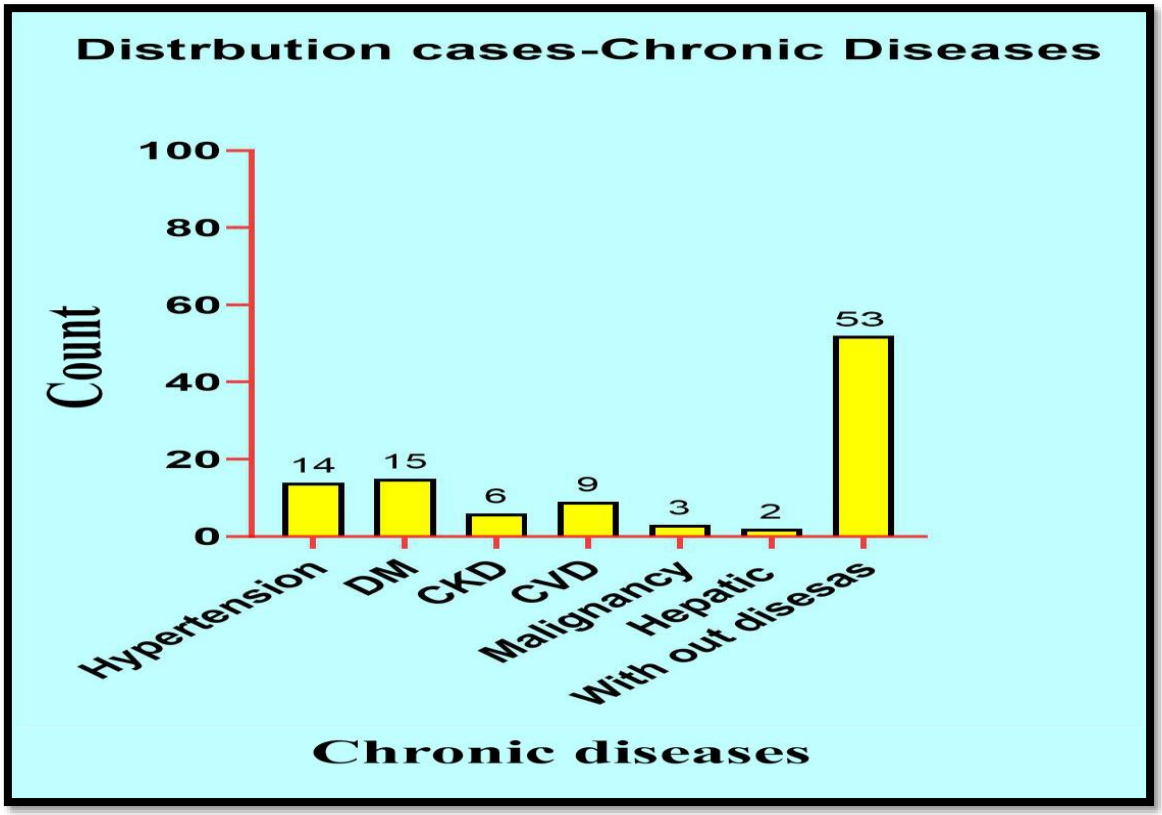


Figure (4-8): COVID-19 cases distributed according to chronic diseases.

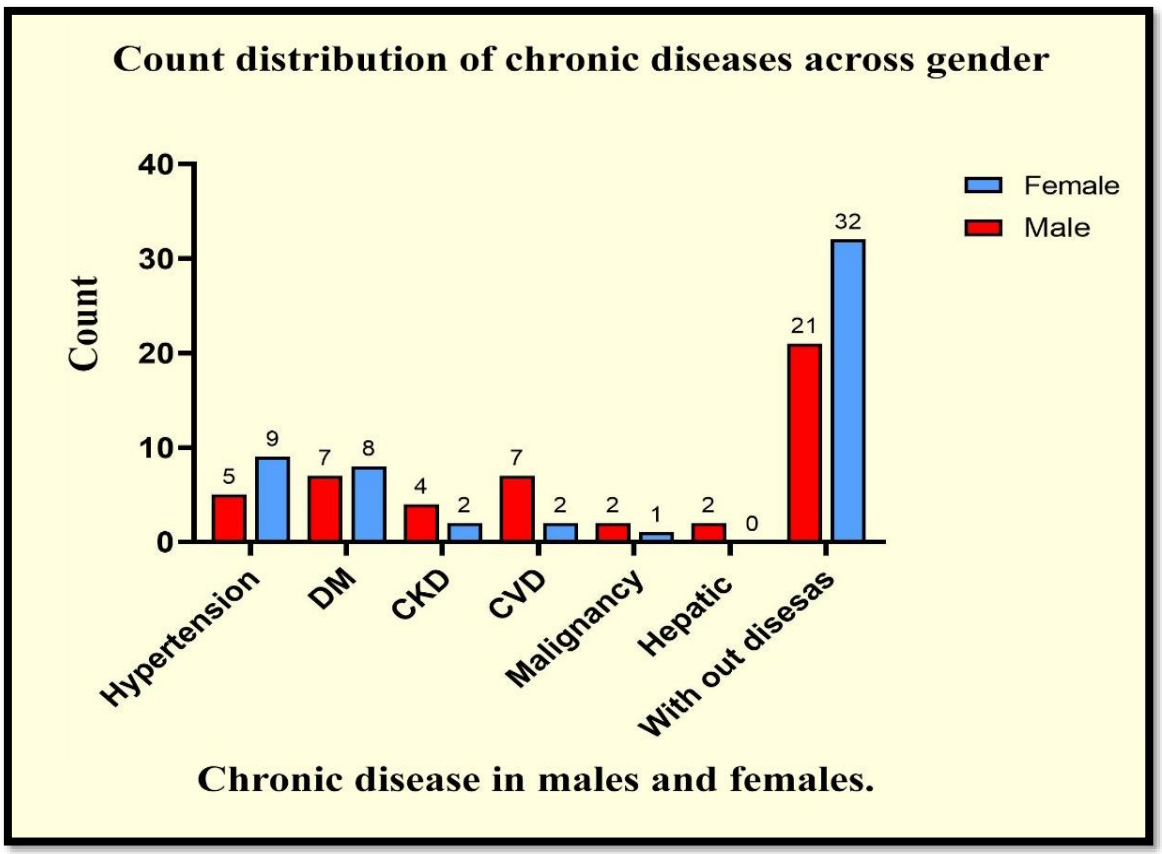


Figure (4-9): COVID-19 cases with chronic disease distributed according to gender.

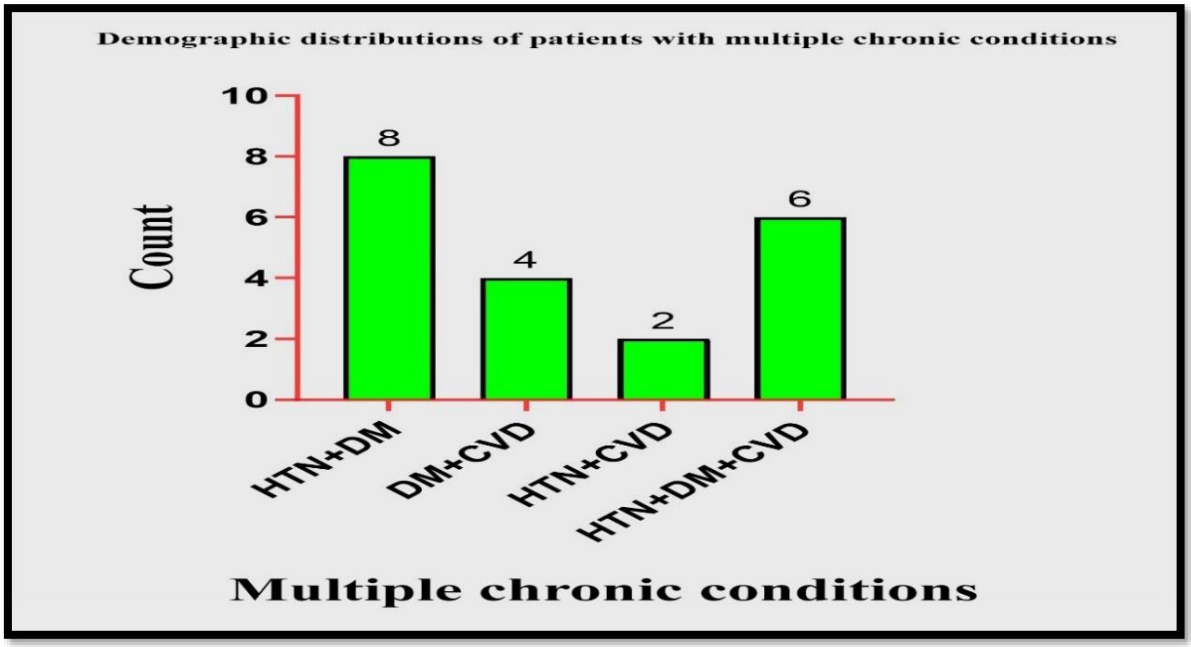


Figure (4-10): COVID-19 cases distributed according to more than chronic diseases in patients.

Table 4-1. Demographic, and clinical characteristics variables among COVID-19 patients.

Characteristics	Status	Total patient N=102	Non-severe group N=45(%)	Severe group N=57(%)	P-value
Age, median (IQR)	-	53(36-69)	37 (28-42)	67(60-75)	**P≤0.001***
Gender	-	Male/48(47.1%) Female/54(52.9 %)	Male/15(31.3) Female/30(55.6)	Male/33(68.8) Female/24(44.4)	**0.01***
Death	Yes No	5(4.9%) 97(94.1%)	0(0) 45(100)	5(8.7) 52(91.3)	0.7
Comorbidity	Yes No	49(48%) 53(52%)	17(37.8) 28(62.2)	32(56.1) 25(43.9)	0.067
Hypertension	Yes No	14(13.7%) 88(86.3%)	3(6.7) 42(93.3)	11(19.3) 46(80.7)	**0.04***
Diabetes Meletus	Yes No	15(14.7%) 87(85.3%)	6(13.4) 39(86.6)	9(15.8) 48(84.2)	**0.02***
Cardiac diseases	Yes No	9(8.8%) 93(91.2%)	5(11.2) 40(88.8)	4(7.1) 53(92.9)	0.4
Kidney diseases	Yes No	6(5.9%) 96(94.1%)	1(2.3) 44(97.7)	5(8.8) 52(91.2)	0.1
Cancer	Yes No	3(3%) 99(97%)	2(4.5) 43(95.5)	1(1.7) 56(98.3)	0.4
Hepatic diseases	Yes No	2(2%) 100(98%)	0(0) 45(100)	2(3.5) 55(96.5)	0.2
Oxygen support	Yes No	47(46%) 55(54%)	0(0) 45(100)	47(82.4) 10(17.6)	**P≤0.001***
Vaccination status	Yes No	41(40.1%) 61(59.9%)	29(64.4) 16(35.6)	12(21) 45(79)	**P≤0.001***

Abbreviations: IQR: Interquartile range, P: probability, *: significant at the 0.05 level.

4.4. Baseline clinical characteristics

Several hematological parameter changes in COVID-19 patients have been observed including, WBCs, platelets, hemoglobin, neutrophils, granulocytes, and levels of D-dimer, patient's COVID-19 illness severity may be determined by these markers. All parameters in this study were compared with the worldwide reference ranges for each parameter based on (https://www.mayoclinic.org) and determined whether or not the measured values were within the normal, elevated, or lowered ranges as in table (4-2).

Table 4-2 Baseline characteristics and clinical outcomes of COVID-19 patients at admission.

Parameters	Total patient N (102) M±SD	Non-severe group N (48)	Severe group N (56)	Normal range	P-value
White blood cell	13.5×10^9/L ±5.5	8.54±3.06	17.41±3.55	4.5 - 11.0 ×10^9/L	P=**0.002*** increase
Lymphocytes	1.55±0.96	2.36±0.62	0.92±0.67	1.5-4.5×10^9/L	P=**0.001*** decrease
Neutrophils	10.88±4.35	7.69±2.68	13.39±3.72	2 - 8×10^9/L	P=**0.0032*** increase
Platelet	276.6×10^9/L±77.45	254.42±33	294±96	150-450×10^9/L	P=0.31 normal
D-dimer	1.37±1.09	0.51±0.25	2±1	≤ 0.50 µg/mL	P=**0.0001*** Increase
NLR	11±9	3.54±1.95	16.9±7.9	Less than 0.7 or greater than 3 =abnormal	P=**0.0034*** Increase
PLR	307.96±271.76	116.5±39.98	435±285	Less than 36 or greater than 150= abnormal	P=**0.006*** increase
Hemoglobin	13.93±1.49- male 12.74±1.94-female	Male/14.86±1.16 Female/13.54±1.49	Male/13.51± 1.44 Female/11.75±2	Male/13.8 to 17.2 Female/ 12.1 to 15.1	P=0.21 normal

Abbreviations: NLR, Neutrophil-Lymphocyte Ratio; PLR; The platelet-lymphocyte ratio, M±SD: mean± Standard deviation, P: probability, *: significant at the 0.05 level.

4.4.1. White blood cell count

During viral infection, WBCs, particularly SARS-CoV-2 in present study, played an important role in maintaining immunological homeostasis. In this study WBCs showed increase in older ages mostly with some extreme values to other ages in both the gender with a reference range between the lowest count (5×10^9/L) to high count (23×10^9/L) with a mean±SD of (13.5±5.5) and a median (IQR) 13.65 (IQR 8.5- 19) at 95% (CI, 11- 15×10^9/L), the comparison of common hematological parameters showed a significant difference in WBC (*p*-value=0.01) was observed in severe cases increased compared with less severity the statistical findings of severe individuals were an odds ratio (OR) of 2.8 (95% CI: 1.135 to 3.287) and relative risk 1.8 (95% CI: 1.135 to 3.287) with mean±SD was 17.41±3.55 and median (IQR) 18 (14.5-20) compared with non-severe mean±SD 8.54±3.06 at 95% (CI:7.62-964). The cut-off value of WBCs among severe and non-sever predictions was ($11.5\times10^{\wedge9}$/L) at the area under the ROC Curve (AUC) was excellent (0.96) as shown in figure (4-11) below with a significant difference (*p*-value=0.001).

WBCs are the most essential immune system cellular component, mediating innate and adaptive immune responses against viral, bacterial, fungal, and parasitic infections. Furthermore, they have a beneficial relationship with inflammatory responses(Tigner *et al.*,2020). WBCs count was correlated with mortality COVID-19, and increased levels of WBCs be taken into account in the treatment of COVID-19(Zhu B. *et al*. 2021). A study by Elshazli *et al*. (2020) demonstrated that raised WBC levels were substantially related to a higher odds ratio between severe, ICU admission and a high mortality rate. It has been found that increased WBC counts appear to be the most significant indicator of a poor outcome(Sobhani *et al.*, 2021). In a study by Fahmi *et al*. (2021) in Iraq the patients had higher WBC in laboratory results and were initially diagnosed among COVID-19 patients.

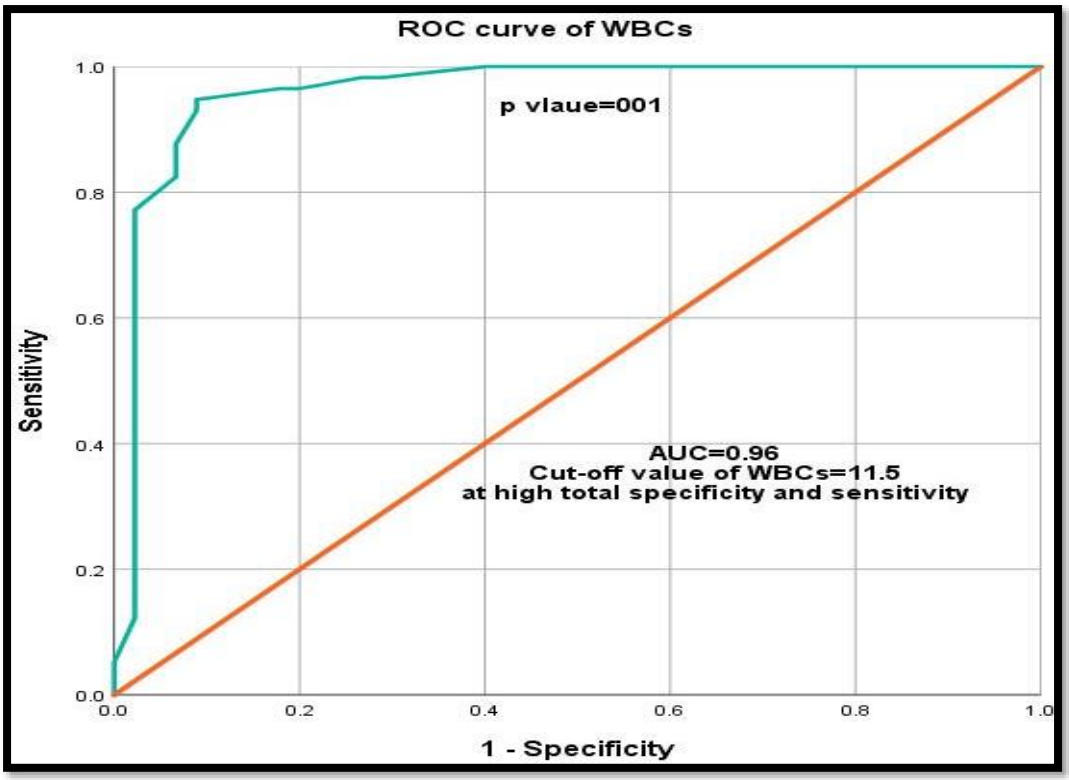


Figure (4-11) The ROC curve of WBC count in predicting critical illness of COVID-19.

4.4.2. Hemoglobin

Anemia is a common symptom of critical illness, and the reason is frequently complex, influencing mortality. Anemia is frequently caused by a combination of factors, anemia is thought to be an independent prognostic factor of coronavirus infection severity in 2019 (COVID-19)(Oh SM. *et al*. 2021).

In the present study, the mean±SD hemoglobin levels at the time of admission were 13.93±1.49 (47.1%) for males, with a median value of 14 (interquartile range [IQR]: 13-15), while females exhibited levels of 12.74±1.94 (52.9%), with a median value of 13 (IQR: 12-14). When considering the severity of cases, the results indicated that non-severe males had a mean±SD hemoglobin level of 14.86±1.16, whereas non-severe females had a mean level of 13.54±1.49. In contrast, severe cases among males and females displayed mean±SD hemoglobin levels of 13.51±1.44 and 11.75±2,

respectively. Importantly, the difference in hemoglobin levels between severe and non-severe cases was not statistically significant (p-value>0.05), except for some of severe cases that exhibited exceptionally low hemoglobin levels. This observation suggests that the severity of COVID-19 infection may contribute to anemia in certain critical patients. The accuracy of using the parameter in this study for predicting the severity of COVID-19 appears to be questionable, as indicated by the area under the curve (AUC) in figure (4-12), which is less than 0.5. This suggests that the parameter may not be a reliable predictor of severity on its own. It's important to note that without considering other factors, it may not be suitable for predicting the severity of the disease. As highlighted by Bergamaschi and Gaetano, anemia is a common symptom of COVID-19. While anemia itself may not directly contribute to mortality, it can have a significant impact on the quality of life, especially in older and weaker individuals. Therefore, when assessing the severity of COVID-19, it's crucial to consider a combination of factors and not rely solely on a single parameter, as the disease's complexity and impact on individuals can vary widely(Bergamaschi *et al.*, 2021).

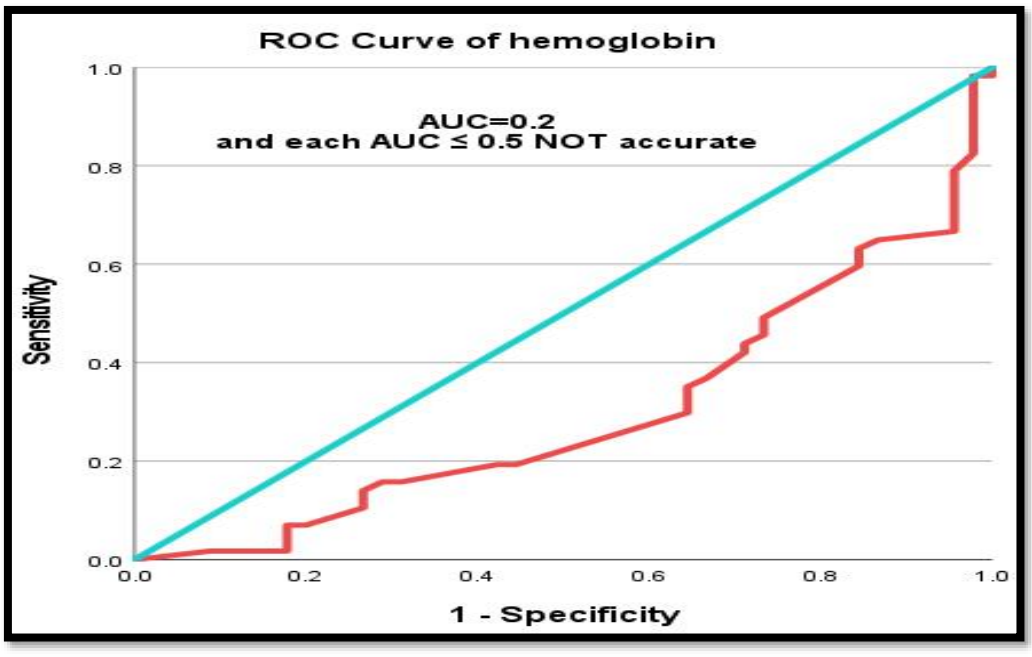


Figure (4-12) The ROC curve of Hemoglobin count in predicting critical illness of COVID-19.

On the other study drop of hemoglobin levels during COVID-19-related hospitalizations was linked to an increased risk of acute kidney injury (AKI) and in-hospital death, patients with low hemoglobin levels were more likely to get therapeutic anticoagulation within two days of hospitalization(Kuno *et al.*, 2021).

Indeed, there seems to be a connection between anemia and an increased risk of experiencing severe COVID-19 infection, as suggested by findings from a meta-analysis. These findings provide valuable insights into the potential pathophysiological link between anemia and the severity of COVID-19. Anemia patients typically exhibit lower hemoglobin levels, which may contribute to their heightened vulnerability to severe forms of the disease(Hariyanto and Kurniawan, 2020).

4.4.3. Platelets count

The platelet levels of COVID-19 patients in this study were characterized by a mean±SD of 276.63×10^9/L±77.45 and a median (IQR) of 256 (IQR 233.75-290). These findings indicated that there was no significant difference between patients with severe and non-severe cases, as reflected by a p-value greater than 0.05.

For severe cases, the mean±SD platelet count was 294±96 with a 95% confidence interval (CI) of 250.82-283.28, and a median (IQR) of 254 (235-282). In contrast, mild to moderate patients had a mean±SD platelet count of 254.42±33.48. However, the platelet count did not prove to be an effective predictor of illness severity, as evidenced by an area under the curve (AUC) of less than or equal to 0.5 in figure (4-13).

It's important to note that some severe patients exhibited thrombocytopenia, characterized by a low platelet count of ≤150×10^9/L. Additionally, in certain cases within this study, a high platelet count exceeding

400×10^9/L may lead to platelet hyperactivity, which represents one of the pathways contributing to the development of increased blood clot formation.

A study has revealed that the platelet count in COVID-19 patients changes between mild to severe infections. Patients with mild manifestations have a normal or a slightly raised platelet count, whereas severe cases COVID-19 infections have thrombocytopenia ≤150×109/L(Rohlfing *et al.*, 2021).

The coagulopathy and platelet hyperactivity are characteristics of COVID-19, and they contribute to the elevated thrombotic risk seen in critical cases(Portier *et al.*, 2021). Platelets played a key role in thrombosis, a physiological process in which arterial damage results in clot formation. However, in a pathological condition, this might lead to vascular blockage, ischemia, and tissue destruction(Lin E. *et al.*, 2009).

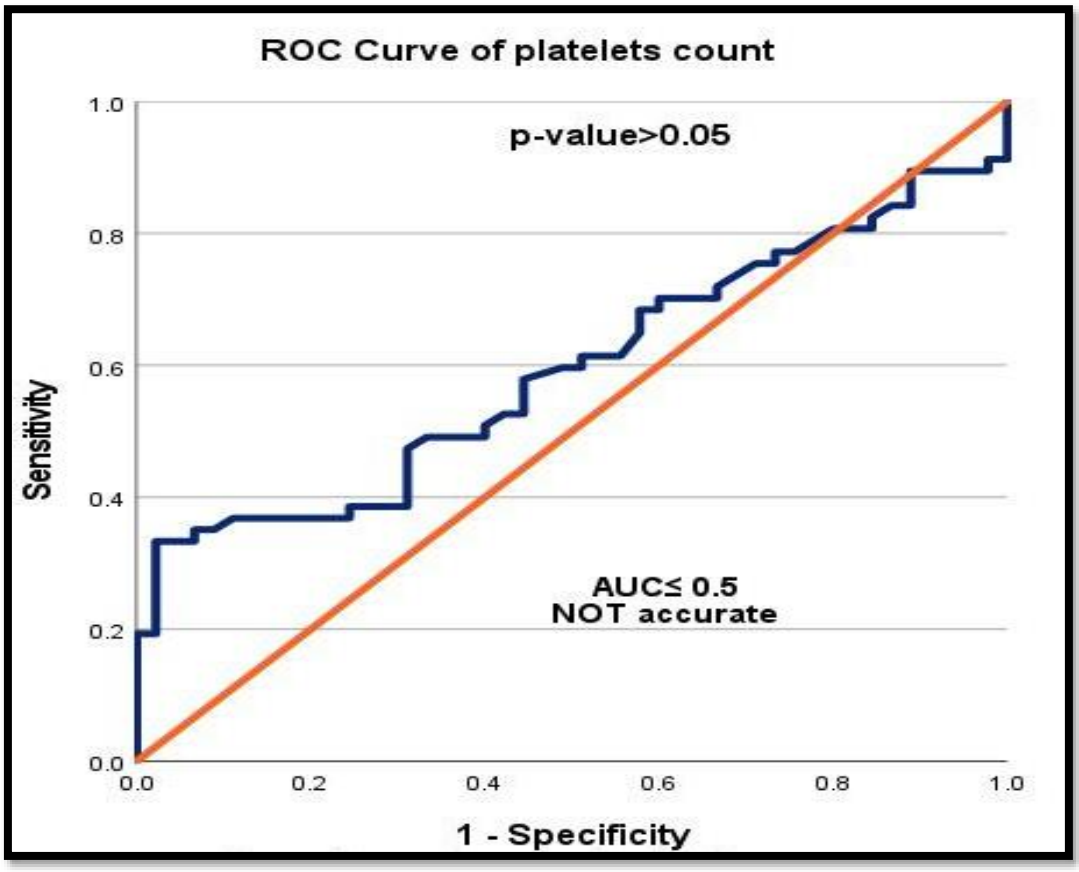


Figure (4-13) The ROC curve of Platelets counts in predicting severity illness of COVID-19.

4.4.4. Neutrophil count

During the COVID-19 pandemic, certain granulocyte biomarkers, including neutrophils, eosinophils, and basophils, were found to be elevated and could effectively distinguish between individuals with mild and severe illness. The study's findings indicated that the neutrophil count in all patients had a mean±SD of 10.88±4.35, with a 95% confidence interval (CI) ranging from 10.02 to 11.7. The median (IQR) value for neutrophils was 10.6 (IQR 6.89-14.45). Notably, when a cut-off value of 8.9x10^9/L was applied, it achieved an area under the curve (AUC) of 0.88, demonstrating strong predictive power. This cut-off value yielded 86% sensitivity and 80% specificity in predicting severe cases, and the difference was statistically significant with a p-value of ≤0.05, as visualized in figure (4-14).

In severe cases, the mean±SD of neutrophils was 3.39±3.72, with a median (IQR) of 13.91 (11.21-16). In comparison, non-severe cases had a mean±SD of 7.69±2.68 and a median (IQR) of 6.9 (6.18-8.41). These findings underline the potential of neutrophil count as a valuable marker for distinguishing between severe and non-severe COVID-19 cases

Neutrophils have been the most common leukocytes in circulation and have long been considered to represent the first line of defense in the immune system's innate arm. As a key mechanism of COVID-19 immunopathy, neutrophil recruitment at the site of infection predicts the severity and outcome of the disease(Rosales, 2018).

The production of neutrophil extracellular traps (NETs) during NETosis, a controlled type of neutrophil cell death, damage caused by neutrophils depends on this effector function. Many COVID-19 patients' neutrophils have abundant NETosis and NET production, resulting in adverse coagulopathy and immunothrombosis(Liu J. *et al*., 2020). According to similar results, the neutrophil count, % neutrophils, and a several parameters increased with severity of COVID-19(Trofin *et al.*, 2023). According to a recent study,

COVID-19 patients' neutrophil counts were significantly higher when compared to controls (p=0.001)(Tiwari *et al.*, 2023).

In recent findings, granulocytes from patients with severe COVID-19 patients had an increase in inflammation and suppressive signatures at the same time, according to research. Whole blood transcriptomes are very relevant for COVID-19 because they capture granulocytes, which are important drivers of disease severity (Aschenbrenner *et al.*, 2021). Another study by Liu J. *et al.*, (2020) in COVID-19 patients, an increased neutrophil blood count predicts poor results. Additionally, Krishnan *et al.* (2022) revealed that a higher number of neutrophils predicted disease severity with COVID-19 liver damage patients. In study found increasing in neutrophils in COVID-19 patients based on the Bayesian inference analysis for lung damage in addition to immune cell profiling using transcriptomes indicated elevated neutrophil markers in the lung and BALF samples of COVID-19 patient (Wang J. *et al.* 2020). The concludes in results confirmed all severe cases in both gender high granulocytes or/and neutrophils was considered a valuable marker in the severity and prognosis of COVID-19.

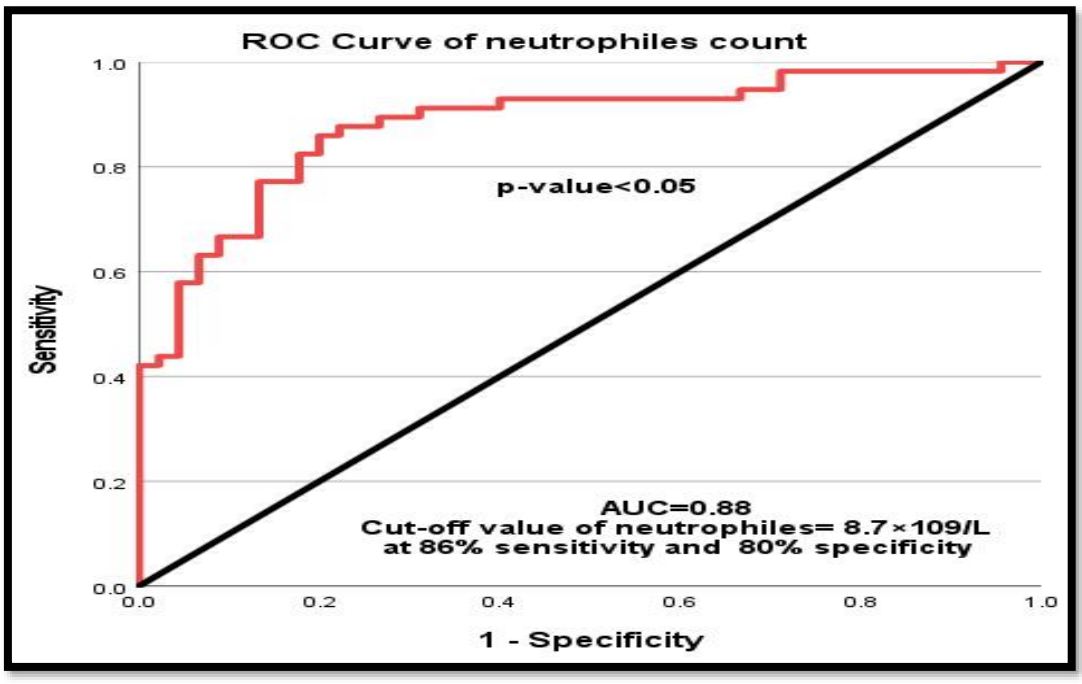


Figure (4-14) The ROC curve of Neutrophiles count in predicting critical illness of COVID-19.

4.4.5. D-Dimer values

The D-dimer antigen is a specific biomarker of fibrin breakdown produced by the serial activity of three enzymes: thrombin, factor XIIIa, and plasmin. D-dimer tests have been shown to be predicting and diagnostic in a variety of disease conditions, including disseminated intravascular coagulation (DIC), pulmonary embolism, deep vein thrombosis, and cancer-associated thrombosis(Chapin and Hajjar, 2015). Recently revealed as a significant biomarker among COVID-19 infection needed to improve early detection for severity of COVID-19(Düz *et al.*, 2020).

The results of this study revealed that the mean±SD D-dimer level was 1.37±1 µg/ml, with a 95% confidence interval (CI) ranging from 1128.13 to 1587.21 µg/ml, for all patients, including both severe and non-severe cases. The median (IQR) D-dimer level was 865.5 (438-2028) ng/mL. Additionally, a cut-off value of D-dimer at 0.78 µg/mL or 780 ng/mL was established to predict severe cases, achieving an impressive area under the curve (AUC) of 0.96, indicating high specificity and sensitivity. This difference was statistically significant, as denoted by a p-value of ≤0.05, as depicted in figure (4-15).

Furthermore, for non-severe cases, the mean±SD D-dimer level was 0.51±0.25 µg/ml, with a median (IQR) of 0.43 (0.38-0.55) µg/ml. In contrast, severe cases exhibited a mean±SD D-dimer level of 2±1 µg/ml, with a median (IQR) of 1.97 (1.19-2.7) µg/ml. These findings underscore the potential utility of D-dimer levels as a valuable marker for distinguishing between severe and non-severe COVID-19 cases.

When compared to non-severe patients, severe patients had higher average D-dimers levels upon admission, as well as patients who needed critical care and those who passed away, according to a meta-analysis of 29 randomized studies including 4328 hospitalized COVID-19 patients, the study found an increased risk of severe illness progression and death among patients

with SARS-CoV-2 infection who had higher D-dimers levels at the start of their hospitalization(Nugroho *et al.*, 2021).

In study by Gustian *et al.* (2021) The d-dimer levels of mild, moderate, and severe COVID-19 patients varied significantly. The severity of COVID-19 was related to higher D-dimer levels. In the previously published study by Wang *et al*, patients needing ICU treatment exhibited comparatively higher D-dimers ($P \leq 0.001$) than less severe cases(Wang. D *et al*., 2020). D-dimer and blood ferritin levels both rise as a result of COVID-19 infection, making it a reliable tool for the early identification of COVID-19 infection(Al Meani *et al.*, 2020). D-dimer and a rise in neutrophil count, the potential of such clinical indicators to identify severe of COVID-19 has been suggested by the effectiveness of these abnormalities in predicting disease severity and progression(Kadhim and Abdullah, 2021).

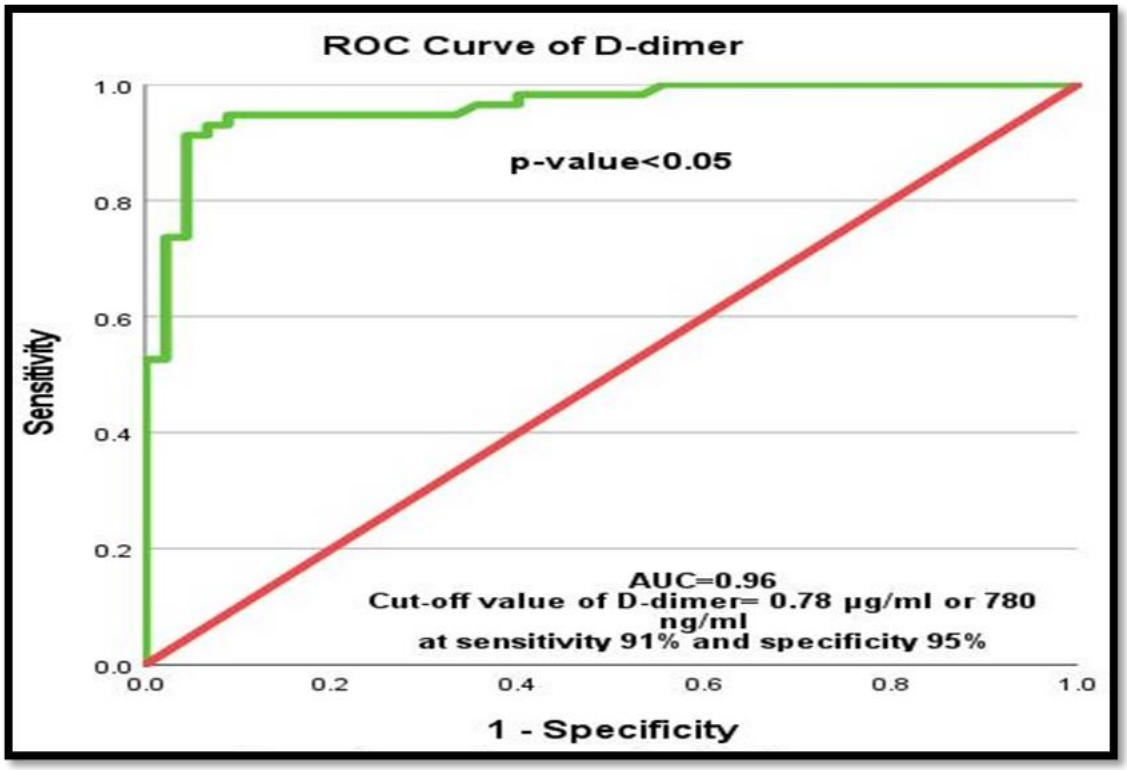


Figure (4-15) The ROC curve of D-dimer values to explore its role in predicting the severity of COVID-19.

4.4.6. Lymphocytes count

Lymphocytes are white blood cells that are immune cells. The cells that fight illness and disease are divided up into three primary groups: T cells, B cells, and natural killer (NK) cells. The T and B cells played important roles in adaptive immunity. NK cells are a subset of lymphocytes that contribute to innate immunity(Omman and Kini, 2019). Lymphocytes are critical components of the immune response to viral infections(Koyasu and Moro, 2012). The lymphocyte number has been studied as a marker. It has been linked to severe COVID-19, with non-survivors having a notably lower lymphocyte number than survivors(Ruan *et al.*, 2020). Lymphopenia can be used to predict poor prognosis in COVID-19. Lymphopenia, defined as a lymphocyte count of 1100 cells/L, is associated with a threefold risk of poor outcome(Huang I. and Pranata 2020).

The lymphocyte count results in the current study indicated that, for all patients, the mean±SD was 1.55±0.96, with a median (IQR) of 1.46 (0.64-2.3) ×10^9/L. A more detailed for severe cases, the mean±SD was 0.92±0.67, with a median (IQR) of 0.71 (0.51-0.93), whereas for non-severe cases, the mean±SD was 2.36±0.62, with a median (IQR) of 2.31 (2-2.74). This study identified a clear association between reduced lymphocyte counts and the presence of severe COVID-19 cases. The study's findings also demonstrated the accuracy of using lymphocyte count as a marker for predicting disease severity, as illustrated in the ROC curve in figure (4-16). The cut-off value for this prediction was determined to be ≤1.72.

In study from China found lymphocyte count may be an consider predictor of COVID-19 severity with share others factors. It may also be beneficial in determining when nucleic acid test findings in COVID-19 patients will become negative(Li Y. *et al.*, 2021). In work the average absolute lymphocyte count (ALC) measured at the time of hospital admission in patients who required ICU admission was lower ($0.8\pm0.11\times10^3$ cells/L) than in those

who did not require ICU admission (1.4±0.15×10^3 cells/L; P =.01). The ICU population was also more likely to have lymphocytopenia at the time of hospital admission (62%) than the non-ICU population (32%; P =0.04)(Wagner *et al.*, 2020). This result was comparable to what found in present study. A study by Zahchary Illg *et al*. (2021) showed that the degree of lymphopenia corresponds with the severity of COVID-19.

Hence, it appears feasible to utilize Absolute Lymphocyte Count (ALC) as a prognostic indicator in COVID-19 patients. This approach could assist healthcare professionals in monitoring individuals who are at a heightened risk of experiencing severe illness. A study conducted in Iraq also revealed that low lymphocyte counts were linked to longer stays in the Intensive Care Unit (ICU) among COVID-19 patients, especially those with severe cases(Nafakhi *et al.*, 2021). The findings of this study, when considered alongside other research, suggest that lymphopenia has become a recognized risk factor in severe cases of COVID-19. It shares certain common factors that can be used to predict the severity of the disease.

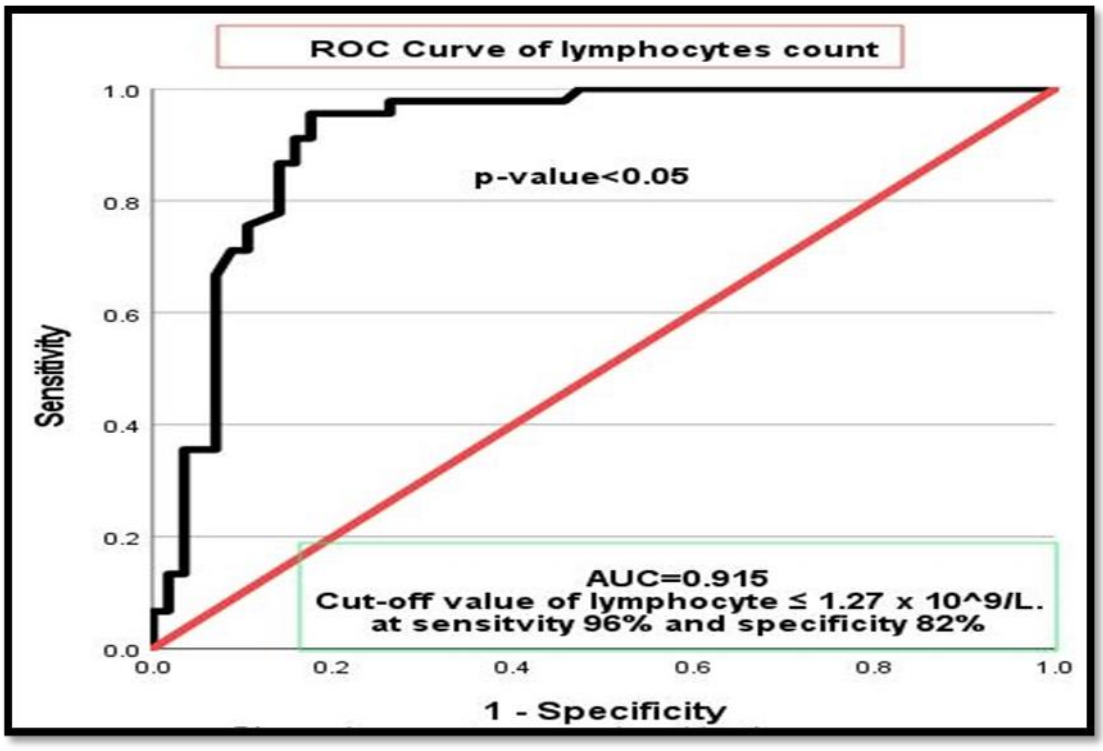


Figure (4-16) The ROC curve of Lymphocytes counts in predicting critical illness of COVID-19

4.4.7. Neutrophil to lymphocyte ratio

The NLR is calculated by dividing the number of neutrophils by the number of lymphocytes. NLR is now routinely employed in practically all medical fields as a dependable and easily accessible marker of immune response to numerous pathogenic agents. The typical range of NLR in people is 1-2; levels more than 3.0 and less than 0.7 are abnormal. NLR may assist distinguish between severe and non-severe illness(Zahorec, 2021).

The study's analysis of Neutrophil-to-Lymphocyte Ratio (NLR) levels in predicting severity among COVID-19 patients yielded significant findings. For all patients, the mean±SD NLR was 11±9, with a median (IQR) of 7.94 (3-17.9). Notably, in the subgroup of severe cases, the mean±SD NLR was substantially higher at 16.95±7.95, with a median (IQR) of 16.75 (11.64-21.88), while non-severe cases exhibited a lower mean±SD NLR of 3.54±1.95, and a median (IQR) of 3.05 (2.42-4.09). These results underscore the marked difference in NLR levels between severe and non-severe COVID-19 cases, highlighting the potential utility of NLR as a predictive marker for disease severity.

Many studies in recent years depend on some ratios to predict and diagnose many diseases and inflammatory causes of infection such as neutrophil/lymphocyte and platelet/lymphocyte, clinical significantly seen tuberculosis(Jeon *et al.*, 2019), tumor disease(Ying *et al.*, 2014) , in addition to autoimmune diseases such as rheumatoid arthritis(Uslu *et al.*, 2015) and pulmonary infection(Nam *et al.*, 2018).

A study in turkey suggested these ratios NLR and PLR might be helpful indicators for identifying individuals who are Sars CoV-2 positive(Seyit *et al.*, 2021). Another study from China verified NLR is a COVID-19 prognostic indicator that may be used. Additionally, increased NLR was found to be a separate predictive biomarker for COVID-19 patients, according to the study's findings. NLR can improve a clinician's ability to evaluate COVID-19

patients(Yang A.-P. *et al*. 2020). Higher NLR levels at admission were associated with a higher prevalence of these events in patients. The overall rate of ARDS was 26.4%, which is consistent with reports from 19% to 29.3% in the past, but it happened to 35.1% of patients who had NLR > 4.94 upon admission in COVID-19 infection(Wang D. *et al*. 2020; Huang C. *et al*. 2020).

In a study conducted by Hongmei Zhang *et al*. (2020) it was observed that Neutrophil-to-Lymphocyte Ratio (NLR) proved effective in identifying and predicting individuals whose COVID-19 conditions were likely to deteriorate within a span of 4 weeks after the onset of the disease. In combination with other clinical indicators, NLR can predict severe COVID-19 better than any other clinical indicator(Shang W. *et al*., 2020).

In study by Yildiz *et al*. (2021) NLR cut-off value of 5.94 in a prospective investigation indicated a significant in-hospital death rate for patients admitted with COVID-19 pneumonia. the result of this study based on ROC curve output, AUC was (0.938) very good value to use for NLR as an indicator of COVID-19 severity with a cut-off value of 5.9 at 95% specificity and 89% sensitivity as illustrated in figure (4-17). Neutrophil to lymphocyte ratios in COVID-19 patients are useful early markers of inflammation dysregulation, according to these findings.

4.4.8. Platelets to lymphocyte ratio

The PLR is determined by dividing the number of platelet cells by the number of lymphocytes, a generalized inflammatory measure, suggests that platelet count and lymphocyte count interact simultaneously and that aggregation and inflammatory pathways are reflected. It has been shown that several acute and chronic proinflammatory diseases cause an increase in this biomarker(Erre *et al*., 2019; Liu. L *et al*., 2020), and it has been linked to a bad prognosis in individuals with COPD(El-Gazzar *et al*., 2020), carcinomas(Tian *et al*., 2018), and recently severe COVID-19 cases were related to high PLR

levels upon admission to the hospital. With a potential predictive function for assessing the severity of COVID-19 patients, the on-admission PLR level is a new, affordable, and easily accessible biomarker(Simadibrata *et al.*, 2022).

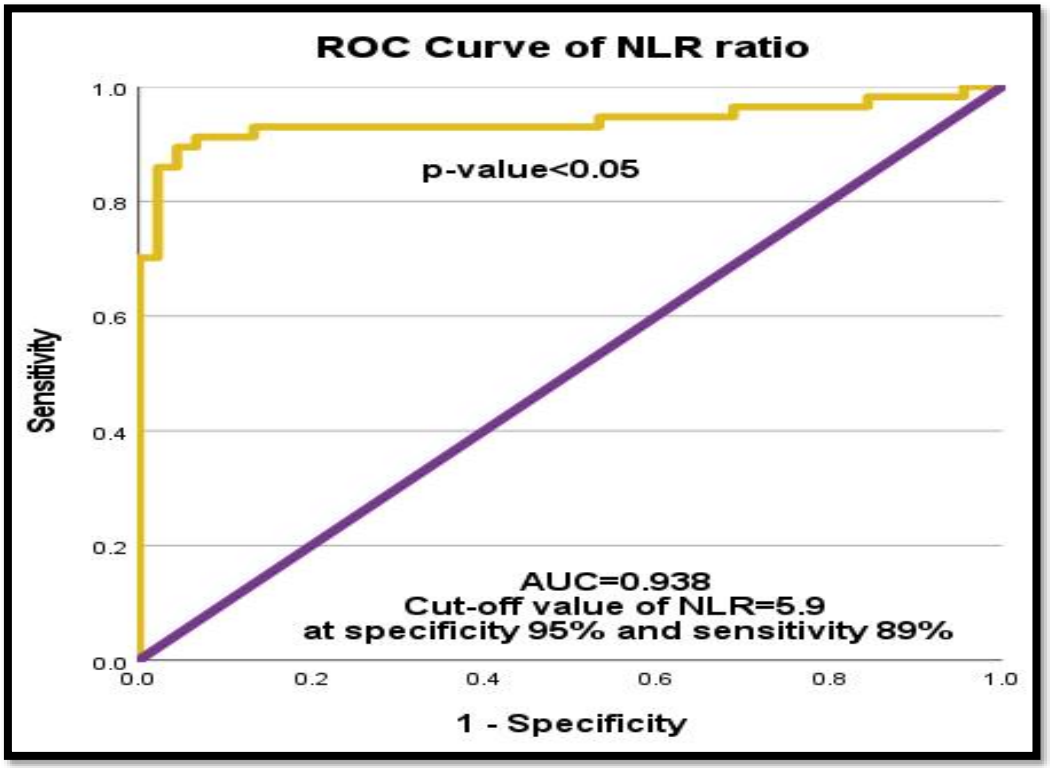


Figure (4-17) A ROC curve showing the association between NLR ratio and COVID-19 severity.

The outcomes of present study for PLR were mean±SD for total patients 307.96±271.76 and median (IQR) was 182.35 (106.46-426.5), knowing that based on significant difference between severe and non-severe since mean±SD and median (IQR) were 453.49±285.09 and 373.61 (274.68-568.62) vs 123.63±62.44 and 107.92 (90.83-130.89), respectively. In study by Yang, Ai-Ping, *et al.*(Yang A-P. *et al*. 2020) found the PLR and NLR ratio were significantly higher in 24 severe patients compared with 69 non-severe of COVID-19. In a study indicated the PLR of patients refers to the level of the cytokine storm, which may serve as a new monitoring sign in patients with COVID-19(Qu *et al.*, 2020). According to the findings of a study conducted in the United States, NLR and PLR can be employed as independent predictive indicators of disease severity in COVID-19(Chan A. S. and Rout 2020).

In a study, PLR exhibited a significant difference ($p \leq 0.001$) including of 415 laboratory-confirmed COVID-19 patients, indicating that verified evaluation of PLR may assist identify high-risk people with COVID-19(Huang S. *et al*., 2020). A study from South China noticed raised platelet to lymphocyte ratio (PLR) values are linked to increase severity and mortality(Wu. L *et al*. 2019). Another study confirmed the PLR are predictors of increased risk of death, and these results indicate might be an accurate and feasible marker for identifying older persons at high risk of mortality using COVID-19(Ortega-Rojas *et al.*, 2022).

The data were all examined, the severe patient group's NLR and PLR values were found to be higher than the non-asymptomatic group's at statistically significant levels(Tumer *et al.*, 2022). The area under curve was 0.91 as in figure (4-18) and cut-off value PLR was 203 at sensitivity 82% and specificity 95%. As a result, the PLR level at the time of admission offers a novel and easily accessible biomarker with a promising predictive role in determining COVID-19 patient severity.

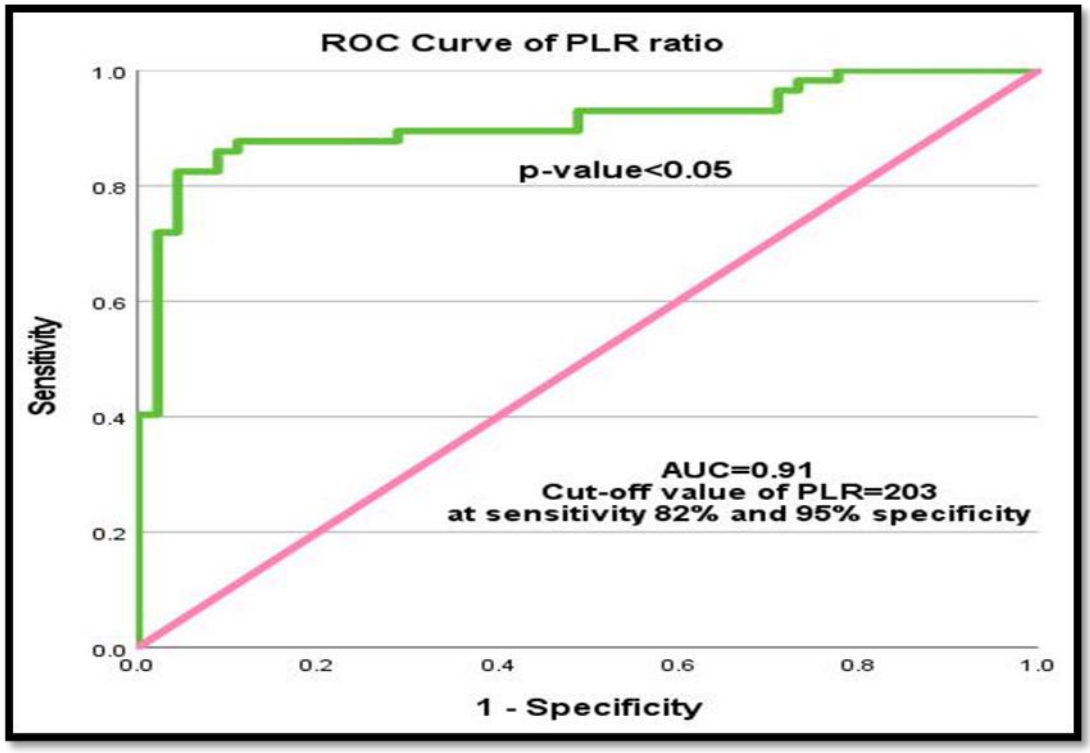


Figure (4-18) The ROC curve of PLR ratio in predicting critical illness of COVID-19.

Hematological parameters WBC, neutrophil, lymphocytes, and D-dimer counts can help predict the worsening progress of COVID-19 patients. In addition, two markers are NLR and PLR inexpensive and widely available in all laboratories. Future research should compare these markers' trends with disease progression. According to the results of further research into laboratory biomarkers, it will become possible to better manage patients with severe diseases and pandemics.

4.4.9. Clinical and Laboratory Findings Correlation

The correlation between samples of each dataset was examined using TWO statistical approaches. Spearman and Pearson correlation coefficients were used in this study as seen in table (4-3) and table (4-4) respectively. The correlation results in the present study to assess laboratory findings with severity of COVID-19. LYM was strongly negatively correlated with NLR, with a correlation coefficient of -0.820, and with other parameters were positively strongly correlated. LYM had a significant negative correlation with all parameters in the study.

PLR was positively correlated with parameters and negatively with LYM as well mentioned above. Selective correlations demonstrated that D-dimer was positively correlated with WBC count, neutrophil counts, PLR, and NLR with the respective correlation of 0.703, 0.645, 0.608, and 0.696, in return was with LYM negative correlation of -0.701. Finally, WBC and NEUT were also positively correlated with PLR, NLR, and D-dimer counts both negative with LYM, in addition positive correlation among it. In similar results found that there was a strong correlation between D-Dimer, NLR and PLR between COVID-19 patients ($p \leq 0.01$)(Ye *et al.*, 2020). A recent study showed negative correlation between higher D-dimer and lower levels of lymphocyte ton increased severity of COVID-19(Zhang *et al.*, 2022).

A study confirmed strong correlation among lymph/monocytes count, D-dimer, and other biomarkers that have an impact on the inflammatory responses to SARS-CoV-2 infection(Biamonte *et al.*, 2021).

Table 4-3 An analysis of Spearman's correlation between key laboratory parameters and clinical characteristics in patients with COVID-19.

Correlations								
			NLR	LYM	PLR	D-dimer	NEUT	WBC
Spearman's rho	**NLR**	Correlation Coefficient	1.000	-.820**	.899**	.708**	.843**	.722**
		Sig. (2-tailed)	.	.000	.000	.000	.000	.000
		N	102	102	102	102	102	102
	LYM	Correlation Coefficient	-.820**	1.000	-.863**	-.701**	-.612**	-.740**
		Sig. (2-tailed)	.000	.	.000	.000	.000	.000
		N	102	102	102	102	102	102
	PLR	Correlation Coefficient	.899**	-.863**	1.000	.680**	.694**	.735**
		Sig. (2-tailed)	.000	.000	.	.000	.000	.000
		N	102	102	102	102	102	102
	D-dimer	Correlation Coefficient	.708**	-.701**	.680**	1.000	.645**	.703**
		Sig. (2-tailed)	.000	.000	.000	.	.000	.000
		N	102	102	102	102	102	102
	NEUT	Correlation Coefficient	.843**	-.612**	.694**	.645**	1.000	.635**
		Sig. (2-tailed)	.000	.000	.000	.000	.	.000
		N	102	102	102	102	102	102
	WBC	Correlation Coefficient	.722**	-.740**	.735**	.703**	.635**	1.000
		Sig. (2-tailed)	.000	.000	.000	.000	.000	.
		N	102	102	102	102	102	102

Abbreviations: NLR, Neutrophil-Lymphocyte Ratio; PLR; The platelet-lymphocyte ratio; WBC, white blood cell; NEUT; Neutrophils; LYM; Lymphocytes ** Correlation is significant at the 0.01 level (2-tailed).

Table 4-4 Pearson correlations between important clinical features and laboratory findings of COVID-19 patients.

Pearson Correlations							
		NLR	LYM	PLR	D-dimer	NEUT	WBC
NLR	Pearson Correlation	1	-.811**	.839**	.635**	.760**	.740**
	Sig. (2-tailed)		.000	.000	.000	.000	.000
	N	102	102	102	102	102	102
LYM	Pearson Correlation	-.811**	1	-.728**	-.618**	-.606**	-.755**
	Sig. (2-tailed)	.000		.000	.000	.000	.000
	N	102	102	102	102	102	102
PLR	Pearson Correlation	.839**	-.728**	1	.572**	.636**	.659**
	Sig. (2-tailed)	.000	.000		.000	.000	.000
	N	102	102	102	102	102	102
D-dimer	Pearson Correlation	.635**	-.618**	.572**	1	.595**	.603**
	Sig. (2-tailed)	.000	.000	.000		.000	.000
	N	102	102	102	102	102	102
NEUT	Pearson Correlation	.760**	-.606**	.636**	.595**	1	.647**
	Sig. (2-tailed)	.000	.000	.000	.000		.000
	N	102	102	102	102	102	102
WBC	Pearson Correlation	.740**	-.755**	.659**	.603**	.647**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	
	N	102	102	102	102	102	102

Abbreviations: NLR, Neutrophil-Lymphocyte Ratio; PLR; The platelet-lymphocyte ratio; WBC, white blood cell; NEUT; Neutrophils; LYM; Lymphocytes ** Correlation is significant at the 0.01 level (2-tailed).

4.5. Molecular study

4.5.1. DNA extraction

The kit used in the extraction of DNA is obtained from Geneaid/Taiwan to be extracted pure DNA from COVID-19 patients and control groups this method depends on several steps included lyses blood cells and removing undesirable contaminants such as (proteins and RNA) in addition to dissolving cell membrane of white blood cells after DNA extraction measured concentration and purity of DNA extracted by nanodrop and were ranged concentrations (from 20-67 ng/uL) and purity (from 1.7-1.9) as in appendix (2). DNA integrity was checked through electrophoresis in agarose gel as seen in figure (4-19).

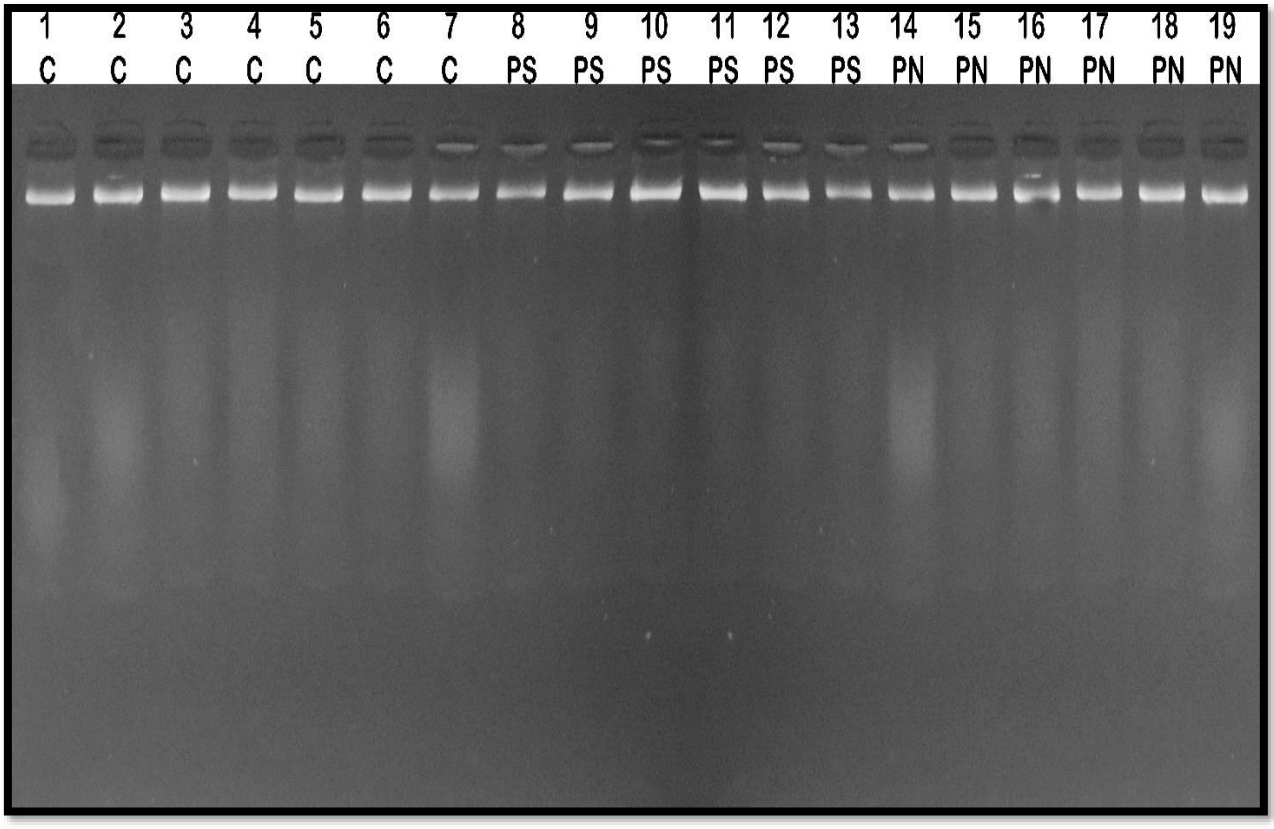


Figure (4-19) Gel electrophoresis for the DNA extract from blood showed genomic DNA bands visualized under UV after staining with ethidium bromide on "Agarose 1%, at 75 voltages, 20 mA, for 30 min," C= control 1-7 lane, PS=patients of severe 7-13 lane, PN= patients of non-severe 14-19 lane.

4.5.1.1. The Ala222Val polymorphism rs1801133 C>T SNPs in *MTHFR* gene

The *MTHFR* gene is found on 1p36.3 of chromosome 1 exon 4 and encodes an enzyme that is necessary for folate-mediated one-carbon metabolism. The common variant C677T (rs1801133) in the *MTHFR* gene which causes valine to be substituted for alanine and results in decreased enzyme activity and increased homocysteine levels(Frosst *et al.*, 1995). The relationship between COVID-19 and coagulopathy has been well-studied to confirmed it the relation (Abou-Ismail *et al.*, 2020). Methylenetetrahydrofolate reductase (*MTHFR)* is a gene that encodes an enzyme that aids in folate metabolism. Systemic homocysteine accumulation is seen in genetic variations that markedly impair *MTHFR* activity. This may cause abnormal clotting and vascular irritation, and is considered a risk factor for blood clots(Undas *et al.*, 2005).

The *MTHFR* gene is one of many genes whose single nucleotide polymorphisms (SNPs) regulate the coagulation cascades was one factor that influences thrombosis occurrences in COVID-19-affected patients or in post-vaccinated people(Balzanelli *et al.*, 2021).

The targeted variant was detected using PCR and done by allele-specific polymerase chain reaction (AS-PCR) as a technique for detecting polymorphisms based on designed three unique primers(Kwok and Chen, 2003). In this method, the specialized primers are created to only allow DNA polymerase amplification when the nucleotide at the mutant or wild-type sequences completely matches the nucleotide at the 3' end of the primer. Following PCR and electrophoresis. The Tetra-ARMS PCR amplification, is a PCR-based approach that may be used to detect SNPs by four primers. In addition to high throughput SNP detection techniques by DNA sequencing.

In exon 4 of the *MTHFR* gene was found the substitution (C677T) and resulted in the change of the amino acid (Ala222Val). The polymorphism was identified using AS-PCR depended on three primers involved one as a common primer and two primers as one wild (C allele) and second mutant (T allele), amplified a region that contains the targeted sequence of the gene-based base present in the region. This method is based on the selection/design of two AS primers that end on a SNP and a common primer to amplify the PCR fragments.

The difference between the two AS-PCR products is made possible by the distinct sequences of the tails; the 5' end of the primer is not regarded as important for specificity. The insertion of a destabilizing mismatch within the last five bases of the 3' primer, which differs between the two AS primers, is essential for effective differentiation of the two alleles.

The size of the targeted polymorphism rs1801133 in this study was 266 bp, in figure (4-20), the PCR bands provided by agarose gel electrophoresis allow the genotype to be identified as homozygous wild type in one band, heterozygous in two bands, or rare homozygous in the other band.

The results of T-ARMS-PCR of the *MTHFR* rs1801133 as mentioned previously involved four primers (two outer region and two inner region), in this variant the outer region was 273 bp while the T allele (mutant) was 176 bp and the C allele (wild) was 139 bp as in figure (4-21) by gel electrophoresis.

Sequencing results to this SNP *MTHFR* rs1801133 amplified 50 PCR products were sent for direct DNA sequencing and found perfect 100% concordance followed analysis the results and alignment to determines the optimal similarity as in figure (4-22) and the appendix (3).

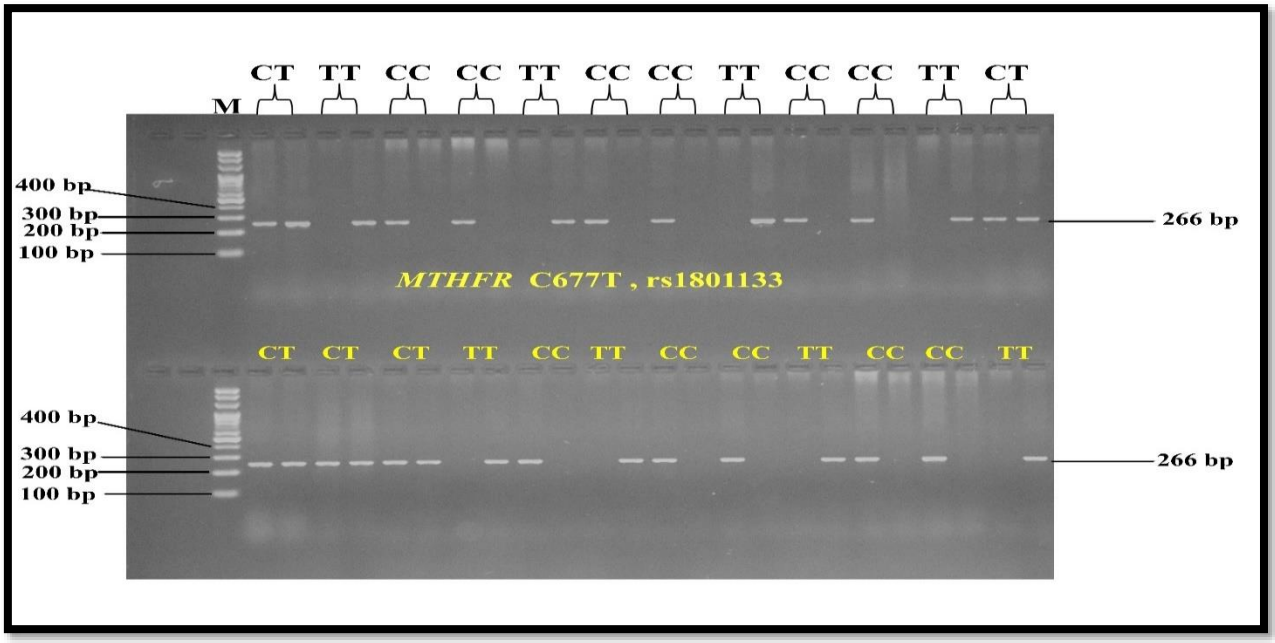


Figure (4-20) An electrophoresis pattern for some of the successful amplifications of rs1801133 (Agarose 2%, at 70 voltages, 20 mA for 60min), "M" denotes the DNA ladder with a size of 100 base pairs, TT= wild-type; TC= heterozygous; CC= homozygous mutant.

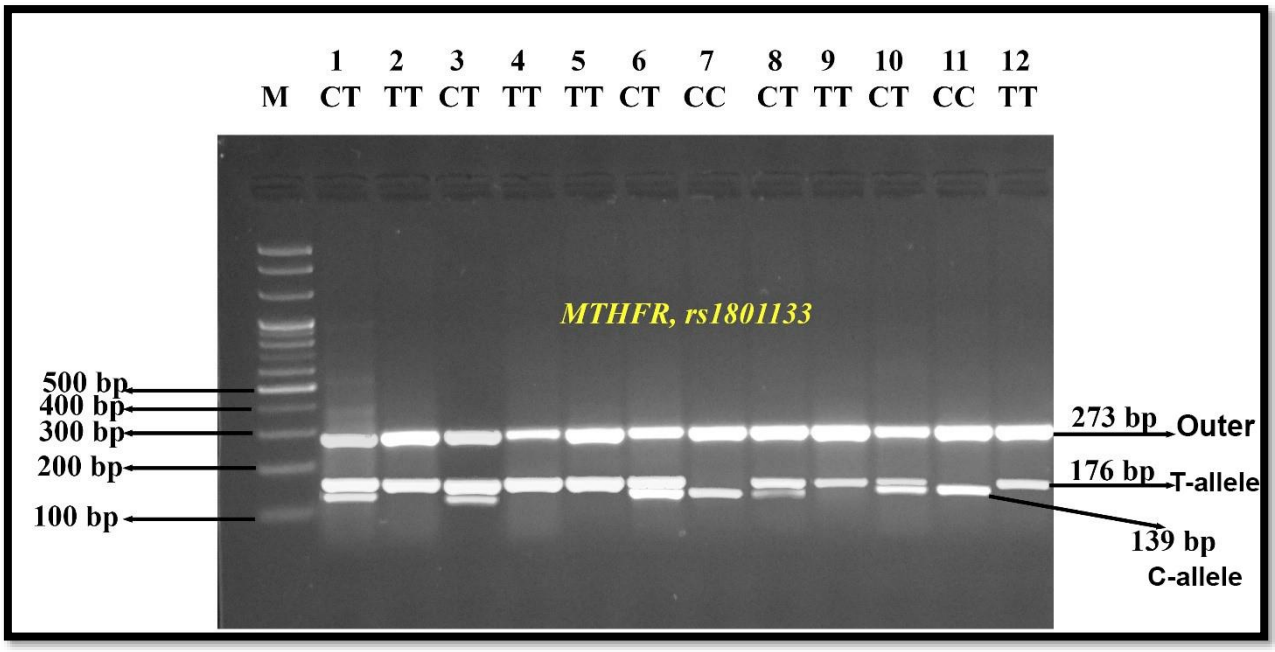


Figure (4-21) illustrates agarose gel electrophoresis conducted with a 2% agarose gel, at 70 volts and 20 mA for 60 minutes. The gel was loaded with PCR products of the T-ARMS PCR for the C677T locus of the *MTHFR* gene. The gel includes three distinct bands: the outer primers resulting in a 273 bp band, the T allele resulting in a 176 bp band, and the C allele resulting in a 139 bp band. The gel bands correspond to specific genotypes: samples 1, 3, 6, 8, and 10 exhibit the CT genotype, samples 2, 4, 5, 9, and 12 exhibit the TT genotype, and samples 7 and 11 exhibit the CC genotype.

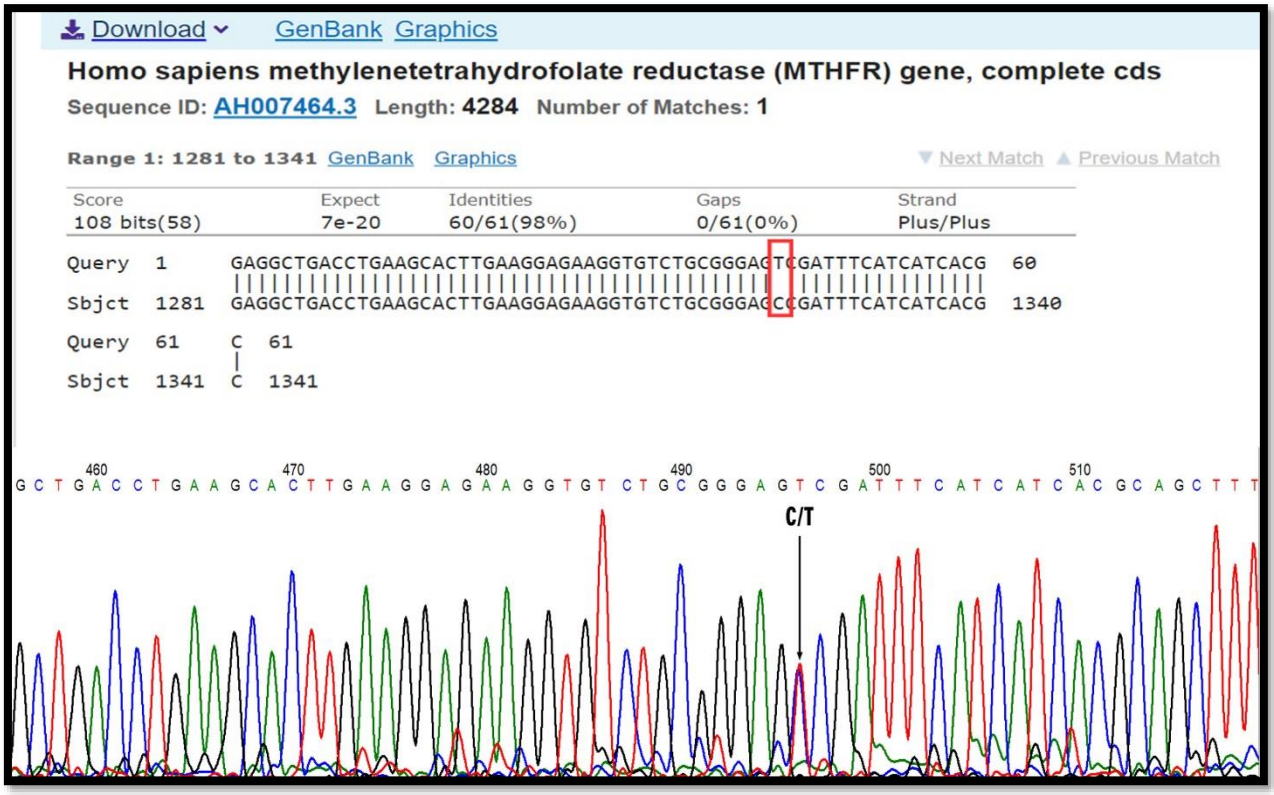


Figure (4-22) *MTHFR* genotyping results of DNA sequencing to indicate heterozygous C/T genotype.

Analysis of the frequency distribution of genotypes of *MTHFR* rs1801133 in the Iraqi population samples revealed their agreement with the Hardy–Weinberg equilibrium for all genotypes in both groups with significant p>0.05 as in table (4-5).

Table (4-5) Hardy–Weinberg equilibrium for *MTHFR* gene rs1801133 genotype among patient groups and controls.

Polymorphism	Group	Genotype, n (%)			Allele, n (Frq.)		HWE	
							χ^2	*p*
		CC	CT	TT	C	T		
MTHFR	**Control**	49(53.3)	33(35.8)	10(10.9)	131(0.711)	53(0.288)	1.44	0.48
rs1801133	**Patients**	36(35.2)	47(46.1)	19(18.7)	119(0.583)	85(0.417)	0.27	0.87

Abbreviations: HWE: Hardy–Weinberg equilibrium, Frq: allele frequency rate, $\chi 2$: chi-square, *p*: The p-value.

In COVID-19 subjects as in table (4-6), Genotypes and alleles distributions in C677T polymorphism in codominant model found CC® (53.3%), CT (35.8%) and TT (10.9%) in the healthy while the frequency of genotype in patients were CC® (35.2%), CT (46.1%) and TT (18.7%) and allele frequency in both groups were (C® 71.1% vs T 28.9% , C® 58.3% vs T 41.7)respectively, and reported T allele increase risk of disease (OR = 1.76; 95% CI = 1.15–2.69; p=0.008).

Analysis of crude odd ratio in codominant revealed *MTHFR* 677 CT genotype associated significantly with a higher risk of infection with COVID-19 (OR = 1.93; 95% CI = 1.04–3.6; p=0.03); in addition, COVID-19 positive patients carrying TT genotype had a 2.58-fold increased risk infection statistically these were significantly different (OR = 2.58; 95% CI = 1.079–6.22; p=0.034), at the same time in dominant model found CT+CC had increased the risk of COVID-19 infection (CC® vs CT+TT; OR=2.08; 95% CI= 1.71–3.71; p=0.01).

According to the results of *MTHFR* mutant variant A222V showed increased virus infection compared to wild-type *MTHFR* ; thus, this variant could increase the risk for COVID-19, and this results in present study similar to study in Uzbek population showed patients had a slightly higher frequency of the T allele than controls, while patients with complications after COVID had a higher frequency of the C/T genotype than controls(Kurbonova and Babadjanova, 2022).

Another study agreed with the present study to show patients with post COVID-19 complications after infection found T allele higher risk of infection than C to increase of COVID-19 infection(Boymuradov *et al.*, 2022).

Table (4-6) association of *MTHFR* rs1801133 polymorphism with COVID-19 in patients and control samples in multiple genetic models.

Polymorphism	Genetic models	Control, n(%)	Patients, n(%)	Odds ratio	
				OR (95%CI)	p
***MTHFR* rs1801133**	**Codominant**				
	CC®	49(53.3)	36(35.2)	1.00 (Ref.)	--
	CT	33(35.8)	47(46.1)	**1.93(1.04-3.6)**	**0.03***
	TT	10(10.9)	19(18.7)	**2.58(1.07-6.22)**	**0.034***
	Dominant				
	CC®	49(53.3)	36(35.2)	1.00 (Ref.)	--
	CT+TT	43(46.7)	66(64.8)	**2.08 (1.17-3.71)**	**0.01***
	Recessive				
	CC+CT®	82(89.1)	83(81.3)	1.00 (Ref.)	--
	TT	10(10.9)	19(18.7)	1.87 (0.82-4.28)	0.13
	Over-dominant				
	TT+CC®	59(64.1)	55(53.9)	1.00 (Ref.)	--
	CT	33(35.9)	47(46.1)	1.52 (0.85-2.72)	0.15
	Allele frequency				
	C®	131(71.1)	119(58.3)	1.00 (Ref.)	--
	T	53 (28.9)	85 (41.7)	**1.76 (1.15-2.69)**	**0.008***

Abbreviations: ® References, OR: odd ratio, p: The p-value, *: Significant.

In this study the patients with severe COVID-19 vs. non-severe, the comparison of distribution of genotypes in the *MTHFR* gene 677 C>T (rs1801133) polymorphism to the Hardy-Weinberg law.

The both the COVID-19 cases, the allelic distribution in the cohort for this SNP was entirely consistent with the Hardy-Weinberg equilibrium as in table (4-7).

Table (4-7) Hardy–Weinberg equilibrium for *MTHFR* rs1801133 genotype among patient groups.

Polymorphism	Group	Genotype, n (%)			Allele, n (Frq.)		HWE	
							$\chi 2$	*p*
		CC	CT	TT	C	T		
***MTHFR* rs1801133**	**Severe**	13(22.8)	28(49.2)	16(28)	54(0.473)	60(0,527)	0.01	0.99
	Non-severe	23(51.1)	19(42.2)	3(6.7)	65(0.722)	25(0.278)	0.12	0.94

Abbreviations: HWE: Hardy–Weinberg equilibrium, Frq: allele frequency rate, $\chi 2$: chi-square, *p*: The p-value.

The association between a genetic variation in the human *MTHFR* (rs1801133) gene with the severity and progression of COVID-19 in the results of this study showed *MTHFR* p. Ala222Val polymorphism (rs1801133) was detected in patients. The genotype and allele frequencies of this SNP are shown in table (4-8), the frequency of genotype CT (49.2%) and TT (28%) in severe patients while in non-severe CT (42.2%) and TT (6.7).

The results showed that the difference of CC vs. CT and CC vs. TT between the severe and non-severe forms of COVID-19 was significant ($P \leq 0.05$). T allele in the codominant model (CC® vs. CT) model conferred an increased severity of COVID-19 in the population of samples (OR = 2.6, 95% CI = 1.06–6.38, P =0.03) and (CC® vs. TT) genotype a 9.431-folds higher risk of severity due to COVID-19 infection (OR = 9.43, 95% CI = 2.3–38.58, $P \leq 0.01$).

The dominant (CC® vs. TT+CC) and recessive (CC+CT® vs. TT) models markedly were associated with a significant increase severity of COVID-19 [OR = 3.53, 95% CI = 1.51–8.28, P = 0.003 and OR = 5.46, 95% CI = 1.48–20.16, P = 0.01] respectively. During the analysis, the following results emerged that the *MTHFR* rs1801133 gene polymorphism in over-dominant Was not significantly correlated with the severity of infection due to COVID-19. Statistical analysis showed that the T allele of rs1801133 caused a marked

to get symptomatic or more severe COVID-19 (OR = 2.88, 95% CI = 0.75–2.34, P ≤0.01). In a study of Uzbek population by Khidoyatovna, *et al*. (2022) confirmed the T allele minor (CT,TT) were factors that induce the severe course of COVID-19 and this results agreed to the results of this study. In a second study, the frequency of the *MTHFR* C677T allele in the Latino community, as well as the incidence and mortality of COVID-19, were greater than those reported for other populations worldwide(Ponti, Pastorino, *et al.*, 2021).

In Portugal research choose 8 SNPs one of them rs1801133 had an important role in the mortality caused by SARS-CoV-2(Cantanhede *et al.*, 2022). The findings of the study by Cappadona *et al*., (2021) demonstrated that evidence for a correlation with severe COVID-19 was established for five loci, including two genes involved in the metabolism of folate (*MTHFR* and MTR).

Most intriguingly, vitamin supplementation is advised for people with the *MTHFR* C677T polymorphism since it has previously been found to predispose to a more severe course of COVID-19(Karst *et al.*, 2020).

Ischemic stroke observed after COVID-19 is one of the main serious complications of this disease. For that should recognize thrombophilia genes as predictors of this serious complication, because some allelic forms of one gene of thrombophilia *MTHFR* (rs1801133), in patients overexpressed this protein causes systemic inflammation in the pathogenesis of COVID-19. This is the reason rs1801133 polymorphism of the thrombophilia gene causes the folate cycle to not pass normally. As a result, induced hyper-homocysteinemia damages the vascular endothelial layer and causes coagulopathy, which appeared as a result of the disease COVID-19(Ибодов *et al.*, 2022), additionally this study determined that the *MTHFR* rs1801133 gene polymorphism was associated with COVID-19 susceptibility and that the SNP rs1801133-T variant was associated with COVID-19 severity.

Table (4-8) *MTHFR* gene (rs1801133) polymorphism under different genetic model and allelic models in severe patient and non-severe.

Polymorphism	Genetic models	Severe, n(%)	Non-severe, n(%)	Odds ratio	
				OR (95%CI)	p
***MTHFR* rs1801133**	**Codominant**				
	CC®	13(22.8)	23(51.1)	1.00(Ref.)	--
	CT	28(49.2)	19(42.2)	**2.6(1.06-6.38)**	**0.03***
	TT	16(28)	3(6.7)	**9.43(2.3-38.58)**	**p≤0.01***
	Dominant				
	CC®	13(22.8)	23(51.1)	1.00(Ref.)	--
	CT+TT	44(77.2)	22(48.9)	**3.53(1.51-8.28)**	**0.003***
	Recessive				
	CC+CT®	41(71.9)	42(93.3)	1.00(Ref.)	--
	TT	16(28.1)	3(6.7)	**5.46(1.48-20.16)**	**0.01***
	Over-dominant				
	TT+CC®	29(50.8)	26(57.8)	1.00(Ref.)	--
	CT	28(49.2)	19(42.2)	1.32(0.6-2.9)	0.48
	Allele				
	C®	54(47.3)	60(52.7)	1.00	--
	T	65(72.2)	25(27.8)	**2.88(0.75-2.34)**	**p≤ 0.01***

Abbreviations: ® References, OR: odd ratio, p: The p-value, *Significant p≤0.05.

It was found that no association was found between rs1801133 genotype and demographic characteristics, clinical and medical history with the exception that female gender in severe cases and vaccination status in non-severe were significantly higher among patients with three different genotypes (p = 0.01 and p = 0.002, respectively) in table (4-9). Recent studies have shown that confirmed vaccination reduces non-severe COVID-19 cases and COVID-19-related deaths in patients with the disease(Bell *et al.*, 2022; Mosconi *et al.*, 2022; Kikuchi *et al.*, 2023). Similar findings indicate full COVID-19 vaccination reduces the likelihood of progression to severe COVID-19 disease(Ichii *et al.*, 2022).

Table (4-9) summarizes the demographics and clinical characteristics of patients with COVID-19, based on genetic variant rs1801133 in the *MTHFR* gene.

Parameter	Severity state	Genotype			P-value
		CC	CT	TT	
Male	Severe	9	15	9	0.33
	Non-severe	7	5	3	0.44
Female	Severe	4	13	7	0.072
	Non-severe	16	14	0	--
Death	Severe	1	1	3	0.44
	Non-severe	0	0	0	--
Hypertension	Severe	1	3	7	0.07
	Non-severe	0	2	1	0.56
Diabetes Mellitus	Severe	3	2	4	0.71
	Non-severe	1	4	1	0.22
Cardiac Diseases	Severe	0	4	0	--
	Non-severe	2	3	0	0.65
Kidney Diseases	Severe	2	1	2	0.81
	Non-severe	0	1	0	--
Malignancy	Severe	0	0	1	--
	Non-severe	1	1	0	--
Hepatic Diseases	Severe	1	0	1	--
	Non-severe	0	0	0	--
Oxygen Support	Severe	11	19	13	0.29
	Non-severe	0	0	0	--
Vaccination Status	Severe	2	6	4	0.36
	Non-severe	12	16	1	**0.002***

Abbreviations: *: significant p≤0.05, p: The p-value.

According to Table (4-10), the hematological parameters of patients with different genotypes of the rs1801133 polymorphism were summarized for severe and non-severe patients. Among hematological parameters, WBC, NEU, LYM, D-dimer, and PLR values were all significant in the total patient population in addition to NLR values in both total and non-severe, while non-significant in HGB and PLT values.

Table (4-10) Hematological parameters in patients with COVID-19 according to genotypes for *MTHFR* (rs1801133) polymorphism.

Parameters		Severity	Mean±SD genotypes of *MTHFR* (rs1801133)			P-value
			CC	CT	TT	
WBC		Total	11.5±5.43	13.97±5.53	16.11±4.54	**0.009***
		Non-Severe	8.5±3.63	8.52±2.46	9±2.64	0.96
		Severe	16.81±3.77	17.67±3.6	17.44±3.44	0.77
PLT		Total	267.58±56.35	279.74±75.02	286.1±113.21	0.658
		Non-Severe	251.82±36.21	259.1±31.75	244.66±6.65	0.68
		Severe	295.46±74.44	293.75±91.72	293.87±122.34	0.99
NEU		Total	9.44±4.12	11.39±4.34	12.32±4.23	**0.034***
		Non-Severe	7.17±2.66	8.22±2.74	8.26±2.51	0.43
		Severe	13.46±3.018	13.54±3.9	13.08±4.1	0.92
D-Dimer		Total	0.98±0.89	1.52±1.17	1.72±1.07	**0.021***
		Non-Severe	0.44±0.1	0.6±0.36	0.51±0.13	0.13
		Severe	1.93±0.88	2.15±1.11	1.95±1.013	0.74
LYM		Total	1.9±0.96	1.39±0.89	1.28±1	**0.023***
		Non-Severe	2.42±0.62	2.22±0.62	2.78±0.66	0.28
		Severe	0.99±0.76	0.84±0.56	1.02±0.78	0.68
NLR		Total	9.73±10.73	20.57±17.13	24.9±22.88	**0.002***
		Non-Severe	3.45±1.28	5.6±3.55	3.16±1.075	**0.02***
		Severe	20.83±11.13	30.73±15.06	28.97±22.72	0.218
PLR		Total	216.63±203.17	329.45±268.09	411.01±349.88	**0.028***
		Non-Severe	108.62±23.83	129.96±52.66	91.93±24.87	0.12
		Severe	407.73±239.67	464.81±271.29	470.84±350.2	0.8
HB	Male	Total	14.33±1.65	13.57±1.6	14.03±0.91	0.31
		Non-Severe	15.2±1.23	14.38±1.18	14.9±1.01	0.51
		Severe	13.65±1.67	13.3±1.67	13.74±0.72	0.73
	Female	Total	13.24±1.8	12.52±1.86	12.17±2.54	0.33
		Non-Severe	13.74±0.72	12.98±1.87	13.54±1.49	0.55
		Severe	10.1±0.78	12.03±1.78	12.17±2.54	0.19

Abbreviations: WBC: white blood cell, PLT: platelet, NEU: neutrophils, LYM: lymphocytes, NLR: Neutrophil-Lymphocyte Ratio, PLR: platelet-lymphocyte ratio, HB: hemoglobin, mean±SD: mean± standard deviation, *: P≤0.05 was considered statistically significant.

4.5.1.2. Association of the *MTHFR* rs1801133 to Serum homocysteine in COVID-19

Homocysteine is an amino acid containing sulfur that cannot be effectively incorporated into proteins. It is a metabolic intermediate produced when the amino acid methionine is converted to homocysteine, and it may be expelled via the urine(Hou H. and Zhao 2021).

Homocysteine may be methylated to produce methionine or transformed to cysteine through a transsulfuration pathway. In the methylation process, vitamin B12, folate, and the enzyme methylenetetrahydrofolate reductase (*MTHFR*) are required, hence deficiency or severe deficiency of these vitamins may result in homocysteine buildup in the blood, particularly in individuals with certain mutations in the *MTHFR* gene(Raghubeer and Matsha, 2021).

It is well known that a high homocysteine plasma level considerably increases the risk of vascular damage both in small and big vessels(Balint *et al.*, 2020). In addition to serological and clinical indicators that are clearly connected with a severe clinical outcome of COVID-19 infection, it has been recently proposed that Hcy played an essential predictive role(Yang Z. *et al*. 2020). In the present study showed Hcy levels was a significant increase in 92 patients serum compared with 92 healthy group was lower, the mean±SD serum Hcy level in COVID-19 patients was16.17±9.33, while that in the controls was 4.46±2.73 ($p \leq 0.01$) as in figure (4-23).

In Iraq study confirmed serum homocysteine levels compared to healthy controls, COVID-19 patients had significantly higher levels(Khalid *et al.*, 2022). Recent study on pediatric indicate the median serum Hcy concentration in COVID-19 patients was 27.5 mol/L, while it was 1.8 mol/L in the control group compared patients to controls, there was a statistically marked increase in the Hcy level ($p \leq 0.001$)(Fouda *et al.*, 2022). Post-COVID-19 patients showed increased homocysteine levels than healthy controls, leading to endothelial dysfunction and inflammatory vasculopathy associated with COVID-19(Keskin *et al.*, 2022).

In patients with severe COVID-19, several studies have suggested that a number of parameters may contribute to the severity of COVID-19. According to the results multiple studies, homocysteine levels are a useful prognostic indicator for predicting the severity of COVID-19 illness.

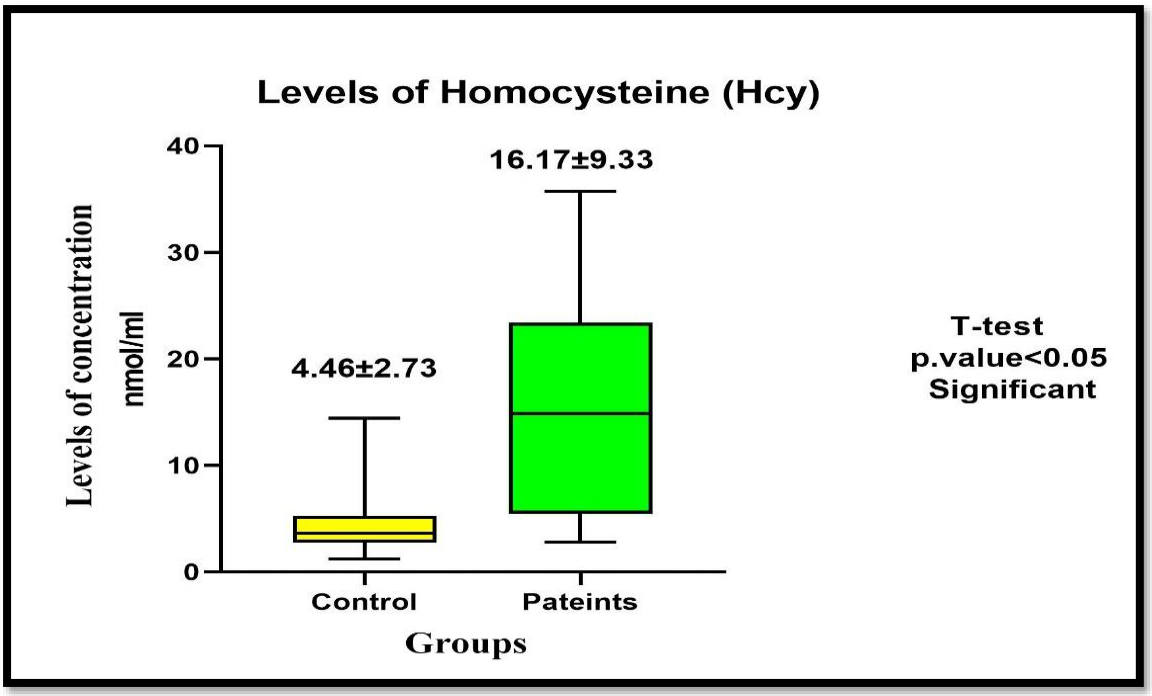


Figure (4-23) Mean ± SD of homocysteine levels in healthy and COVID-19 patient groups.

When compared the results between severe, non-severe, and dead patients there was significantly differences ($p \leq 0.01$) with mean±SD (10.42±6.5, 3.25±1.21, and 12.5±5) respectively, as in figure (4-24). In addition, the median (IQR) was severe 9.160 (IQR 5.72-13.91), non-severe 3.27 (IQR 2.45-4.2), and dead 10 (IQR 7-19). In a study including 304 hospitalized COVID-19 patients similar to the results of present study, plasma homocysteine levels were measured and found to be significantly higher in non-survivors compared to survivors(Ponti, Roli, *et al.*, 2021).

In a study done by Keskin *et al*. (2022) and colleagues, plasma homocysteine levels were evaluated in 117 COVID-19 hospital patients. Blood oxygen saturation levels over 90% on room air were used to divide patients into a moderate disease group, and those below 90% into a severe disease group (patients with blood oxygen saturation lower) There was a significant elevation in homocysteine levels in the severe disease group compared to the mild disease group.

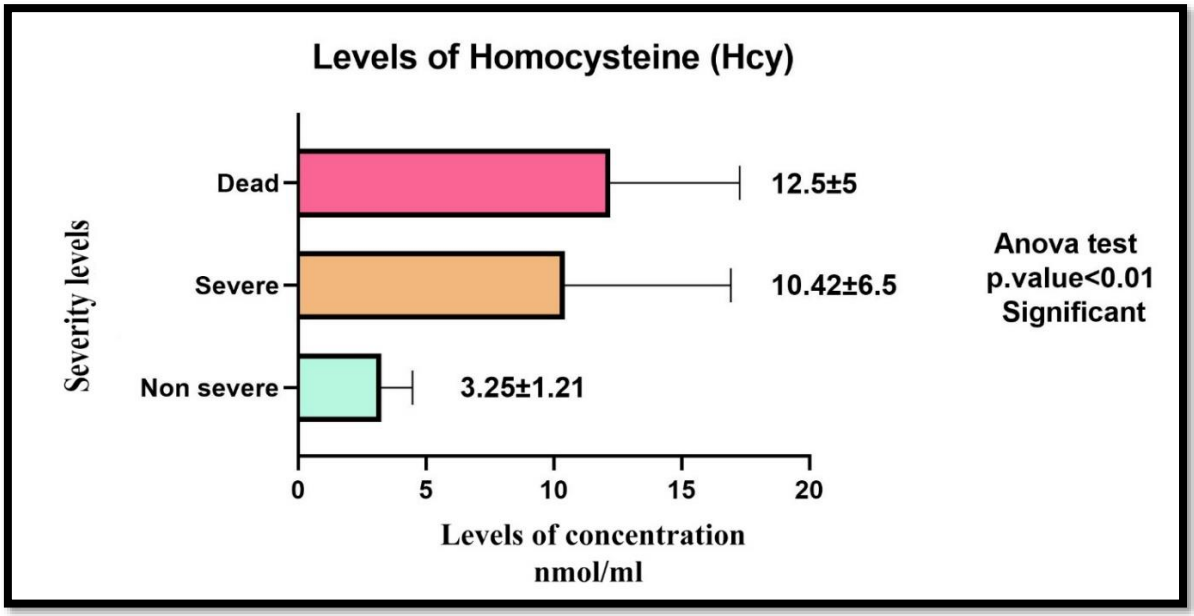


Figure (4-24) Mean ± SD of homocysteine levels in COVID-19 patients group including (severe *vs* non-severe).

Increased homocysteine levels have been reported in patients with advanced disease or those whose COVID-19 was severe(Yang. Z *et al*. 2020). The results of a study have shown that Hcy is an effective biomarker for predicting the outcome of COVID-19 patients who have been hospitalized. Hcy may be a useful biomarker for assisting clinicians in the identification of individuals at increased risk for severe COVID-19 infection(Oliva *et al.*, 2021). Higher Hcy was associated with higher in COVID-19 incidence and severity and clinical outcome in individuals with ischemic stroke(Syahrul *et al.*, 2022). Serum homocysteine levels are associated with the diagnostic progression of pulmonary disease on chest CT imaging for COVID-19 patients(Yang Z. *et al*. 2020). Hyper-homocysteinemia, an increase in homocysteine levels, is a risk factor for several diseases, including cardiovascular and neurological conditions, and may contribute to the severe course of COVID-19(Karst *et al.*, 2020). According to the Brazilian study, higher serum levels of homocysteine are major risk factors for the severity of COVID-19(Dos Reis *et al.*, 2022).

Based on several investigations and studies homocysteine is a promising biomarker of COVID-19 infection severity, and it can also indicate the mortality rate. The ROC curve analysis was performed on the serum Hcy levels for the experimental groups included (severe vs non-severe). The results showed that the area under the curve (AUC) of Hcy is 0.938 (95% CI 0.854~1). The best critical point was 10.3 nmol/ml, the specificity was 100%, and the specificity was 81% as in figure (4-25).

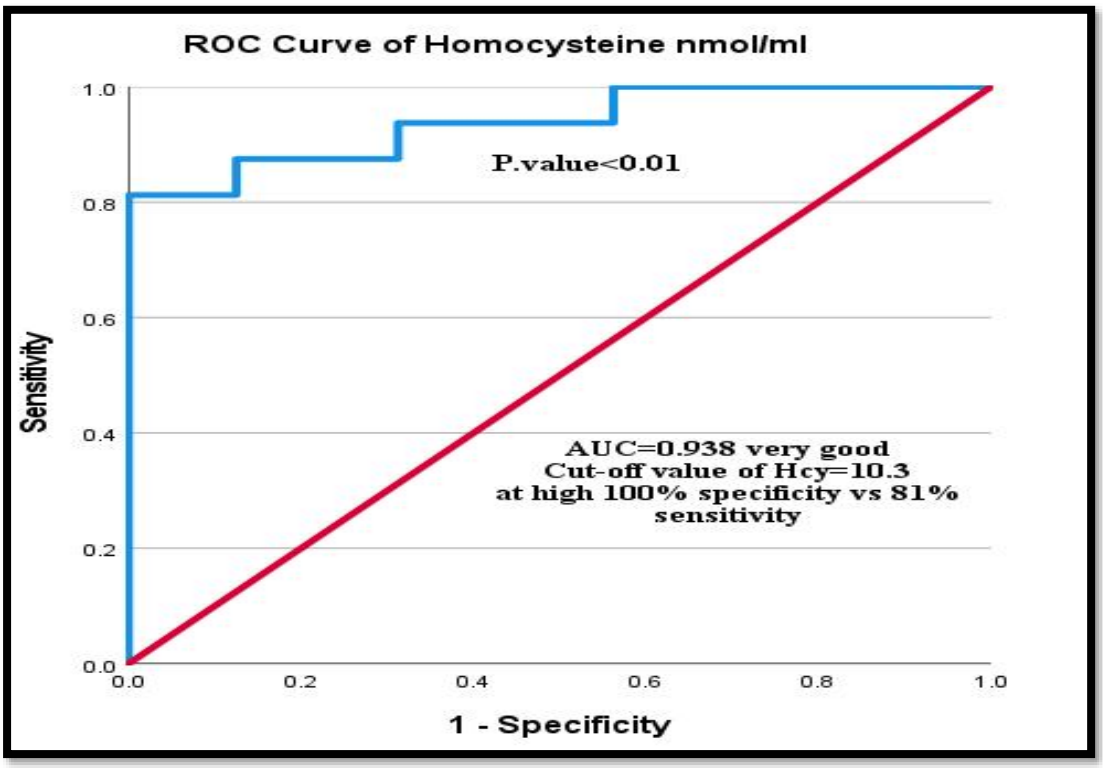


Figure (4-25) The ROC curve of homocysteine levels in predicting severity of COVID-19.

One of the most frequent genetic causes of high homocysteine levels is a mutation in the *MTHFR* gene. The most common *MTHFR* variant is the C677T mutation, also known as the "thermolabile" *MTHFR* mutation(Moll and Varga, 2015). The investigations of present study based on different genotypes and impact of Hcy levels by the variant, the serum homocysteine levels with a CT and TT genotype (11.3 ± 4.6 and 8.6±6.1 nmol/mL, respectively) were

significantly higher than with CC genotype (3.9 ± 1.8 nmol/mL, p = 0.002) as in figure (4-26).

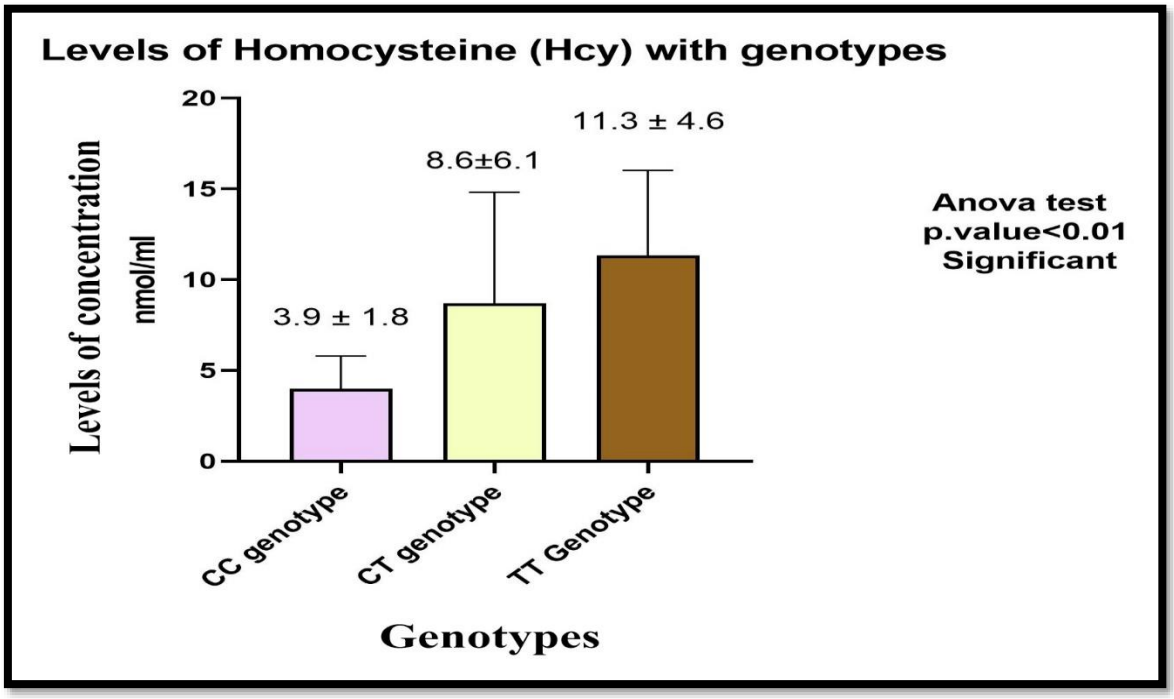


Figure (4-26) Mean ± SD of homocysteine levels in participants individuals based on genotypes carriers.

A study by Karst, *et al*(2020) assumed a theory of special susceptibility to a severe course of COVID-19 caused by higher Hcy, which might be activated by the presence of the C677T polymorphism. The presence of the rare allele (T) decreases *MTHFR* enzyme activity to 35% (per copy of the allele), resulting in increased homocysteine levels(Shane *et al.*, 2018). The variant of C677T (rs1801133) in the Uzbek population and their impact on the etiology of COVID-19 and in patients with hyper-homocysteinemia it was crucial to evaluate the severity of COVID-19 infection, enzyme activity reduces by 70% in homozygous mutants and 35% in heterozygotes as a result of the impact(Khidoyatovna *et al.*, 2022). In similar findings to the results of this study, rare homozygous carriers of this polymorphism had elevated homocysteine levels compared to normal subjects, the mutation's wild-type.

Heterozygotes showed a modest rise, midway between the uncommon homozygotes and the wild type(Liew and Gupta, 2015).

In study on *MTHFR* rs1801133 on women proved patients with the TT genotype had substantially higher homocysteine levels (8.3 3.9 nmol/mL) than those with the CC and CT genotypes (6.3 1.4 and 6.6 1.4 nmol/mL, respectively, p = 0.002)(Kuroda *et al.*, 2021). Accordingly, increased levels of homocysteine may cause diseases seen in COVID-19 and raise the risk of thrombosis.

4.5.1.3. The Met268Thr polymorphism rs699 T>C SNPs in *AGT* gene

The *AGT* gene, located at 1q42.2, codes for the peptide hormone angiotensinogen. The rs699 c.803T>C is missense polymorphism in exon 2 changed Methionine (M) to threonine(T) in the protein at p. Met268Thr. This rs699 is also known as M235T since it was previously found at amino acid 235. Numerous human tissues and organs express the *ACE2* cellular receptor for SARS-CoV-2, the renin-angiotensin system (RAS) is associated with COVID-19's pathogenicity. The *AGT* gene is considered one of the candidate genes of the RAS system(El-Arif *et al.*, 2021). Renin, which is released by the kidneys, cleaves Angiotensinogen (*AGT*), released by the liver, to produce Angiotensin I, last-mentioned cleaved by ACE to create Angiotensin II (Ang II), which serves as the substrate of *ACE2*(Annweiler *et al.*, 2020).

Several studies have evaluated the role of the renin-angiotensinogen system in the development of COVID-19 severity in many patient groups(Wiese *et al.*, 2020; Akbari *et al.*, 2022). There was growing evidence that an imbalance in the RAS played a key role in the development of ARDS(Vrigkou *et al.*, 2017), and contributes significantly to the pathogenesis of COVID-19(Henry *et al.*, 2020). Generally, dysregulated RAAS in the vasculature of COVID-19 patients may trigger a cascade of events that result

in an increase in coagulopathy across oxidative stress damage, endothelial dysfunction, and subsequent activation of the clotting cascade by a possible role for von Willebrand factor (vWF) in the coagulopathy caused by COVID-19.

Genotyping of rs699 was conducted using the primers tetra amplification refractory mutation system (T-ARMS-PCR), Allele Specific-PCR (ARMS-PCR), and sequencing techniques. The allele specific-PCR by used three primers one of common primer and the other two for each base as reference allele (wild primer) and alternative allele (mutant primer), the polymorphism rs699 in *AGT* gene was identified PCR fragment was 142 bp for each allele, after the amplification, electrophoresis was performed the presence of PCR bands in the agarose gel in wild variant considered the (TT) genotype carriers, whereas the (TC) genotype carriers (heterozygous) two band in gel included wild and mutant, and the (CC) genotype carriers (homozygous mutant) had one band in mutant line as in figure (4-27).

The T-ARMS-PCR in a single PCR, four primers are used, and gel electrophoresis followed it: outer forward (OF), outer reverse (OR), inner forward (IF), and inner reverse (IR). As an internal control for the PCR, the OF/OR primer combination produces the SNP locus's outer fragment. The inner primer gives the two allele-specific primers (inner primers), which will make the allele-specific fragments. According to Figure (4-28), outer fragment (319 bp), T allele, and C allele (194,168) respectively, in heterozygous genotype carriers (TC), the fragments were 319, 194, and 168 bp, while in wild-type carriers (TT), they were 319 and 194, whereas in (CC) mutant genotype carriers, they were 319 and 168).

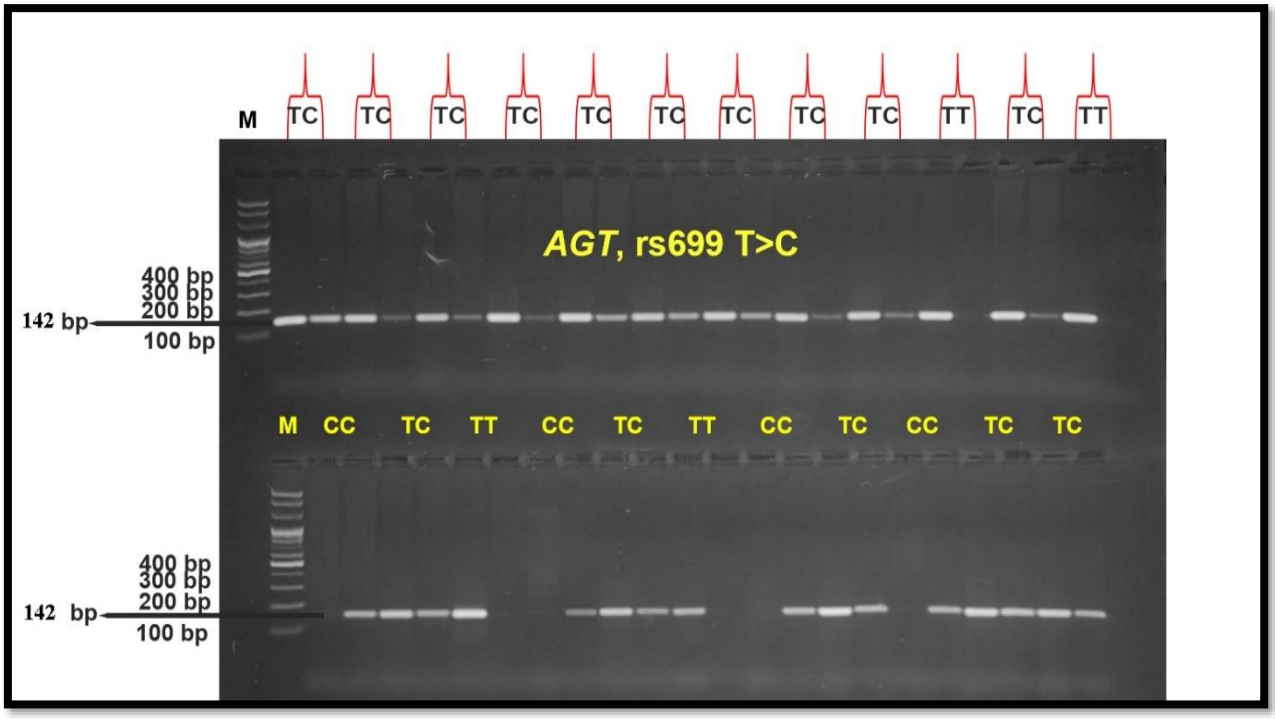


Figure (4-27): Allele specific-PCR analysis of the PCR product that contains position T4072C of the *AGT* gene separated on (75 voltages, separated on agarose 1%, at 75 voltage, 20 mA for 60min), M=DNA ladder 100 bp, TT= wild-type; TC= heterozygous; CC= homozygous mutant.

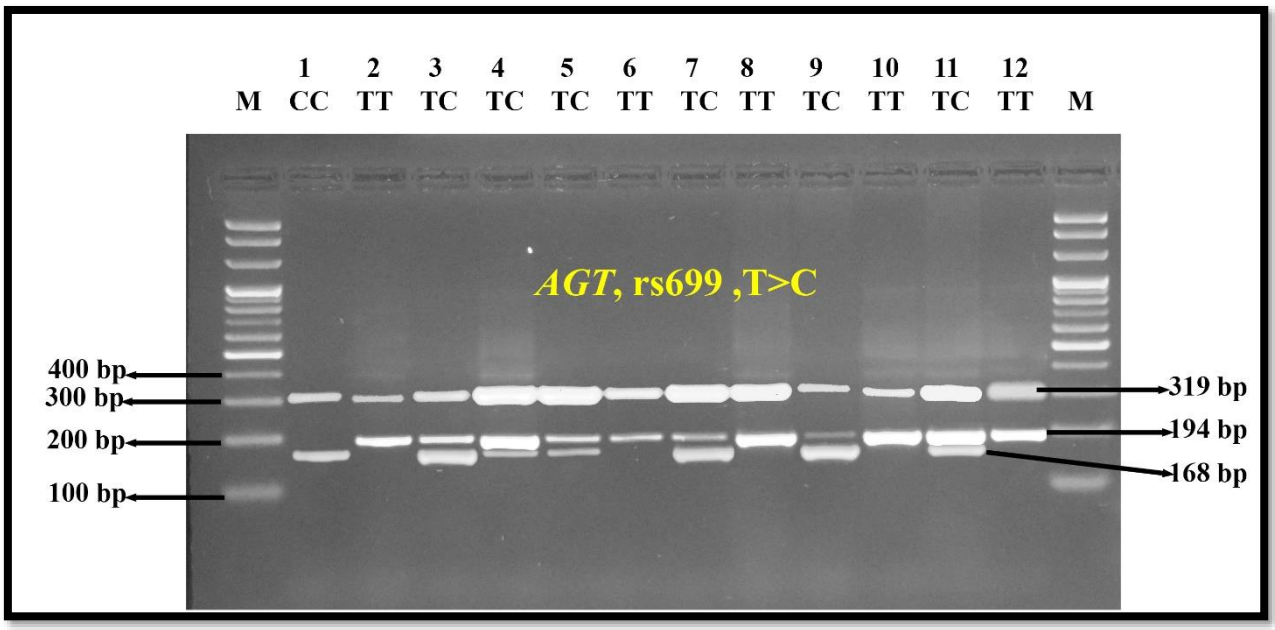


Figure (4-28) SNP rs699 genotyping by T-ARMS-PCR resolved on (75 voltages, separated on agarose 1%, at 75 voltage, 20 mA for 60min), 219 bp band represents the outer amplicon, whereas the T and C alleles bands are represented by the 194 and 168 bp amplicons, respectively.

After amplifying 50 PCR products for the *AGT* rs699 SNP, direct DNA sequencing was performed, which showed perfect 100% concordance. Subsequently, the sequencing results were analyzed and aligned to determine the optimal level of similarity as in figure (4-29) and the appendix (4).

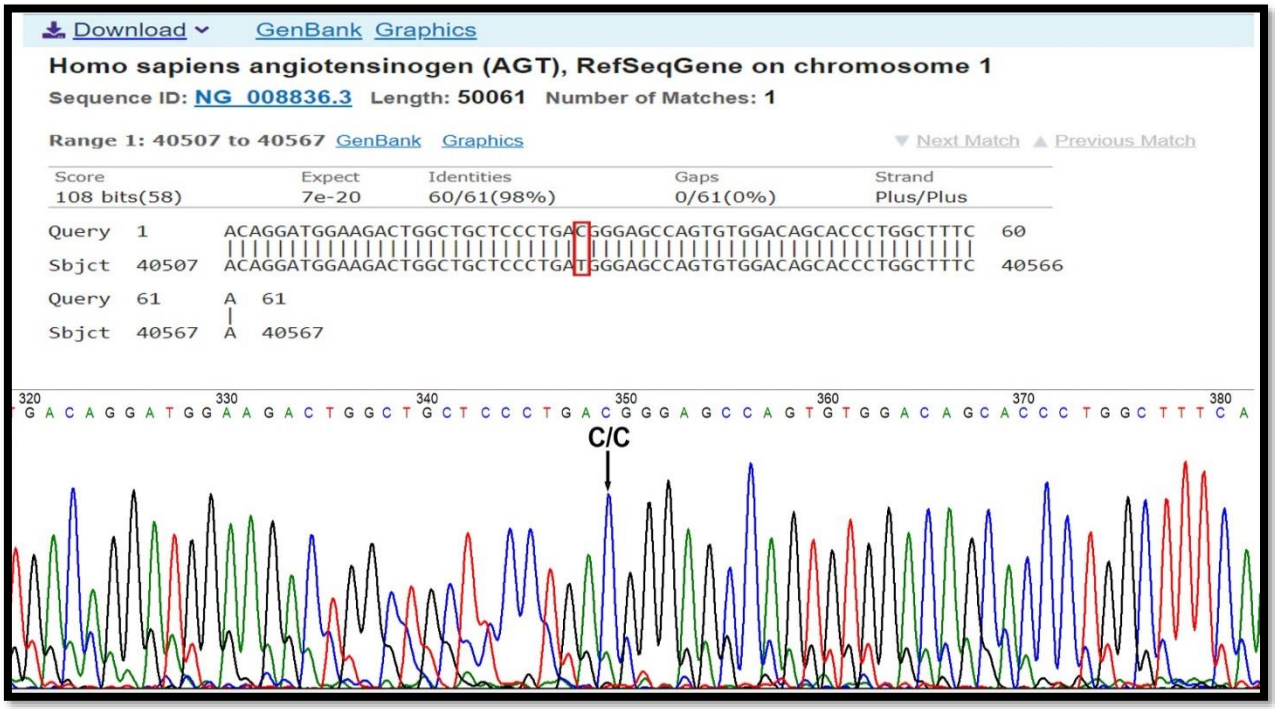


Figure (4-29) Electropherograms of rs699 (TT) homozygous genotypes detected by Sanger sequencing.

The rs699 polymorphism genotype and allelic frequency distributions the data in table (4-11) shows the number of COVID-19 patients and apparently healthy. The HWE was estimated for SNP *AGT* (rs699) genotype frequencies, which did not match with HWE among the control group with a significant difference, and was non-significant statistically matching with HWE among patients. Results revealed significant differences statistically and impact of the rs699 variant The risk of infection with COVID-19 is increased between patient groups compared to control groups in codominant (OR = 2.69, 95% CI = 1.12–6.44, P ≤0.02) and over dominant (OR = 1.69, 95% CI = 1.1–3.51, P ≤0.022) as in table (4-12).

A study on 104 positive patients by Cafiero *et al*. (2021) T/C genotype was found to be significantly prevalent in patients with no symptoms. According to a study by Kouhpayeh *et al*., (2021) carrying the C allele and TC genotype of *AGT* rs699 increase the risk of contracting COVID-19.

Table (4-11) Hardy–Weinberg equilibrium for *AGT* gene rs699 genotype among patient groups and controls.

Polymorphism	Group	Genotype, n (%)			Allele frequency, n (Frq.)		HWE	
							χ^2	*p*
AGT (rs699)		TT	TC	CC	T	C		
	Control	18(19.5)	31(33.7)	43(46.8)	67(0.364)	117(0.636)	6.82	**0.03***
	Patients	11(10.8)	51(50)	40(39.2)	73(0.358)	131(0.642)	0.78	0.67

Abbreviations: *: Significant p≤0.05, HWE: Hardy–Weinberg equilibrium, $\chi 2$: chi-square, *p*: The p-value, Frq: allele frequency rate.

Table (4-12) association of *AGT* rs699 polymorphism with COVID-19 in patients and control samples.

Polymorphism	Genetic models	Control, n (%)	Patients, n (%)	Odds ratio	
				OR (95%CI)	p
***AGT* rs699**	**Codominant**				
	TT®	18(19.5)	11(10.8)	1.00 (Ref.)	--
	TC	31(33.7)	51(50)	**2.69(1.12-6.44)**	**0.02***
	CC	43(46.8)	40(39.2)	1.52(0.64-3.61)	0.34
	Dominant				
	TT®	18(19.5)	11(10.8)	1.00 (Ref.)	--
	TC+CC	74(80.5)	91(89.2)	2.01 (0.89-4.52)	0.09
	Recessive				
	TT+TC®	49(53.3)	62(60.8)	1.00 (Ref.)	--
	CC	43(46.7)	40(39.2)	0.73 (0.41-1.3)	0.29
	Over-dominant				
	CC+TT®	61(66.3)	51(50)	1.00 (Ref.)	--
	TC	31(33.7)	51(50)	**1.96 (1.1-3.51)**	**0.022***
	Allele frequency				
	T®	67(36.4)	73(35.8)	1.00 (Ref.)	--
	C	117(63.6)	131(64.2)	1.02 (0.67-1.55)	0.89

Abbreviations: ® References, OR: odd ratio, p: The p-value.

As for distribution based on HWE law between patients group including severe and non-severe, the results showed as in the table (4-13), the severe and non-severe cases were consistent and match with HWE law.

Table (4-13) Hardy–Weinberg equilibrium for *AGT* gene rs699 genotype among patient groups.

Polymorphism	Group	Genotype, n (%)			Allele, n (Frq.)		HWE	
							χ^2	*p*
		TT	TC	CC	T	C		
AGT (rs699)	Severe	6(10.5)	28(49.1)	23(40.4)	40(0.35)	74(0.65)	0.35	0.83
	Non-severe	5(11.1)	23(51.1)	17(37.8)	33(0.367)	57(0.633)	0.45	0.79

Abbreviations: *: Significant p≤0.05, HWE: Hardy–Weinberg equilibrium, Frq: allele frequency rate, $\chi 2$: chi-square, *p*: The p-value.

Table (4-14) compared the allelic frequency of *AGT* genotypes in rs699 in several genetic models based on severe versus non-severe cases. Statistically, the differences between the two groups were not significant in genotypes in all models of *AGT* rs699 gene polymorphisms and severity of the disease.

Even though patients in this study with severe disease had a higher frequency of the C allele, no statistical significance was found, possibly a need for a larger sample size. So far, no data much-studied polymorphism rs699 in order to explain its impact severity of COVID-19, there was one study in Iran mentioned above, that did not observe any genotype associated with severe outcomes or mortality of COVID-19(Kouhpayeh *et al.*, 2021). According to the findings in table (4-15), rs699 genotype did not correlate with demographics, clinical history, and medical history except that female gender, diabetes mellitus, hypertension, and oxygen therapy in severe cases were significantly higher among patients with three different genotypes (p = 0.001, p=0.003, p=0.045 and p = 0.001, respectively).

Table (4-14) *AGT* gene (rs699) polymorphism under different genetic model and allelic models in severe patient and non-severe.

Polymorphism	Genetic models	Severe, n(%)	Non-severe, n(%)	Odds ratio	
				OR (95%CI)	p
***AGT* (rs699)**	**Codominant**				
	TT®	6(10.5)	5(11.1)	1.00(Ref.)	--
	TC	28(49.1)	23(51.1)	1.01(0.27-3.75)	0.98
	CC	23(40.4)	17(37.8)	1.12(0.29-4.31)	0.86
	Dominant				
	TT®	6(10.5)	5(11.1)	1.00(Ref.)	--
	TC+CC	51(89.5)	40(88.9)	1.06(0.3-3.73)	0.92
	Recessive				
	TT+TC®	34(59.6)	28(62.2)	1.00(Ref.)	--
	CC	23(40.4)	17(37.8)	0.89(0.4-2)	0.79
	Over-dominant				
	TT+CC®	29(50.9)	22(48.9)	1.00(Ref.)	--
	TC	28(49.1)	23(51.1)	1.08(0.49-2.36)	0.84
	Allele				
	T®	40(35)	33(36.7)	1.00	--
	C	74(65)	57(63.3)	1.07(0.6-1.9)	0.81

Abbreviations: ® References, OR: odd ratio, p: The p-value.

Among diabetic patients, COVID-19 is more common and more severe(Singh *et al.*, 2020). In a study, it was found that patients with COVID-19 are more likely to be hospitalized and to die as they age and have diabetes mellitus, hypertension, and obesity(Muniyappa and Gubbi, 2020). The severity of complications associated with COVID-19 is high in patients with diabetes(Li G. *et al.*, 2021). COVID-19 patients with hypertension have a high mortality rate compared to patients without hypertension. An increase in the severity of the COVID-19 infection may be caused by dysregulation of the RAAS, immune response, gastrointestinal tract, and inflammation(Peng *et al.*, 2021).

Compared to COVID-19 patients without hypertension, patients with hypertension often suffered from severe pneumonia, excessive inflammatory reactions, organ and tissue damage, and disease progression.

In order to prevent hypertension from worsening, patients should receive additional care(Songjiang Huang *et al.*, 2020). Specifically, COVID-19 requiring oxygen is assessed as either a noninvasive respiratory therapy or invasive mechanical ventilation(Kayem *et al.*, 2020). A chest X-ray showed bilateral alveolar or interstitial infiltrates was more common in patients with severe pneumonia needing additional oxygen(San-Juan *et al.*, 2020).

Table (4-15) summarizes the demographics and clinical characteristics of patients with COVID-19 based on the *AGT* genotype rs699.

Parameter	Severity state	Genotypes			P-value
		TT	TC	CC	
Male	Severe Non-severe	5 5	12 6	16 4	0.6 0.81
Female	Severe Non-severe	1 0	16 17	7 13	**0.001*** 0.46
Death	Severe Non-severe	2 0	3 0	0 0	0.65 --
Hypertension	Severe Non-severe	1 1	9 2	1 0	**0.003*** 0.56
Diabetes Mellitus	Severe Non-severe	1 2	7 3	2 1	**0.045*** 0.6
Cardiac Diseases	Severe Non-severe	1 3	1 2	0 0	-- 0.65
Kidney Diseases	Severe Non-severe	0 1	4 0	1 0	0.18 --
Malignancy	Severe Non-severe	0 1	1 1	0 0	-- --
Hepatic Diseases	Severe Non-severe	0 0	1 0	1 0	-- --
Oxygen Support	Severe Non-severe	3 0	22 0	20 0	**0.001*** --
Vaccination Status	Severe Non-severe	2 4	4 14	6 11	0.36 0.06

Abbreviations: *: significant p≤0.05, p: The p-value.

To investigate whether the rs699 polymorphism modulates hematological parameters, the hematological parameters were compared between severe and non-severe subjects, based on their genotypes table (4-16). Among the study parameters, the rs699 polymorphism did not significantly affect any of them, except for Hb in females was significant (p=0.01).

Table (4-16) Analysis of hematological values (Mean ± SD) according to the *AGT* (rs699) genotypes in patients.

Parameters		Severity	Mean±SD genotypes of *AGT* (rs699)			P-value
			TT	CT	CC	
WBC		Total	13.78±4.28	13.73±5.58	13.12±5.87	0.86
		Non-Severe	10.2±1.3	8.47±2.34	7.72±2.79	0.42
		Severe	16.76±3.44	18.04±3.23	16.8±3.94	0.43
PLT		Total	246.72±37.28	284.47±91.04	274.87±64.92	0.33
		Non-Severe	258.6±24.13	254.52±36.65	253.05±31.56	0.94
		Severe	236.83±45.34	309.07±113.42	291±78.15	0.24
NEU		Total	8.7±3.53	11.46±4.59	10.73±4.11	0.15
		Non-Severe	6.14±1.55	8±2.76	7.72±2.79	0.38
		Severe	10.83±3.33	14.3±3.78	12.96±3.47	0.08
D.DIMER		Total	0.96±0.8	1.47±1.14	1.38±1.09	0.38
		Non-Severe	0.48±0.148	0.55±0.34	0.47±0.09	0.61
		Severe	1.36±0.93	2.22±1	2±1.04	0.17
LYM		Total	1.66±1.06	1.54±0.95	1.54±0.97	0.93
		Non-Severe	2.53±0.55	2.24±0.75	2.47±0.421	0.43
		Severe	0.93±0.79	0.97±0.69	0.86±0.63	0.85
NLR		Total	16.07±16.88	18.49±17.77	16.76±16.97	0.85
		Non-Severe	2.77±0.96	4.79±3.21	4.2±2.09	0.31
		Severe	27.14±19.35	29.75±16.88	26.04±17.19	0.74
PLR		Total	243.44±201.2	299.74±261.75	328.19±303.07	0.65
		Non-Severe	104.27±14.89	127.59±50.89	105.15±20.46	0.16
		Severe	359.41±212.8	441.15±280.68	493.05±309.3	0.57
HB	Male	Total	14.92±1.15	13.68±1.62	13.67±1.36	0.06
		Non-Severe	15.4±0.65	14.13±1.37	15.3±0.87	0.13
		Severe	14.44±1.4	13.46±1.75	13.26±1.15	0.29
	Female	Total	13±0.1	12.53±2.15	13.07±1.56	0.62
		Non-Severe	14.01±0.91	12.92±1.87	--	0.04
		Severe	13.26±0	10.96±1.99	13.35±0.74	**0.01***

Abbreviations: WBC: white blood cell, PLT: platelet, NEU: neutrophils, LYM: lymphocytes, NLR: Neutrophil-Lymphocyte Ratio, PLR: platelet-lymphocyte ratio, HB: hemoglobin, mean±SD: mean± standard deviation, *: P≤0.05 was considered statistically significant.

4.5.1.4. Association of the *AGT* rs699 to Serum Angiotensinogen in COVID-19

The serum level of angiotensinogen was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for concentration measurement in study samples. The present results in the study showed significant ($P \leq 0.05$) increase in angiotensinogen level of COVID-19 patients as compared to healthy subjects, The results, as mean ± SD of concentration

level, are presented in figure (4-30) showed increase in angiotensinogen in patients compared with healthy individuals, in addition the results of median (IQR) 76.56 (63.62-9219) vs 69.6 (55.66-84.34) for 95 patients and 95 healthy control respectively.

There are still a limited number of studies evaluating the association between rs699 (M268T) and other *AGT* levels and biomarkers of COVID-19 in general populations. No data for comparison with present results, only one study confirmed the concentration of angiotensinogen in the serum increased COVID-19 severity(Tepasse *et al.*, 2022).

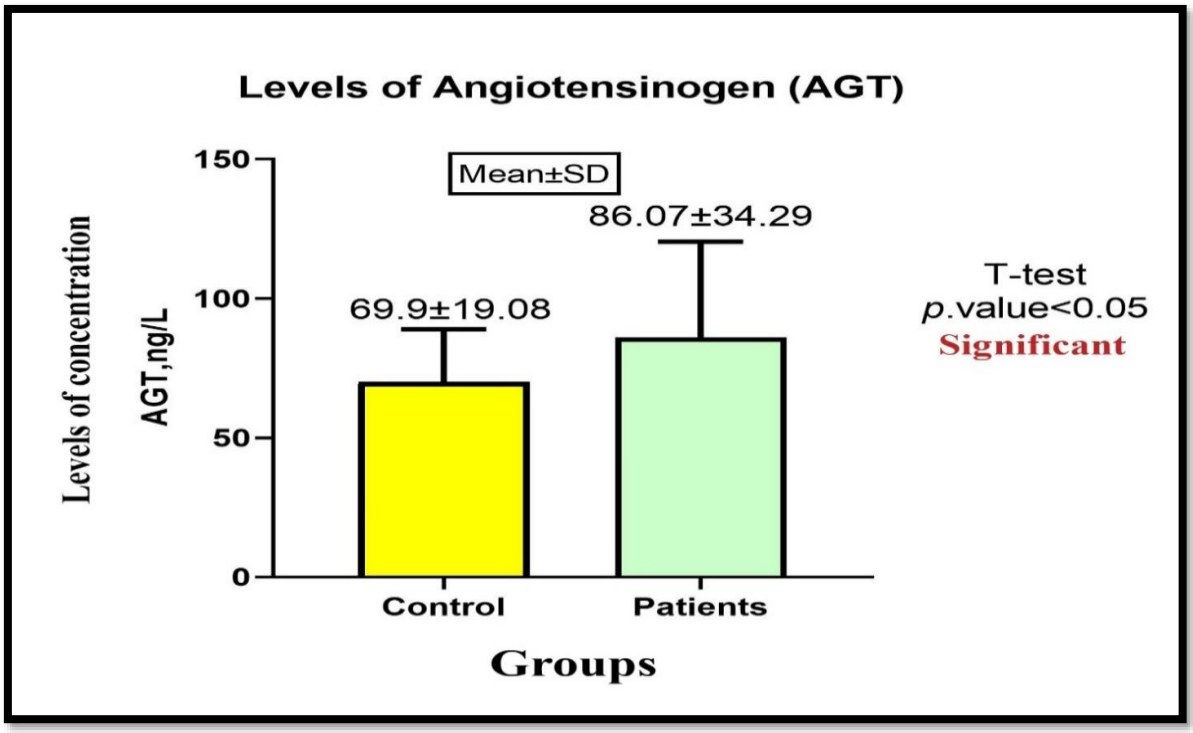


Figure (4-30) Mean ± SD of hormone angiotensinogen levels in healthy and COVID-19 patients.

There has been much of attention on how the renin-angiotensin signaling (RAS) pathway is involved in COVID-19. The involvement of the RAAS system in viral entry across Angiotensin-converting enzyme 2 (*ACE2*) has been recognized as a potential entry receptor for SARS-CoV-2 infection(Bian and Li, 2021), therefore may be a need for the replenishment of *ACE2* in the system

by increased of angiotensinogen concentration because the activity of the RAS depends on the availability of angiotensinogen and may be to it as.

In study by Wu. Z *et al*. (2020), 12.2 % of SARS-CoV-2 patients showed higher than normal renin levels, in advance the renin is secreted by juxtaglomerular cells in the kidney and converts angiotensinogen to angiotensin-I(Komukai *et al.*,2010), therefore, increased angiotensinogen concentration may be for this reason. In a letter to the editor, it was found that a decrease in renin may lower RAS system activity and *ACE2*, so decreasing SARS-CoV-2 viral entrance into the host cell(Vasanthakumar, 2020). As previously described RAS system is known best for its role in the maintenance of blood pressure and electrolyte and based on significant results by Taheri *et al*. (2021) were obtained after measuring the levels of electrolytes and micronutrients in the bodies of patients with COVID-19.

It was discovered that the levels of trace elements and electrolytes in the blood circulation play important roles in either lowering or increasing the severity of the disease, and thus the mortality rates of patients, therefore this may be one of reason to higher angiotensinogen concentration in present study of patients compared with apparently healthy individuals. Based on the above evidences could compare the levels of the *AGT* between severe vs non-severe and their slight differences in levels were in severe cases than in non-severe without significant differences ($p>0.05$) as in figure (4-31). In results of the ROC curve in present study was drawn and the area under the curve (AUC) was calculated to determine the accuracy of predicting the angiotensinogen levels, AUC in *AGT* was (0.437) not accurate because less than 0.5 and non-significant $p>0.05$ as in figure (4-32).

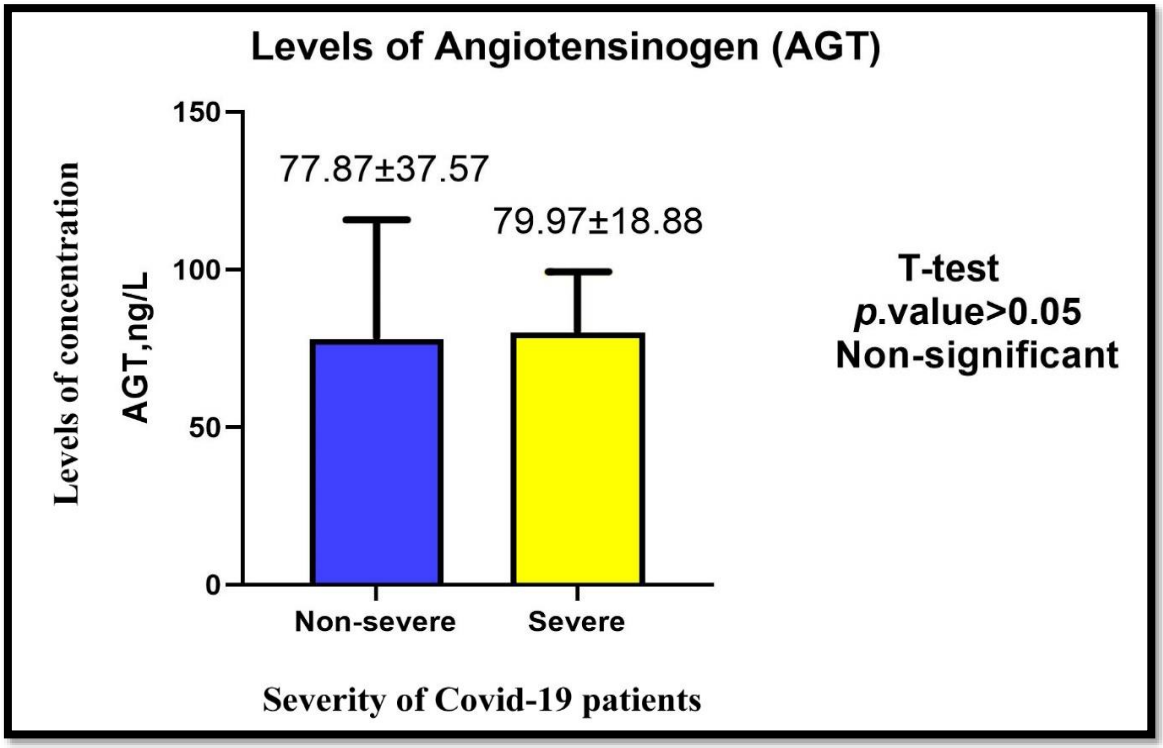


Figure (4-31) Mean ± SD of hormone angiotensinogen levels in COVID-19 patients group including (severe *vs* non-severe).

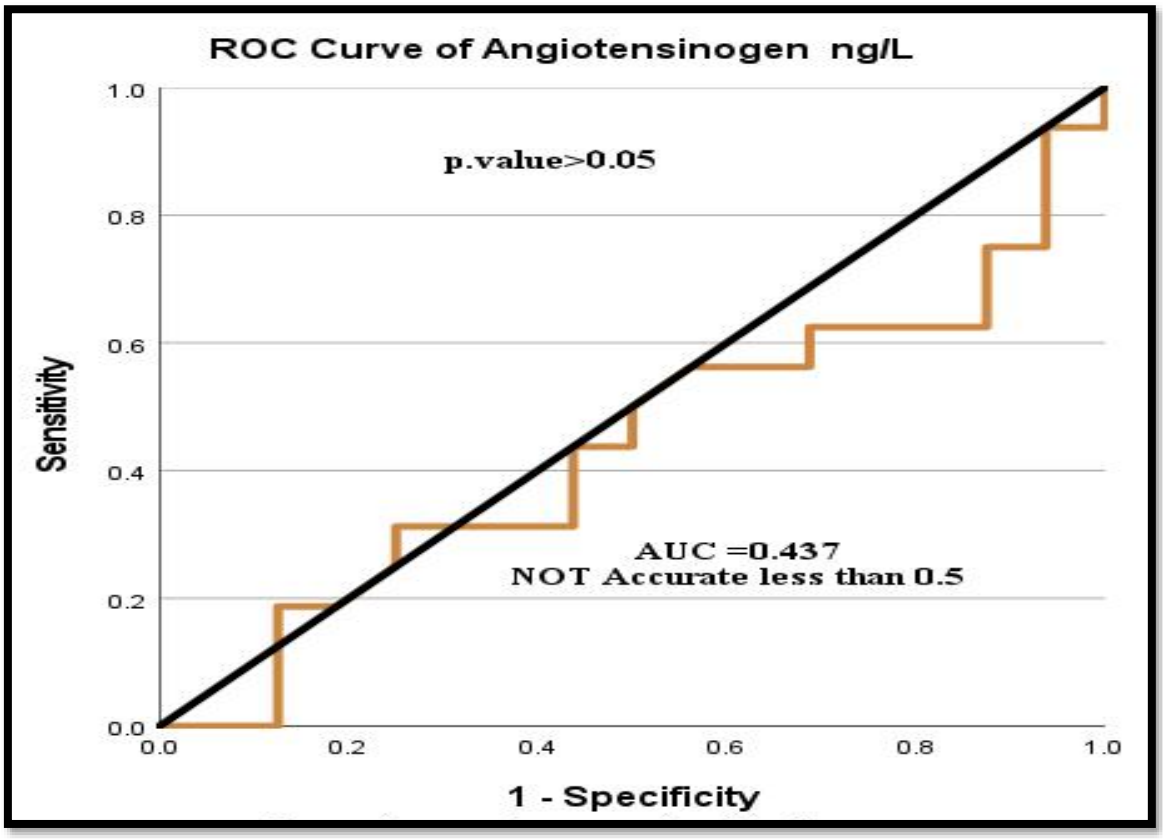


Figure (4-32) ROC curve of serum angiotensinogen level.

The relationship between genotype and serum levels of *AGT* appears to be present. Studies have indicated that there exists an association between the presence of the 235T allele in the *AGT* M235T (rs699) polymorphism and a notable elevation in plasma *AGT* concentrations(Jeunemaitre *et al.*, 1992; Sethi *et al.*, 2003).

The results of present study to assessed *AGT* levels in present homozygous TT genotype individuals were lowest circulating *AGT* levels was median 62.24 (IQR 53.37-75.99) in comparison with C-allele (TC+CC) carriers where higher levels CC was median 75.89 (IQR 59.64-90.48) and TC 70.46(IQR 56.53-87.17), but there were no statistically significant differences between genotypes as in figure (4-33), but still when changed Methionine to a threonine will change the *AGT* concentrations. In study found homozygous for the wild allele (M235 or T4072) had the lowest circulating *AGT* levels, while those mutant homozygous for the T235 (Thr) allele (C4072) had the highest(Zarebska *et al.*, 2013).

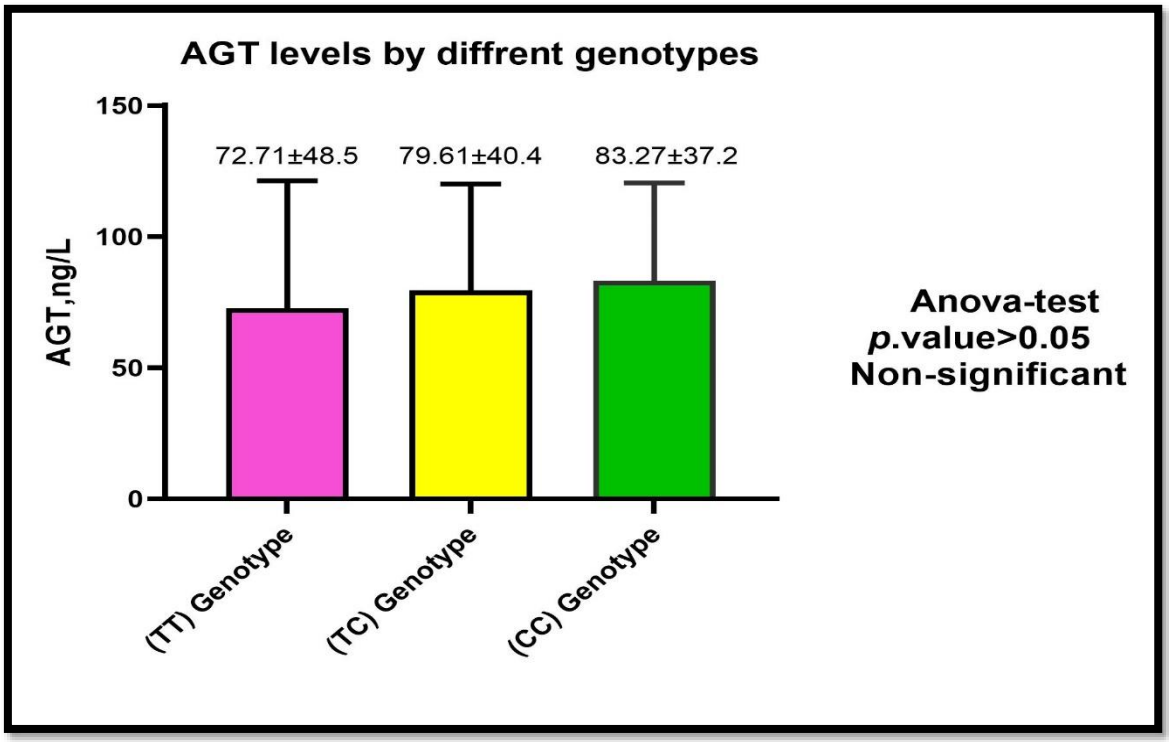


Figure (4-33) Mean ± SD of protein angiotensinogen levels in participants individuals based on genotypes carriers.

The results in present study were similar to a study confirmed the *AGT* rs699 C allele (TC+CC) is related with increased angiotensinogen (*AGT*) levels(Pringle *et al.*, 2016). Increased plasma *AGT* levels by 10–20% have been associated to the mutant genotype, which has also been related with an increased risk of developing hypertension(Jeunemaitre *et al.*, 1992). The *AGT* rs699 C allele (threonine variant) has been linked to increased plasma angiotensinogen levels, according to previous studies(Sethi *et al.*, 2003; Takeuchi *et al.*, 2012).

4.5.1.5. The Intron Polymorphism rs2106809 T>C SNPs in *ACE2* Gene

The *ACE2* gene is located on chromosome Xp22, spans 39.98 kb of genomic DNA, and has 20 introns and 18 exons, as mentioned in the results *AGT* gene was considered Angiotensin-converting enzyme 2 (*ACE2*) played a significant role in the RAS system by converting Ang II into angiotensin-(1-7)/mitochondrial assembly receptor [*ACE2*/Ang-(1–7)/MasR] axis.

The rs2106809 variant (g.7132T>C) is located at the beginning of the intron 2 of the *ACE2* gene. The *ACE2*-Ang (1-7)-MAS axis has been identified as a key mediator of the impacts an association between COVID-19 and infection severity. *ACE2* expression levels affect how successfully a virus enters the human body, complications following COVID-19 are associated with decreased *ACE2* expression(Sahu *et al.*, 2022).

Genotyping of rs2106809 was identified using the primers amplification refractory mutation system (T-ARMS-PCR), Allele Specific-PCR (AS-PCR), and sequencing techniques. In Allele specific-PCR the polymorphism 7132T>C in *ACE2* gene was identified PCR fragment was 196 bp for each allele, PCR reactions were performed in two reactions involved wild and mutant to predicted the results amplifies the wildtype and mutant the PCR products were separated and detected according to the difference bands that

appear between wild (W) type and mutant (M) type was used to determine the genotype of samples in figure (3-34).

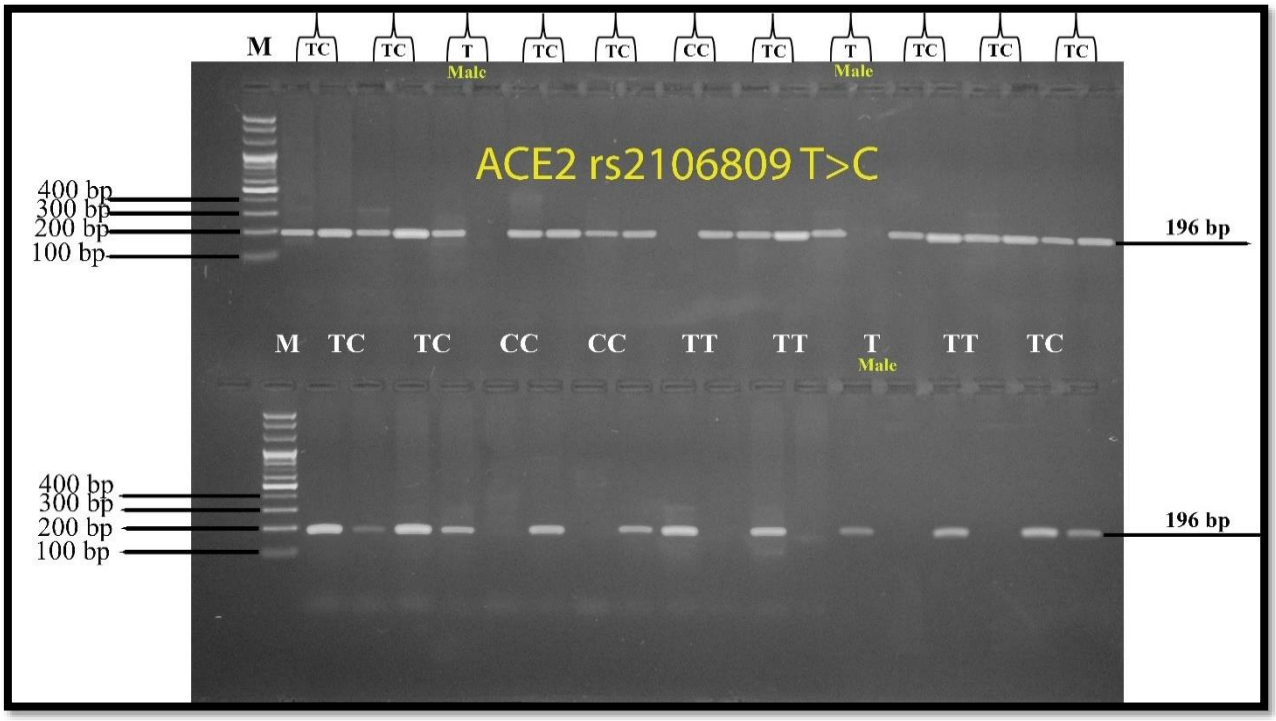


Figure (4-34) Gel electrophoresis separated on agarose 2%, at 75 voltage, 20 mA for 60min for allele specific PCR for *ACE2* SNP-7132T>C (rs2106809). M: 100 bp DNA ladder from Promega. PCR product upon using control forward primer and using C primer and T primer, with some samples for males contain only allele because *ACE2* on X-chromosome.

The ARMS–PCR technique for identification of SNP rs2016809 was collected and used to identification of gene sequence and position of single polymorphism. The fragment size of the ARMS PCR product was used to tell the difference between the mutant allele and the wild type allele.

The optimization of primer Amplification Refractory Mutation System Polymerase Chain Reaction (T-ARM-PCR) was applied by using different ratios of outer and inner primer concentration. The genotype variation of rs2106809 determined by control fragment or outer was (312 bp), specific fragment of TT wild allele (312+159 bp) and CC mutant allele (312+196 bp) as in figure (4-35).

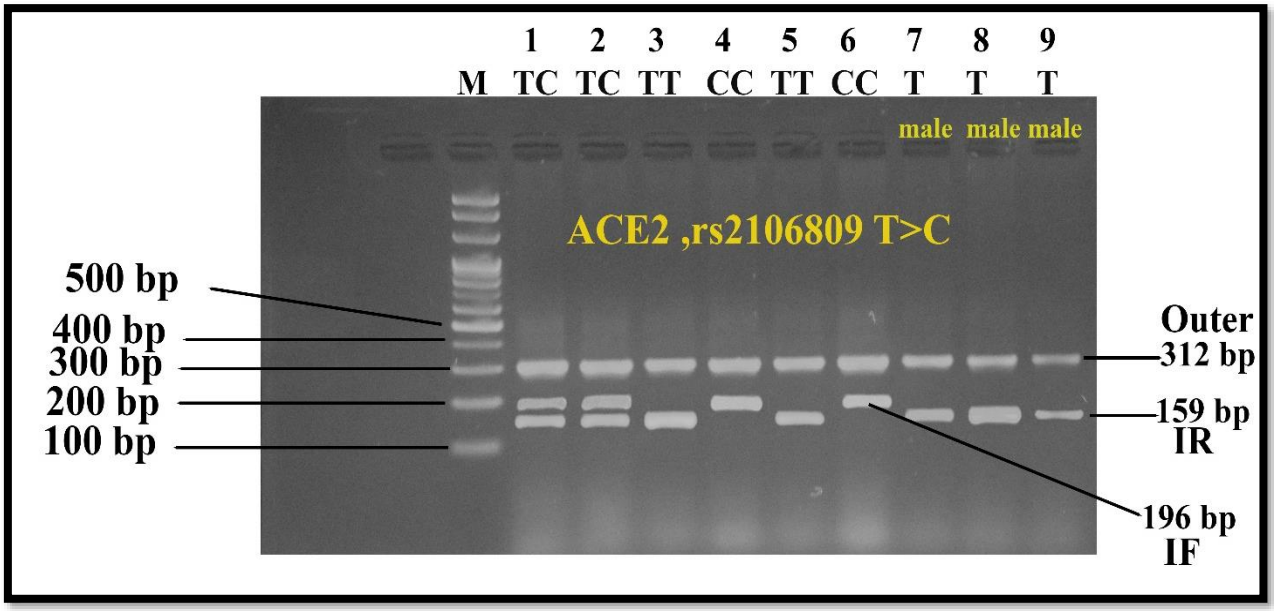


Figure (4-35) the T-ARMS-PCR for rs2106809 detection, M=100 bp ladder DNA, outer primer, IR=inner reverse, IF=inner forward, male=only T OR C allele because *ACE2* on X-chromosome, separated on agarose 2%, at 75 voltage, 20 mA for 60min.

Results of sequencing showed the genotypes of rs2106809 of *ACE2* gene wild in figure (3-36) while heterozygous and mutant in appendix (5).

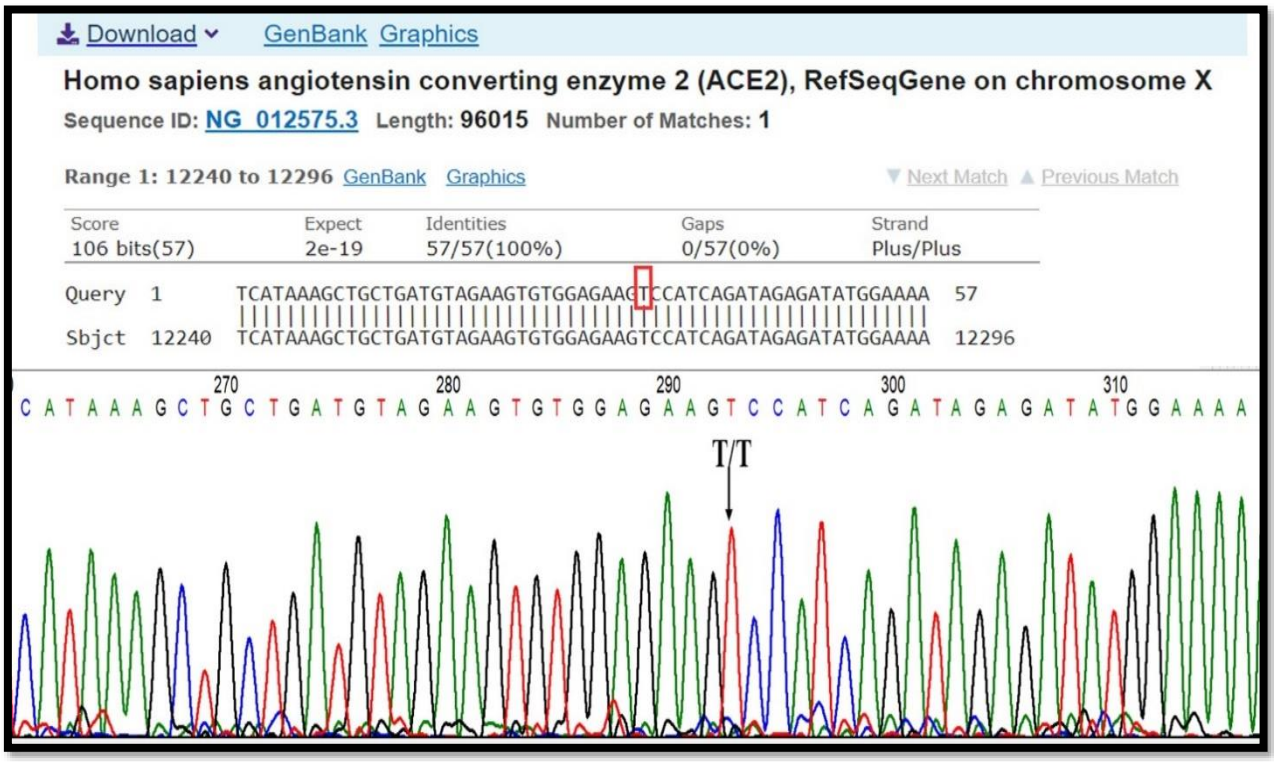


Figure (4-36) Sequence alignment and sequencing analysis to confirmed of *ACE2* rs2106809 sample TT genotype.

The genotype distribution frequencies were in agreement with the Hardy-Weinberg Equilibrium in the patients' and control groups (p>0.05) in females as in table (4-17). Table (4-18) showed no relation between the outcome of COVID-19 infection and rs2106809 in findings of present study in females, and these results come similar to some investigations in other populations.

In a study by, Çelik *et al.* examined 155 COVID-19 patients according to the infection of the disease and found no association between *ACE2* rs2106809 variation in females and the result of COVID-19 disease(Karakaş Çelik *et al.*, 2021). A case-control investigation that looked at the relationship between rs2106809-tag SNP rs2285666 (and different exonic *ACE2* variants) and COVID-19 (and its severity) could not find a statistically significant correlation. This may be because the study design and sample size were not accurate(Gómez *et al.*, 2020).

Table (4-17) Hardy–Weinberg equilibrium for *ACE2* gene rs2106809 genotype among patient groups and controls and different gender (male and female).

Polymorphism	Gender	Group	Genotype, n (%)			Allele, n (Frq.)		HWE	
								χ^2	*p*
ACE2 rs2106809	Female	Control	TT® 22(57.8)	TC 15(39.4)	CC 1(2.6)	T 59(0.648)	C 32(0.351)	0.7	0.7
		Patients	34(62.9)	16(29.6)	4(7.4)	84(0.777)	24(0.222)	1.1	0.57
	Male	Control	T® 43(79.6)		C 11(20.3)	T 43(0.796)	C 11(0.203)	--	
		Patients	27(56.2)		21(43.8)	27(0.562)	21(0.438)	--	

Abbreviations: ® References, HWE: Hardy–Weinberg equilibrium, p: The p-value, $\chi 2$: chi-square.

In a study carried out by Cafiero *et al*. (2021) the researchers looked at a number of SNPs, one of which was rs2106809, and discovered that there was no correlation between it and COVID-19 infection. While in males there were new findings in the present study indicated (C) allele genotype as a key concern in association with likely to acquired COVID-19 infection, there is significant statistically at p≤0.05 between patients and control groups at threefold (OR, 3.04, 95% CI 1.26–7.28).

Table (4-18) Association of *ACE2* rs2106809 polymorphism patients with COVID-19 as well as controls under different genetics models.

Polymorphism	Genetic models	Control, n (%)	Patients, n (%)	Odds ratio	
				OR (95%CI)	p
***ACE2* rs2106809**	**Codominant female**				
	TT®	22(57.9)	34(62.9)	1.00 (Ref.)	--
	TC	15(39.5)	16(29.7)	0.69 (0.28 - 1.67)	0.41
	CC	1(2.6)	4(7.4)	2.58 (0.27 - 24.7)	0.4
	Dominant female				
	TT®	22(57.9)	34(62.9)	1.00 (Ref.)	--
	TC+CC	16(42.1)	20(37.1)	0.8 (0.34-1.88)	0.6
	Recessive female				
	TT+CT®	37(97.3)	50(92.6)	1.00 (Ref.)	--
	CC	1(2.7)	4(7.4)	2.96 (0.31-27.5)	0.34
	Over-dominant female				
	CC+TT®	23(60.5)	38(70.3)	1.00 (Ref.)	--
	TC	15(39.5)	16(29.7)	0.64 (0.26-1.54)	0.32
	Allele frequency female				
	T®	59(77.7)	84(77.8)	1.00 (Ref.)	--
	C	17(22.3)	24(22.2)	0.99 (0.49-2)	0.98
	Male alleles				
	T®	43(62.9)	27(56.8)	1.00 (Ref.)	--
	C	11(20.3)	21(43.2)	**3.04 (1.26 - 7.28)**	**0.01***

Abbreviations: ® References, OR: odd ratio, Frq: allele frequency rate, p: The p-value, *Significant p≤0.05.

In addition, the predicted genotypic frequencies in females between severe and non-severe cases follow the Hardy-Weinberg equilibrium, the

genotype frequencies in the same generation in the present data and allele frequencies remained constant in generations and no significant difference as seen in table (4-19).

Table (4-19) Hardy–Weinberg equilibrium for *ACE2* gene rs2106809 genotype among patient groups.

Polymorphism	Gender	Patients Group	Genotype, n (%)			Allele, n (Frq.)		HWE	
								χ^2	*p*
***ACE2* rs2106809**	**Female**	**Severe**	**TT®** 13(62.9)	**TC** 10(29.6)	**CC** 1(7.4)	**T** 84(0.777)	**C** 24(0.223)	0.29	0.86
		Non-severe	21(57.8	6(39.4)	3(2.6)	59(0.648)	32(0.352)	4.2	0.12
	Male	**Severe**	**T®** 15(45.5)		**C** 18(54.5)	**T** 15(0.455)	**C** 18(0.545)	--	
		Non-severe	12(80)		3(20)	12(0.8)	3(0.2)	--	

Abbreviations: ® References, $\chi 2$: chi-square, Frq: allele frequency rate, p: The p-value, HWE: Hardy–Weinberg equilibrium.

Regarding the severity, the present study assessed the (C) genotype in males to increase the risk of severe COVID-19 as in table (4-20), In similar results by Franczyk *et al*. (2022)showed the presence of SNPs rs2106809 and rs2285666 was associated with an increased risk of hospitalization and disease severity.

In second a study with recessive inheritance patterns in females, there was an association between the SNP rs2106809 and the risk of hospitalizations and a more severe progression of disease (C/C vs. T/C-T/T, OR = 11.41, 95% CI: 1.12-115.91; p =0.012). Furthermore, COVID-19 patients who showed the (C)for the rs2106809 SNP had a greater probability of being admitted to the

ICU or dying (OR = 11.41, 95%IC: 1.12-115.91, p = 0.012)(Sabater Molina *et al.*, 2022).

Table (4-20) *ACE2* gene (rs2106809) polymorphism under different genetic model and allelic frequency in severe patient and non-severe.

Polymorphism	Genetic models	Severe, n (%)	Non-severe, n (%)	Odds ratio	
				OR (95%CI)	p
***ACE2* rs2106809**	**Codominant Females**				
	TT®	13 (54.1)	21 (70)	1.00 (Ref.)	--
	TC	10 (41.6)	6 (20)	2.69 (0.79-9.17)	0.11
	CC	1 (4.1)	3 (10)	0.53 (0.05-5.74)	0.6
	Dominant Females				
	TT®	13 (54.2)	21 (70)	1.00 (Ref.)	--
	TC+CC	11 (45.8)	9 (30)	1.97 (0.64-6.05)	0.23
	Recessive Females				
	TT+TC®	23 (87.7)	27 (57.7)	1.00(Ref.)	--
	CC	1 (12.2)	3 (42.2)	0.39 (0.038-4.02)	0.43
	Over-dominant Females				
	TT+CC®	14(58.4)	24(80)	1.00(Ref.)	--
	TC	10(41.6)	6(20)	2.85 (0.85-9.56)	0.08
	Allele Females				
	T®	84(77.7)	59(64.8)	1.00 (Ref.)	--
	C	24(22.3)	32(35.2)	0.62 (0.35-1.09)	0.1
	Allele Male				
	T®	15 (45.5)	12 (80)	100 (Ref.)	--
	C	18 (54.5)	3 (20)	**4.8 (1.13-20.25)**	**0.03***

Abbreviations: ® References, OR: odd ratio, p: The p-value, *Significant p≤0.05.

In present results this variant was risk in male more than female and this is due to the human *ACE2* gene is located on the X chromosome (Xp22.2 chromosomal region), in males, who carry only one copy of X and females have two, in addition higher *ACE2* expression was reported in men's lungs more than women(Fawzy *et al.*, 2022).

In this study, it was found that genotypes of rs2106809 *ACE2* were significantly associated with severe and non-severe cases in women (p=0.008 and p=0001 respectively), and the *ACE2* rs2106809 C and T allele were significant with non-severe in men (p=0.02), whereas *ACE2* genotypes in females and alleles in males were statically significant with oxygen-requiring cases (p=0.001 and p=0.02 respectively). The vaccination status in non-severe patients was correlated with *ACE2* rs2106809 genotypes in females (p=0.03) as shown in table (4-21).

Table (4-21) shows the effect of *ACE2* rs2106809 genotype on the demographic and clinical features of patients with COVID-19.

Parameter	Severity state	Genotype Female			P-value	Allele Male		P-value
		TT	TC	CC		T	C	
Male	Severe	--	--	--		15	18	0.6
	Non-severe	--	--	--		12	3	**0.02***
Female	Severe	13	10	1	**0.008***	--	--	
	Non-severe	21	6	3	**0.001***	--	--	
Death	Severe	2	1	0	0.56	0	2	--
	Non-severe	0	0	0	--	--	--	--
Hypertension	Severe	5	1	1	0.1	1	3	0.3
	Non-severe	1	1	0	--	0	1	--
Diabetes Mellitus	Severe	1	3	0	0.31	1	4	0.1
	Non-severe	2	1	1	0.77	2	0	--
Cardiac Diseases	Severe	1	0	0	--	2	1	0.5
	Non-severe	0	1	0	--	4	0	--
Kidney Diseases	Severe	1	0	0	--	1	3	0.3
	Non-severe	1	0	0	--	0	0	--
Malignancy	Severe	0	0	0	--	0	1	--
	Non-severe	1	0	0	--	1	0	--
Hepatic Diseases	Severe	0	0	0	--	0	2	--
	Non-severe	0	0	0	--	0	0	--
Oxygen Support	Severe	14	9	1	**0.001***	6	17	**0.02***
	Non-severe	0	0	0	--	0	0	--
Vaccination Status	Severe	1	1	0	--	4	6	0.5
	Non-severe	11	5	2	**0.03***	8	3	0.1

Abbreviations: *: significant p≤0.05, p: The p-value.

In females, hematological characteristics did not differ significantly among 54 patients with different genotypes of the *ACE2* rs2106809

polymorphism on chromosome X, while only Hb differed significantly among the non-severe patients as in table (4-22).

Table (4-22) Hematologic parameters associated with *ACE2* polymorphism rs2106809 in females genotypes.

Parameters	Gender	Severity	Mean±SD Female genotypes *ACE2* (rs2106809)			P-value
			TT	CT	CC	
WBC	Female	Total	11.99±6.06	13.86±5.19	10.25±5.96	0.42
		Non-Severe	8.19±3.8	8.5±1.51	7.33±1.52	0.86
		Severe	18.13±3.27	17.09±3.6	19±0	0.71
PLT		Total	278.11±96.15	270±57.6	254±24.01	0.84
		Non-Severe	249.8±41.14	258.5±26.6	253.3±29.36	0.88
		Severe	323.84±137.62	276.9±70.69	256±0	0.57
NEU		Total	10.1±4.76	10.63±3.81	10.19±3.92	0.92
		Non-Severe	7.54±3	7.17±0.83	8.32±1.43	0.83
		Severe	14.24±4.09	12.7±3.33	15.82±0	0.54
D-DIMER		Total	1.04±1.03	1.44±1.02	1.21±1.53	0.47
		Non-Severe	0.45±0.11	0.54±0.1	0.44±0.075	0.18
		Severe	2±1.15	1.9840 .94538	3.51±0	0.39
LYM		Total	1.92±0.91	1.3870 ±0.87	1.62±0.9	0.15
		Non-Severe	2.38±0.65	2.32±0.41	1.99±0.6	0.6
		Severe	1.18±0.8	0.82±0.49	0.49±0	0.36
NLR		Total	11.91±15.43	18.56±13.74	17.27±24.85	.035
		Non-Severe	3.83±1.63	5.57±2.52	4.84±0.41	0.11
		Severe	24.96±18.77	26.36±11.45	54.55±0	0.22
PLR		Total	215.11±221.77	304.46±221.75	232.27±196.81	0.41
		Non-Severe	112±32.29	112.97±13.47	135.54±44.35	0.47
		Severe	381.66±291.31	419.35±206.75	522.44±0	0.84
HB		Total	12.86±1.95	12.85±1.66	11.3±2.75	0.36
		Non-Severe	13.86±0.93**a**	13.46±1.3**a**	11.4±3.36**b**	**0.02***
		Severe	11.23±2.1	12.49±1.816	11±0	0.32

Abbreviations: WBC: white blood cell, PLT: platelet, NEU: neutrophils, LYM: lymphocytes, NLR: Neutrophil-Lymphocyte Ratio, PLR: platelet-lymphocyte ratio, HB: hemoglobin, mean±SD: mean± standard deviation, *: P≤0.05 was considered statistically significant.

In table (4-23), hematological parameters and the rs2106809 polymorphism are presented by type of allele in males. WBC and Hb have statistically significant differences in total samples and severe cases, whereas NEU, LYM, NLR, and PLR are significant in total and non-severe samples. D-dimer in a total sample with p≤0.05 significant. Meanwhile, PLT value was non-significant.

Table (4-23) Hematologic characteristics associated with *ACE2* polymorphism rs2106809 in males Alleles.

Parameters	Gender	Severity	Mean±SD Male Alleles *ACE2* (rs2106809)		P-value
			T	C	
WBC	Male	Total	12.64±4.59	17.38±4.28	**0.001***
		Non-Severe	9±2.44	10.5±3.27	0.38
		Severe	15.55±3.74	18.52±3.24	**0.02**
PLT		Total	262.37±51.83	301.95±88.77	0.059
		Non-Severe	255.25±19.59	276.33±36.11	0.17
		Severe	268.06±67.89	306.22±94.77	0.2
NEU		Total	9.86±4.15	13.75±3.35	**0.001***
		Non-Severe	6.79±1.63	12.7±1.46	**0.0001***
		Severe	12.32±3.91	13.92±3.57	0.23
D-DIMER		Total	1.22±0.97	2.06±1.075	**0.007***
		Non-Severe	0.52±0.23	0.91±0.84	0.15
		Severe	1.77±0.99	2.25±1	0.18
LYM		Total	1.66±1.03	0.93±0.76	**0.01***
		Non-Severe	2.61±0.56	1.6800 .74081	**0.03***
		Severe	0.91±0.6	0.81±0.71	0.67
NLR		Total	13.84±13.76	30.74±20.02	**0.001***
		Non-Severe	3.02±0.93	10.24±6.44	**0.001**
		Severe	22.5±13.05	34.16±19.5	0.05
PLR		Total	267.93±232.13	511.63±344.22	**0.005***
		Non-Severe	103±29.5	190.02±86.87	**0.009***
		Severe	399.8±239.71	565.23±342.36	0.12
HB		Total	14.53±1.04	13.17±1.64	**0.001***
		Non-Severe	14.99±1.032	14.36±1.76	0.42
		Severe	14.17±0.93	12.97±1.59	**0.01***

Abbreviations: WBC: white blood cell, PLT: platelet, NEU: neutrophils, LYM: lymphocytes, NLR: Neutrophil-Lymphocyte Ratio, PLR: platelet-lymphocyte ratio, HB: hemoglobin, mean±SD: mean± standard deviation, *: P≤0.05 was considered statistically significant.

4.5.1.6. Association of the *ACE2* rs2106809 to Serum Ang (1-7) Levels in COVID-19 Patients

The results were measured by ELISA of samples in this study showed 93 patients with reduced levels of Ang-(1–7) the median was 70.96 (IQR 58.6-91.93) compared with elevated levels in 93 control group the median was 83.69 (IQR 68.96- 120.84) with significant difference at (p-value≤0.05) as shown in figure (4-37). Fang *et al.* Ang (1-7) has an anti-inflammatory and anti-thrombotic impact through activation the MasR and antioxidant role protecting

the lungs injury(Fang, Y. *et al.*,2019). In present study revealed lower Ang (1-7) in patients because as mentioned it previously *ACE2* is receptor to entry the virus and *ACE2* converts angiotensin II into angiotensin 1-7 (Ang 1-7), therefore the *ACE2* utilized by virus and that may be lead reduced the Ang (1-7) peptide levels consequently lead to increase of pathogenicity of infection. In study with similar results found Ang 1-7 levels in COVID-19 patients were lower than in controls, and lower levels of this peptide were associated with disease severity. Patients with COVID-19 had lower Ang 1-7 levels after several days of symptom onset(Carpenter *et al.*, 2022).

Conclude that the SARS-CoV-2 had the same receptor with the SARS-CoV-1, which was *ACE2*, and that this shared receptor might cause enhanced activity of ACE-Ang II compared to *ACE2*-Ang-(1-7) to cause acute lung damage in SARS-CoV-2 patients(Kuba *et al.*, 2005).

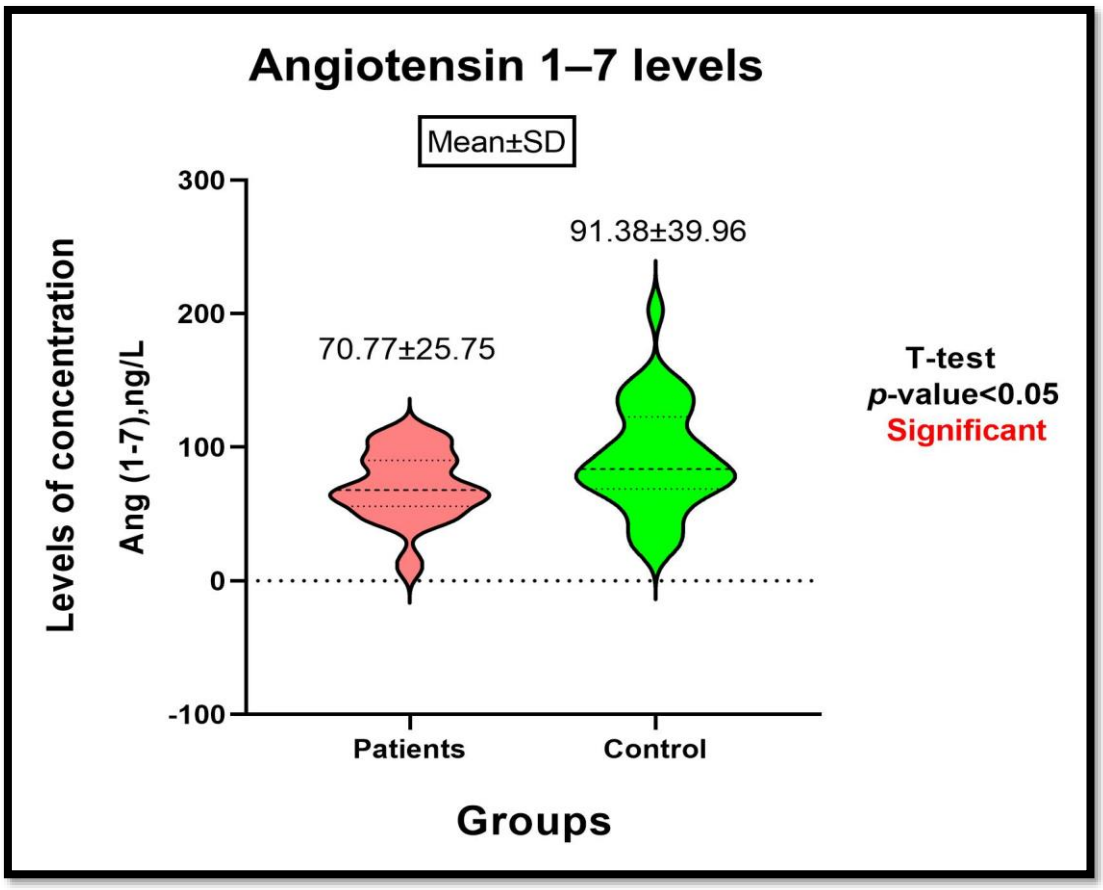


Figure (4-37) Mean ± SD of peptide Ang 1–7 levels in control (healthy individuals) and (COVID-19 patients) groups.

The present results agreed with those published by Henry *et al*. (2021). who discovered that Ang 1-7 levels were significantly lower in COVID-19 patients compared to controls, and in those hospitalized to the ICU vs those who did not require critical care. The recent study by Amezcua-Guerra *et al*. (2022) serum from 74 patients was tested compared with healthy group, The concentration of Ang II in patients was higher while that of Ang(1-7) was lower, despite the fact that *ACE2* activity in patients was higher. In study with similar results to this study confirmed *ACE2* activity and Ang (1-7) levels were significantly lower in COVID-19 plasma samples than in healthy group, despite increased s*ACE2* protein levels. This displays that *ACE2* activity is functionally inhibited in COVID-19 patients. Cell-surface *ACE2* receptors are internalized when SARS-CoV-2 attaches to them, which may lead to less *ACE2* cleavage action. Plasma *ACE2* may involve both active and inactive versions, potentially as a result of spike protein binding(Daniell *et al.*, 2022).

In one study found the S protein-*ACE2* complex is internalized by endocytosis, resulting in a partial or total loss of *ACE2*'s enzymatic function in alveolar cells and, as a result, an increase in the tissue concentration of pro-inflammatory angiotensin II and decreasing the concentration of its physiological antagonist angiotensin 1-7(Rothlin *et al.*, 2020). The data from various papers compared to present results in this study found similar. When it comes to the severity of COVID-19, ranges widely from asymptomatic infection to severe disease with lethal complications, there was a significant difference in Ang (1-7) levels between severe patients compared to non-severe in addition to 5 dead individuals as in figure (4-38), Ang 1-7 levels were lower in severe more than non-severe and were more reduction among the dead. Median (IQR) were in severe 68.96 (IQR 62.43- 91.93) vs. non-severe were 108.39(IQR 70.96-136.92) vs 44 (IQR 34-70) in dead with statistical differences.

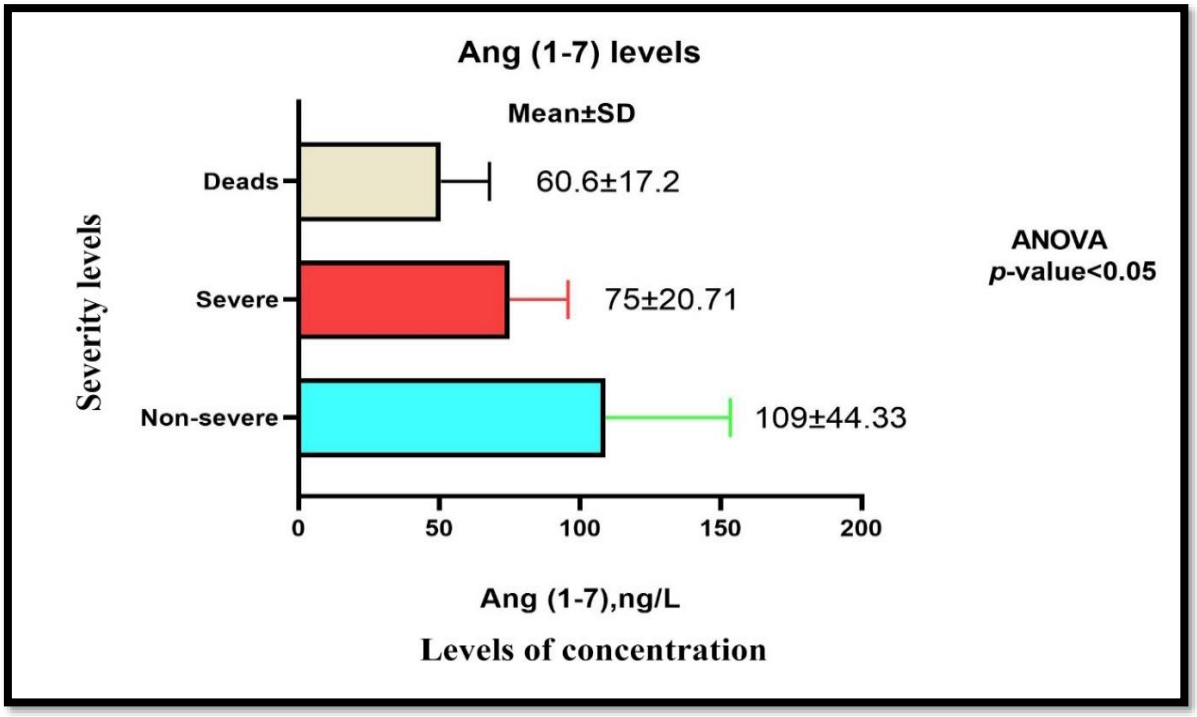


Figure (4-38) Mean ± SD of peptide Ang 1–7 levels in severe vs non-severe vs dead comparisons.

The area under the curve (AUC) is a crucial psychometric that provides the likelihood that the instrument would provide a higher score of the cut-off value at high 87.5% specificity and 75% sensitivity to serum Ang (1-7) level used to identify the severity of COVID-19 within the study population. The results of this study found the AUC was 0.848 with a significant difference ($p \leq 0.05$) with cut-off value 80.6 ng/L as in figure (4-39). Ang 1–7 levels were lower in COVID-19 patients than in control subjects, according to a study that agreed with the present results, lower levels of this peptide were linked to the severity and progression of the disease(Carpenter *et al.*, 2022). In another study, it was shown that higher levels of Ang (1–7) are associated to less severe disease on the contrary of the results of present study based on the conclusions(Seyedmehdi *et al.*, 2022). In consequence, a study suggests that severe symptoms and high mortality rates in COVID-19 patients may be related to several factors including vasoconstriction, vascular damage, fibrosis, proliferation, and inflammation caused by Ang reduction (1–7)(Khodarahmi *et*

al., 2021). In a recent study unveiled importance it is to enhance the angiotensin-(1-7)/MasR axis to control the severity of disease(Shete, A., 2020). It should be noted selected the polymorphism rs2106809 and associated it with Ang (1-7) levels, there was many of evidence to assess it with this variant. In a recent study rs2106809 was associated with significantly lower levels of angiotensin-(1-7) in the blood (P = 0.006)(Chen and Yu, 2018). In a paper, it was speculated that the rs2106809 SNP would down-regulate *ACE2* expression and reduce Ang- (1-7)(Fan *et al.*, 2019).

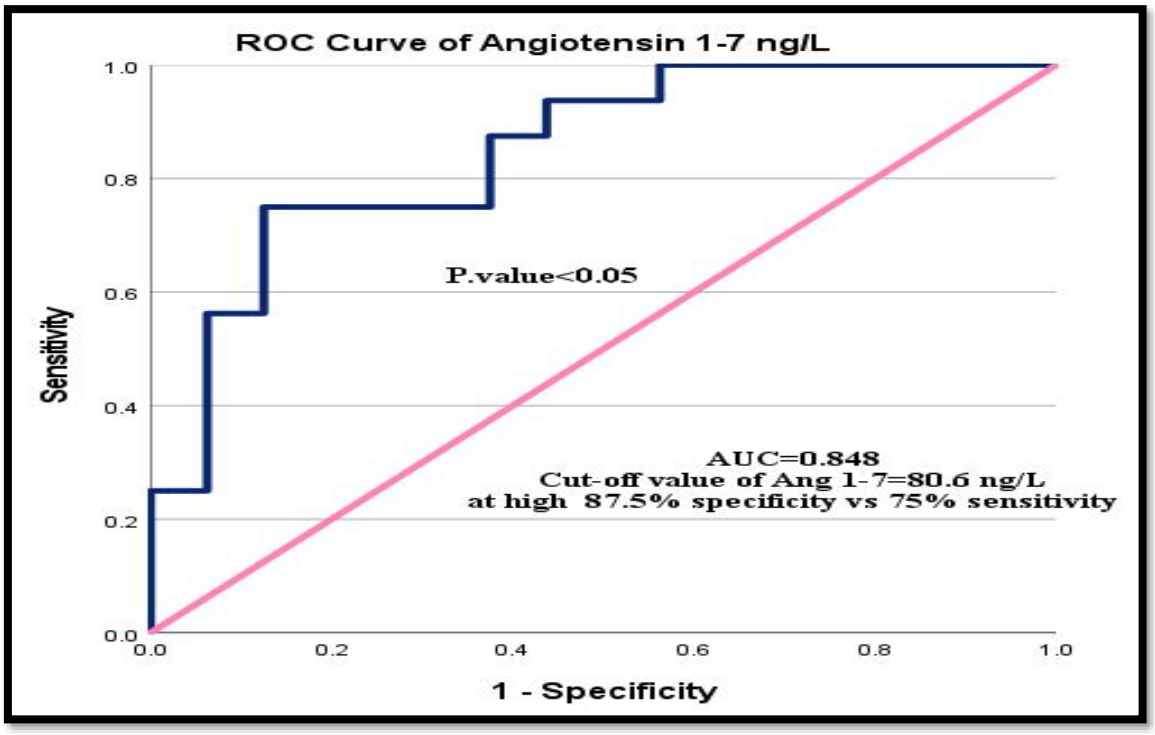


Figure (4-39) ROC curve analysis for Ang 1-7 levels.

The T-allele of *ACE2* rs2106809) leads to reduction in *ACE2* levels/activity higher Ang II levels and makes COVID-19 pathogenesis worse(De *et al.*, 2021), and based on these results and many studies conclude lower *ACE2* and higher Ang II levels were accompanied by a decrease in Ang (1-7). Last but not least, linkage disequilibrium is expected between *ACE2* rs2106809 and other enzymes that might affect Ang-(1–7) level. The results found in female *ACE2* rs2106809 TT or CT genotype carriers showed greater

circulating Ang (1-7) levels than CC genotype carriers according to figure (4-40), although the difference was not statistically significant, while in male there was increase T allele and decrease C allele with differences significant as in table (4-24).

Table (4-24) showed the Ang 1-7 levels based on different genotype related rs2106809.

Polymorphism	Gender	Genotype	Mean ±SD, ng/L	P-value
rs2106809 Ang (1-7) levels	**Female**	**TT**	77.72±15.79	p>0.05
		TC	73.63±20.72	
		TT	43.83±9.43	
	Male	**T**	69.87±16.54	**p≤0.05***
		C	41.65±8.92	

Abbreviations: *: significant p≤0.05, p: The p-value, mean±SD: mean± standard deviation,ng/L: nanograms (ng) per liter

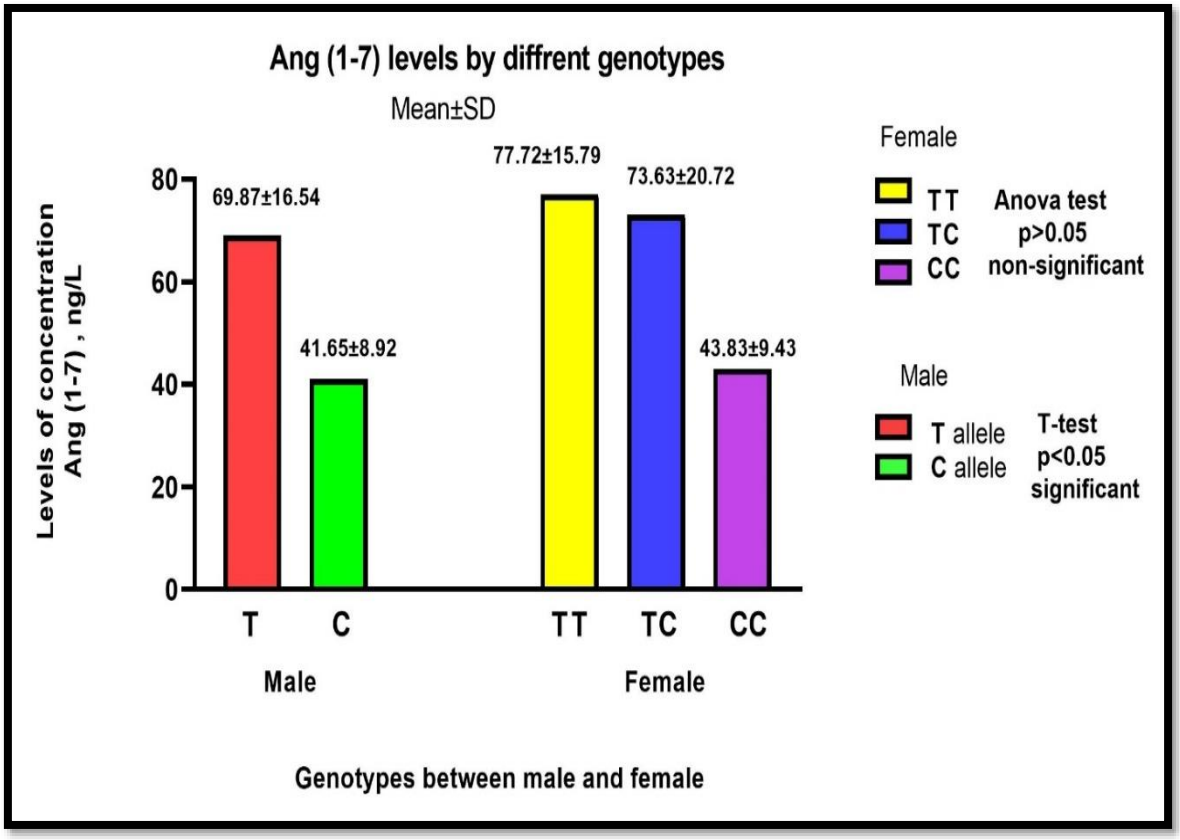


Figure (4-40) showed the mean±SD of Ang (1-7) levels between rs2106809 genotypes in males and females.

4.5.1.7. The Promoter Polymorphism rs1799889 4G/5G Variant in *SERPINE1* Gene

Plasminogen activator inhibitor 1, a master inhibitor of fibrinolysis and protease activity (PAI-1/ *SERPINE1*), on chromosome 7q21.3-q2, the *SERPINE1* (SERine Proteinase INhibitor, clade E, member 1) gene provides instructions for making PAI-1 polypeptide played a significant role in thrombotic events. The rs1799889 4G/5G insertion/deletion polymorphism was found in the gene promoter region. This genetic variation, called the 4G/5G polymorphism, is about 675 base pairs (bp) upstream from where the gene starts to be transcribed, in the 4G/5G polymorphism, there may be four (4G) or five (5G) guanosine residues(Baglin, 2012). PAI-1 gene variants at 675 bp included wild-type 4G/5G, mutant homozygous 5G/5G, and heterozygous 4G/5G.

The genotyped all subjects for 4G/5G polymorphisms of PAI-1, based on the single nucleotide polymorphism (SNP) site, allele specific-PCR markers were designed, allowing effective discriminating of SNP polymorphisms in two reactions under PCR conditions. The finding was indicated by detecting 4G/5G polymorphism by mutant and wild-type alleles in electrophoresis.

A common reverse primer and two forward allele-specific primers with varying length tails to distinguish the two SNP genotypes by the size of amplification products on an agarose gel. The size of 4G/5G bands in the study were 255 bp as in figures (4-41 and 4-42) to detected the genotype wild 4G/4G, heterozygous 4G/5G, and rare homozygous mutant 5G/5G.

The T-ARMS PCR was used to detect the genotype SNP of the *SERPINE* 1 4G/5G (rs1799889) gene. This variant had three genotype types (4G/4G, 4G/5G, and 5G/5G) with sizes (wild 315+145 bp, heterozygous 315+213+145 bp, and mutant 315+213 bp respectively) as in figure (4-43).

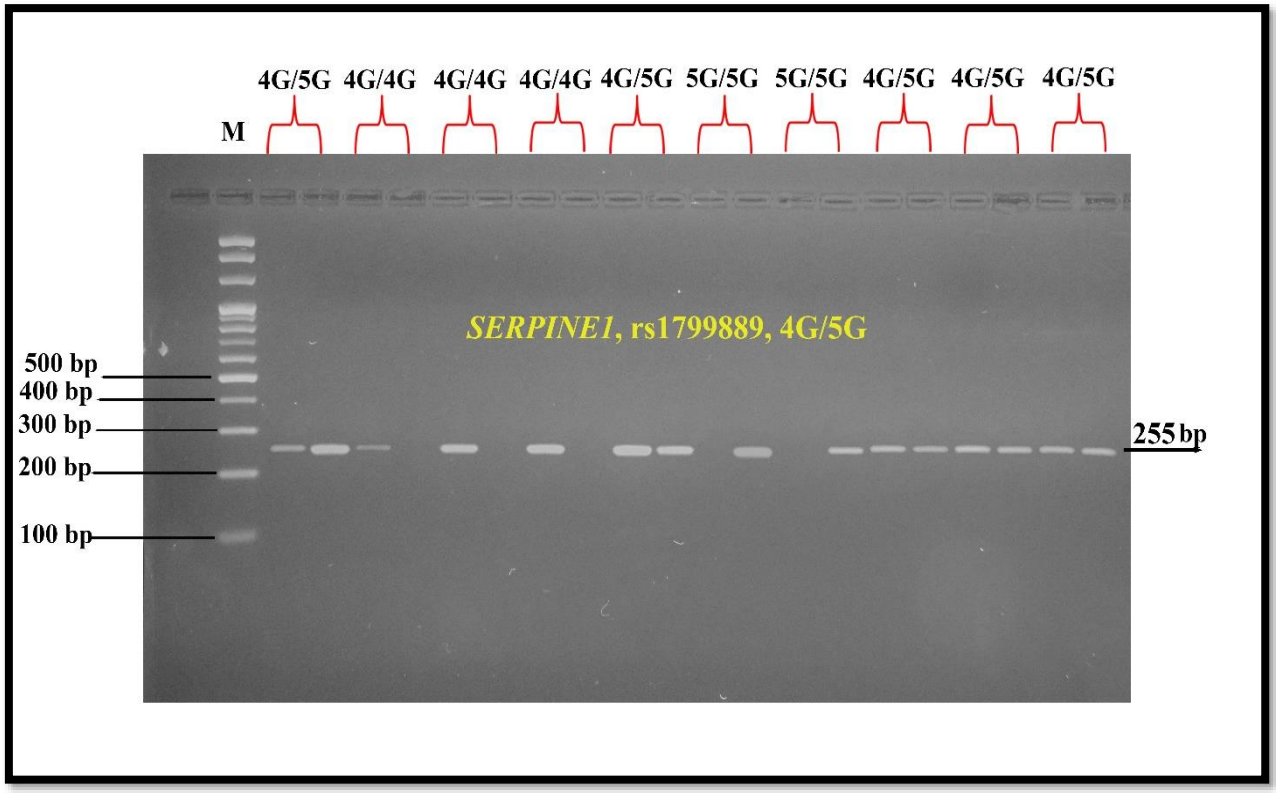


Figure (4-41): PCR product of SNP 4G/5G rs1799889 (255bp) visualized under UV light. An agarose gel 2% was electrophoresed at 75 voltages, 20 mA for one hour; M = 100 bp marker (ladder).

Figure (4-42) Electrophoresis of AS-PCR results of rs1799889 was performed with a 2% agarose, at 75 voltages, 20 mA for 60min, Lane M is the DNA size standard marker; lane 4G/4G is the homozygous genotype, 4G/5G heterozygous, and 5G/5G rare homozygous with size bands at 255 bp.

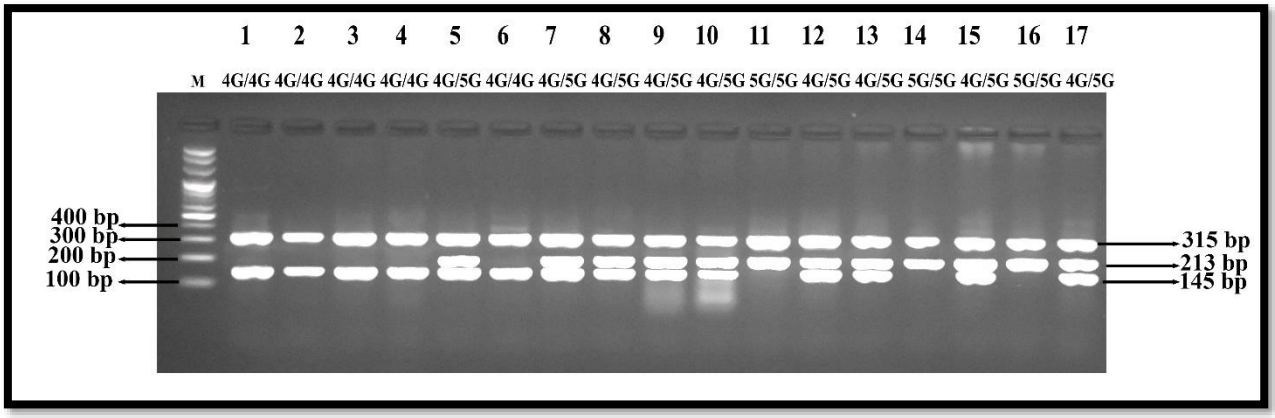


Figure (4-43) T-ARMS-PCR pattern for detecting 4G/5G rs1799889 polymorphism. It involved two outer primers and two inner primers. The 4G allele product size was 145 bp, the 5G allele product size was 213 bp, and the outer band size was 315 bp, M stands for DNA marker, separated on agarose 2%, at 75 voltages, 20 mA for 60min.

Results of sequencing samples of this study were submitted to the sequencer to detect rs1799889 and the raw sequencing data were alignment by blast NCBI to find regions of local similarity between sequences and indicated the genotypes of SNP *SERPINE*-1 rs1799889 as in figure (4-44) and the appendix (6). The law of Hardy-Weinberg equilibrium (HWE) is a fundamental concept in population genetics. It is normal to check to see if the observed genotypes meet the HWE law in both the control population and the patients. The calculated HWE for the *SERPINE1* (rs1799889) SNP genotype frequencies agreed with the law in the patient's group but differed substantially from the HWE in the control group ($p \leq 0.05$) as seen in the table (4-25).

Table (4-25) Hardy–Weinberg equilibrium for *SERPINE1* gene rs1799889 genotype among patient groups and controls.

Polymorphism	Group	Genotype, n (%)			Allele, n (%)		HWE	
							χ^2	*p*
SERPINE1 rs1799889	**Control**	**4G/4G®**	**4G/5G**	**5G/5G**	**4G**	**5G**	8.4	**0.01***
		39(42.3)	31(33.6)	22(23.9)	109(0.592)	75(0.407)		
	Patients	29(28.4)	47(46)	26(25.4)	105(0.514)	99(0.485)	0.61	0.71

Abbreviations: ® References, HWE: Hardy–Weinberg equilibrium, p: The p-value, $\chi 2$: chi-square.

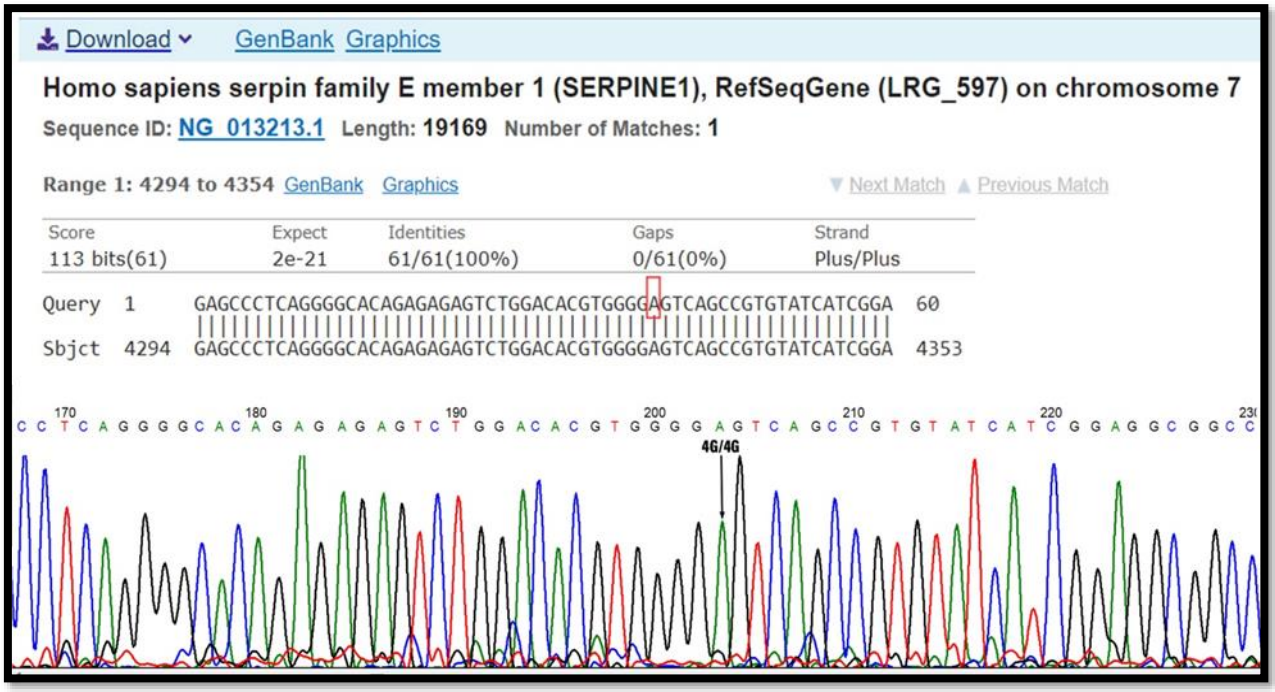


Figure (4-44) DNA sequencing results and alignment of *SERPINE-1* rs1799889 in samples of present study to showed wild genotype 4G/4G.

Table (4-26) Association of *SERPINE1* rs1799889 polymorphism with both controls and patients with COVID-19.

Polymorphism	Genetic models	Control, n (%)	Patients, n (%)	Odds ratio	
				OR (95%CI)	p
SERPINE1 rs1799889	**Codominant**				
	4G/4G®	39(42.4)	29(28.4)	1.00 (Ref.)	--
	4G/5G	31(33.6)	47(46.1)	**2 (1.05-3.94)**	**0.03***
	5G/5G	22(24)	26(25.5)	1.58 (0.75-3.34)	0.22
	Dominant				
	4G/4G®	39(42.4)	29(28.4)	1.00 (Ref.)	--
	4G/5G+5G/5G	53(57.6)	73(71.6)	1.85 (1.01-3.36)	**0.04***
	Recessive				
	4G/4G+4G/5G®	70 (76)	76(74.5)	1.00(Ref.)	--
	5G/5G	22 (24)	26(25.5)	1.08 (0.56-2.09)	0.79
	Over-dominant				
	4G/4G+5G/5G®	61(66.3)	55(54)	1.00(Ref.)	--
	4G/5G	31(33.7)	47(46)	1.68 (0.93-3)	0.08
	Allele frequency				
	4G®	109(59.2)	105(51.5)	1.00(Ref.)	--
	5G	75 (40.8)	99(48.5)	1.37 (0.91-2.04)	0.12

Abbreviations: ® References, OR: odd ratio, p: The p-value, *Significant p≤0.05.

Regarding the impact of variants on participants individuals, found statistically significant differences between the patient and control groups at the p≤0.05 level, the study concludes that individuals with the 4G/5G genotype are at an elevated risk of acquiring COVID-19 infection by in codominant model (OR:2; 95% CI =1.05-3.94; p=0.03) according to noticed the table (4-26). In similar results to this study heterozygous genotypes of PAI-1 4G/5G were more prevalent in patients with COVID-19 and coagulation events(Badulescu *et al.*, 2022). In dominant model results there is significant differences by increased risk of infection (OR:1.85; 95% CI =1.01-3.36; p=0.04).

The Hardy-Weinberg theorem describes the distributions of genotype frequencies in this results and identifies severe cases that are not evolving and are consistent with the HWE law whereas the population of non-severe individuals is not in Hardy-Weinberg equilibrium and the allele frequencies differ from the original frequencies after one cycle of random mating; hence, the population is not in Hardy-Weinberg equilibrium, and evolution has actually occurred within the population as in the table (4-27).

Table (4-72) Hardy–Weinberg equilibrium for *SERPINE1*gene rs1799889 genotype among patient groups.

Polymorphism	Groups	Genotype, n (%)			Allele, n (Frq.)		HWE	
							χ^2	*p*
***SERPINE1* rs1799889**	**Severe**	**4G/4G®** 15(26.3)	**4G/5G** 35(61.4)	**5G/5G** 7(12.2)	**4G** 65(0.57)	**5G** 49(0.429)	3.64	0.1
	Non-severe	14(31.1)	12(26.6)	19(42.2)	40(0.44)	50 (0.55)	9.52	**0.008***

Abbreviations: ® References, HWE: Hardy–Weinberg equilibrium, R: allele frequency rate p: The p-value, $\chi 2$: chi-square.

Table (4-28) shows in addition to the risk of infection, the 4G/5G genotype has been an associated increase in the risk of severity and progression of COVID-19 and a higher mortality rate in two genetic models codominant and over-dominant at (OR:2.72; 95% CI =1.02-7.25 p≤0.05 and OR:4.37; 95% CI =1.87-10.22 p≤0.05) respectively. While in the recessive model found 5G/5G genotype was positively associated with protective factor at (OR:0.19; 95% CI =0.07-0.51 p≤0.01). There were no large-scale studies on the polymorphisms rs1799889 and their association with COVID-19 infection and severity.

Table (4-28) *SERPINE1* gene (rs179989) polymorphism under different genetic model and allelic models in severe patient and non-severe.

Polymorphism	Genetic models	Severe, n (%)	Non-severe, n (%)	Odds ratio	
				OR (95%CI)	p
SERPINE1 rs1799889	**Codominant**				
	4G/4G®	15(26.3)	14(31.1)	1.00 (Ref.)	--
	4G/5G	35(61.4)	12(26.6)	**2.72 (1.02-7.25)**	**0.04***
	5G/5G	7 (12.2)	19(42.2)	0.34 (0.015-1.57)	0.06
	Dominant				
	4G/4G®	15(26.3)	14(31.1)	1.00 (Ref.)	--
	4G/5G+5G/5G	42(73.6)	31(68.8)	1.26 (0.53-2.99)	0.59
	Recessive				
	4G/4G+4G/5®	50 (87.7)	26(57.7)	1.00(Ref.)	--
	5G/5G	7 (12.2)	19(42.2)	**0.19** (0.07-0.51)	**0.001***
	Over-dominant				
	4G/4G+5G/5®	22(40.4)	33(64.4)	1.00(Ref.)	--
	4G/5G	35(59.6)	12(35.5)	**4.37** (1.87-10.22)	**0.001***
	Allele				
	4G®	65(57.1)	40(44.4)	1.00(Ref.)	--
	5G	49(42.9)	50(55.5)	0.62 (0.35-1.09)	0.1

Abbreviations: ® References, OR: odd ratio, p: The p-value, *Significant p≤0.05.

It was recently confirmed in a Turkish thesis that PAI-1 4G/5G polymorphism is associated with increased risk SARS-CoV-2 infection, Delta variants as opposed to the Omicron BA.1 variant. Moreover, according to the findings of the study, the PAI-1 polymorphism at 6754G/5G polymorphism of the PAI-1 gene shows that 64.7% of those with positive SARS-CoV-2 infection (the "case group") had at least one mutant allele (whether it was homozygous or heterozygous), while only 3% of patients in the "control group" were homozygous. Only 21% of those infected with the Delta variant were found to have the "wild type" genotype, whereas 79% of those infected carried at least one mutant allele (homozygous or heterozygous)(MOĞOL, 2022).

A second study revealed PAI-1-675 5G/4G was related to COVID-19 infection and especially 4G/4G genotype may be a predictor of severe COVID-19 disease(Vatseba and Virstyuk, 2021). A study showed a strong correlation between critical or severe COVID-19 and heterozygous 4G/5G PAI-1 polymorphism(Sezer *et al.*, 2022).

In Russia, a study with different findings from present study investigations and due to genetic polymorphisms determine the diversity of individuals within populations, Patients with mild cases of COVID-19 (91%) were more likely to display the 4G allele (4G/4G, 4G/5G variants) in the PAI-I gene (rs1799889), which leads to impaired fibrinolysis than patients with extremely severe COVID-19 (70%). The 4G/4G and 4G/5G polymorphisms may therefore have a protective effect on individuals who are severely ill and are experiencing hyperfibrinolysis. This can cause pulmonary COVID-19 to become sepsis(Городин *et al.*, 2021).

Among the 2 groups studied (severe and non-severe), no significant differences were observed in chronic diseases correlated to genotypes rs1799889 4G/5G. The gender male and female were higher in patients with severe symptoms compared to patients with mild or no severe who did not

require hospitalization (p≤0.001 Hospital ward male and severe female; p≤0.03 respectively) related to genotypes of 4G/5G. Moreover, oxygen requirements were significantly higher in the severe group than in the non-oxygen-requiring group (p≤0.001) as show in table (4-29).

Table (4-29) presents the impact of the SERPIN 1 4G/5G genotype on demographics and clinical characteristics of patients with COVID-19.

Parameter	Severity state	Genotype			P-value
		4G/4G	4G/5G	5G/5G	
Male	Severe Non-severe	10 8	21 3	2 4	**0.001*** 0.2
Female	Severe Non-severe	5 6	14 9	5 15	**0.03*** 0.1
Death	Severe Non-severe	1 0	4 0	0 0	0.1 --
Hypertension	Severe Non-severe	3 1	8 1	0 1	0.1 --
Diabetes Mellitus	Severe Non-severe	6 3	1 1	2 2	0.09 0.6
Cardiac Diseases	Severe Non-severe	4 1	0 2	0 2	-- 0.8
Kidney Diseases	Severe Non-severe	2 1	2 0	1 0	0.8 --
Malignancy	Severe Non-severe	0 1	1 0	0 1	-- --
Hepatic Diseases	Severe Non-severe	1 0	1 0	0 0	-- --
Oxygen Support	Severe Non-severe	12 0	31 0	4 0	**0.001*** --
Vaccination Status	Severe Non-severe	4 9	7 7	1 13	0.1 0.3

Abbreviations: *: significant p≤0.05, p: The p-value.

In the table (4-30) different genotypes of polymorphism rs1799889 had different hematological parameters. According to the comparisons of hematological parameters with genotypes, severe and non-severe cases were identified, and the WBC, NEU, and NLR values were significant both in the total patient sample and in non-severe cases, while the LYM, D-dimer, and PLR

values were significant in the total sample. The hemoglobin levels of all cases differed significantly.

Table (4-30) Hematological characteristics of COVID-19 patients in accordance with their genotypes on the *SERPINE*1 polymorphism rs1799889.

Parameters		Severity	Mean±SD genotypes of *SERPINE*1 (rs1799889)			P-value
			4G/4G	4G/5G	5G/5G	
WBC		Total	13.26±4.67	15.76±5.57	9.66±4.14	**0.001***
		Non-Severe	10.1±4.32	8.5±2.32	7.42±1.71	**0.04***
		Severe	16.21±2.67	18.25±3.91	15.77±1.84	0.07
PLT		Total	270.31±59.92	288.06±95.55	263.03±54.11	0.36
		Non-Severe	252.35±25.6	267.08±34.88	247.94±35.93	0.28
		Severe	287.06±77.13	295.25±108.4	304±75.14	0.92
NEU		Total	9.21±3.83	12.86±3.93	9.15±4.22	**0.001***
		Non-Severe	6.52±2.05	10±3.23	7.09±1.78	**0.001***
		Severe	11.72±3.38	13.84±3.69	14.77±3.83	0.1
D.DIMER		Total	1.29±1.07	1.68±1.05	0.87±1	**0.008***
		Non-Severe	0.51±0.21	0.6±0.41	0.46±0.12	0.32
		Severe	2.03±1.04	2.06±0.94	2±1.47	0.98
LYM		Total	1.62±0.9	1.2±0.89	2.13±0.9	**0.001***
		Non-Severe	2.18±0.61	2.33±0.8	2.51±0.5	0.33
		Severe	1.09±0.81	0.81±0.51	1.093±0.962	0.3
NLR		Total	12.29±12.51	24.43±18.55	10.98±15.8	**0.001***
		Non-Severe	3.34±1.23	6.68±3.9	3.6±1.52	**0.001***
		Severe	20.65±12.53	30.52±17.64	30.99±19.86	0.15
PLR		Total	264.64±234.33	396.65±306.5	183.66±178.15	**0.003***
		Non-Severe	123.45±33.59	130.4±58.52	102.65±25.11	0.12
		Severe	396.43±265.01	487.93±303.97	403.52±230.62	0.52
HB	Male	Total	13.99±1.74	13.74±1.36	14.55±1.17	0.49
		Non-Severe	15.36±0.62	14.36±1.76	14.25±1.38	0.21
		Severe	12.9±1.56	13.65±1.33	15.15±0.21	0.1
	Female	Total	13.86±0.83	11.67±2.09	13.35±1.59	**0.001***
		Non-Severe	14±1.09	12.3±1.99	14.1±0.69	**0.007***
		Severe	13.7±0.44	11.27±2.13	11.12±1.42	**0.042***

Abbreviations: WBC: white blood cell, PLT: platelet, NEU: neutrophils, LYM: lymphocytes, NLR: Neutrophil-Lymphocyte Ratio, PLR: platelet-lymphocyte ratio, HB: hemoglobin, mean±SD: mean± standard deviation, *: P≤0.05 was considered statistically significant.

4.5.1.8. PAI-1 rs1799889 Genotype Associated with PAI-1 Levels.

PAI-1 is a protein that is encoded by the *SERPINE1* gene. It has been observed that a polymorphism known as 4G/5G in the gene promoter of the plasminogen activator inhibitor type 1 (PAI-1) gene has an effect on the plasma levels of PAI-1. PAI-1 is the most essential component of the fibrinolytic system, accounting for about 60% of inhibitor action.

PAI-1 played a vital role in the control of fibrinolysis; hence, its increase may induce thrombosis of events. Various lines of evidence indicated that PAI-1 interacts to increase coagulopathy and thrombosis in COVID-19 patients. The results in this study exhibited significantly increased levels in 95 COVID-19 patients compared with 95 apparently healthy control was lower (median COVID-19: 5.56 [IQR 4.34– 10.93] mg/mL, Control: 3.37 [IQR 2.73– 3.85] ng/mL, $P \leq 0.05$) in addition to mean±SD was (3.56±1.34 and 6.5±5) for healthy and patients, respectively as in figure (4-45). In similar findings, the study by Whyte *et al.* found a group of 113 hospitalized COVID- 19 patients, levels of PAI- 1 antigen was higher compared to 24 patients with healthy controls(Whyte *et al.*, 2022). In a second study included 37 individuals with positive COVID-19 and 23 healthy individuals, in the COVID-19 group, the levels of PAI-1, tPA, and fibrinogen were all significantly higher(Cabrera-Garcia *et al.*, 2021).

The results from Cuba showed that moderately and severely sick COVID-19 patients who were diagnosed during the B.1.617.2 variant wave in Cuba had high serum levels of PAI-1(Saavedra *et al.*, 2022). A study that agreed with results of this study showed that patients with moderate or mild COVID-19 had significantly higher levels of PAI-1 in their blood. These levels increased with the severity of the disease, with patients in the death group having the highest levels(Lopez-Castaneda *et al.*, 2021). There was strong correlation evidence about how higher PAI-1 affects the formation of thrombosis in COVID-19 patients with three types of obesity and BMI-related disease severity(Mahdi, 2021). Several studies had indicated the fibrinolytic shutdown in COVID-19, which has been associated with increased plasma levels of plasminogen activator inhibitor 1 (PAI-1)(Zuo *et al.*, 2021).

Therefore, higher levels of PAI-1 induction were seen in COVID-19 patients, showed resistance to thrombus breakdown and hence promoting thrombosis.

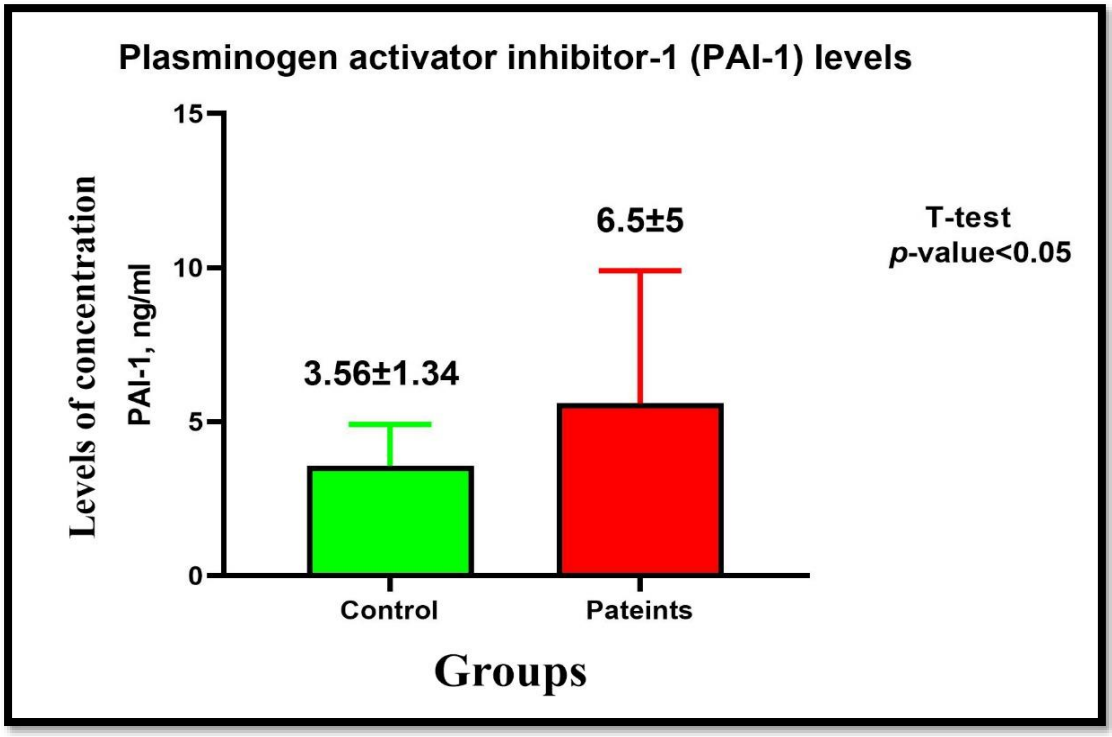


Figure (4-45) COVID-19 cases and control group measured differences according to serum PAI-1 levels.

The relationship between PAI-1 marker with COVID-19 disease progression was studied by grouping according to the level of severity included severe, non-severe cases and dead. In results of this study revealed that was higher PAI-1 levels in severe patients compared with non-severe and dead mean±SD were (8.63±4.62 *vs* 3.34±0.75 *vs* 13.2±4) respectively according to figure (4-46) at (p-value≤0.05), the median in severe was 6.54 (IQR 4.63-11) while in non-severe was 3.55(2.86-4.37) in addition in dead was 13 (IQR 9-19).

Pine *et al*. (2020) showed Plasma levels of PAI-1 were normal in individuals with mild to moderate illness, but were elevated in patients with severe COVID-19 infection and strongly predictive of mortality. Furthermore, PAI-1 plasma concentrations have been identified as a possible biomarker for

predicting disease development in ARDS and a favorable predictor of death. Functional studies showed that in COVID-19 disease, PAI-1 activity was elevated whereas plasmin production and clot lysis were significantly reduced(Whyte *et al.*, 2022). Consistent with this study, a study found high PAI-1 correlated with decreased oxygen supply in COVID-19 patients and that tPA, as an activator of plasminogen, ultimately led to its consumption, making it the strongest predictor of mortality(Zuo *et al.*, 2021). In a new study, it was confirmed that PAI-1 levels are likely to be a biomarker of poor clinical outcomes in COVID-19 cases. Due to COVID-19's prothrombotic risk, early detection allows for prophylactic anticoagulation to be initiated in patients with high prothrombotic risk(Baycan *et al.*, 2023). Plasminogen activator and inhibitor levels have both been associated to the severity of COVID-19(Cugno *et al.*, 2021). In similar results to present study PAI-1 is considered an endothelial damage biomarker, it has been shown that its concentration increases in severe cases of COVID-19(Khan S.S. 2021).

There was a correlation between the concentration of plasminogen activation inhibitor-1 (PAI-1) and lung injury. individuals with severe infections showed a reduction in fibrinolysis in a different cohort. a study demonstrated a substantial association between plasminogen levels and death in patients with COVID-19. Increased PAI-1 is connected with pneumonia that is more severe, a major cause of mortality in COVID-19 patients(Cheng *et al.*, 2021). The concentration of serum PAI-1 to predicted severity in the study was calculated using receiver operating characteristic curves and the area under the curve (AUC) at 0.766 in a study and cut-off value was 6.14 ng/ml at high 75% sensitivity and 75% specificity were plotted on a curve as in figure (4-47). In this study, investigated the effect of PAI-1 gene polymorphism (-675 4G > 5G rs1799889) on the level of PAI-1. One of the most well-known *SERPINE1* polymorphisms that influences PAI-1 levels is the 4G/5G insertion/deletion (rs1799889)(Bayram *et al.*, 2021). The genotypes in present study may impact

on the PAI-1 concentration, the results showed elevated mean±SD in 4G allele (4G/4G, 4G/5G) were 6.73±3.19 and 5.94±1.16 respectively, while in 5G/5G was lower 3.22±0.7 with significant difference (p-value≤0.05) as in figure (4-48).

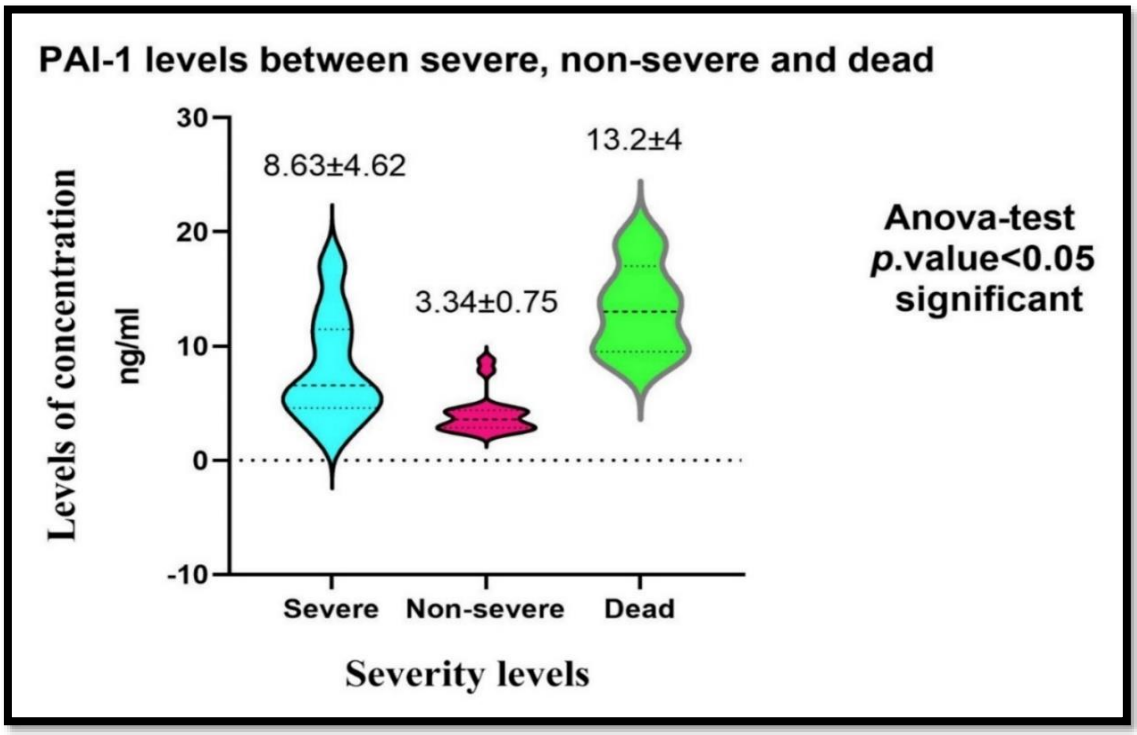


Figure (4-46) measured PAI-1 levels to COVID-19 cases according to severe, non-severe, and dead.

Notably, there is no published data of serum PAI-1 data to confirm PAI-1 levels in people linked to the 4G/5G genotype in COVID-19 infection so that may be compare the present results with other studies. Although some studies have associated the 4G allele to increased plasma levels of PAI-1(Kucukarabaci *et al.*, 2008). A study revealed when compared to other PAI-1 genotypes, the 4G/4G had considerably higher PAI-1 levels(Ezzidi *et al.*, 2009). In another study it was the 4G allele carriers(4G/4G,4G/5G) that showed the significant increases in PAI-1(Abboud *et al.*, 2010). In a study done by Gilabert-Estellés (2012) higher PAI-1 concentrations in endometrial tissue seem to be related to the 4G/5G polymorphism.

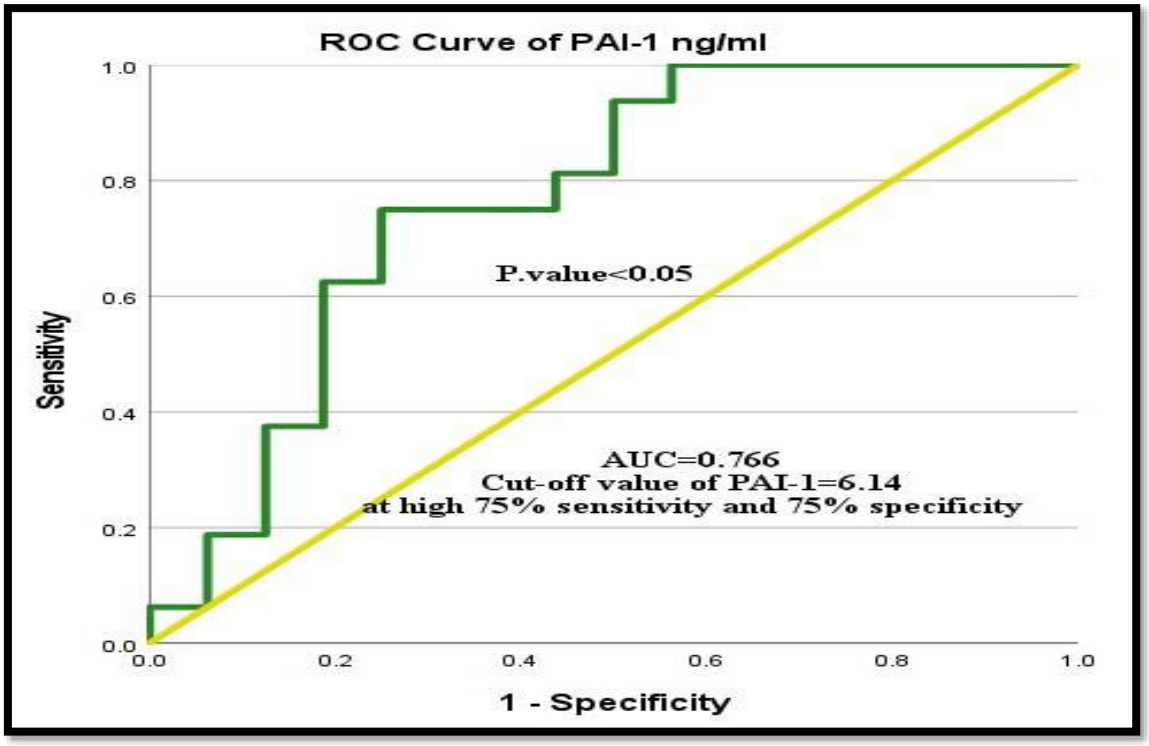


Figure (4-47) Receiver Operating Characteristic (ROC) curves for serum PAI-1 in study.

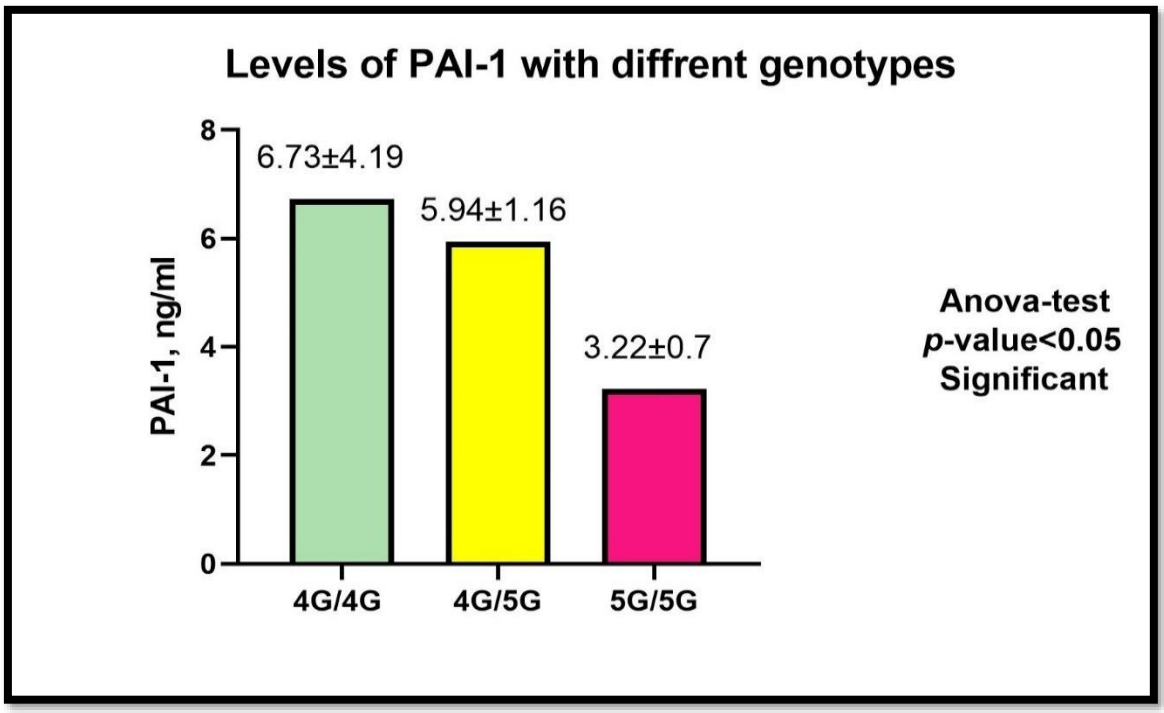


Figure (4-48) showed the mean±SD of PAI-1 levels between rs1799889 genotypes in samples.

4.6. In Silico Study

4.6.1. Interaction Genes and Proteins

Genetic interactions in model organisms differ depending on the type of interaction being studied, with the goal of finding functional relationships between genes in order to gain a deeper understanding of what role each gene plays within the organism. There are two levels of interaction in organisms, the first interaction depends on the genes and the second one relies on the product of genes intended as proteins.

The results of the present study figure (4-49) showed by using the GENEMANIA network(Warde-Farley *et al.*, 2010), a type of interactions between genes of the study such as physical Interaction if two gene products were discovered to link in protein-protein interaction research, they are related to each other.

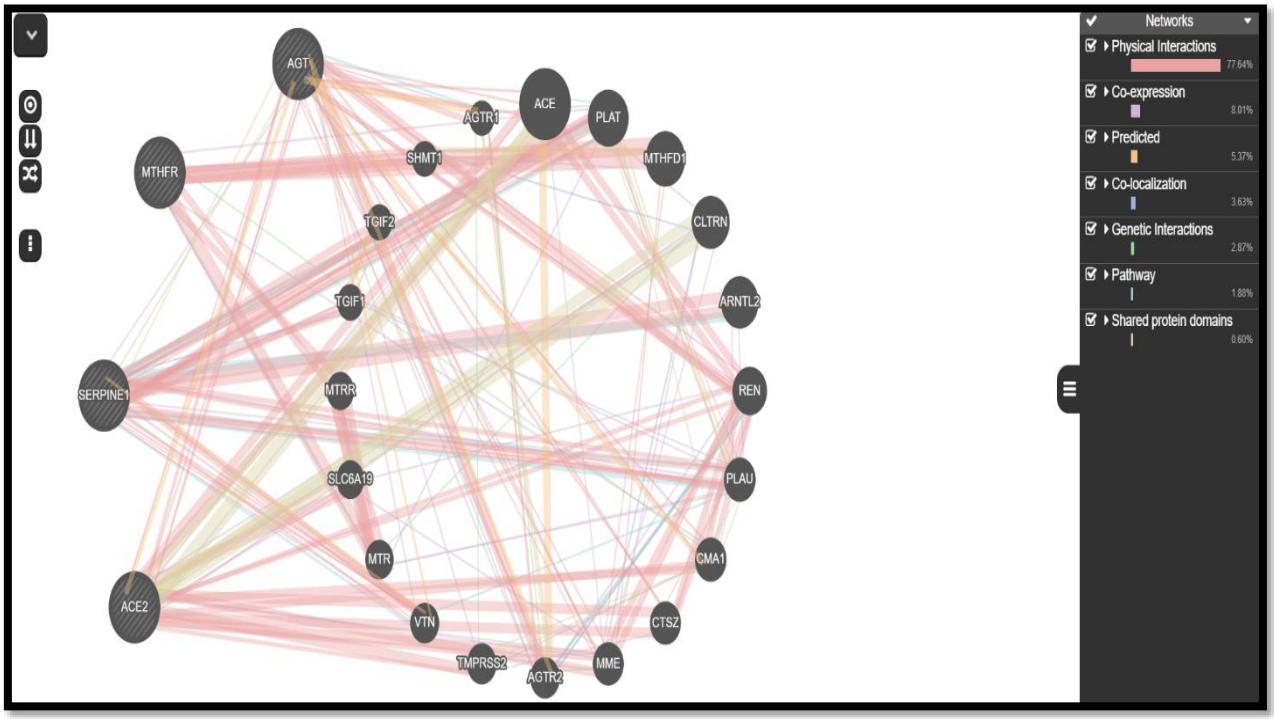


Figure (4-49) Genemaina network predicts function and relationships between the genes included in the study.

The results of the present study in silico showed physical interaction between *AGT* and *ACE2*, in addition to predicted interaction type between both genes which means connections are the mapping of known functional links from another organism via ortholog is an important source of expected data. Further, that co-expression interaction if two genes are related their expression levels are equal across conditions such as co-expression between *AGT* and *SERPINE-1* and both have a shared protein domain which included if two gene products have the same protein domain, they are said to be related.

As for protein interactions, the results of STRING database(Szklarczyk *et al.*, 2015) in the study of genes, there were several connections shown between the products of the study of genes. In addition to stable complexes, metabolic pathways, and a dizzying array of direct and indirect regulatory interactions between proteins, proteins can form a wide range of functional connections with one another.

In human genetics, protein networks can provide a number of benefits, including aiding in drug discovery, filling knowledge gaps about metabolic enzymes, and the prediction of phenotypes and the function of genes, to name a few(Szklarczyk *et al.*, 2010).

The results of this study found interactions on proteins level included knowing, predicted and other interactions. Figure (4-50) clarified that between *AGT* and *ACE2* experimentally determined, gene neighborhood and protein homology in addition to curated databases with regard to other genes there is textmining interactions figure (4-51), one of their comparison of polymorphisms for the genes associated with thrombotic disease or another disease such as cancer for example.

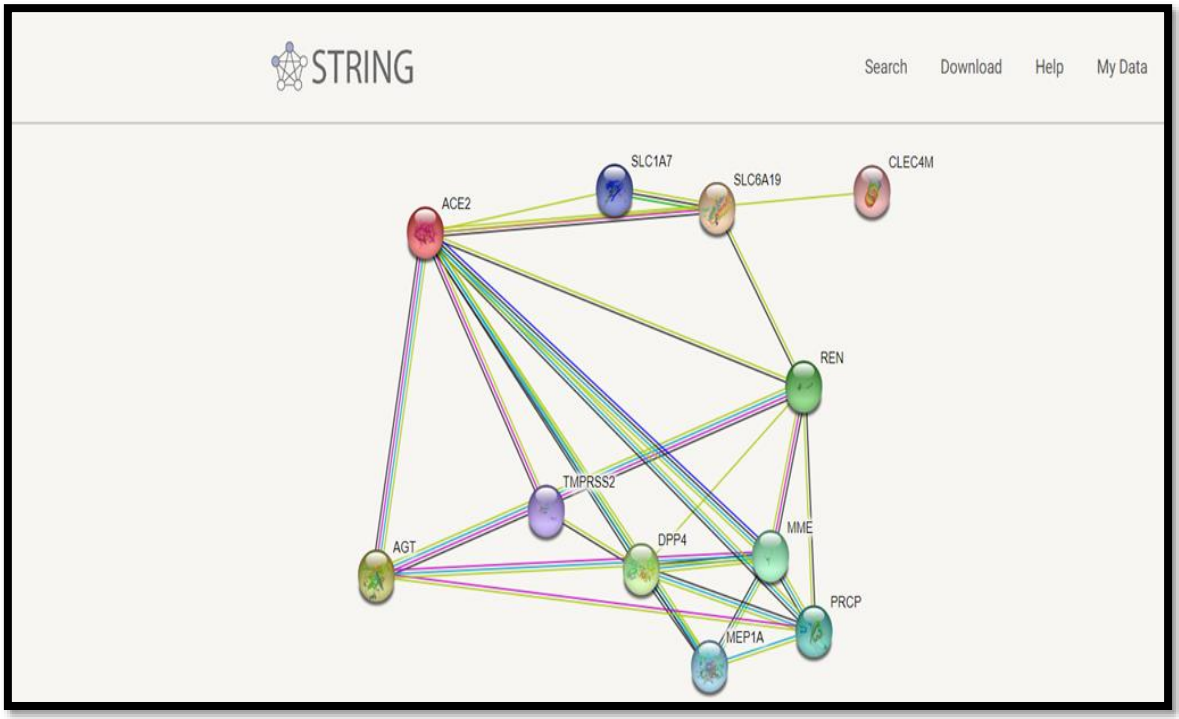


Figure (4-50) The string database to viewed the interaction between the *AGT* and *ACE2* proteins.

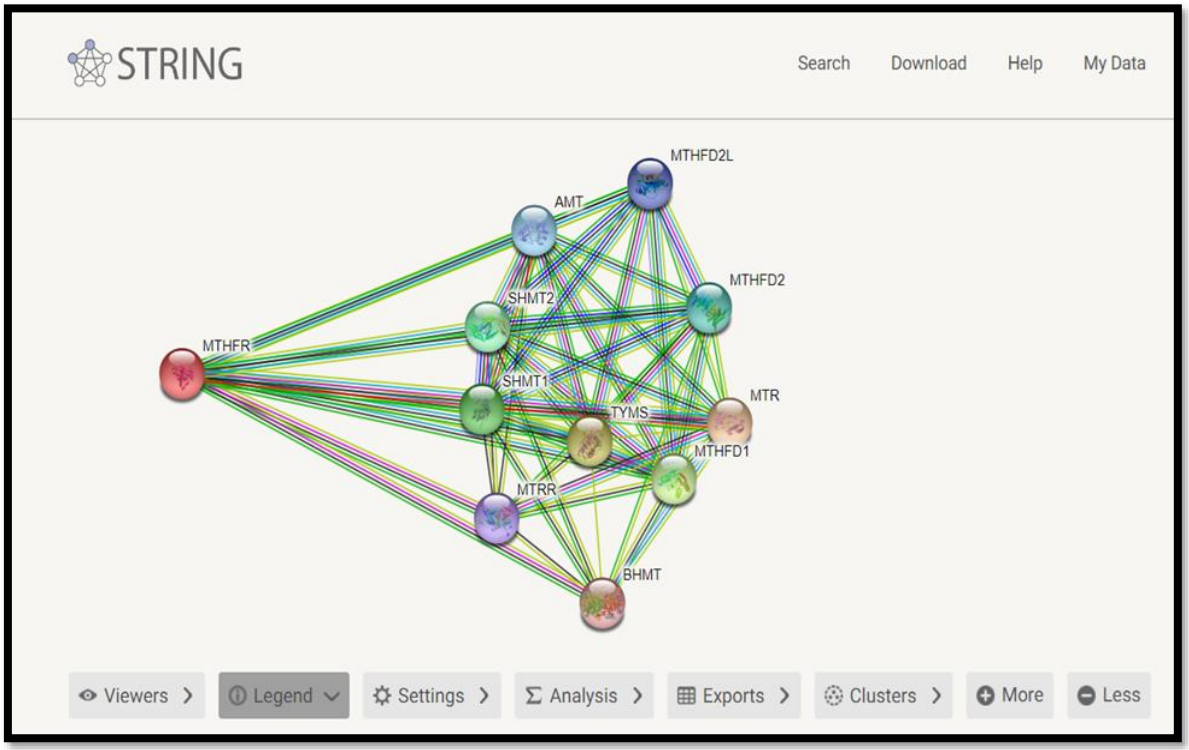


Figure (4-51) The interaction of *MTHFR* gene and related to thrombosis genes network.

In figure (4-52) string relationships and interactions how to observed between four the genes of the present study.

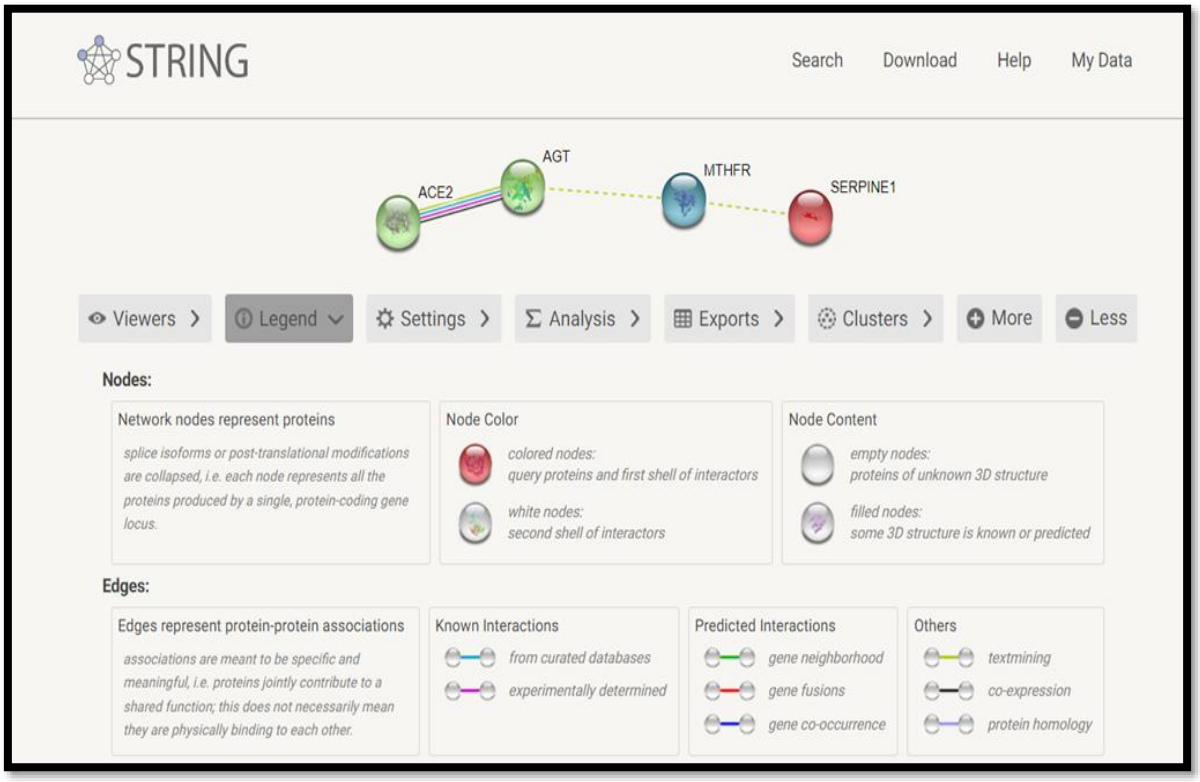


Figure (4-52) The interactions types between of proteins of the study.

4.6.2. Tools Prediction of Polymorphisms

A number of single nucleotide polymorphisms (SNPs) in genes encoding various proteins were discovered in COVID-19 patients. The information was retrieved from the National Biotechnology Information Center's website (using the database dbSNP). The genetic variations relating to COVID-19's pathogenesis and severity can be identified using bioinformatics techniques. The present study subjected four genetic polymorphisms in four genes in Iraqi patients for in vitro detection. Interestingly, these SNPs are worth analyzed by in silico study to further elucidate they’re affected on patients with COVID-19.

Classified the variations in this work as described in the previous, there are two polymorphisms in the exon site, one in the intron site, and finally one upstream site as following table (4-31).

Table (4-31) SNPs details includes gene, rs number, and region of genome.

No.	Gene name	Reference number of SNP	Region of genome	Amino acid change
1	***MTHFR***	rs1801133 T>C	Exon site	A222V
2.	***AGT***	rs699 A>G	Exon site	M268T
3.	***ACE2***	rs2106809 A>G	Intron site	-
4.	***SERPINE1***	rs1799889 4G>5G	Promoter site	-

4.6.2.1. In Silico of Exon SNPs rs1801133 and rs699 SNPs

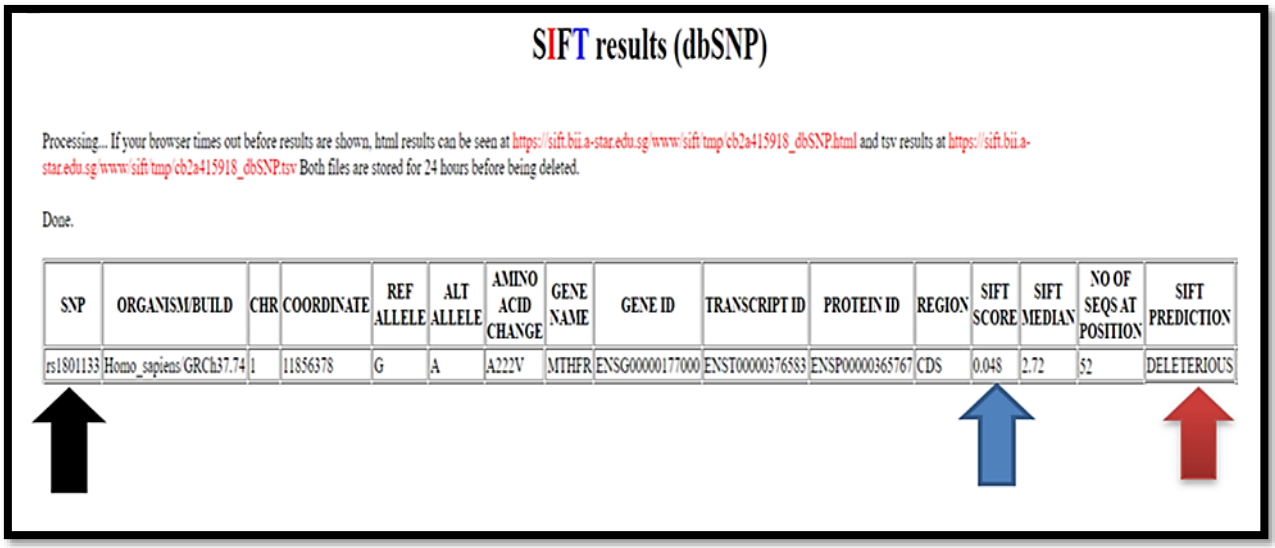
The rs1801133 polymorphism (677C > T DNA label) and alanine to valin (A222V protein label) is located in the *MTHFR* gene (exon 4) and is formed by conversion from cytosine (C) to thymine (T). This SNP viewer all information by dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) as seen in appendix (7).

While the rs699 polymorphism as DNA label T to C substitution in the exon 2 or label protein methionine to threonine at (M268T). Previously, rs699 was positioned to the amino acid 235 varied based on the numbering in today's databases and the SNP is therefore also referred to as M235T(El-Garawani *et al.*, 2021). The SNP can viewer by dbSNP included all information about the rs699 as in appendix (8), the SNP prediction is promising in current genomic studies. Many web servers and software analyzed the polymorphisms in this study as following:

4.6.2.1.1. SIFT Prediction Tool

Using the SIFT algorithm (sorting intolerants from tolerants), the gap between mutations and phenotypic variations is bridged by predicting the likelihood of amino acid substitutions being deleterious and the likelihood of amino acid alterations having a detrimental effect on protein function. This

predicts the potential impact that amino acid substitutions may have on protein functions(Sim *et al.*, 2012). In order to calculate the SIFT scores, a range of 0 to 1 is taken into account, where damage can be predicted if the score is below 0.05, and tolerable if it is above 0.05. The result for rs1801133 was predicted deleterious as shown in the figure (4-53).



SIFT results (dbSNP)

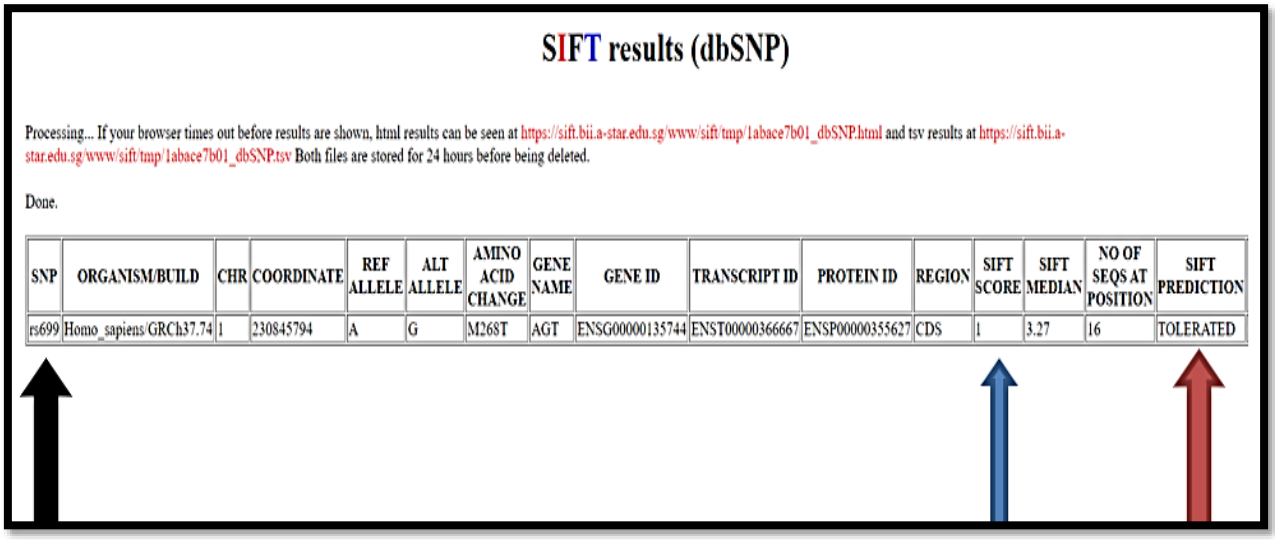
Processing... If your browser times out before results are shown, html results can be seen at https://sift.bii.a-star.edu.sg/www/sift/tmp/cb2a415918_dbSNP.html and tsv results at https://sift.bii.a-star.edu.sg/www/sift/tmp/cb2a415918_dbSNP.tsv Both files are stored for 24 hours before being deleted.

Done.

SNP	ORGANISM/BUILD	CHR	COORDINATE	REF ALLELE	ALT ALLELE	AMINO ACID CHANGE	GENE NAME	GENE ID	TRANSCRIPT ID	PROTEIN ID	REGION	SIFT SCORE	SIFT MEDIAN	NO OF SEQS AT POSITION	SIFT PREDICTION
rs1801133	Homo_sapiens/GRCh37.74	1	11856378	G	A	A222V	MTHFR	ENSG00000177000	ENST00000376583	ENSP00000365767	CDS	0.048	2.72	52	DELETERIOUS

Figure (4-53): The SIFT prediction for the effect of the SNP (rs1801133) *MTHFR* gene.

While the result for rs699 was predicted tolerated with score=1 and this change in amino acid may not involve in protein function or structure for that has been tolerated as in figure (4-54).



SIFT results (dbSNP)

Processing... If your browser times out before results are shown, html results can be seen at https://sift.bii.a-star.edu.sg/www/sift/tmp/1abace7b01_dbSNP.html and tsv results at https://sift.bii.a-star.edu.sg/www/sift/tmp/1abace7b01_dbSNP.tsv Both files are stored for 24 hours before being deleted.

Done.

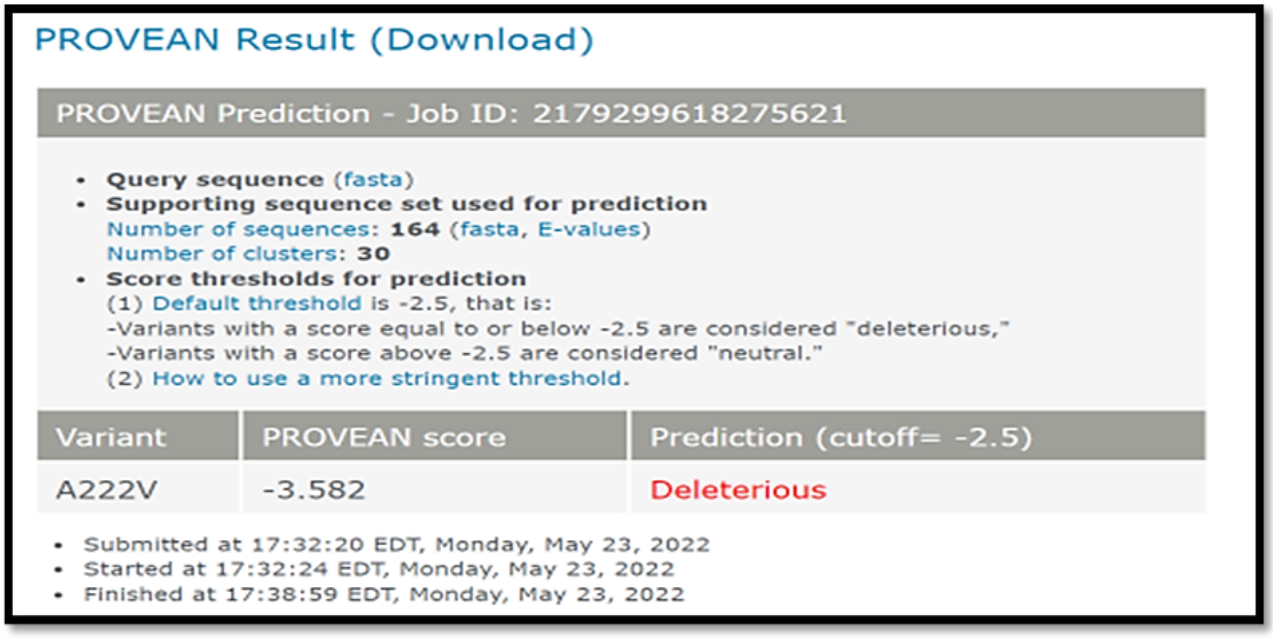
SNP	ORGANISM/BUILD	CHR	COORDINATE	REF ALLELE	ALT ALLELE	AMINO ACID CHANGE	GENE NAME	GENE ID	TRANSCRIPT ID	PROTEIN ID	REGION	SIFT SCORE	SIFT MEDIAN	NO OF SEQS AT POSITION	SIFT PREDICTION
rs699	Homo_sapiens/GRCh37.74	1	230845794	A	G	M268T	AGT	ENSG00000135744	ENST00000366667	ENSP00000355627	CDS	1	3.27	16	TOLERATED

Figure (4-54): The SIFT prediction for the effect of the SNP (rs699) *AGT* gene.

Sequence profiles offer evolutionary information that can be used to determine whether amino acids are suitable with a given of a protein. While certain residues are tolerated, others may cause structural disruption(Altschul *et al.*, 1997). Change to residues (charged or polar) usually predicted to change protein function but change to other neutral amino acid will be predicted tolerated. The protein can tolerate certain amino acid changes at certain positions if these positions are not critical to function or structure(Pauline and Henikoff 2001).

4.6.2.1.2. PROVEAN Tool

A web server that uses PROVEAN to assess the function of proteins when they are mutated was used to predict the effect of mutations on protein function and determine the functional impact of single or multiple amino acid changes, insertions, and deletions. Additionally, the server offers a high-throughput analysis of human and mouse genetic and protein variations at both the genomics and protein levels. Scores less than 2.5 are known to be harmful, while scores greater than 2.5 are considered tolerant. The rs1801133 figure (4-55) was considered deleterious and this linked to disease state in human.


PROVEAN Result (Download)

PROVEAN Prediction - Job ID: 2179299618275621

- **Query sequence** (fasta)
- **Supporting sequence set used for prediction**
  Number of sequences: **164** (fasta, E-values)
  Number of clusters: **30**
- **Score thresholds for prediction**
  (1) Default threshold is -2.5, that is:
  -Variants with a score equal to or below -2.5 are considered "deleterious,"
  -Variants with a score above -2.5 are considered "neutral."
  (2) How to use a more stringent threshold.

Variant	PROVEAN score	Prediction (cutoff= -2.5)
A222V	-3.582	Deleterious

- Submitted at 17:32:20 EDT, Monday, May 23, 2022
- Started at 17:32:24 EDT, Monday, May 23, 2022
- Finished at 17:38:59 EDT, Monday, May 23, 2022

Figure (4-55): The PROVEAN prediction for the effect of the SNP rs1801133 *MTHFR* gene.

While in rs699 analyzed by provean tool figure (4-56) predicted to be neutral with provean score above -2.5 and this may be to change in protein structure but Sandell and Sharp (2022) noted that while the standing frequency of a detrimental allele is determined by its fitness effect as much as it is by the rate at which it is created by mutations, which is dependent not only on the fitness effect.

There is a possibility that alleles categorized as neutral by PROVEAN due to their high frequency could actually have modestly detrimental effects, while the underlying mutation rate might be high. However, the extent of these adverse effects is unclear.

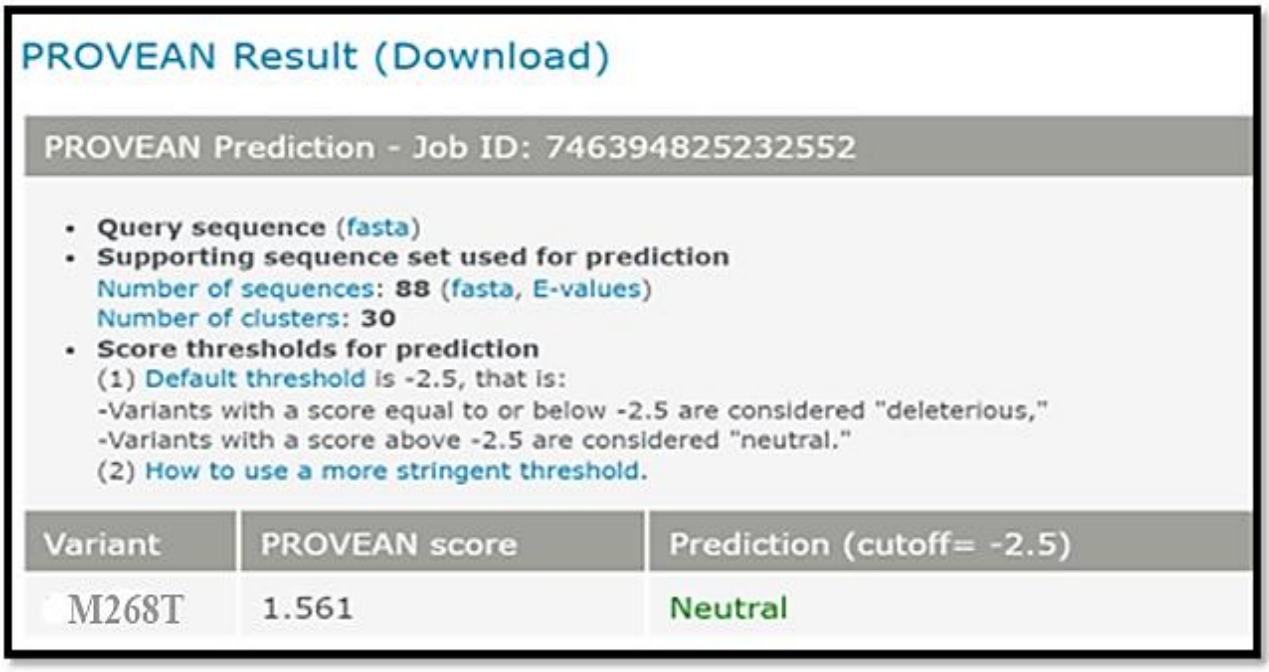


Figure (4-56): The PROVEAN prediction for the effect of the SNP rs699 *AGT* gene.

The interaction between amino acids is useful for predicting secondary and tertiary structures of proteins as well as predicting their folding and stability. when calculate the likelihood of each amino acid residue being the target of a mutation using methionine as an example, should have to take into account the mutational distance as well as the physicochemical similarity between the two residues.

4.6.2.1.3. PolyPhen-2 Software

The polymorphism phenotyping program, known as PolyPhen-2 (Polymorphism Phenotyping v2) predicts how amino acid variations affect human protein structure and function through software and a web server, Polyphen2 predicts based on factors like as sequencing, phylogeny, and structural information(Adzhubei *et al.*, 2010). In this case, a score of 0.9 is considered a potentially harmful mutation. The rs1801133 in the *MTHFR* gene was considered damaging in figure (4-57).

Figure (4-57): Prediction possible impact of the rs1801133 *MTHFR* gene using PolyPhen-2.

In addition, using the PDB database for all proteins and 3D visualization the substitution of A222V in the protein structure of *MTHFR* appendix (9). Based on results of polyphen-2 server size differences between wild-type and mutant amino acids in A222V. There is a larger residue in the mutant compared to the wild-type residue. In the core of the protein, the wild-type residue was hidden. Due to its size, the mutant residue is unlikely to fit. Mutated residues are located within domains that are crucial in contact with other domains and

necessary for protein activity. The mutation may have an effect on the function of the protein by altering the interaction between these domains.

As to rs699 in *AGT* analyzed by polyphen-2 tool the mutation is predicted benign in figure (4-58).

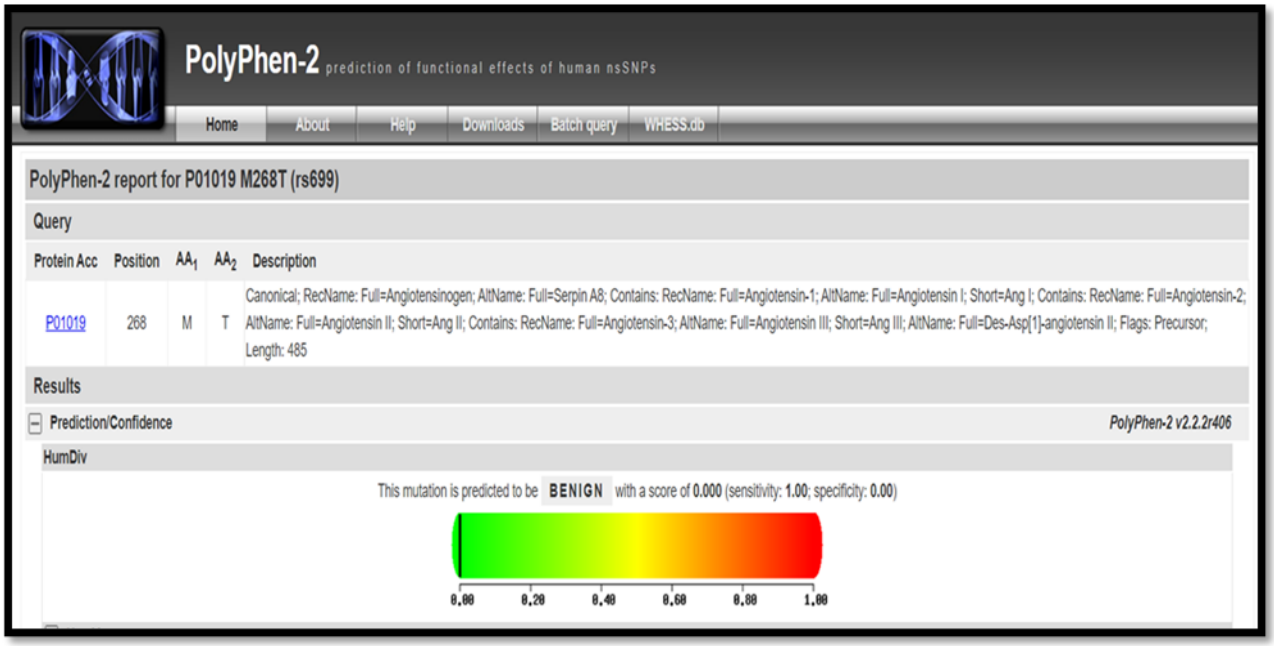


Figure (4-58): Prediction possible impact of the rs699 SNP *AGT* gene using PolyPhen-2.

As for using the PDB file to viewed the protein with mutation M268T for appendix (10), The protein is colored grey based on results from polyphenol-2 server, a wild-type residues are colored green and mutant residues are colored red. An amino acid's size varies between wild-type and mutant. A wild-type residues are larger than mutant residues. It is possible that external interactions will be lost as a result. Wild-type residues are more hydrophobic than mutant residues. Hydrophobic interactions between the protein surface and other molecules might be lost due to the mutation.

4.6.2.1.4. I-Mutant2.0 Tool

This web server is used for automatically predicting the stability of proteins when they are mutated at a single site. Depending on the structure or,

more importantly, the sequence of the protein, I-Mutant2.0 predicts the protein structure(Capriotti *et al.*, 2005). Training and testing were conducted using ProTherm, which is currently the largest resource of thermodynamic experimental data on changes in protein stability resulting from mutations. Basically, the input code for determining G values is the same by selecting two labels, one indicates increased protein stability (ΔΔG > 0, label is +), whereas the other indicates a destabilizing mutation (ΔΔG< 0, label is −) cause causes decreased stability. A single point mutation's effect on protein stability is predicted using Delta G (DDG). This metric is an indicator of a change in Gibbs free energy, otherwise known as the DDG (double digit change). The rs1801133 A222V in the present work showed by I-Mutant2.0 decreased protein stability and the ΔΔG less than 0 as seen in figure (4-59) by using a sequence file of protein. Depend on the structure of protein by used PDB file to check if the protein decreased same in used sequence, and the result figure (4-60) below the stability of protein decreased.

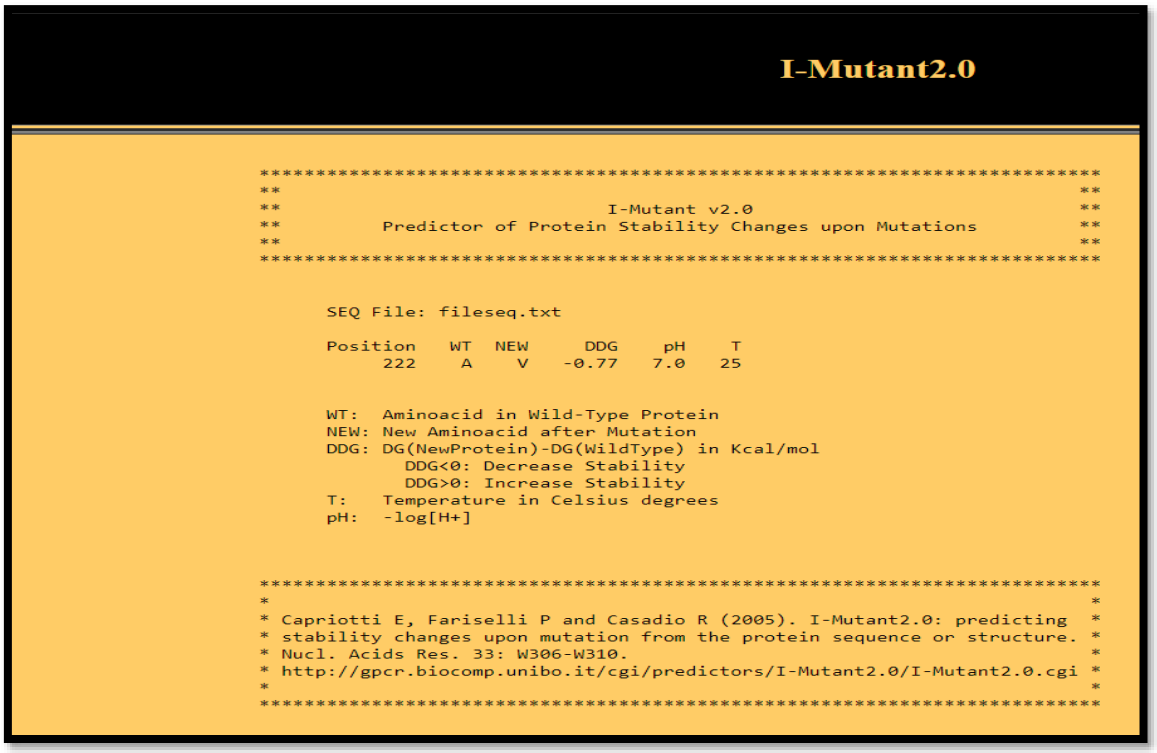


Figure (4-59): The predicted protein stability by ΔΔG value of *MTHFR* gene as affected by the rs1801133 SNP using I-mutant2.0.

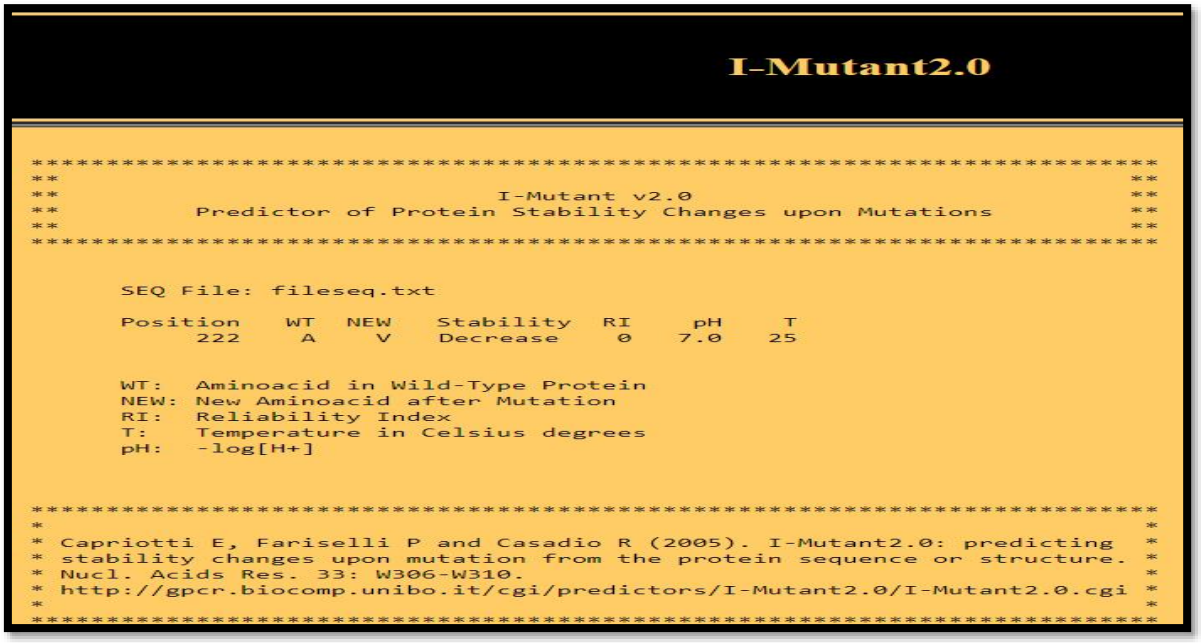


Figure (4-60): The predicted protein stability of *MTHFR* gene as affected by the rs1801133 SNP using I-mutant2.0.

As for rs699 in present study led to decrease of stability of protein because methionine changed to threonine affected on protein stability figure (4-61).

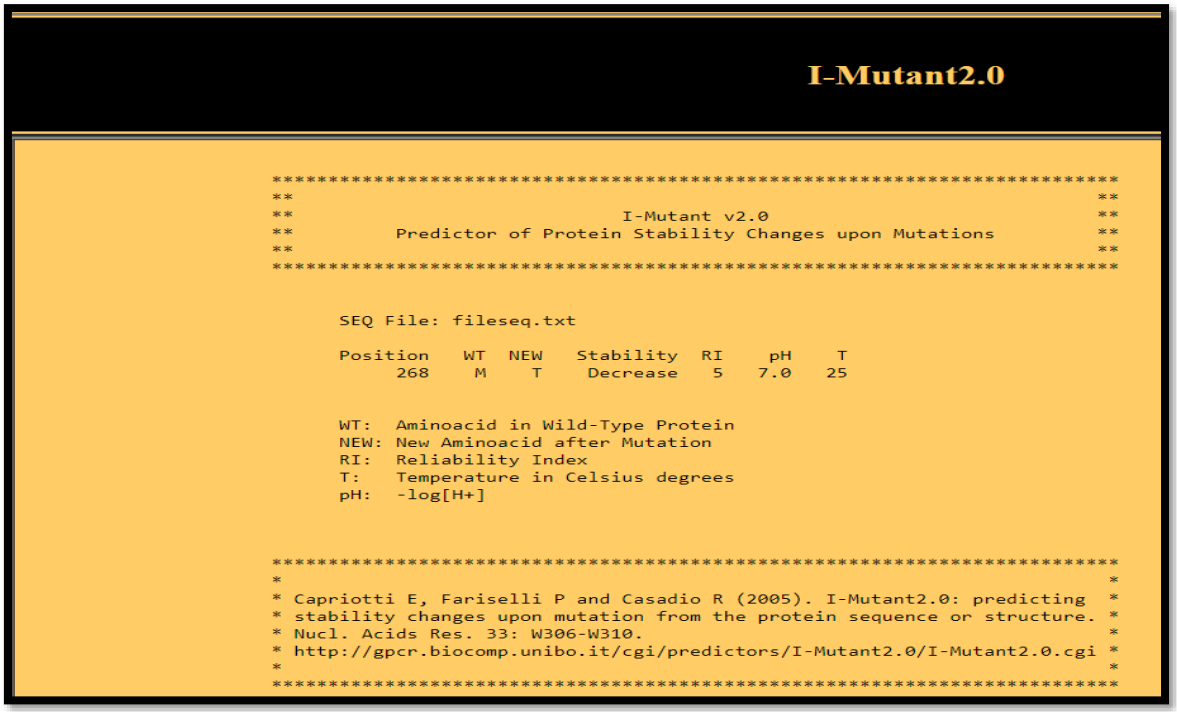


Figure (4-61): The predicted protein stability of *AGT* gene as affected by the rs699 SNP using I-mutant2.0.

While in figure (4-62) showed rely on the DDG less than 0 equal to (-1.48) the result was decreased stability of protein.

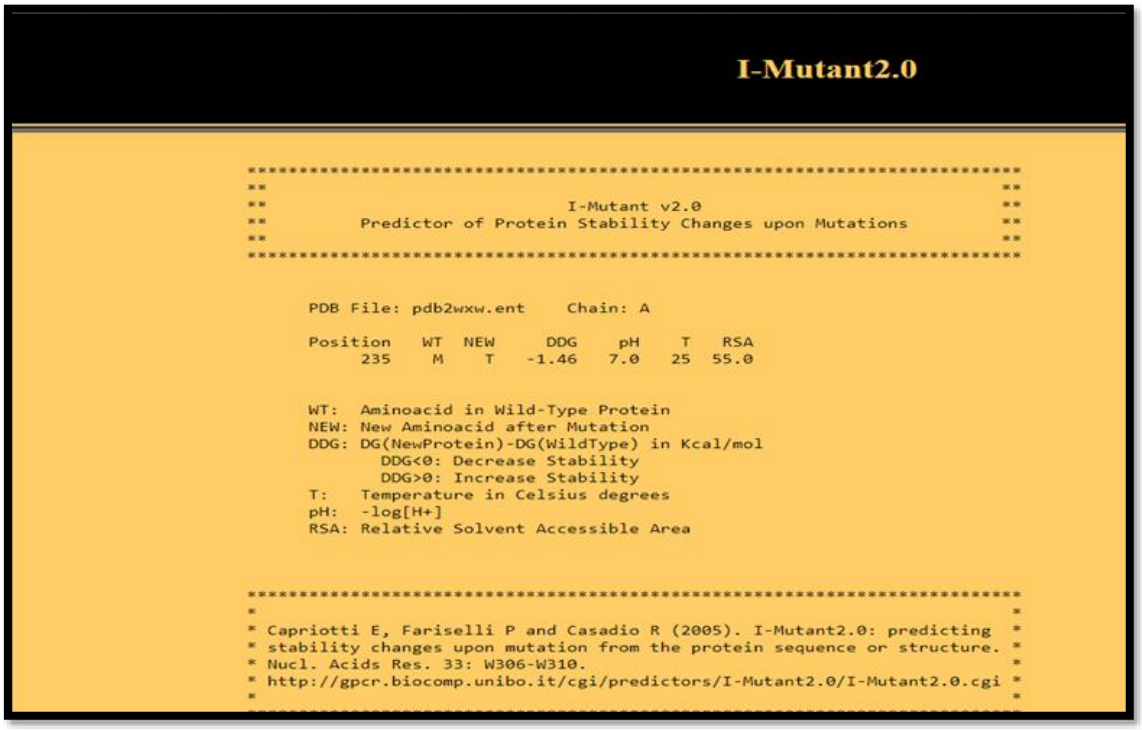


Figure (4-62): The predicted protein stability by ΔΔG value of *AGT* gene as affected by the rs699 SNP using I-mutant2.0.

Many changes must be explained by the cumulative effects of several factors, including (1) A change in the dihedral angles of the main chains and rotomers on the side chains, (2) close interactions between some atoms, and (3) adverse electrostatic interactions between the Asp side chains and main chain carbonyls(Pokkuluri *et al.*, 2002).

4.6.2.1.5. MUpro Tool

A set of machine learning programs for predicting how single amino acid mutations affect protein stability is prediction of protein stability changes for single-site mutations from sequences. Two machine learning methods were developed: support vector machines and neural networks. The two methods were tested using a large mutation dataset and showed an accuracy above 84% via 20 fold cross validation(Cheng *et al.*, 2006). According to the results of polymorphisms by the MUpro tool, *AGT* rs699 (p.M268T) and *MTHFR*

rs1801133 (p.A222V) both decrease the stability of protein when changed to mutated amino acids as depicted in figures (4-63 and 4-64).

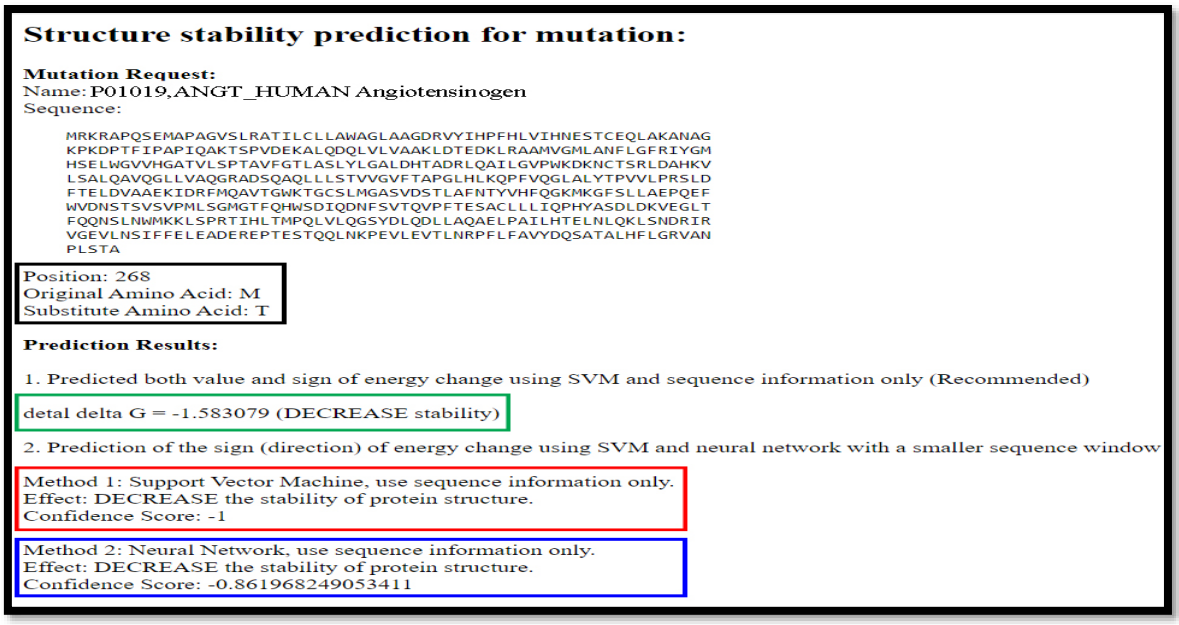


Figure (4-63) The effect of the rs699 SNP on the protein stability was determined through energy change predictions that utilized both support vector machine (SVM) and neural network algorithms using the MUpro tool.

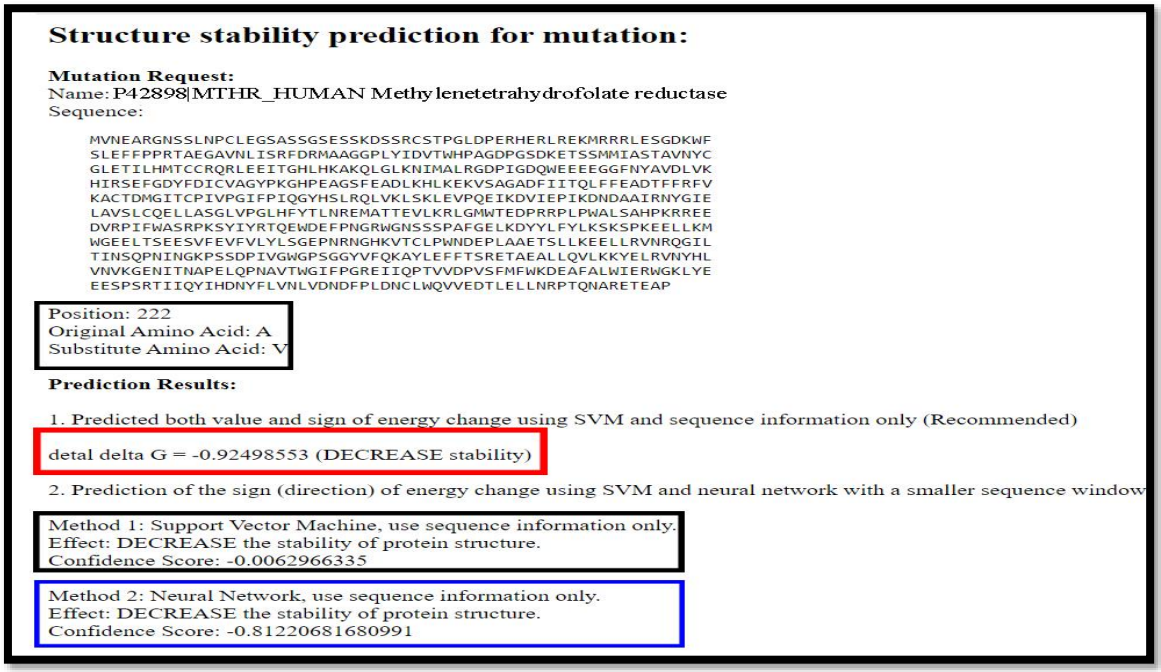


Figure (4-64) The MUpro tool was used to evaluate the impact of the rs1801133 SNP on the predicted protein stability of *MTHFR* through energy change predictions utilizing both SVM and neural network algorithms.

4.6.2.1.6. MutPred2 Tool

The MutPred2 is a tool for classifying pathogenic and benign amino acid changes in humans. It also predicts their effect on over 50 different protein properties, allowing for the inference of pathogenic molecular pathways. It is a machine learning-based method that includes packaged software combined to predict probabilities about the pathogenicity of amino acid alterations. It incorporates genetic and molecular data(Pejaver *et al*. 2017).

The general score to which protein pathogen is between (0.5-1) or not damaging (0.0-0.5), the protein score with change A222V in rs1801133 is (0.892) score that leads to pathogenic and most of the properties of protein after changed p-value was significant as in figure (4-65). Whereas to M268T Angiotensinogen mutation was score is predicted less than 0.5 that equal to (0.028) was considered benign as in figure (4-66) with no result for protein properties after changing it.

It is difficult to identify pathogenic mutations and underlying functional changes. To that aim, there is MutPred2, a tool that enhances the prioritizing of pathogenic amino acid changes over previous approaches, develops molecular processes that may be disease-causing, and produces interpretable pathogenicity score ranges on individual genomes. Providing a probabilistic model for predicting the influence of variants on specific structural and functional elements of proteins, MutPred2 facilitates the study of variations that alter phenotypes(Pejaver *et al.*, 2020).

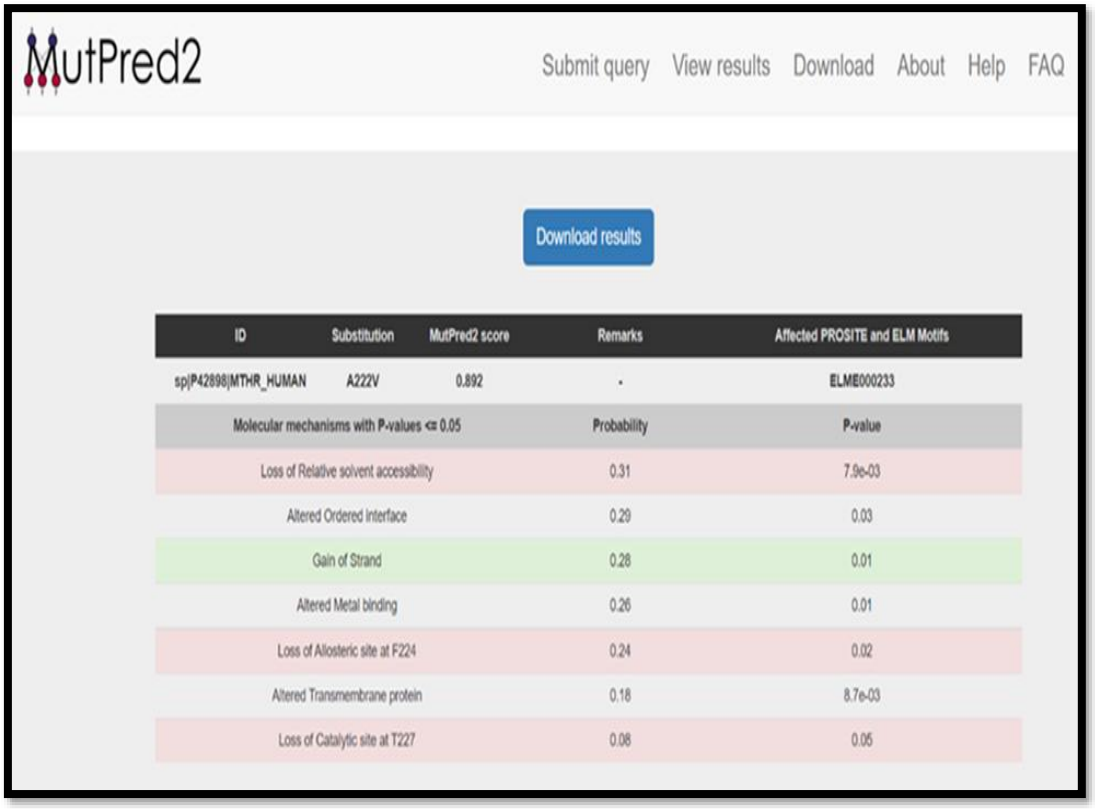


Figure (4-65): The predicted protein probabilities about the pathogenicity of amino acid of *MTHFR* gene as affected by the rs1801133 SNP using MutPred2.

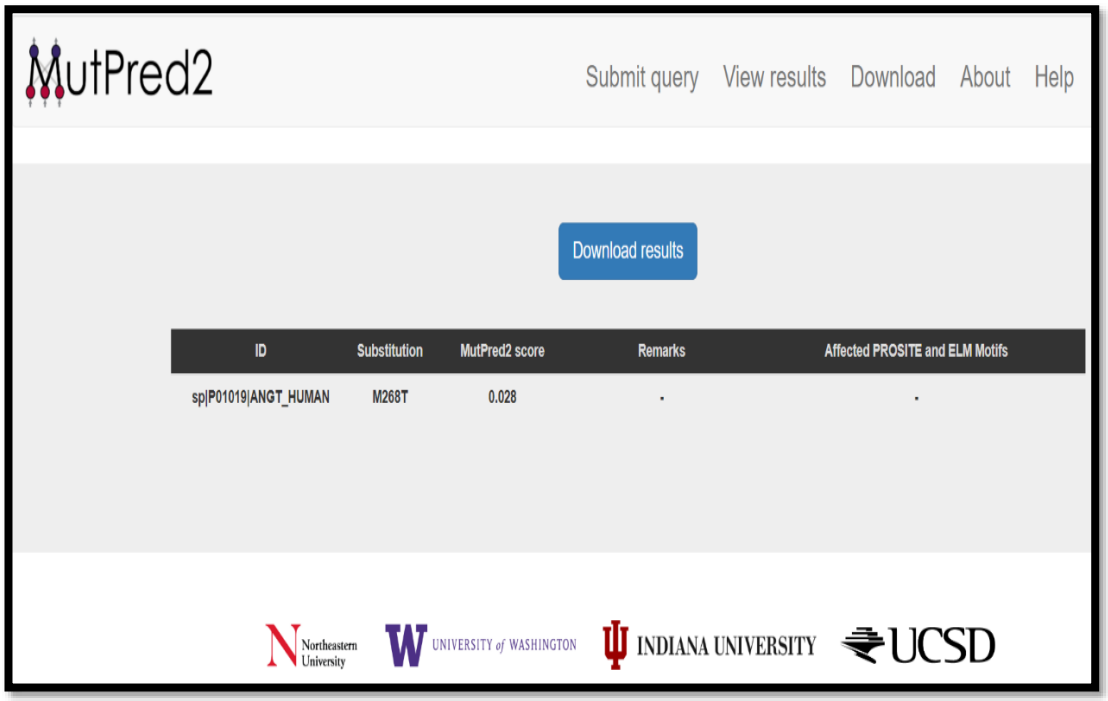


Figure (4-66): The predicted protein probabilities about the pathogenicity of amino acid of *AGT* gene as affected by the rs699 SNP using MutPred2.

4.6.2.1.7. TMHMM Tool

In TMHMM (Transmembrane Prediction Using Hidden Markov Models), hidden Markov models are used to predict transmembrane helices. Using a FASTA formatted protein sequence, it predicts the intracellular, extracellular, and transmembrane domains(Krogh *et al.*, 2001).

Given the very modest number of high-resolution structures for membrane proteins (about 0.5 % of the PDB), predicting transmembrane (TM) helices is critical in the study of membrane proteins. To find out the area of the inside, transmembrane, and outside depend on the changed between the wild and mutant. The rs699 as in figure (4-67) showed there are little changed where in present wild amino acid more transmembrane part and increase outside with mutant on the contrary as shown in figure (4-68), and this may be led to decreased of stability as mentioned previously by Mutant.2 tool.

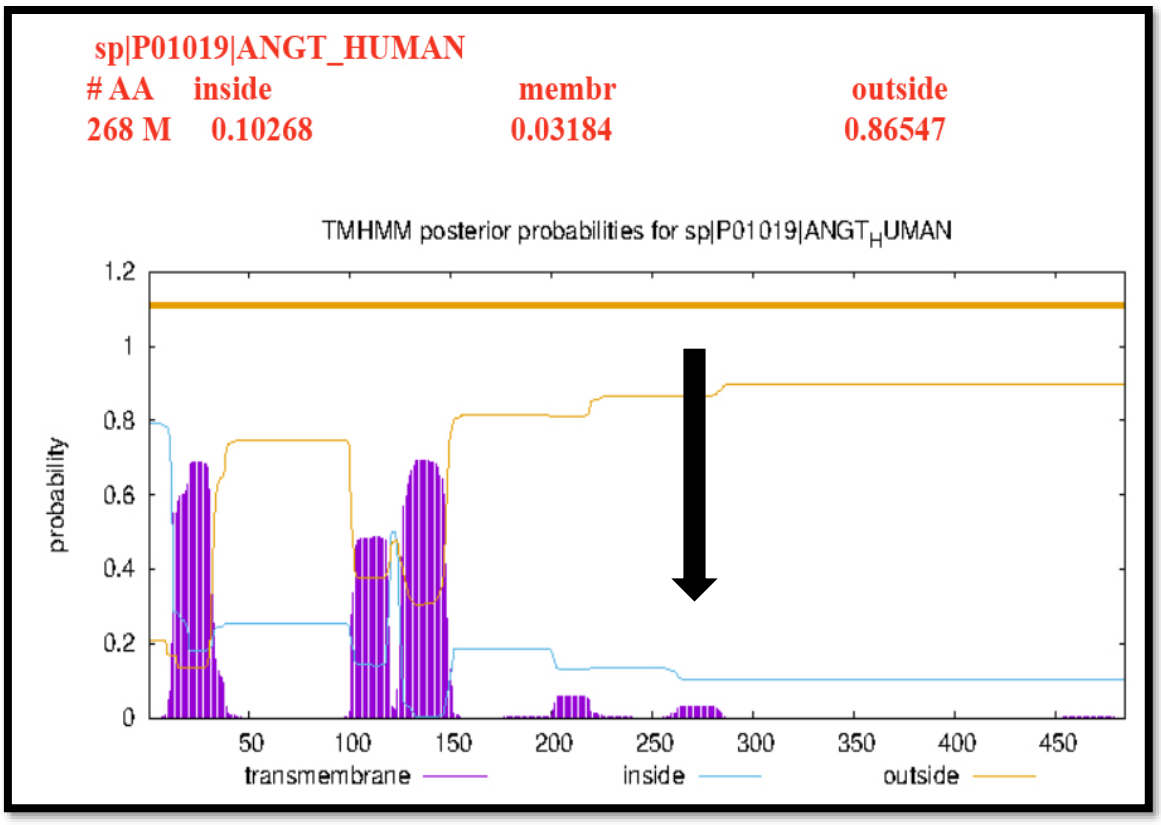


Figure (4-67): The predicted protein transmembrane wild amino acid of *AGT* gene as affected by the rs699 SNP using TMHMM.

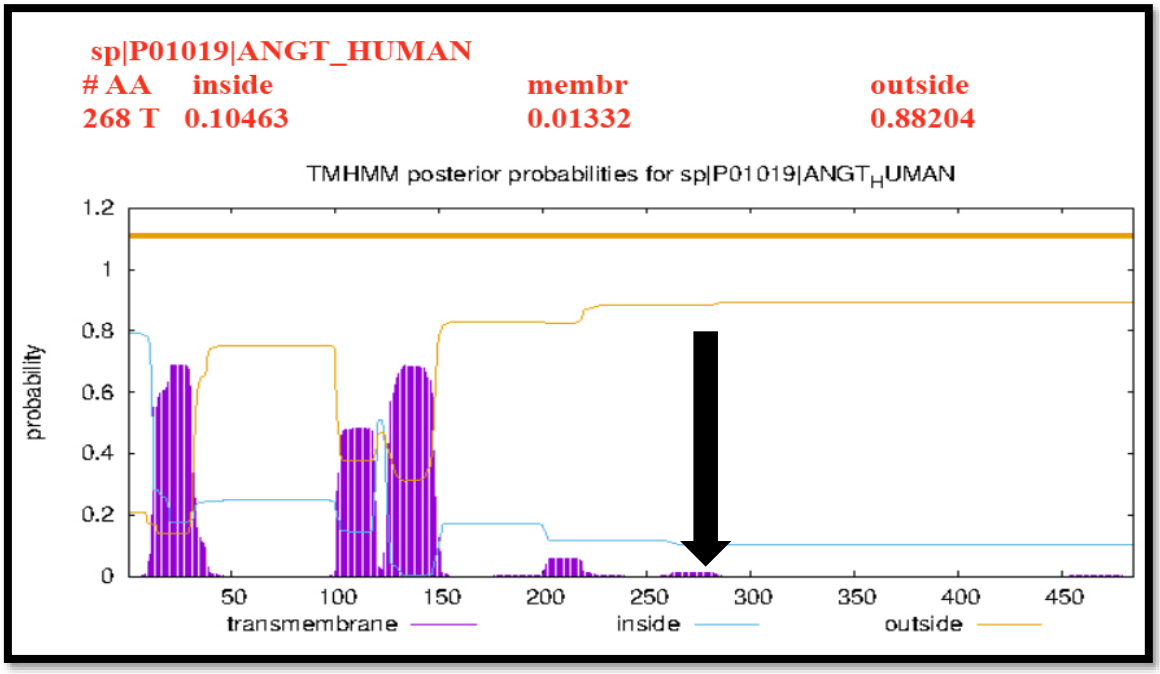


Figure (4-68): The predicted protein transmembrane with mutant amino acid of *AGT* gene as affected by the rs699 SNP using TMHMM.

As for the rs1801133 most of the membrane of outside for that effected on the changed the outside of the protein figure (4-69) with wild amino acid, while figure (4-70) in present of the mutant increased probability to external.

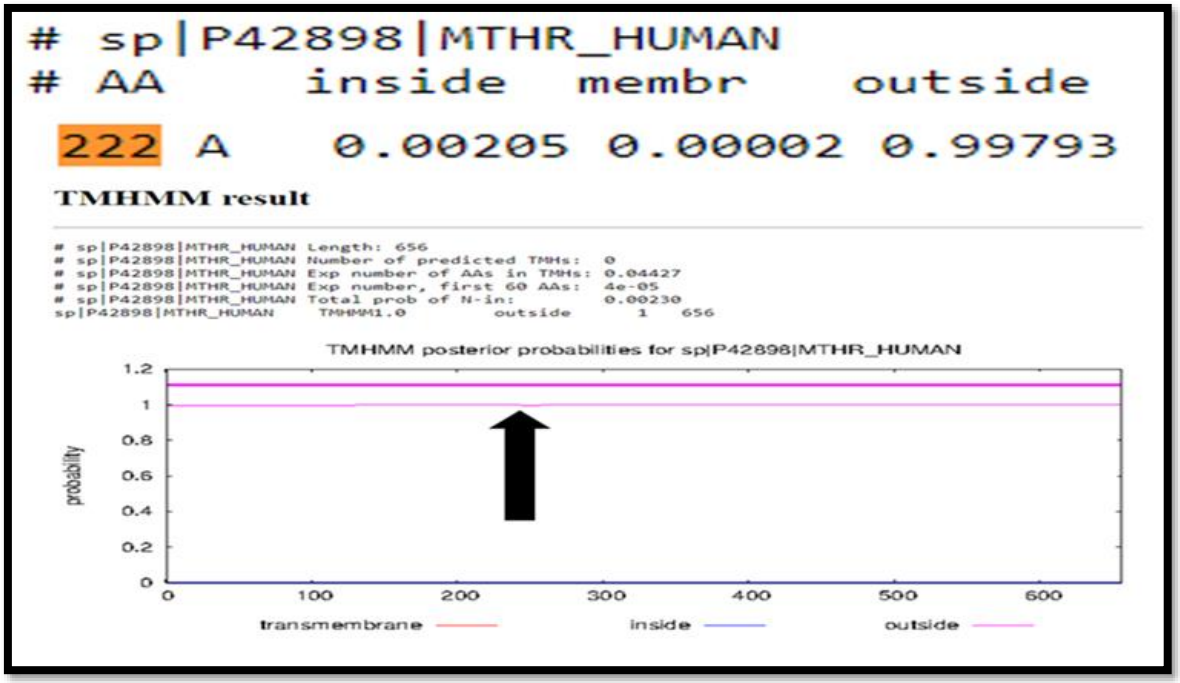


Figure (4-69): The predicted protein transmembrane with wild amino acid of *MTHFR* gene as affected by the rs1801133 SNP using TMHMM.

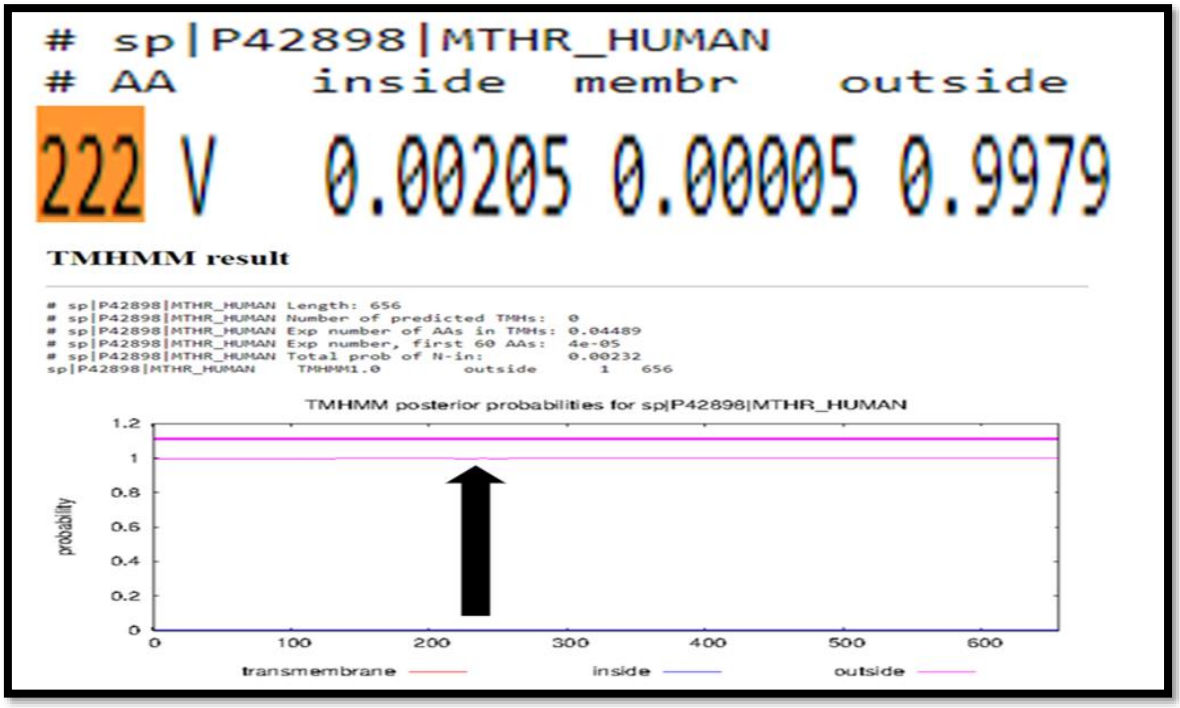


Figure (4-70): The predicted protein transmembrane with mutant amino acid of *MTHFR* gene as affected by the rs1801133 SNP using TMHMM.

4.6.2.1.8. Grand average of hydropathicity

(GRAVY) is a metric used to quantify the hydrophobicity of a peptide, computed as the sum of hydropathy values for its amino acids divided by the peptide's length. Positive GRAVY values signify hydrophobicity, while negative values denote hydrophilicity. These physicochemical values can be directly acquired from the ExPASy website (http://www.expasy.org/).

For the rs1801133 variant where the wild-type amino acid is Alanine:

- Aliphatic index: 81.01
- Grand average of hydropathicity (GRAVY): -0.414

However, when the wild-type Methionine is changed to the mutant Valine:

- Aliphatic index: 80.72
- Grand average of hydropathicity (GRAVY): -0.418

This change results in hydrophilic properties.

For the rs699 variant where the wild-type amino acid is Methionine:

Aliphatic index: 99.77

Grand average of hydropathicity (GRAVY): 0.065

However, when the wild-type Methionine is changed to the mutant Threonine:

Aliphatic index: 99.77

Grand average of hydropathicity (GRAVY): 0.059

This changed in hydrophobic properties.

4.6.2.1.9. PhD-SNP Tool

The PhD-SNP is web server designed to determine single point mutations in proteins are either diseases-related or neutral polymorphisms(Capriotti and Fariselli, 2017). In this work, the Uniprot protein sequence was entered into the computer after the mutation site and the new amino acid residue were specified. The technique uses a threshold of 0.5 as the point at which the nsSNVs are expected to be disease-associated. In changed ALA to VAL at 222 site PhD-SNP tool reported this mutation as deleterious in study and related to disease as figure (4-71).

As for rs699 in *AGT* gene and because not bigger effect on the angiotensinogen protein consequently, the algorithm analyzed by PhD-SNP was neutral figure (4-72). The PhD-SNP has multiple modes that strike concluded that different between runtime and performance. Both sequence and evolutionary characteristics should be taken into account when choosing the most precise mode. In addition to the living systems have many interaction networks. As a result, changed in the protein may affected on the other protein in same network on the protein of study as mentioned in the types of interactions.

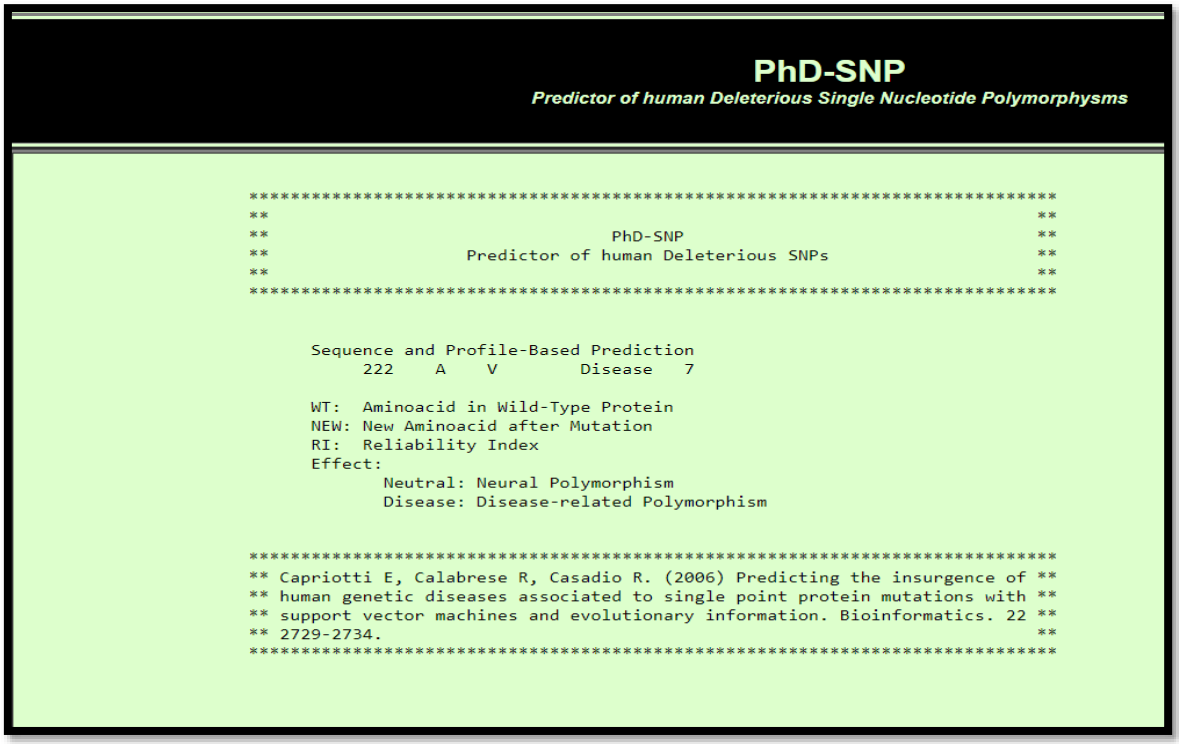


Figure (4-71): The predicted protein stability of *MTHFR* gene as affected by the rs1801133 SNP of gene using PhD-SNP.

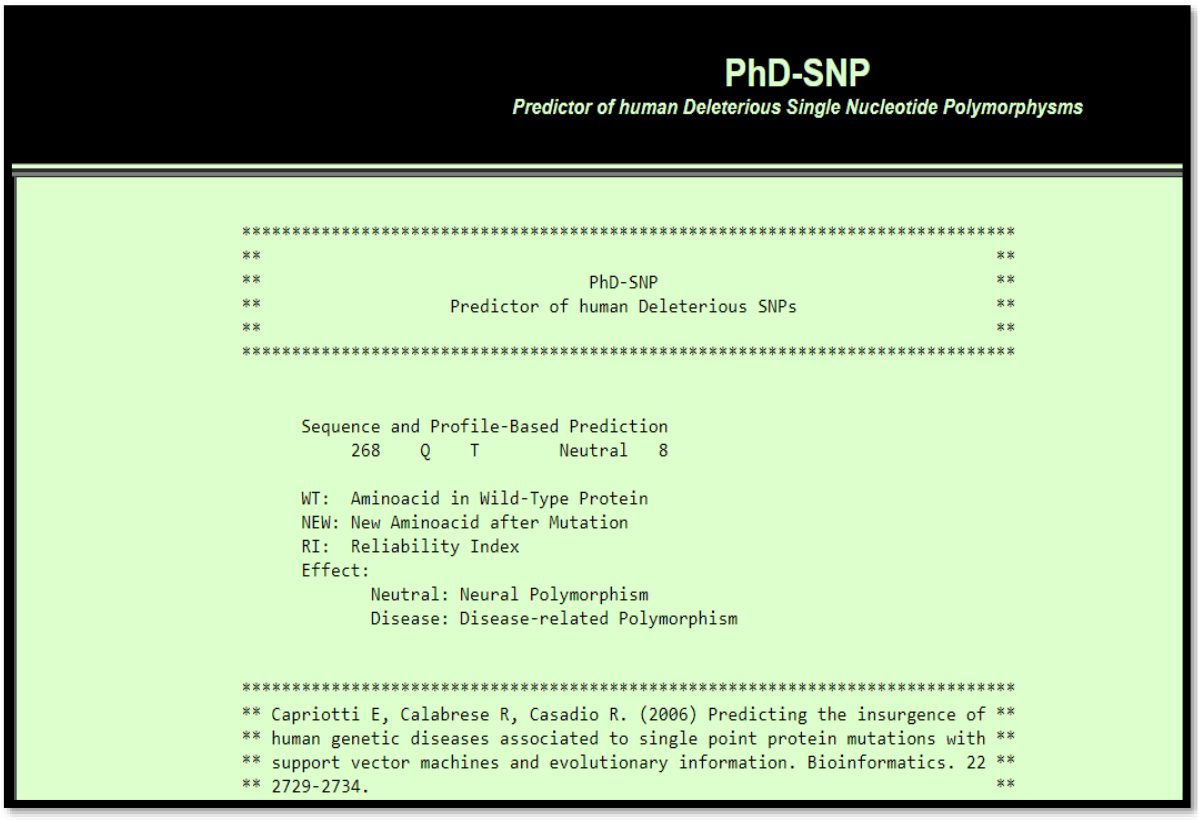


Figure (4-72): The predicted protein stability of *AGT* gene as affected by the rs699 SNP of gene using PhD-SNP.

4.6.2.2. In Silico of Intron Polymorphisms rs2106809 and rs1799889

The polymorphisms of rs2106809 T>C substitution located in intron of *ACE2* gene as in appendix (11). Introns can also include functional polymorphisms that affect the expression of the genes that contain them, intron-specific SNPs may enhance illness risk and modulate the genotype-phenotype association. The impact of intronic SNPs on splicing and transcription factor binding was investigated using some of tools. As for the rs1799889 4G/5G in promoter region polymorphism of *SERPINE1* gene as seen in appendix (12). A promoter is a sequence of DNA that acts upstream or at the 5′ end of a transcription start site, regulating the transcription of genes. Researchers found that promoters affect RNA splicing and polyadenylation in addition to transcription. Among multiple-promoter genes, about 91.9% of human and 87.5% of mouse genes were alternatively spliced genes(Kolathur, 2021).

4.6.2.2.1. SpliceAid 2 Tool

The SpliceAid 2 is a database that contains information on the expression of human splicing factors as well as RNA target motifs. SpliceAid 2 can help predict splicing pattern changes, lead the discovery of molecular effects caused by mutations, and explain tissue-specific alternative splicing. It 2 is a tool that helps researchers figure out which proteins can bind to a given RNA sequence and so induce RNA maturation in certain biological contexts.

The results of rs2106809 showed changed the proteins that bind to wild and mutant variant as in figure (4-73), with A base there is only SRp30c protein bind to the site A base with score (2), SRp30c's function in CE9 activity, an interaction between recombinant SRp30c and CE9 can suppress splicing in vitro by CE9-dependent mechanisms(Simard and Chabot, 2002). Despite substituting sites of ASF/SF2 binding with high affinity for intronic SRp30c binding sites, ASF/SF2, the most closely related homologue of SRp30c, does not bind to CE9 and does not inhibit splicing. Accordingly, SRp30c may

repress the use of the 3' splice site in hnRNP A1, suggesting SRp30c-CE9 interactions may regulate alternative splicing of HnRNP A1(Dai *et al.*, 2012).While with replaced to G base as shown in figure (4-74) there were different proteins involved hnRNP (H1, H2, H3 and F) with score (-5, -5, -2, and-2) sequentially. Nuclear ribonucleoproteins (hnRNPs) are RNA-binding proteins (RBPs) that play a variety of roles in nucleic acid metabolism, including alternative splicing, stabilizing mRNA, controlling transcription and translation, and regulating gene expression. While many hnRNPs have similar characteristics, their domain content and functional aspects vary(Geuens *et al.*, 2016).

Regrading to the results of rs1799889 with 4G contain this site involved hnRNP (F, H1, H2, and H3) and score (-4, -5, -5, and -5) respectively as in the figure (4-75), while in figure (4-76), in addition to hnRNP (F, H1, H2, and H3) and score (-4, -6, -6, and -6) respectively. A mutant type -675 5G creation of new ones splicing site (GGGGGG) and this led to new proteins RNA-binding motif protein 5 (RBM5) at score (-10) participated in new site dependent on results of spliceaid2 tools. It has been reported in the previous that controlling RBM5 expression alters the transcript levels of many genes that regulate cell growth and apoptosis(Mourtada-Maarabouni *et al.*, 2006).

In heterogeneous nuclear RNA, HnRNPs form complexes with RNA-binding proteins. These proteins, which are connected with pre-mRNAs in the nucleus, govern several processes in organisms on the cell and molecular levels, including alternative splicing, polyadenylation, and other aspects of mRNA metabolism and transport (Glisovic *et al.*, 2008). In terms of function, hnRNP F/H has been found to be involved in regulating telomerase activity and telomere length (Xu C. *et al*. 2020). RNA-binding protein 5 (RMP5) is part of the spliceosome a complex. Manages alternative splicing of several mRNAs.

When the U1 and U2 snRNPs are recruited to the 5' and 3' splice sites of the intron, they may modulate splice site pairing(Dvinge, 2018).

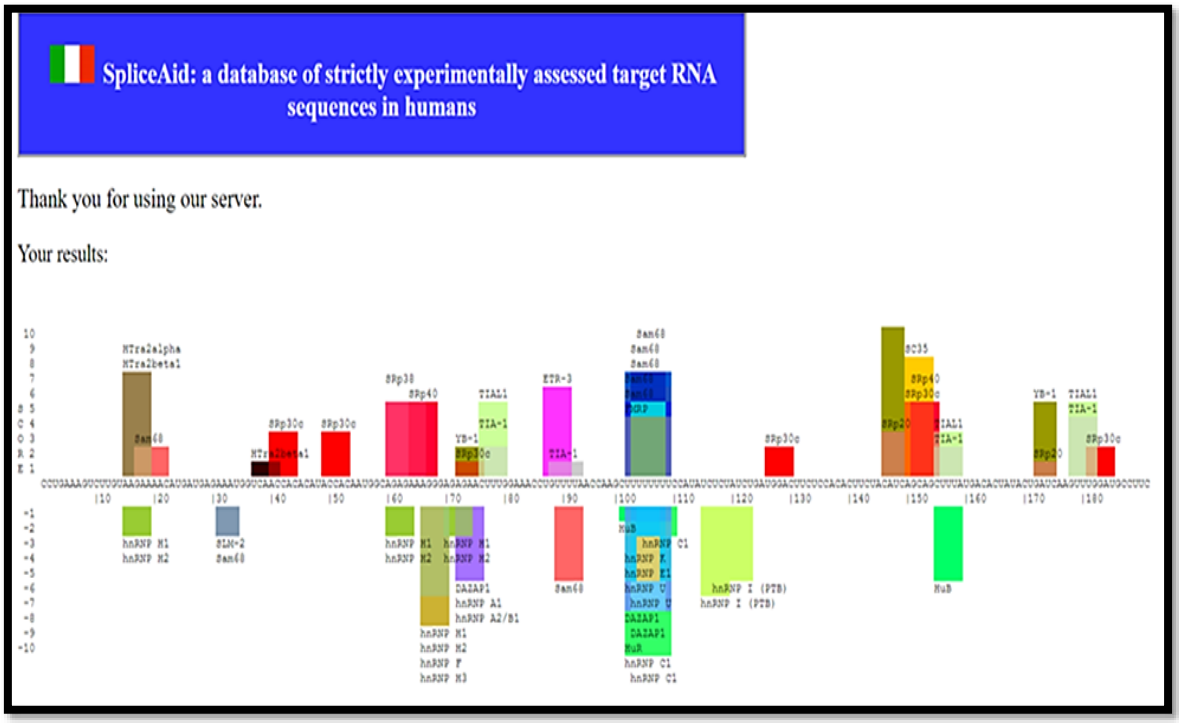


Figure (4-73): The predicted splicing factor of *ACE2* gene as changed by wild base the rs2106809 SNP of gene using SpliceAid.

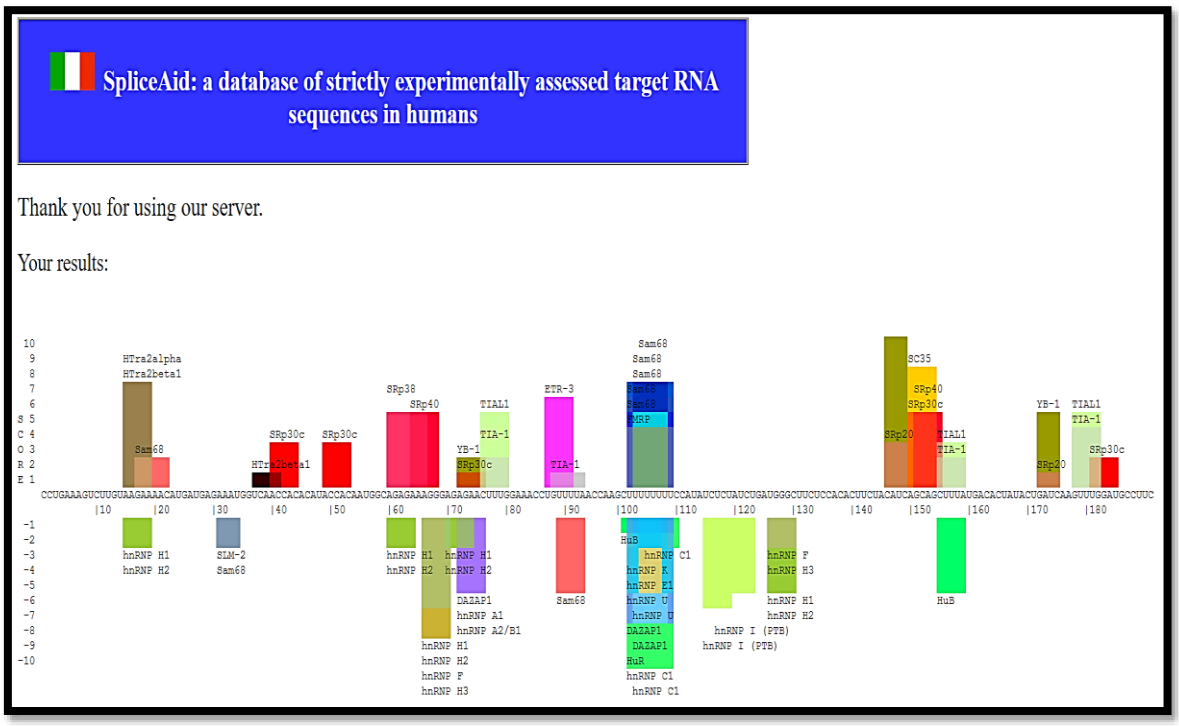


Figure (4-74): The predicted splicing factor of *ACE2* gene as changed by mutant base the rs2106809 SNP of gene using SpliceAid.

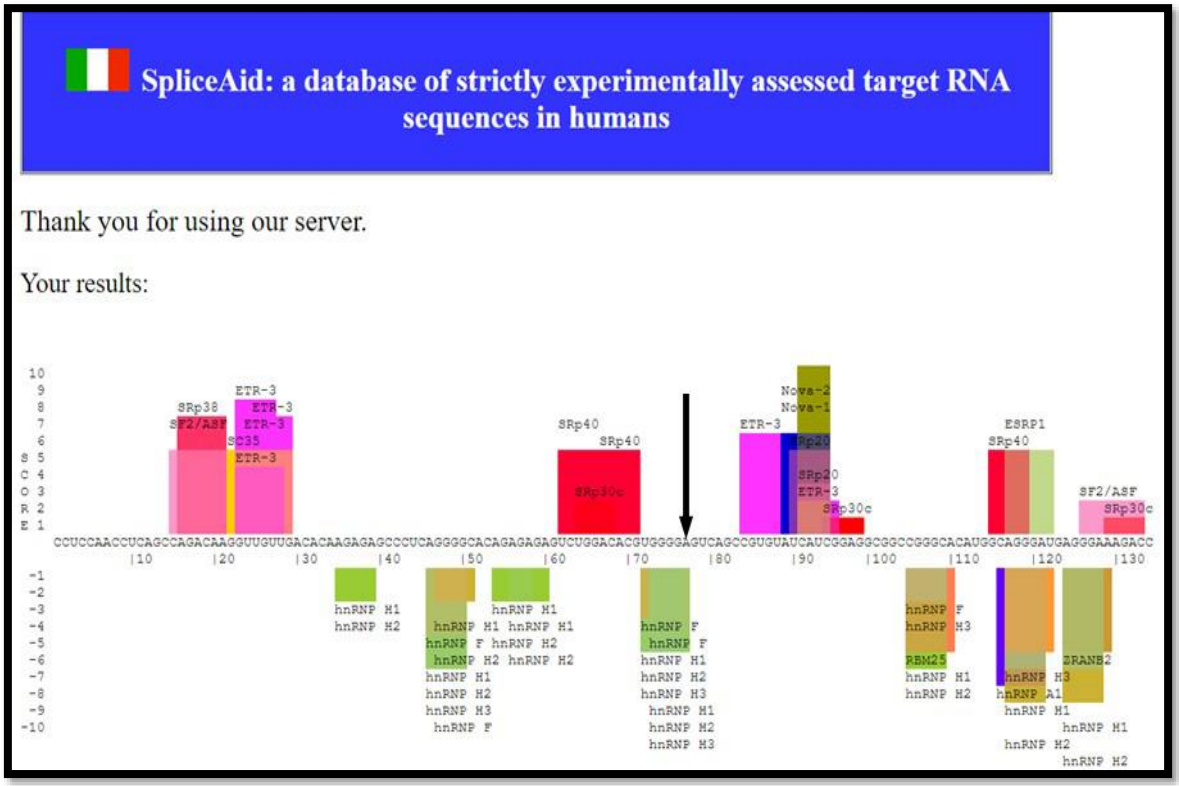


Figure (4-75): The predicted splicing factor of *SERPINE1* gene as changed by wild the rs1799889 SNP of gene using SpliceAid.

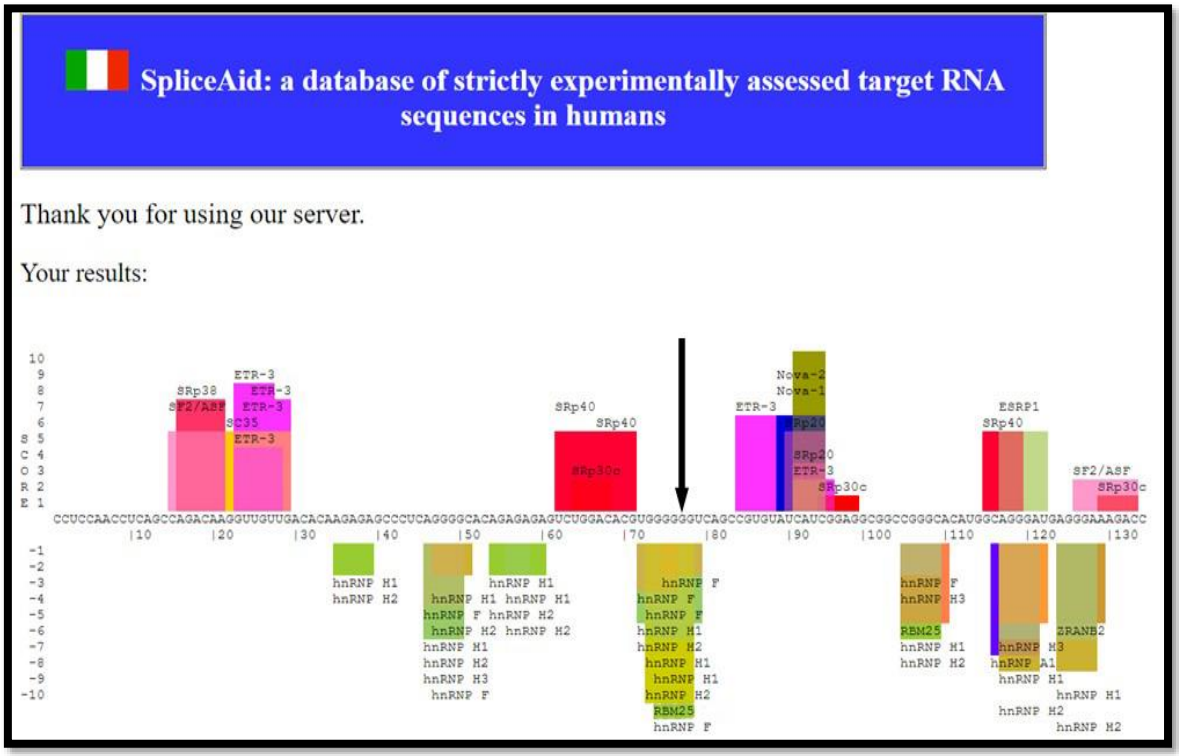


Figure (4-76): The predicted splicing factor of *SERPINE1* gene as changed by mutant the rs1799889 SNP of gene using SpliceAid.

4.6.2.2.2. HaploReg v4.1 Tool

The HaploReg tool explores haplotype blocks for candidate regulatory SNPs at disease-associated loci, as well as annotations of the noncoding genome. Based on SNPs and small indels linked to linkage disequilibrium (LD) data from Phase 1 of the 1000 Genomes Project, there are four ancestral superpopulations available (AFR, AMR, ASN and EUR) and chromatin state and protein binding annotations from ENCODE and Roadmap Epigenomics can be visualized(Abecasis, G. R., *et al* 2012).

A comprehensive repository of bioinformatics information, available online at (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php). An analysis of the polymorphism rs2106809 by HaploReg v4.1 software revealed a number of details about that mutation, such as its location, its reference allele and its alternative allele, 1000 Genomes Phase 1 frequencies in Africans of 0.10, American of 0.34, Asian of 0.52, and European of 0.24. The dbSNP functional annotations include motifs altered, promoters, DNAse and enhancers of histones in figure (4-77).

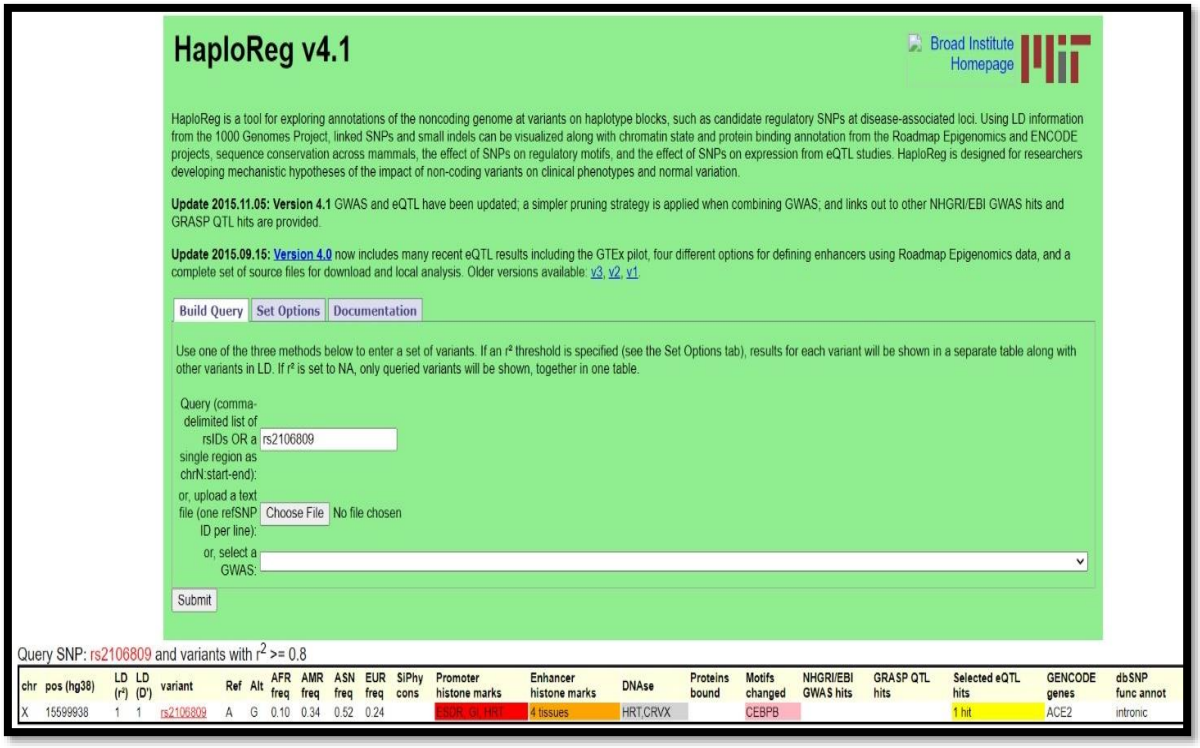
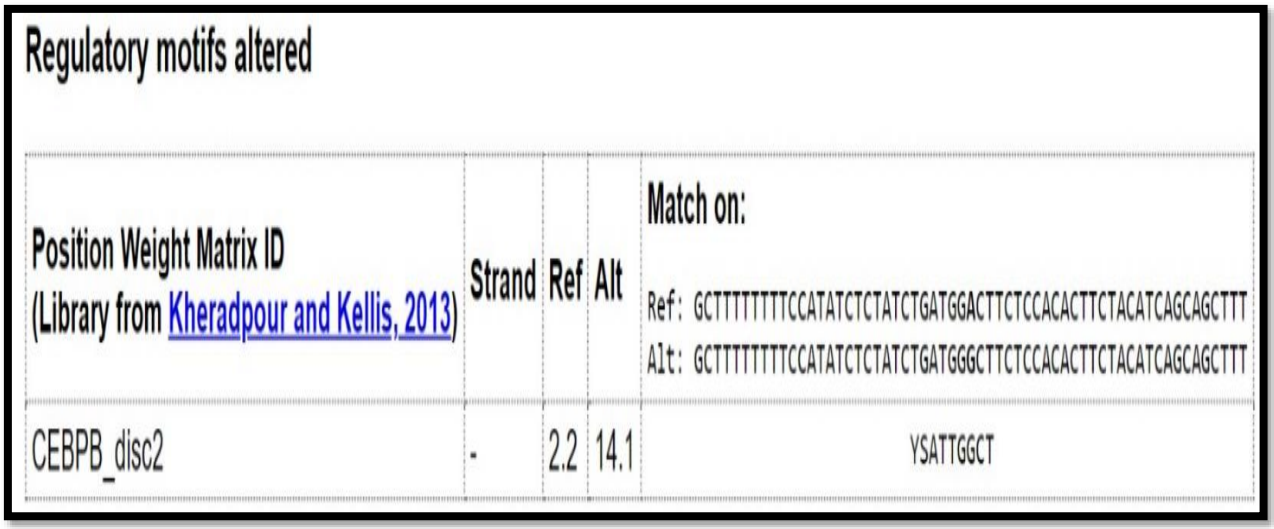


Figure (4-77): The polymorphism rs2106809 details are represented in the HaploReg v4.1 software.

According to HaploReg v4.1 software, a change on the regulatory motif CCAAT Enhancer Binding Protein Beta (CEBPB_disc2) was predicted. This intronless gene encodes a transcription factor with a basic leucine zipper domain (bZIP). According to study by Ruminy (2001), this transcription factor plays an important role in regulating the expression of genes implicated in immune responses as well as inflammation. A study based on current findings, it was found that the risk allele (C) results in the generation of a second CCAAT/enhancer binding protein beta motif (CEBPB) and leads to an increase in this motif, this is due to a polymorphism in the *ACE2* gene (rs2106809) as in figure 4-78. According to the new study, CEBPB is strongly expressed in lung tissue(Consortium, 2020). This lead may be to important effects of the *ACE2* rs2106809 on developing COVID-19.



Regulatory motifs altered

Position Weight Matrix ID (Library from Kheradpour and Kellis, 2013)	Strand	Ref	Alt	Match on:
				Ref: GCTTTTTTTTCCATATCTCTATCTGATGGACTTCTCCACACTTCTACATCAGCAGCTTT Alt: GCTTTTTTTTCCATATCTCTATCTGATGGGCTTCTCCACACTTCTACATCAGCAGCTTT
CEBPB_disc2	-	2.2	14.1	YSATTGGCT

Figure (4-78): In HaploReg v4.1, one regulatory motif was predicted as altered.

Based on the results of the rs1799889 polymorphism, the variant overlaps with enhancers located on 17 bound proteins, such as GR, USF1, POL2, E2F6, RXRA and so on. There is also an association between the variant rs1799889 and several of the markers that indicate DNase I hypersensitivity and histone modification as in figures (4-79 and 4-80).

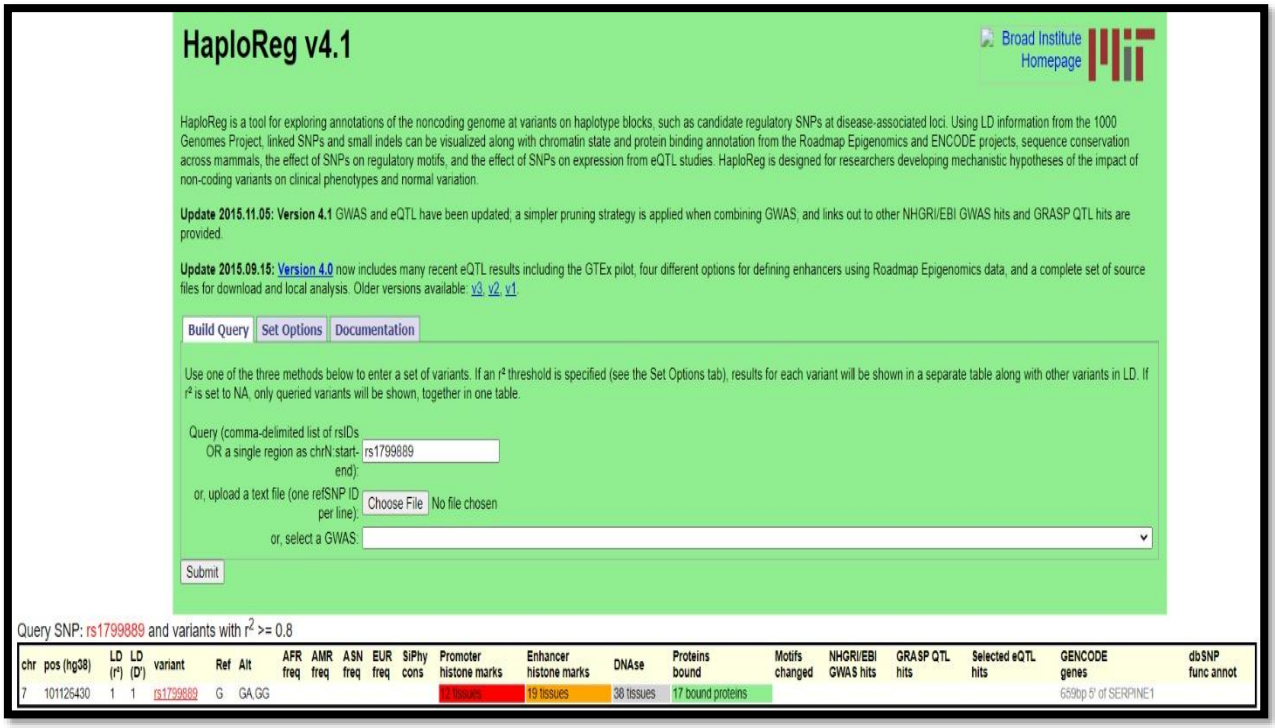


Figure (4-79): A representation of the polymorphism rs1799889 details in the HaploReg software v4.1.

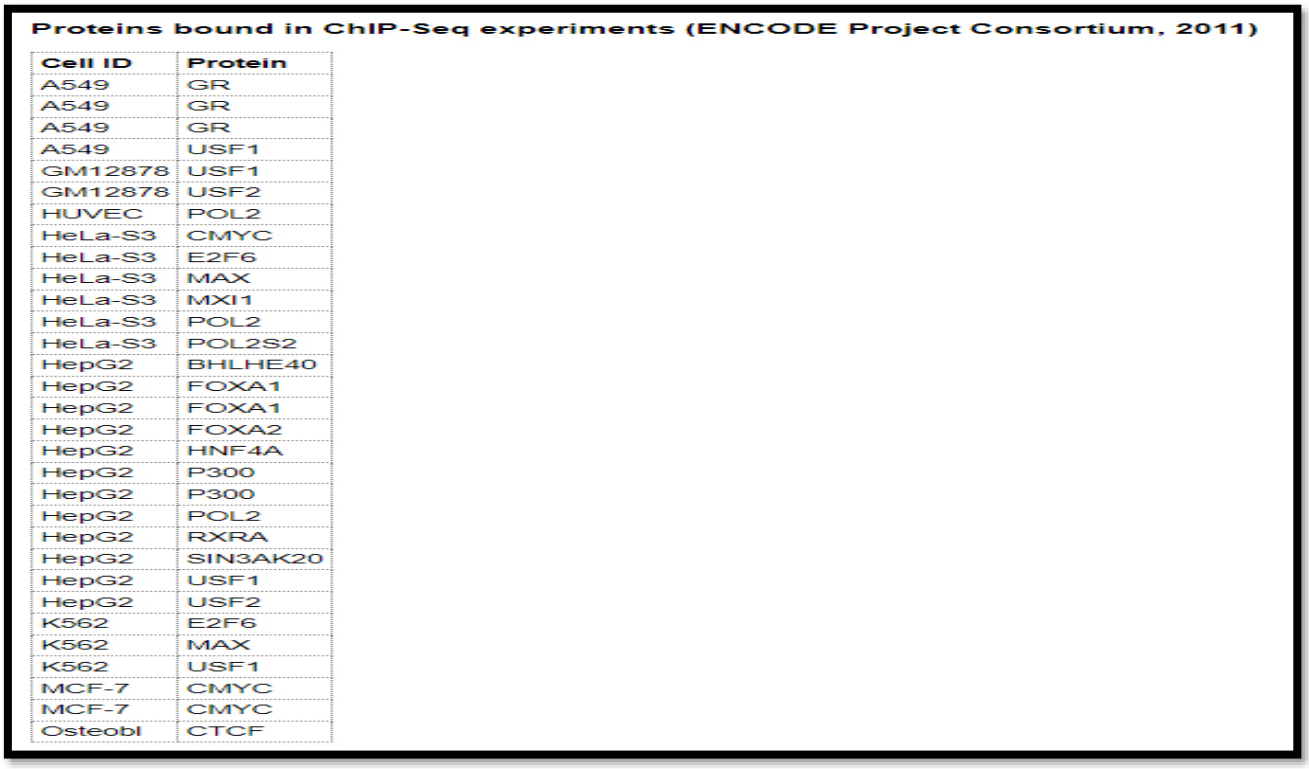


Figure (4-80) Protein binding is detected in the rs1799889 polymorphism using HaploReg software.

Conclusions and Recommendations

Conclusions

Based on the findings and data from the present study and data, the following conclusions can be drawn:

1. The severity and the progression of COVID-19 infection depend on the age was an independent risk factor of patients; moreover, gender may play a role as the disease was more severe in male patients because ACE2 on X-chromosome was a single copy. Chronic conditions like hypertension, cardiovascular disease, and diabetes increase COVID-19 severity and mortality risk. Vaccines can reduce severity and potentially alter the course of the pandemic.
2. Elevated white blood cells, neutrophils, NLR, PLR, and D-dimer along with decreased lymphocytes have clinical significance for predicting disease severity and outcomes in severe patients.
3. The MTHFR gene polymorphism (rs1801133, C677T) suggests that CT and TT genotypes, along with the T allele, are associated with a higher risk and increased severity of COVID-19 infections. Serum homocysteine levels were notably elevated in individuals with TC and TT genotypes compared to those with CC genotypes.
4. The variant (rs699) of the *AGT* gene; CT genotype may contribute to COVID-19 infection, but no genotype and alleles contribute to infection severity. Serum levels of angiotensinogen were not significantly different among carriers of the TT, TC, and CC genotypes.
5. The genotypic distribution of (rs1799889, 4G/5G) of *SERPINE-1* gene, indicated that the genotype 4G/5G was a risk factor for infection and disease progression. It has been found that carriers of the 4G/4G genotype have significantly higher levels of PAI-1 in their serum than carriers of the 4G/5G or the 5G/5G genotypes.
6. The polymorphism *ACE2* rs2106809 does not contribute to increased risk of SARS-CoV-2 infection and severity in females. In contrast, the C

allele contributes to Covid-19 infection and severe illness among males. Ang 1-7 levels in the serum of carriers of the female genotypes were not significant, although the CC genotype had slight levels. Ang 1-7 levels were significantly affected by the alleles, with C alleles lower than T alleles in males.

7. The levels of angiotensinogen may be a factor in the likelihood of contracting COVID-19, but they do not appear to play a role in worsening the severity of the disease. Conversely, the serum levels of homocysteine and PAI-1 are reduced in COVID-19 patients, while Ang 1-7 levels are elevated. These proteins may play a role in the risk and severity of the disease and show potential as promising biomarkers for assessing the likelihood of contracting the infection and tracking its progression.
8. According to in silico results, the polymorphism in exon rs1801133 p.A222V *MTHFR* contributes to a change in protein structure and damage leading to related disease with decreased protein stability. In contrast, rs699 p.M268T *AGT* affects protein stability slightly, but it is benign in causing disease. Meanwhile, in silico results of intron polymorphisms rs2106809 and rs1799889 in promoter lead to altered transcription activity and the creation of new splicing factors.

Recommendations

1. Hopefully, the Ministry of Health will consider the following points:
    - **A.** In addition to traditional laboratory tests, the use of molecular techniques for critical polymorphisms of genes to each patient early to avoid any worsening for them with COVID-19 or any epidemic.
    - **B.** Study therapy of thrombosis in instances of COVID-19 using the available trials all around the world.
2. Larger samples are required to confirm viral infection behavior by demonstrating substantial differences in viral loads among age groups.
3. Further research is warranted to investigate additional sets of genetic variations (polymorphisms) among individuals with COVID-19.
4. The COVID-19 infection may be mapped in the human genome by using the transcriptomic data method, which can give useful information on differential gene and transcript expression patterns.
5. Proteomic study provides more concrete data and a complete view of COVID-19 infection and severity.
6. Detection of other biomarkers and determining their specificities, such as homocysteine beside D-dimer and strong accuracy rely on our findings, PAI-1, and Ang 1-7 levels can be served as early detection markers, especially if they are correlated with the severity of the disease.
7. Investigate the risk factors associated with post-COVID-19 conditions as well as all human organs after infection.
8. Study risk of acute myocardial infarction after COVID-19 recovery.
9. The COVID-19 viral load test assesses the concentration of RNA copies per milliliter of serum or plasma and requires further study.

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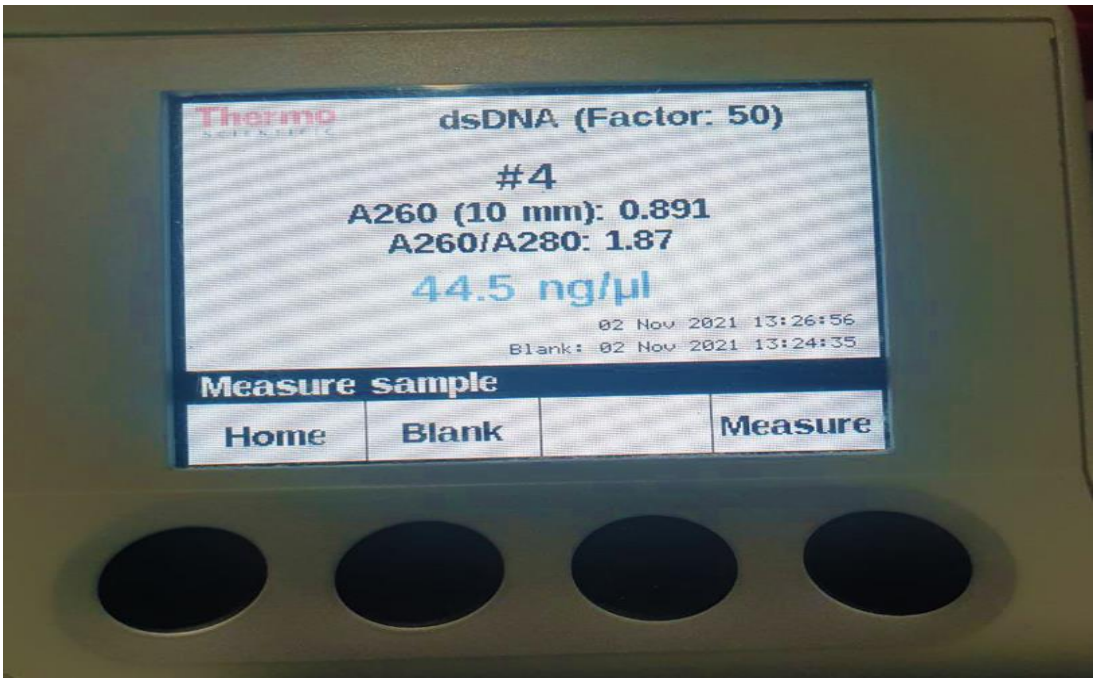
Appendices

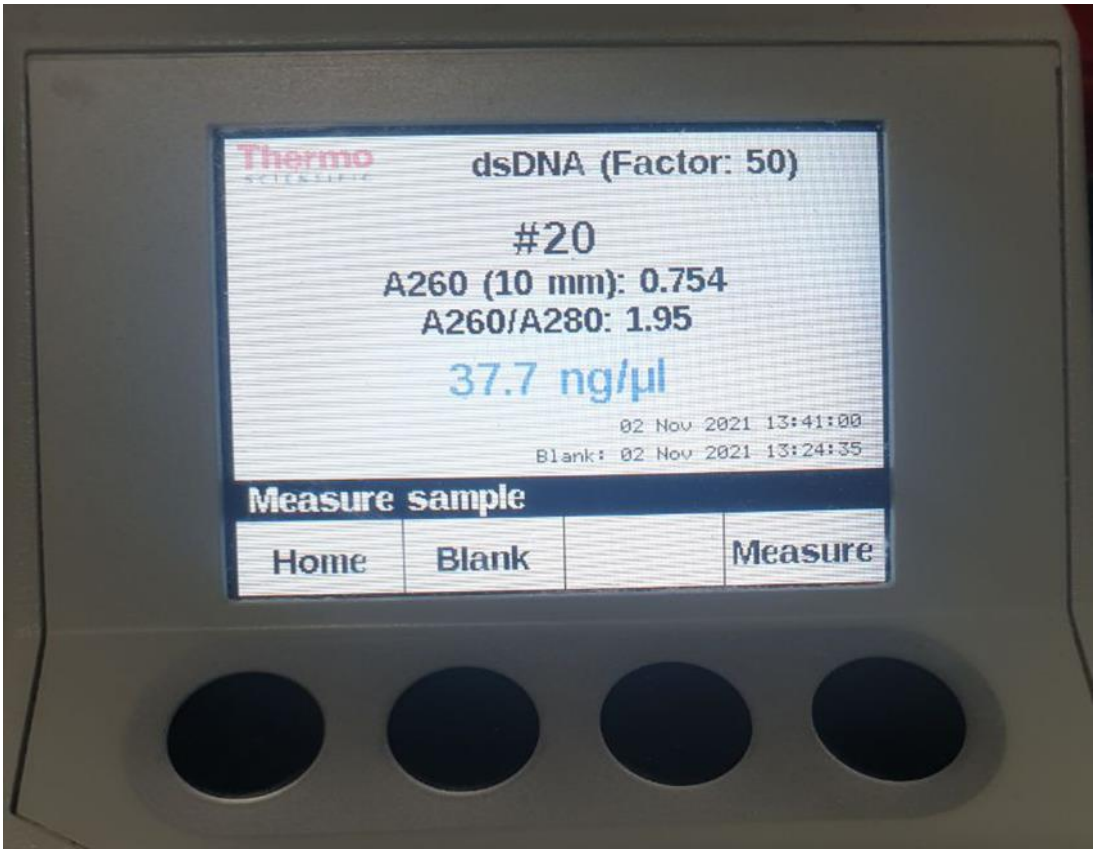
Appendices

Appendix (1): Patient's questionnaire

Number of sample			
Name			
Age			
Gender	○ **Male**	○ **Female**	
Chronic of diseases • **Hypertension** • **DM** • **CKD** • **CVD** • **OTHERS**			
Diagnosis method	• **RT-PCR**	• **CT**	• **Rapid Antibody-Covid-19**
Data admission			
Case severity	• **Severe**	• **non-severe**	
Date sample collection			
Type of sample	• **Blood**	• **Serum**	• **Plasma**
Laboratory tests	• **Hematology**	• **other**	

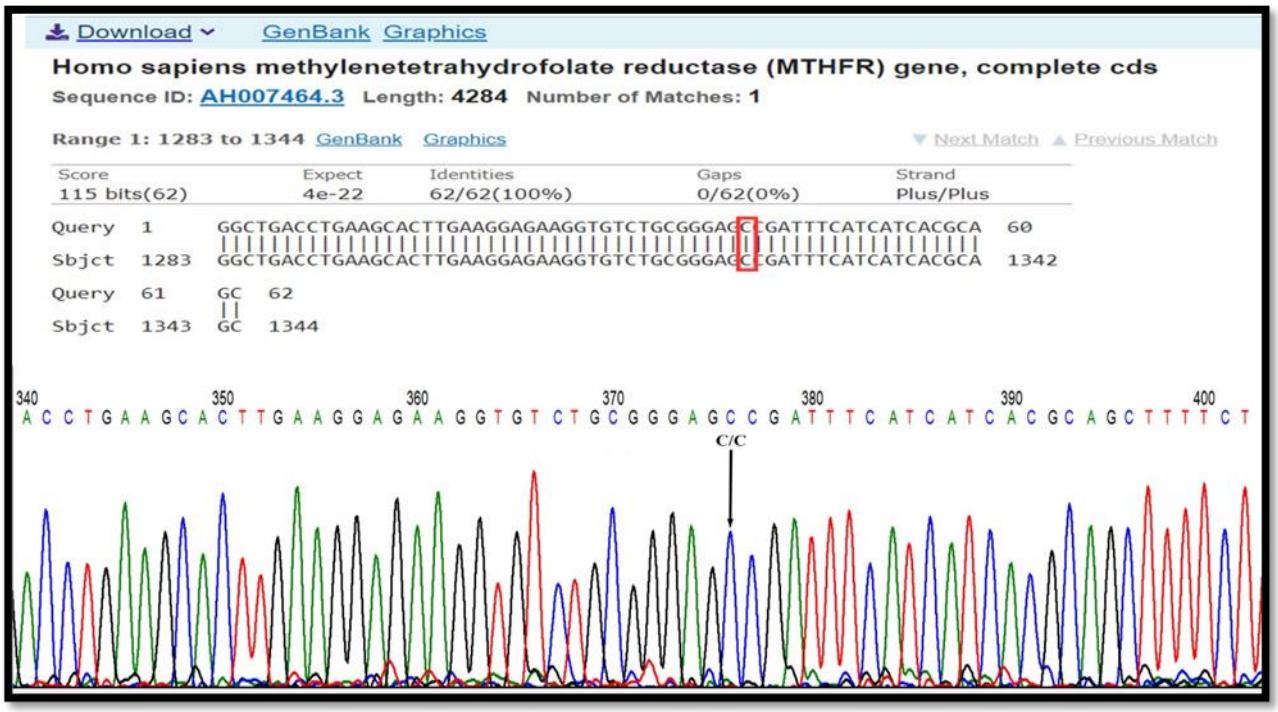
Appendix (2): Measurement of DNA concentration

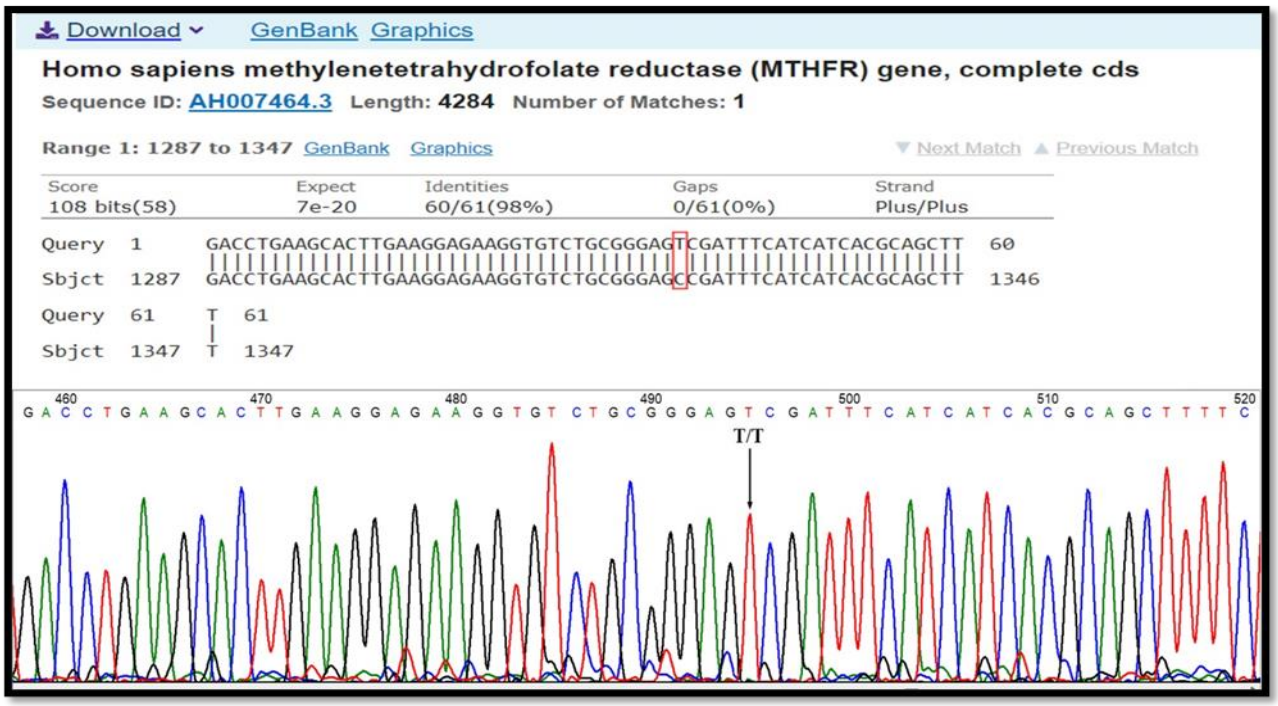




Appendix (3): *MTHFR* rs1801133 sequencing

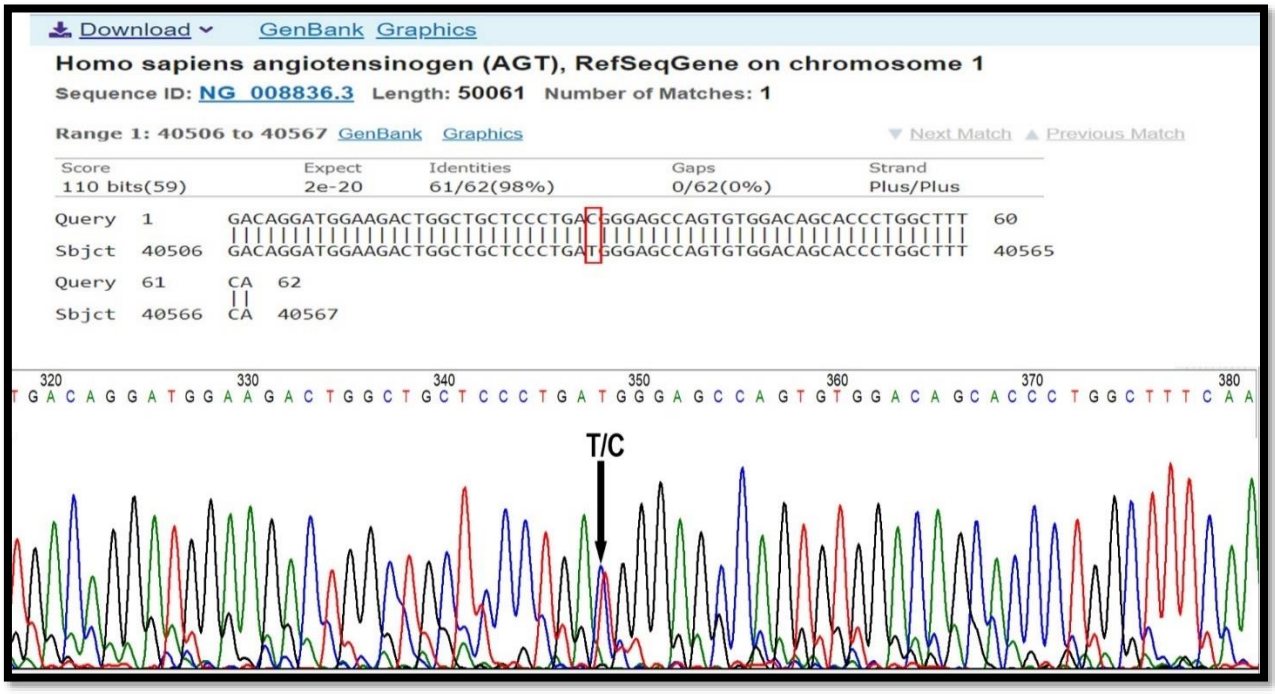
Sequence results and alignment of rs1801133 polymorphism (wild and mutant)

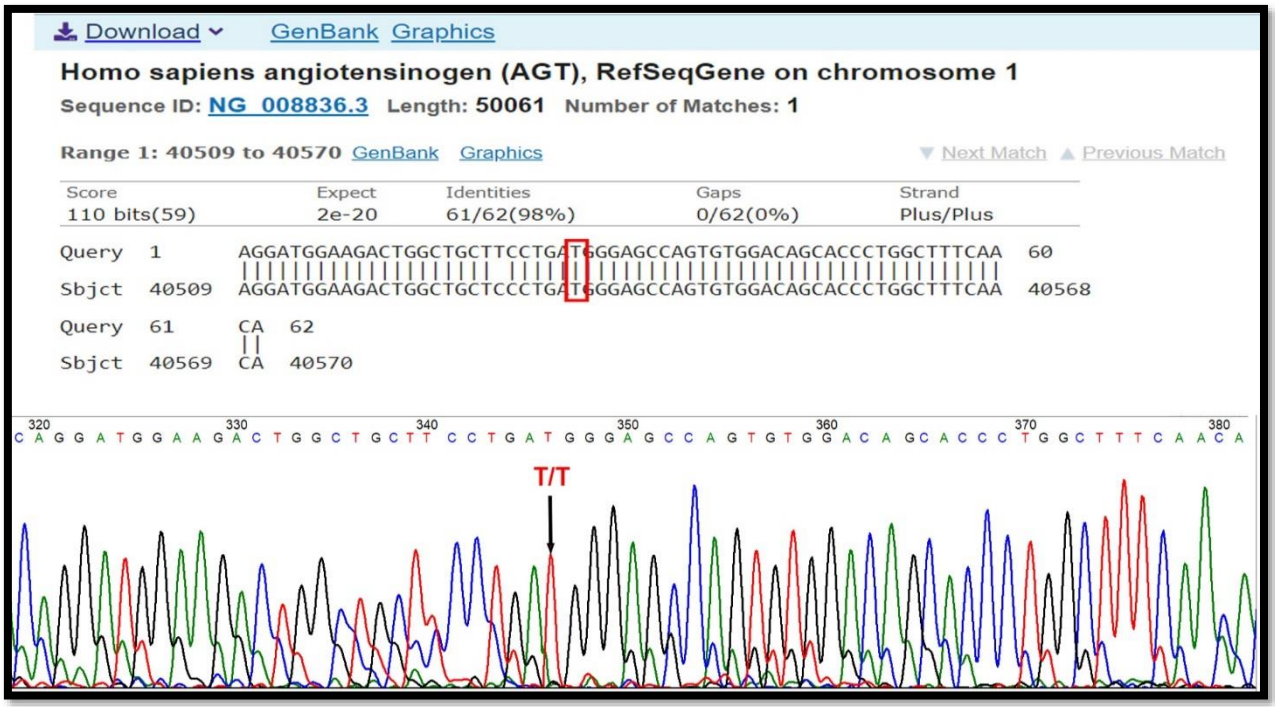




Appendix (4): *AGT* rs699 sequencing

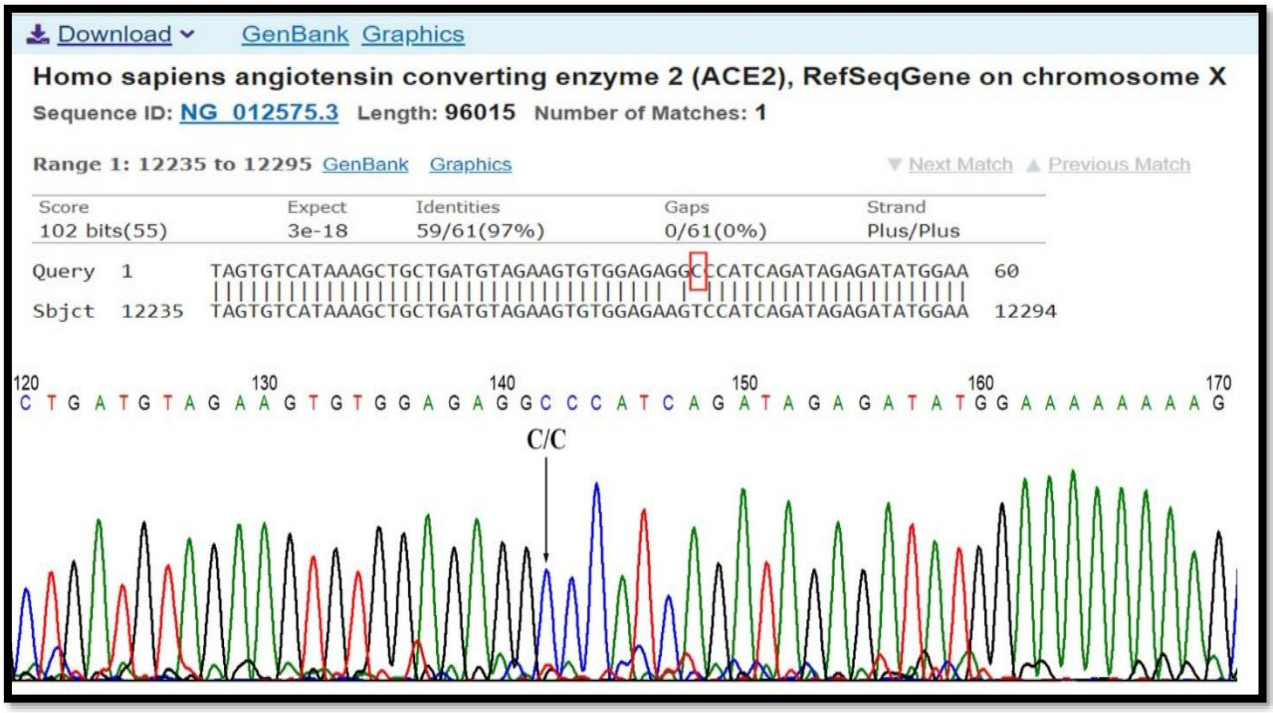
Sequence results and alignment of rs699 polymorphism (heterozygote and wild)

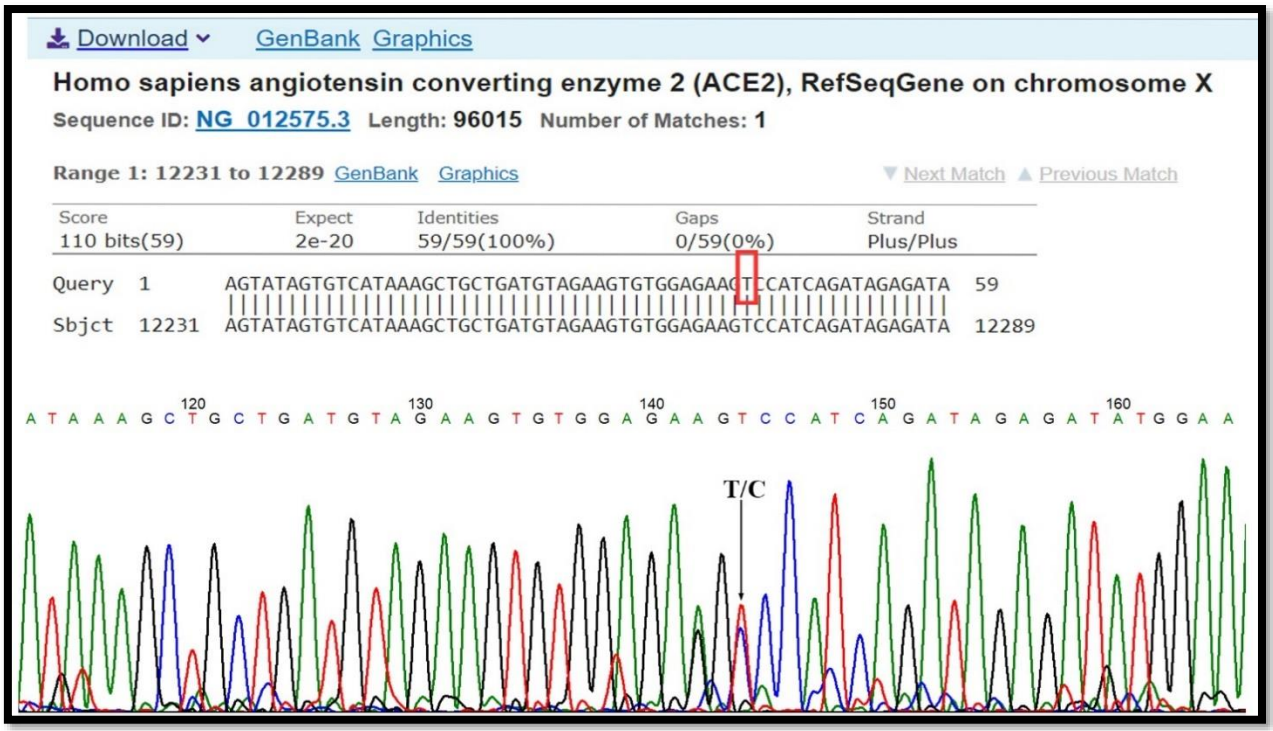




Appendix (5): *ACE2* rs2106809 sequencing

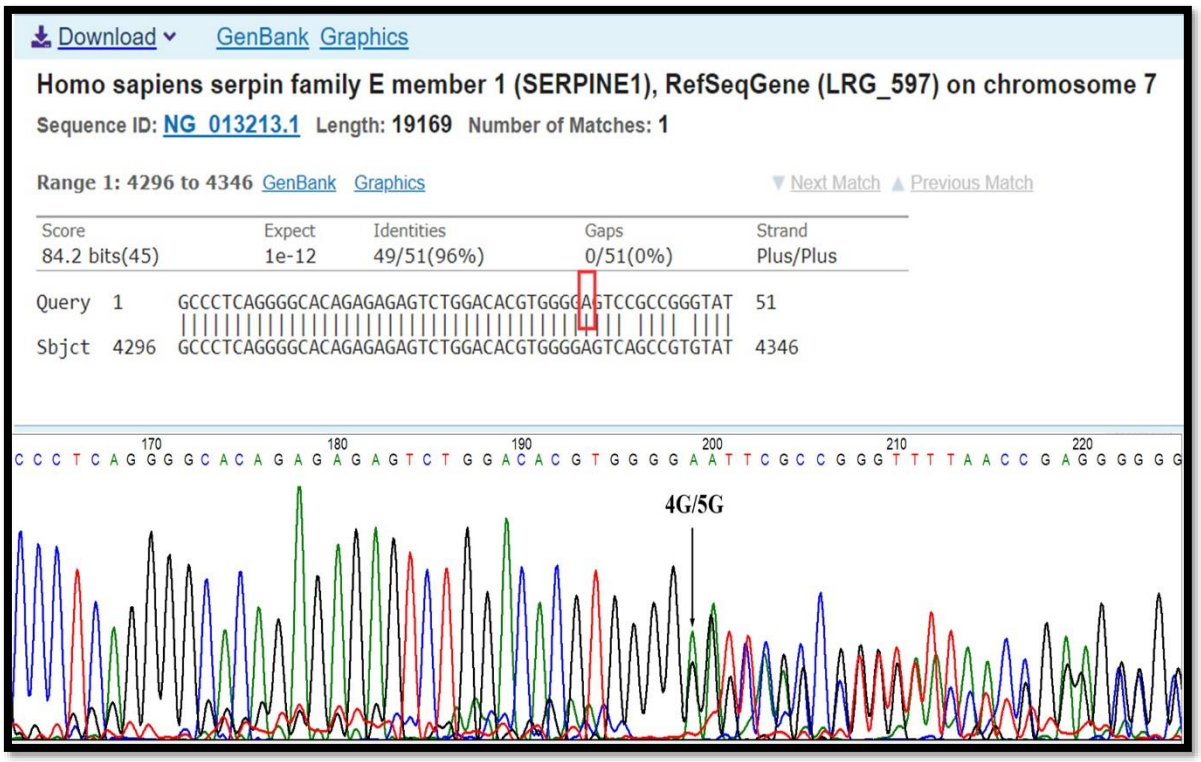
Sequence results and alignment of rs2106809 polymorphism (mutant and heterozygote)

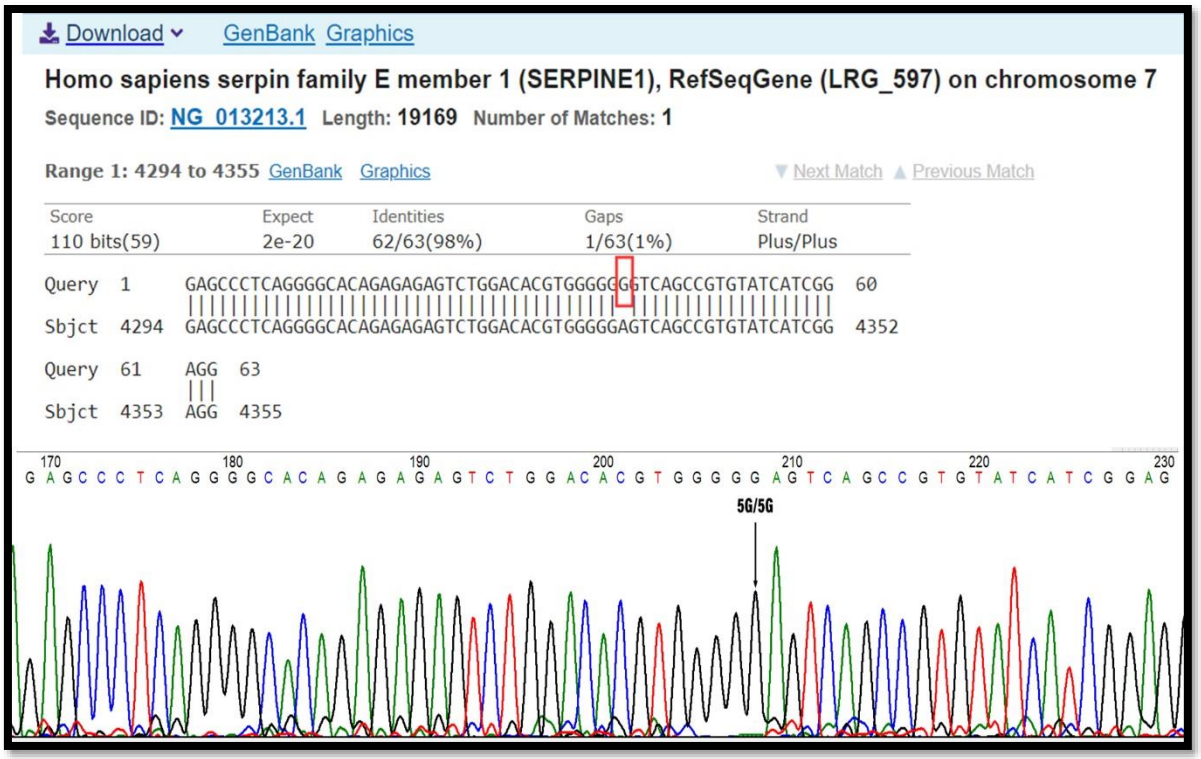




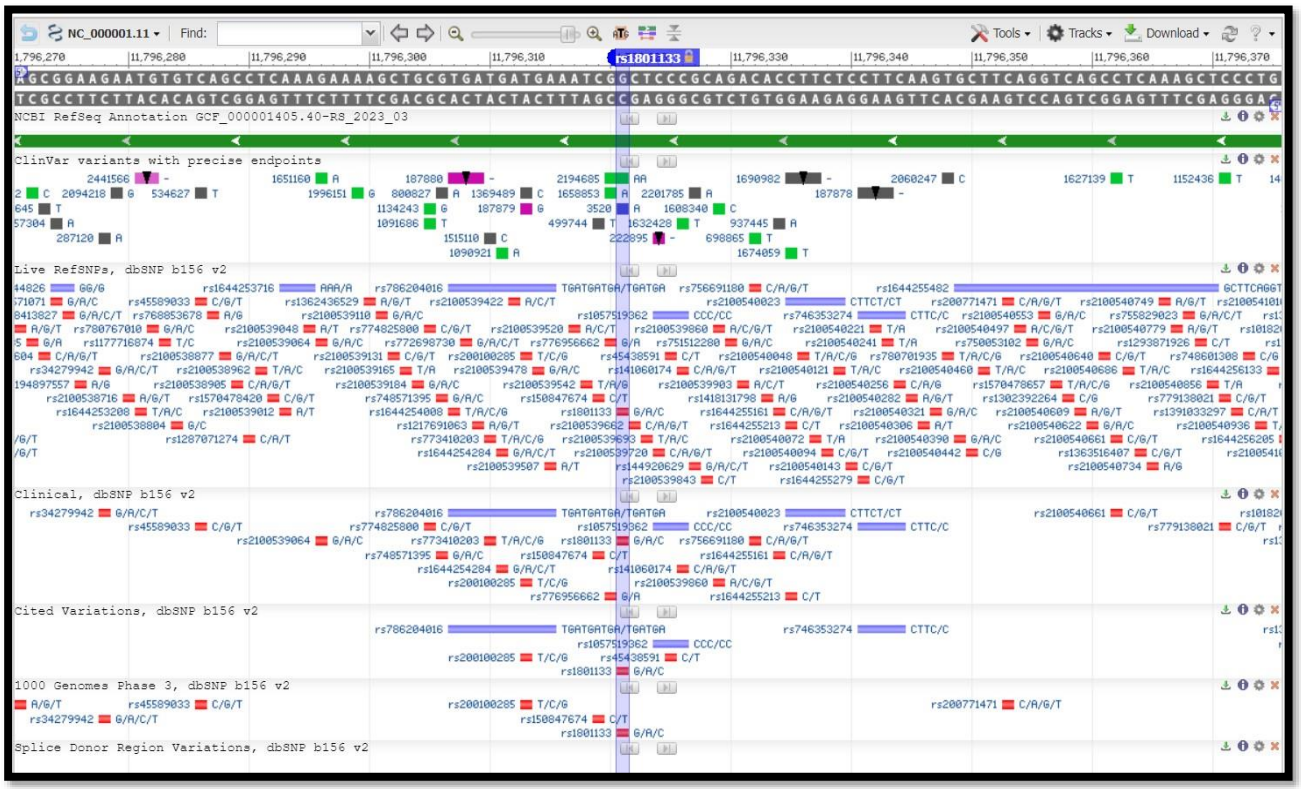
Appendix (6): Appendix (5): *SERPINE1* rs1799889 sequencing

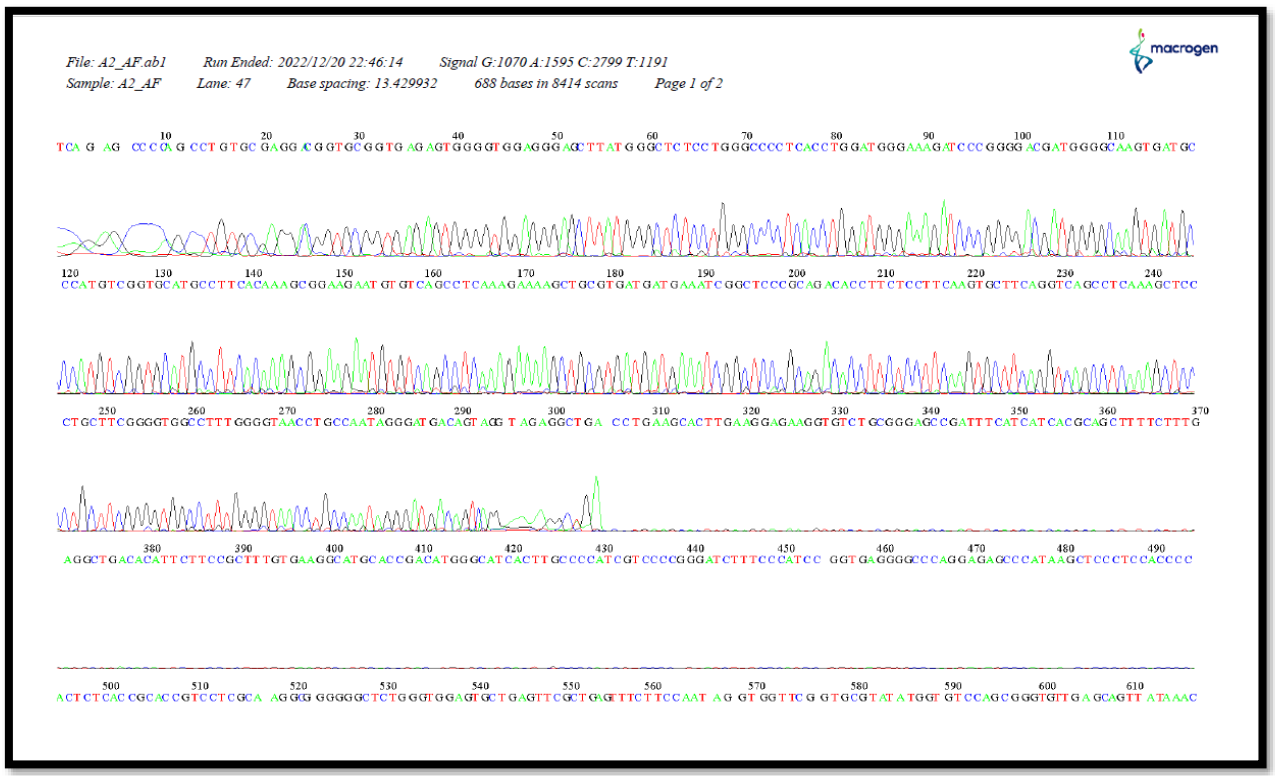
Sequence results and alignment of rs1799889 polymorphism (heterozygote and mutant)



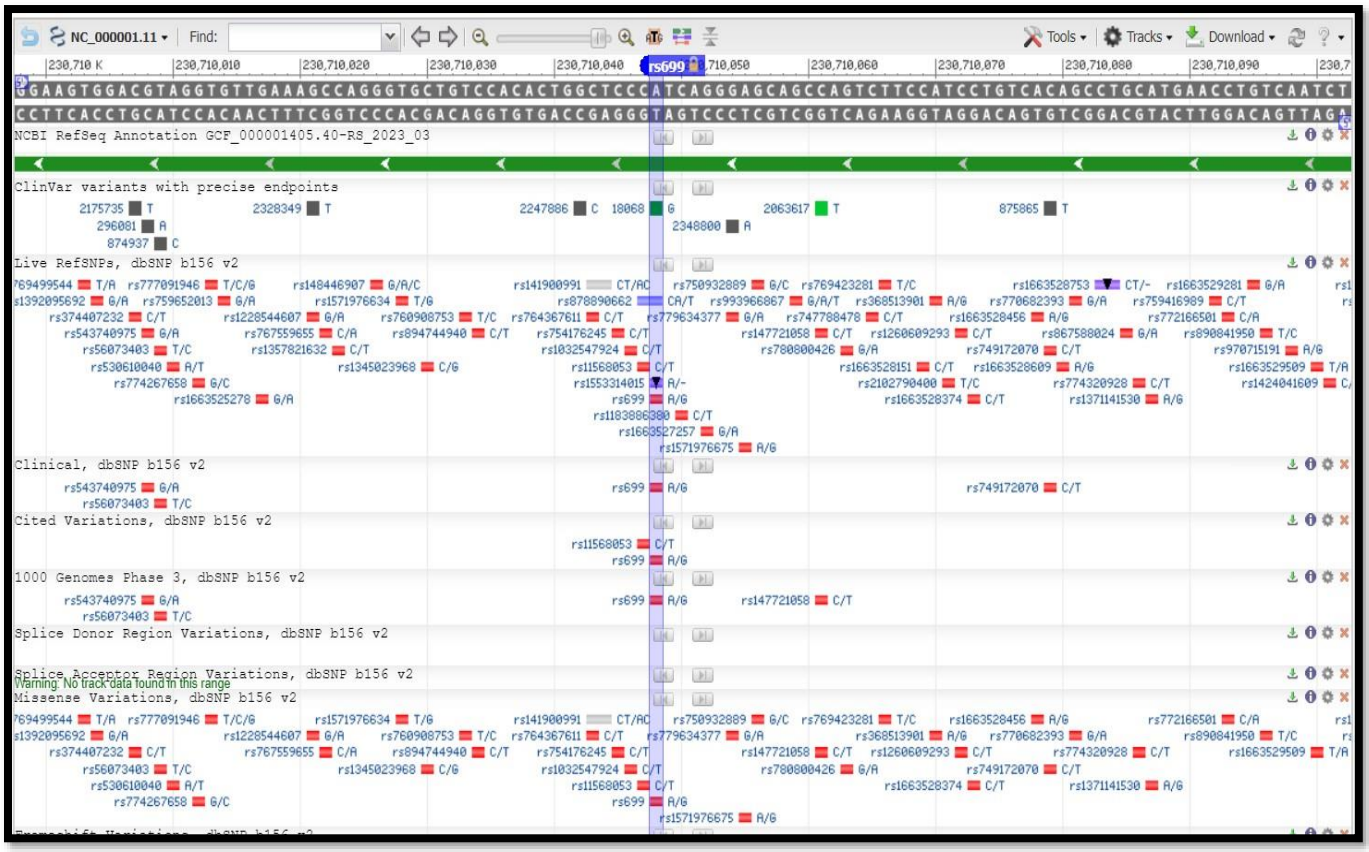


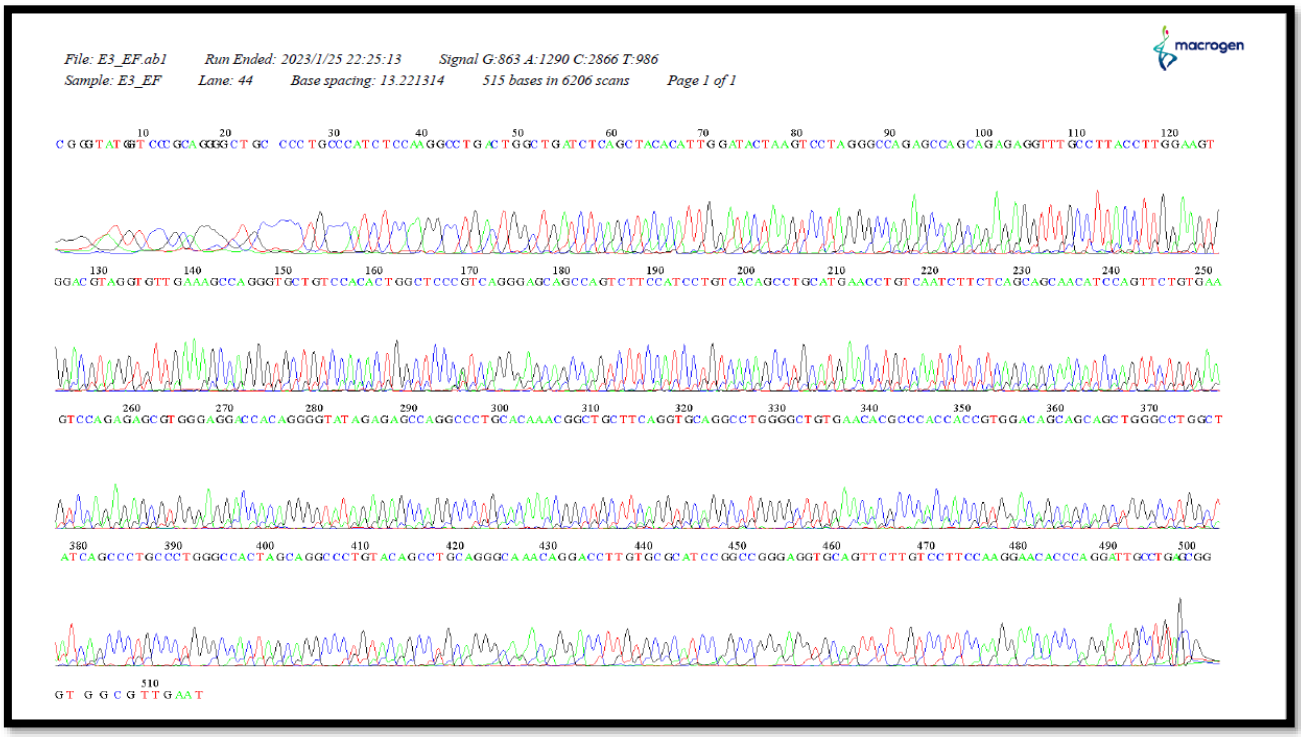
Appendix (7): SNP database to view rs1801133 included most of details variant:



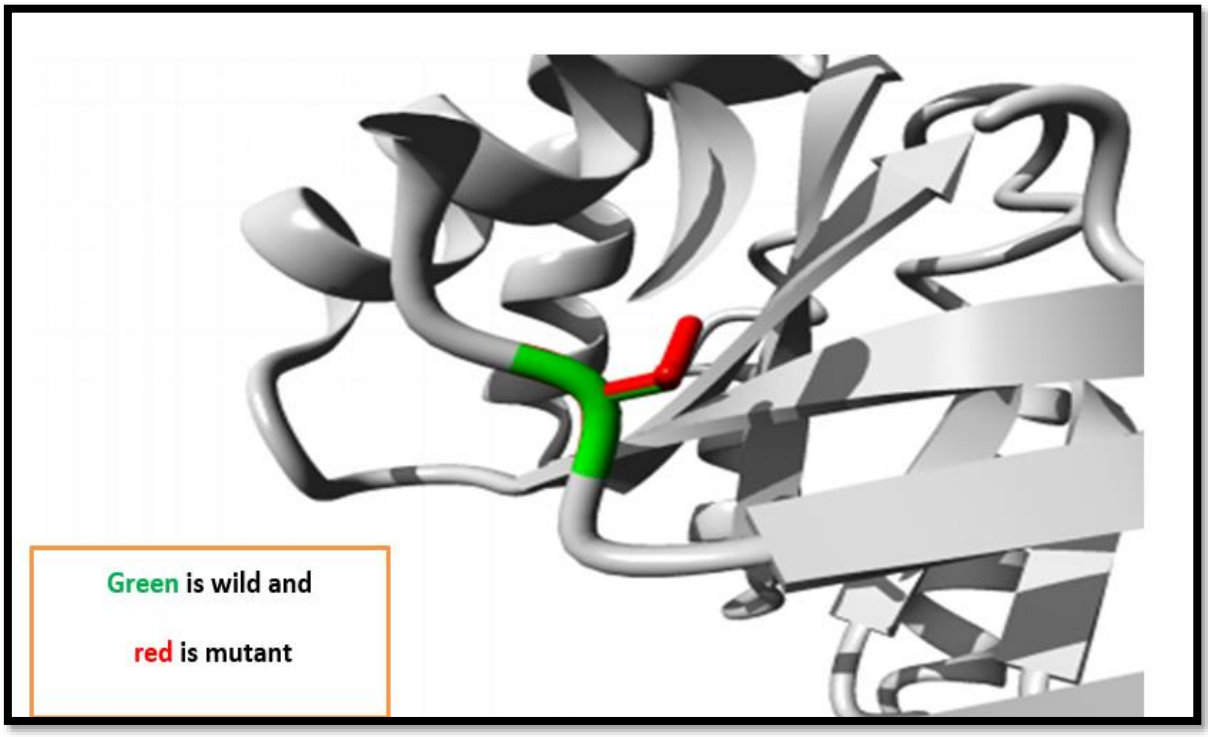


Appendix (8): SNP database to view rs699 included the details of variant

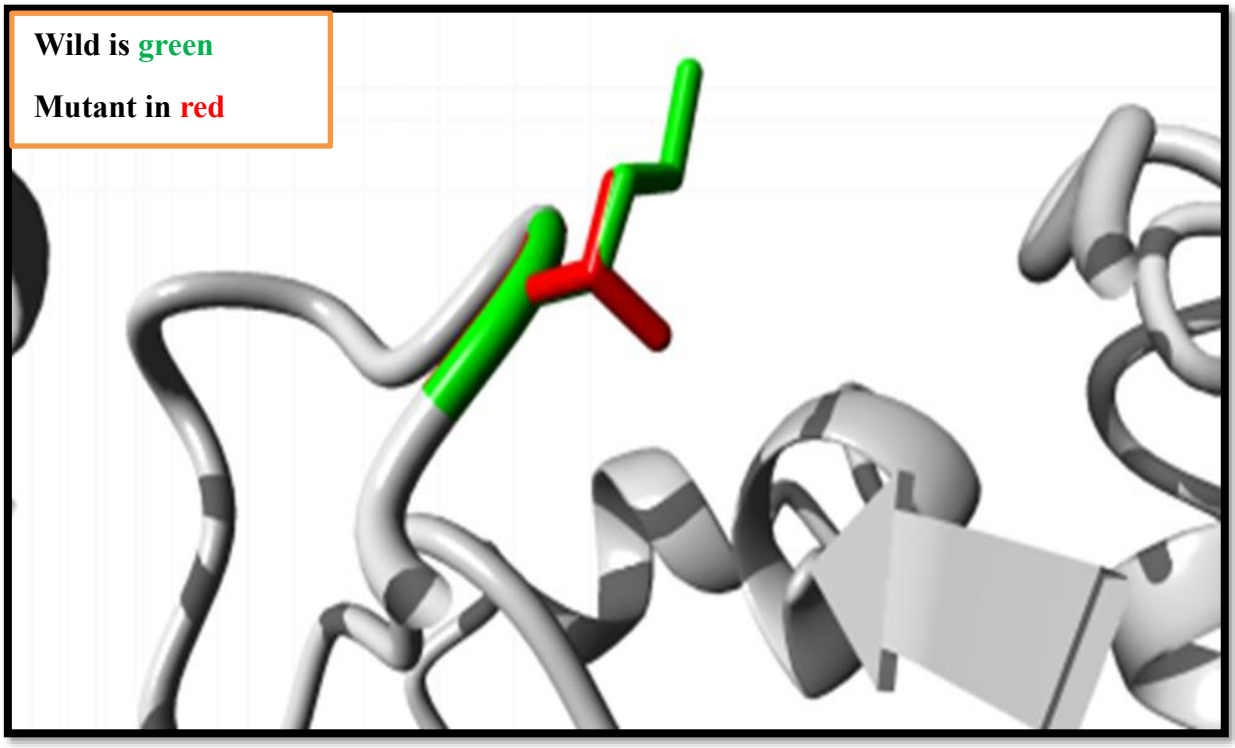




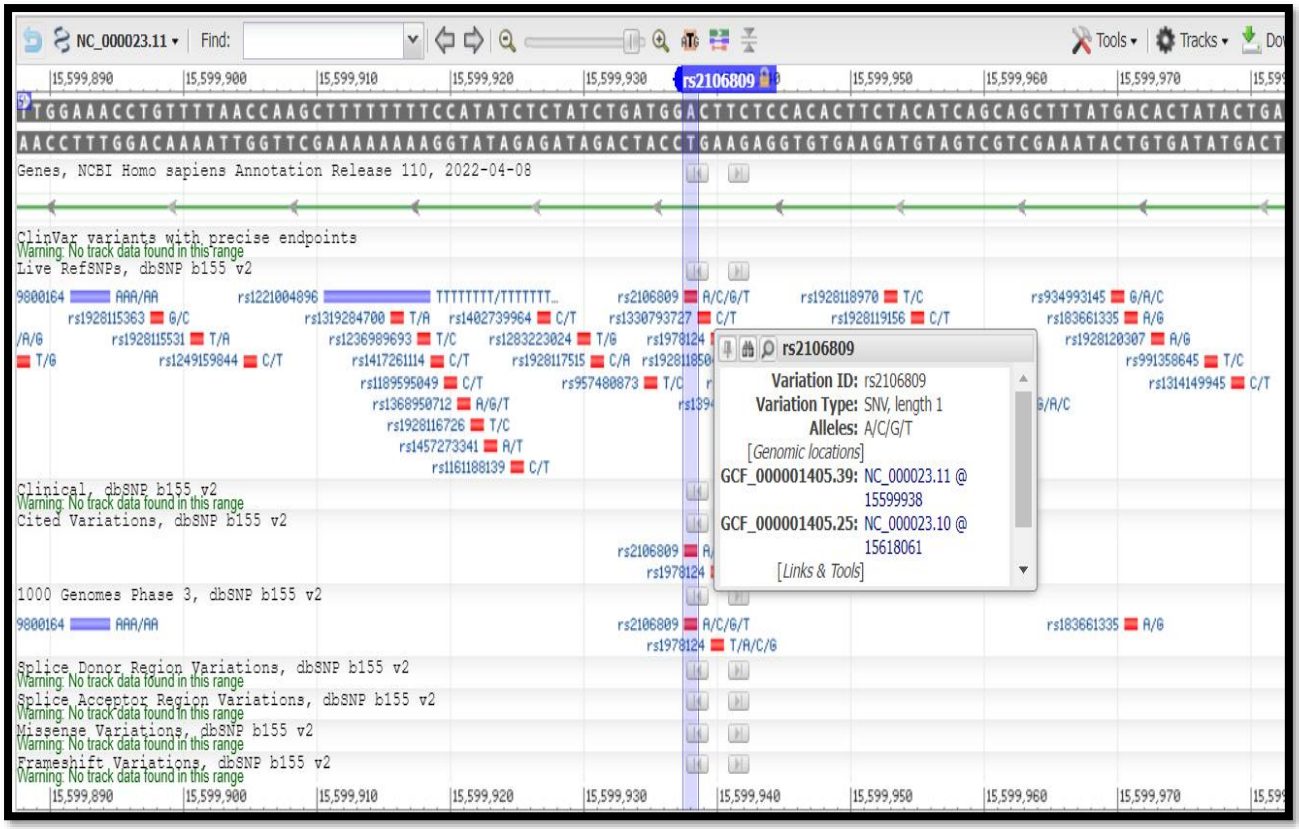
Appendix (9): PDB file to view three-dimensional shape substitution of amino acid A222V.

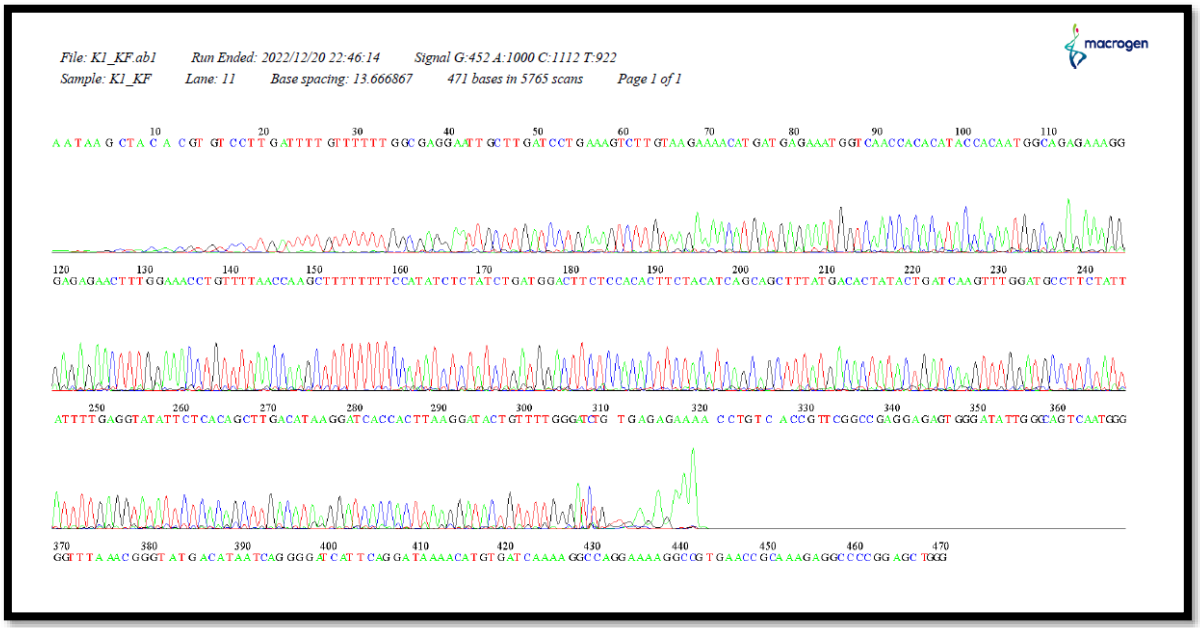


Appendix (10): PDB file to viewed three-dimensional shape substitution of amino acid M268T

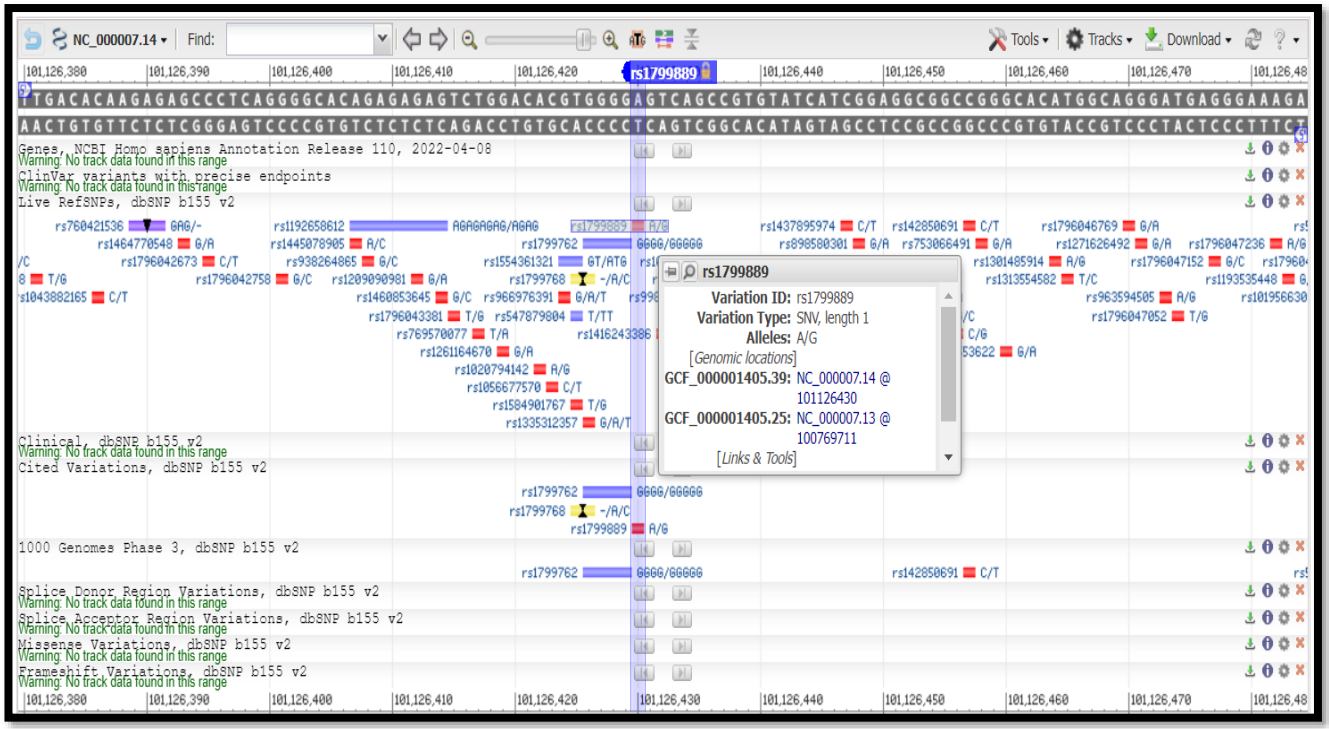


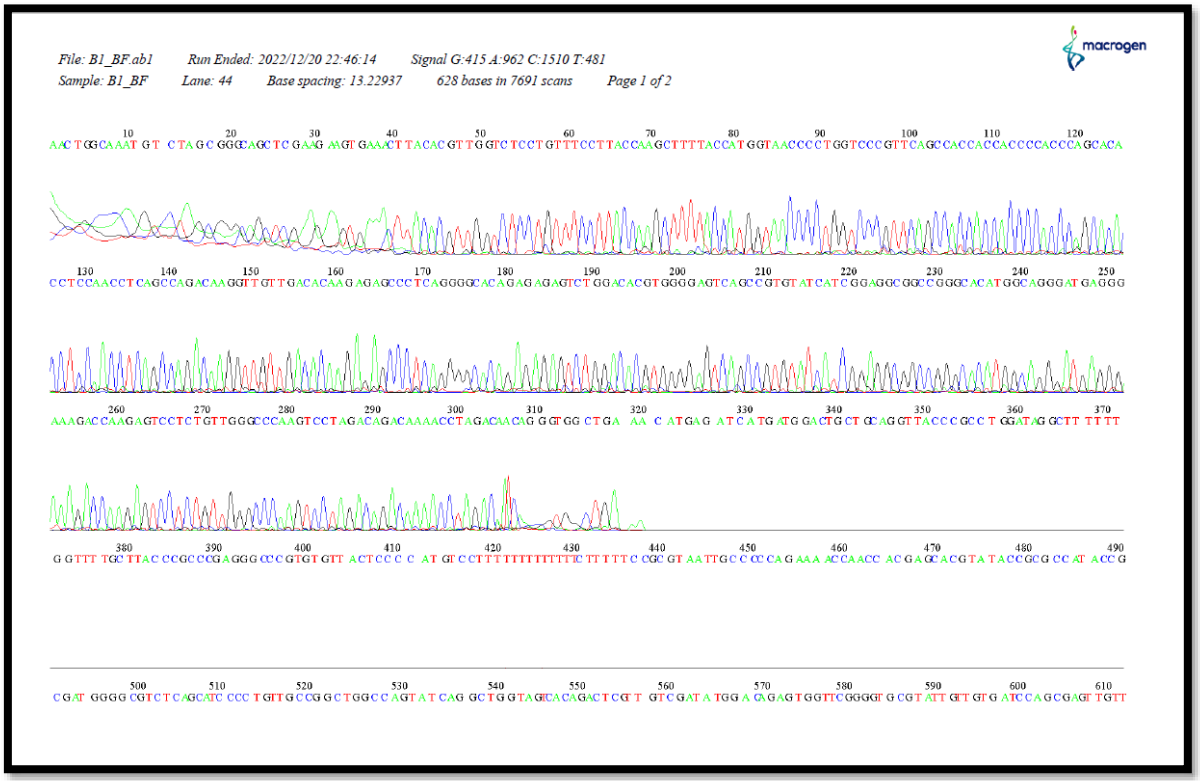
Appendix (11): SNP database to view rs2106809 included the details of polymorphism





Appendix (12): SNP database to view rs1799889 included details.





الخلاصة

لقد ظهر وباء فايروس كورونا (COVID-19) في العالم وأصبح جائحة عالمية ناجما عن متلازمة الالتهاب التنفسي الحاد الفيروسي (SARS-CoV-2) حيث ارتبط معدل ارتفاع الوفيات وسوء الحالة الصحية للمرضى ذو الإصابة الحادة بالزيادة والافراط في التخثر والالتهاب.

تهدف الدراسة إلى تحديد ما إذا كان يمكن استخدام بعض التغايرات الوراثية ومستويات بعض البروتينات كمؤشرات حيوية محتملة لشدة مرض كوفيد-19 في المرضى العراقيين. اشتملت دراسة الحالات والضوابط 102 مريضا (أعمارهم بين 52.66 ± 18.82 سنة) وقسمت إلى مجموعتين فرعيتين (الحالات غير الحادة = 45 والحالات الحادة = 57) على أساس "الحادة" إذا تم إدخالها إلى المستشفيات ووحدة العناية المركزة لفشل في الجهاز التنفسي بينما "الغير حادة" إذا لم تتطلب دخول المستشفى، و92 المجموعة الضابطة (أعمارهم 37.88 ± 14.19 سنة) وخلال هذه الدراسة جمعت عينات من بعض المستشفيات العراقية وهي (مستشفى الديوانية التعليمي، ومستشفى الحسين التعليمي، ومدينة مرجان الطبية ومستشفى الحمزة العام) ، حيث كان المرضى إيجابيين لـفحصCOVID-19 ، إلى جانب المجموعة الضابطة.

تم الكشف عن تعدد الأشكال باستخدام تقنية ARMS ، والمعروفة أيضًا باسم طريقة الاليل المتخصص (AS-PCR) ، بالإضافة الى تقنية نظام الطفرة الحرارية للتضخيم الرباعي-T-ARMS) (PCR، وبعدها تم ارسال العينات الى تقنية تسلسل الحمض النووي، وتضمنت أربعة من تعدد أشكال النوكليوتيدات الفردية (SNP) ،منها الواقعة على جين اختزال ميثيلين تتراهيدروفولات (MTHFR) C677 rs1801133 p.Ala222Val، وجين انجيوتنسينوجين (AGT) rs699 T> C p.Met268Thr، وجين الإنزيم المحول للأنجيوتنسين 2 (ACE2) ،وجين مثبط السيرين بروتياز(Serpin E1) وهو تعدد الأشكال (4G/5G rs1799889) ،وكان هناك ثلاثة بادئات مستخدمة في تقنية AS-PCR، وأربعة بادئات مستخدمة في تقنية T-ARMS-PCR ،لتشخيص المتغايرات الوراثية في الدراسة ،وبعد هذه الخطوة تم تقديم الحمض النووي المستهدف لتقنية تسلسل سانجر. علاوة على ذلك، تم تحديد تركيزات المؤشرات الحيوية لمصل المشاركين (مرضى وأصحاء) باستخدام تقنية الـ ELISA للكشف عن الأجسام المضادة المحددة في المصل لأربعة مؤشرات حيوية مختارة (الهوموسيستين، مولد الأنجيوتنسين، الأنجيوتنسين 1-7، ومنشط البلازمينوجين المانع-1). بالإضافة إلى ذلك، تم استخدام أدوات المعلوماتية الحيوية بطريقة المحاكاة بالبرامج الحاسوبية لفحص تأثير تعدد الاشكال الوراثية على بنية البروتين ووظيفته، بينما يؤثر تعدد الاشكال في المناطق غير المشفرة على التنظيم الجيني ونشاط المحفز للمتغايرات المختارة بالدراسة.

أشارت نتائج الدراسة إلى أن متوسط عمر الحالات المصابة بـ COVID-19 قد زاد بشكل ملحوظ مقارنة بالمجموعة الضابطة حيث كان (52.6±18.84 مقابل 37.88±14.19 على التوالي عند دلالة إحصائية معتدة $p<0.05$) لم يختلف توزيع الإناث والذكور في المرضى والمجموعة الضابطة غير دالة احصائياً($p = 0.99$) كانت البيانات المجمعة عن المرضى مكونة من[58.6٪ ذكور و47.1٪ إناث]والاصحاء [52.9٪ ذكور و (41.3٪) أناث] . وجمعت بيانات ثمانية متغيرات معملية ، وهي مستوى الهيموغلوبين ، والصفائح الدموية ، وخلايا الدم البيضاء (WBC) ، والعدلات ، والخلايا الليمفاوية ، واختبار D-dimer ، ونسبة العدلات إلى اللمفاويات (NLR) ، ونسبة الصفائح الدموية إلى الخلايا الليمفاوية (PLR) وبمقارنة هذه المعلمات بالقيم المرجعية ، وجدت خمس معلمات ذو تأثير في مرض كورونا فايروس ليتسبب بزيادة شدته وتطوره ؛ حيث ان عدد كرات الدم البيضاء (13.5 × 109 / لتر ± 5.5) ، (1.37 ± 1) D-Dimer، العدلات (4.35 ± 10.88)، (9 ± 11) NLR، و PLR (271.76 ± 307.96) كما تم قياس الخلايا الليمفاوية ولوحظ انخفاضها بشكل ملحوظ 1.55). ± 0.96)

استنادًا إلى التمييز الأليلي لـ(rs1801133) في منطقة الترميز لجينMTHFR ، حيث ترتبط الأنماط الجينية CT و TTبزيادة خطر الإصابة بعدوى COVID-19 مقارنةً بـالمجموعة الضابطة، ويرتبط كلا النمطين الجينيين بزيادة شدة العدوى. اما في جين(rs699) AGT ، أظهر تباين الانماط الوراثية وجود فرق معتد به إحصائيًا في النمط الجينيTC ، مما يشير إلى عامل خطر للإصابة بفيروس الكوفيد-19، بينما لم يكن لأي نمط وراثي في تعدد الأشكال هذا أي تأثير على شدة المرض. لم يؤثر التباين في الانماط الجينية لجين (rs2106809) ACE2 الموجود على كروموسوم X في الإناث في للمساهمة بخطر الإصابة بفيروس كوفيد -19 أو شدته، بينما كان الأليل C في الذكور عامل خطر للمرض ويزيد من شدة وتطور العدوى. وارتبط النمط الجيني (rs1799889) 4G/5G لجين SERPINE1بخطر الإصابة بالمرض وتفاقم شدة العدوى.

على الجانب الاخر كشفت النتائج عن مستويات أعلى بكثير من الهوموسيستين في المرضى (16.17 نانومول/لتر) مقارنة بالمجموعة الضابطة (4.46 نانومول/لتر). وبالمثل، كانت مستويات الهوموسيستين أعلى بشكل ملحوظ في الوفيات والحالات الشديدة مقارنة بالحالات غير الشديدة (12، 10.4، مقابل 3.2 على التوالي). بينما كان هناك اختلاف كبير في مستويات مولد الأنجيوتنسين في الدم بين مرضى COVID-19 والمجموعة الضابطة (69.9 مقابل 86.07) نانوغرام/ لتر، ولكن لم تكن هناك فروق ذات دلالة إحصائية بين الحالات الحادة وغير الحادة. كان متوسط مستوى Ang 1-7 لمرضى COVID-19 أقل بكثير من المتوسط بالنسبة للأصحاء (70.77 مقابل 91.38 نانوغرام/لتر).

وكانت مستويات Ang1-7 في المصل في الحالات الميتة والحادة أقل بكثير من تلك التي لوحظت في الحالات غير الحادة (60.6 و75.2 مقابل 109.44 نانوغرام/ لتر، على التوالي). وكانت مستويات PAI-1 في المرضى أعلى بكثير من تلك الموجودة في الاصحاء (6.5 مقابل 3.56) نانوغرام/مل على التوالي. بالإضافة إلى ذلك، كان لكل من الحالات الميتة والحادة مستويات PAI-1 أعلى بكثير من الحالات غير الحادة (13.2 و8.36 مقابل 3.43) نانوغرام/مل، على التوالي. كما تم العثور على ارتباط بين ثلاثة مستويات من البروتين (Hcy، Ang 1-7، وPAI-1)، وكلها مرتبطة بشكل كبير بالأنماط الجينية والأليلات لتعدد الاشكال الـــ (rs1801133، rs2106809، و1799889 على التوالي) وعلى العكس من ذلك، لم تكن مستويات AGT مرتبطة بالأنماط الجينية المرتبطة بـ rs699.

أُستخدام عدد كبير من خوادم الويب للتحقق من تعدد الأشكال وتأثيرها في الدراسة وإظهارها في نتائج المحاكاة بالحاسوب حيث كان تعدد الأشكال rs1801133 p.A222V لـ MTHFR وباستخدام عدة خوادم في هذه الدراسة ووجد ان التغاير الوراثي بتغير الحامض الاميني يقلل من استقرار البروتين ويؤدي إلى المرض وتلف البروتين. في حين أن AGT rs699 p.M268T قلل من استقرار البروتين بطريقة حميدة، وتم تحمل التغيير دون التسبب في المرض. بالإضافة إلى ذلك، يغير كل من rs2106809 في ACE2-intron وrs1799889 SERPINE1 في المحفز بتعديل نشاط نسخ الجينات عن طريق إضافة او حذف عوامل استنساخ الحمض النووي.

خلصت هذه الدراسة الى أن تعدد الاشكال في الجينات (MTHFR, ACE2, and SERPINE1) بجانب مستويات المعلمات الحيوية (Hcy, Ang 1-7, and PAI-1)، وجدت انها مؤشرات واعدة تستدعي الدراسة والتحقيق بشكل أوسع في ارتباطها في خطورة وشدة ألـــ COVID-19.

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

دراسة تعدد الأشكال الوراثية لبعض الجينات في الانسان المرتبطة بخطورة وتطور عدوى الكوفيد-19

أطروحة
مقدمة الى مجلس كلية العلوم – جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/علوم الحياة

من قبل

غزوان فيصل حسين ليلون

بكالوريوس علوم الحياة/جامعة القادسية/٢٠١٢
ماجستير التقنيات الاحيائية/الجامعة العثمانية /٢٠١٥

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