T.C.

TOKAT GAZIOSMANPASA UNIVERSITY

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DETERMINATION OF ACE GENE POLYMORPHISM IN COVID-19 PATIENTS

MASTER'S THESIS

SHADI HAMID SIDIQ

Supervisor: Prof. Dr. Hacı Ömer ATEŞ

TOKAT – 2024

ETHICS CONTRACT

T.C.

TOKAT GAZİOSMANPAŞA UNIVERSITY

TO THE INSTITUTE OF GRADUATE STUDIES DIRECTORATE

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The Student Who Prepared the Thesis

SHADI HAMID SIDIQ

JURY ACCEPTANCE AND APPROVAL

The defense examination of the thesis study titled "Determination of ACE gene polymorphism in COVID-19 patients", which is prepared by **SHADI HAMID SIDIQ**, was held on the 02/01/2024. It has been accepted by jury members as a Master's Thesis at Tokat Gaziosmanpasa University, Institute of Graduate Studies, Department of Medical Biology.

Jury Members (Title, Name Surname)	Signature
Chairman : Dr. Öğr. Üyesi. Nihan BOZKURT	.....
Member : Prof. Dr. Haci Omer ATES	.....
Member : Dr. Öğr. Üyesi. Sumeyya Deniz AYBEK	.....

CONFIRMATION

Assoc. Dr. Yusuf TEMUR

Director of Institute of Graduate Studies

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ÖZET

COVID-19 HASTALARINDA ACE GEN POLIMORFIZMININ BELIRLENMESI

Shadi H. Sidiq

Yüksek Lisans, Tokat Gaziosmanpaşa Üniversitesi, Lisansüstü Eğitim Enstitüsü, Tıbbi Biyoloji Anabilim Dalı

Tez Danışmanı: Prof. Dr. Hacı Ömer ATEŞ

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Şiddetli Akut Solunum Sendromu Koronavirüs 2 (SARS-CoV-2), solunum yolu rahatsızlığı COVID-19'un nedeni olan etiyolojik ajandır. Bu virüs, yüksek morbidite ve mortalite oranları göz önüne alındığında, son yüzyıllarda insan sağlığı için en zorlu zorluklardan birini teşkil etmektedir. Bu durum, Dünya Sağlık Örgütü'nün Mart 2020'de COVID-19 salgınını küresel bir pandemi olarak ilan etmesine yol açmıştır. SARS-CoV-2, anjiyotensin dönüştürücü enzim-2 (ACE2) reseptörüne karşı yüksek bir afinite sergilemektedir. ACE2 ve anjiyotensin dönüştürücü enzim-1 (ACE1), renin-anjiyotensin-aldosteron sisteminin (RAAS) ayrılmaz bileşenleridir ve yerel doku homeostazının korunmasında çok önemli bir rol oynarlar. Bu araştırmada, polimeraz zincir reaksiyonu tekniğini kullanarak 100 COVID-19 hastası ve 41 sağlıklı kontrolden oluşan örneklem seti olmak üzere toplam 141 katılımcıda ACE I/D (Insertion/Deletion) polimorfizmini araştırdık. Bu araştırma, ACE polimorfizmi ile SARS-CoV-2 semptomlarının şiddeti arasındaki olası bağlantıyı incelemektedir. Bu çalışmada, anjiyotensin dönüştürücü enzim (ACE1) geni I/D polimorfizminin (rs1799752) COVID-19 şiddeti ile ilişkisinin değerlendirilmesi amaçlanmıştır. Katılımcıların yaş ortalaması 49.5'dır (17 ile 94 arasında değişmektedir). Veri analizi, hasta kohortundaki ACE gen polimorfizmini kontrol grubuyla karşılaştırırken p-değerinin 0,989 olduğunu, dolayısıyla istatistiksel olarak anlamlı olmadığını ortaya koymuştur. Bu çalışmanın potansiyel bir kısıtlaması, örneklem büyüklüğünün nispeten küçük olmasıdır. Özet olarak, ACE genindeki I ve D alel polimorfizmi ile çalışılan popülasyondaki SARS-CoV-2 hastalarında semptomların şiddeti arasında fark edilebilir bir korelasyon tespit edilmemiştir.

Anahtar Kelimeler: ACE gen, gen polimorfizm, Covid-19, anjiyotensin dönüştürücü enzim

ABSTRACT

DETERMINATION OF ACE GENE POLYMORPHISM IN COVID-19 PATIENTS

Shadi H. Sidiq

Master's program, Tokat Gaziosmanpaşa University, Graduate Education Institute, Department of Medical Biology

Thesis supervisor: Prof. Dr. Hacı Ömer ATEŞ

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The causal factor behind the respiratory illness COVID-19 is the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This virus poses one of the most formidable challenges to human health in recent centuries, given its high morbidity and mortality rates. In March 2020, the international proclamation by the World Health Organization designated the worldwide dissemination of COVID-19 as a pandemic. The SARS-CoV-2 virus demonstrates an increased proclivity for binding to the angiotensin-converting enzyme-2 (ACE2) receptor. ACE2 and angiotensin-converting enzyme-1 (ACE1) are integral components of the renin-angiotensin-aldosterone system (RAAS) and play a pivotal role in preserving local tissue homeostasis. In the current research, we investigated the ACE I/D (Insertion/Deletion) polymorphism in a total of 141 participants, the sample set of 100 COVID-19 patients and 41 healthy controls employing the polymerase chain reaction technique. The research examines the potential link between ACE genetic variations and the intensity of symptoms caused by SARS-CoV-2. This study aimed to evaluate the association of angiotensin-converting enzyme (ACE1) gene Insertion/Deletion (I/D) polymorphism (rs1799752) with the COVID-19 severity. The mean age of the participants was 49.5 years (ranging from 17 to 94). The data analysis revealed that the p-value to be 0.989 when comparing ACE gene polymorphism in the patient cohort to the control group, hence statistically non-significant. A potential limitation of this study was the relatively small sample size. In summary, no discernable correlation was detected between the I and D allele polymorphism in ACE gene and the severity of symptoms in SARS-CoV-2 patients within the population studied.

Keywords: ACE gene, gene polymorphism, Covid-19, angiotensin converting enzyme

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LIST OF ABBREVIATIONS

SARS-CoV	Severe acute respiratory syndrome
+ssRNA	Positive-strand RNA virus
ACE1	Angiotensin-converting enzyme 1
ARDS	Acute respiratory distress syndrome
S-protein	Spike protein
ACE2	Angiotensin-converting enzyme 2
COVID-19	Coronavirus disease 2019
TMPRSS2	Transmembrane serine protease 2
Ang-I	Angiotensin I
Ang-II	Angiotensin II
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
AT-2	Angiotensin II type 2 receptor
Ang1-7	Angiotensin-(1-7)
HCoVs	Human coronavirus
AT-1	Angiotensin II type 1 receptor
RAAS	Renin-angiotensin-aldosterone system
TLR4	Toll like receptor 4
MERS	Middle East Respiratory Syndrome
S	Spike glycoprotein
E	Envelope glycoprotein
PCR	Polymerase chain reaction
N	Nucleocapsid protein
Pol	Polymerase
RT-PCR	Real-time reverse transcription-polymerase chain reaction
M	Membrane glycoprotein
ALT	Alanine transaminase
AST	Aspartate aminotransferase
GGT	Gamma-glutamyl transferase
CNS	Central nervous system
R_0	Reproduction number

S2	Spike protein S2 subunit
PAMP	pathogen-associated molecular pattern
AT1R	Angiotensin II type 1 receptor
ICTV	The International Committee on Taxonomy of Viruses
VEGF	Vascular endothelial growth factor
BALF	Bronchoalveolar lavage fluid
DAMP	Damage-associated molecular pattern
EDTA	Ethylenediamine tetra acetic acid
TNF	Tumor necrosis factor

1. INTRODUCTION

Coronaviruses are a type of RNA virus surrounded by an envelope, their size ranging from 60 to 140 nm in diameter. They are named after their spike-like protrusions on the outer surface that resemble a crown under electron microscopy. In December 2019, Chinese authorities reported a new virus spreading in their communities and this novel virus was identified as a Coronavirus on January 7, 2020, and had a genetic similarity of over 70% with SARS-CoV and over 95% with the bat Coronavirus. By January and February 2020, the virus had spread to other countries (Hussain et al., 2022; Verma et al., 2021). SARS-CoV-2 is the name of the virus that causes the respiratory condition known as COVID-19. It is a member of the genus *Betacoronavirus*. It is regarded as one of the greatest threats to mankind due of its high rates of sickness and mortality. Due to its intensity and widespread distribution, in March 2020, the World Health Organization (WHO) announced the COVID-19 outbreak as a pandemic (Yamamoto et al., 2021). Since this proclamation, the virus has relentlessly invaded global territories, with the statistics as of early May 2023 standing at over 764 million infections and surpassing 6.9 million fatalities (World Health Organization, accessed on May 2023).

SARS-CoV-2 instigates severe illnesses such as viral pneumonia, which is notorious for its high morbidity and mortality rates, and possesses the capacity to trigger global pandemics. It is estimated that each year, 450 million individuals globally are affected by pneumonia, resulting in approximately 3 million deaths, as per the WHO (Goren et al., 2022). The clinical manifestations in patients diagnosed with COVID-19 span from asymptomatic presentations, through mild symptoms, and in grave scenarios, precipitate acute respiratory distress syndrome (ARDS). These severe cases often present with increased mortality due to complications such as respiratory failure, stroke, multiple organ failure, and thrombotic occurrences. Factors amplifying the disease's severity include advanced age (specifically individuals over 65 years) and pre-existing conditions encompassing chronic respiratory diseases, diabetes, hypertension, and obesity (Verma et al., 2021; Rezaiezadeh et al., 2022).

Several studies have confirmed that SARS-CoV-2 utilizes the angiotensin converting enzyme 2 (ACE2) receptor to enter into host cells (Albashir, 2021;

Rezaiezadeh et al., 2022; Goren et al., 2022). The viral entry mechanism involves the contact between the ACE2 receptor and the spike protein (S-protein) of SARS-CoV-2. Additionally, successful invasion by the virus requires the activation of the S- proteins through the action of a cell-surface serine protease called transmembrane serine protease 2 (TMPRSS2) (Yamamoto et al., 2021; Verma et al., 2021).

Angiotensin converting enzymes (ACE1/ACE2) constitute integral components within the renin-angiotensin-aldosterone system (RAAS), playing pivotal roles in the regulation of vascular physiology and exerting diverse effects on immune system, brain, heart, kidney, and lung functions, either through direct or indirect means (Rezaiezadeh et al., 2022; Oscanoa et al., 2021). Specifically, angiotensin-converting enzyme 1 (ACE1) facilitates the conversion of angiotensin-I (Ang-I) to angiotensin-II (Ang-II), the latter of which exerts its physiological impact by activating AT-1 and AT-2 receptors. Conversely, ACE2 functions as a counterbalancing enzyme, degrading Ang-II, a potent vasoconstrictor, into angiotensin 1-7 (Ang1-7), a vasodilator. The crucial role of Ang1-7 in maintaining homeostasis stems from its multifaceted properties, including anti-inflammatory, anti-coagulant, anti-proliferative, and anti-fibrotic attributes (Möhlendick et al., 2021; Oscanoa et al., 2021; Yamamoto et al., 2021).

ACE2 is distributed across various anatomical sites within the human body and exhibits considerable resemblance to ACE1. The advantageous biological effects of ACE1 can offset the deleterious consequences of the renin-angiotensin-aldosterone system in numerous pathological conditions. Conversely, ACE2 serves as a modulator of ACE1's effects by acting as a vasodilatory factor. These enzymes engage in competitive activities, and the maintenance of equilibrium between ACE1 and ACE2 assumes pivotal significance in the context of coronary disease severity (Rezaiezadeh et al., 2022). Any perturbation in this delicate balance and disruption in the regulatory dynamics of the renin-angiotensin-aldosterone system appear to exert a notable impact on the initiation of COVID-19 (Yamamoto et al., 2021).

Amid the COVID-19 pandemic, a notable gene that has been subjected to intense scrutiny is ACE1 (OMIM 106180), situated at chromosome 17q23.3, and also referred to as DCP, DCP1, and CD143, consisting of 26 exons. A specific ACE1 variant garnering attention in the context of COVID-19 is the insertion/deletion (I/D) polymorphism,

associated with a 287-bp ALU repeat in intron 16. This polymorphism culminates in three genotypes: homozygous II, homozygous DD, and heterozygous ID. Previous investigations have established an association of ACE1 polymorphism with several comorbidities, including hypertension, hypertrophic cardiomyopathy, obesity, and myocardial infarction, all factors known to augment the severity of COVID-19 (Aziz & Islam, 2022; Rezaiezadeh et al., 2022).

2. LITERATURE REVIEW

2.1 General Information on SARS-COV-2

2.1.1 Definition

SARS-CoV-2, identified as the etiological agent responsible for the COVID-19 disease, is recognized as a prominent menace to global human health in the contemporary era, attributed to its significant rates of morbidity and mortality (Yamamoto et al., 2021; Aziz & Islam, 2022). Analogous to other human coronaviruses (hCoVs), SARS-CoV-2 is an enveloped entity that possesses a positive-sense RNA genome, which is a single-strand (+ssRNA) in nature (Figure 2.1) (Rossi et al., 2020; Aziz & Islam, 2022; Albashir, 2021). The host range of this virus encompasses humans and a variety of animal species (Albashir, 2021). SARS-CoV-2, like other viruses that cause respiratory infections, propagates chiefly through the respiratory pathway, demonstrating high transmission efficiency and infectivity. While droplet transmission is the primary mode of spread, aerosols could also play a significant role in its propagation (Ciotti et al., 2020). Infections originating from human coronaviruses have the capacity to impact a broad range of human organ systems, encompassing the respiratory, gastrointestinal, liver, and central nervous systems (Rossi et al., 202).

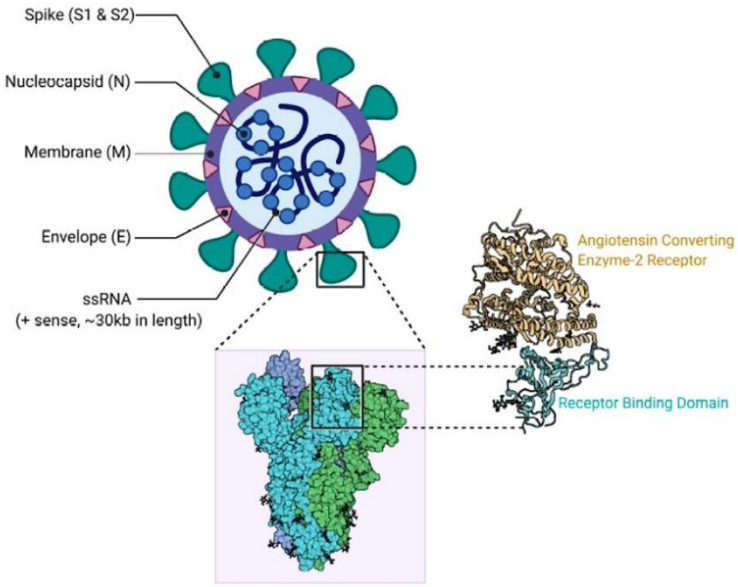

Figure 2.1. Structure of SARS-COV-2 and ACE2 (Hasöksüz et al., 2020).

SARS-CoV-2 gains entry into the human body by attaching to the angiotensin-converting enzyme 2 (ACE2) receptors situated on the surface of host cells (Aziz & Islam, 2022; Yamamoto et al., 2021; Albashir, 2021; Goren et al., 2022; Shoily et al., 2021; Hussain et al., 2022). ACE2 is a decisive enzyme in the renin-angiotensin-aldosterone system (RAAS) (Figure 2.1) (Salamanna et al., 2020). The ACE2 protein is present in various organs, such as the heart, testis, kidney, lungs, nasal passages, oral mucosa, and nasopharynx (Salamanna et al., 2020; Möhlendick et al., 2021). To maintain physiological balance, ACE2 destroys angiotensin II (Ang II) which is a product of ACE, into angiotensin 1-7 (Möhlendick et al., 2021; Aziz & Islam, 2022; Verma et al., 2021). However, SARS-CoV-2's entry into cells prevents this degradation process and results in increased levels of Ang II (Salamanna et al., 2020; Shoily et al., 2021). Elevated levels of Ang II can lead to direct pro-atherosclerotic effects and promote monocyte and endothelial cell activation, which contributes to atherosclerotic plaque formation. Moreover, Ang II induces the generation of proinflammatory cytokines and chemokines via Toll-like receptor 4 (TLR4) (Figure 2.2) (Shoily et al., 2021). The clinical spectrum of the disease encompasses diverse presentations, ranging from asymptomatic and mild cases to the development of severe pneumonia leading to acute respiratory distress syndrome (ARDS) (Albashir, 2021; Rezaiezadeh et al., 2022; Verma et al., 2021; Goren et al., 2022).

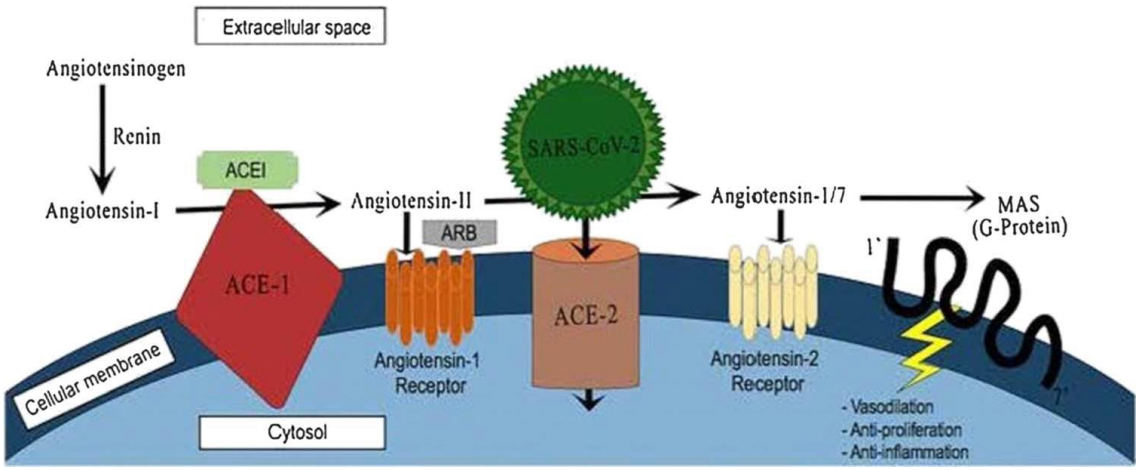

Figure 2.2. Mechanism of working ACE1/ACE2 (Albashir, 2021).

2.1.2 Classification

Human coronaviruses are viruses that have a membrane surrounding them and a genome is a single-stranded RNA that is non-segmented and has a positive sense. They

are mainly found in vertebrates (Kirtipal et al., 2020; Singh & Yi, 2021). Compared to other RNA viruses, the genome of hCoVs is relatively large, with an average size of about 30 kilobases (Rossi et al., 2020; Hasöksüz et al., 2020; Astuti, 2020). The current taxonomic framework categorizes 39 coronavirus species into 27 subgenera, distributed among 5 genera and 2 subfamilies within the family *Coronaviridae*. These taxonomic assignments fall within the suborder *Cornidovirineae*, order *Nidovirales*, and realm *Riboviria* (Kirtipal et al., 2020). The family *Coronaviridae* includes the subfamily *Coronavirinae*, consisting of four genera: Alphacoronavirus (Alpha-CoV), *Betacoronavirus* (Beta-CoV), *Gammacoronavirus* (Gamma-CoV), and *Deltacoronavirus* (Delta-CoV), as stipulated by the International Committee for Taxonomy of Viruses. Notably, both SARS-CoV and SARS-CoV-2 are classified within the *Betacoronavirus* genus (Kirtipal et al., 2020; Hasöksüz et al., 2020; Singh & Yi, 2021).

Alphacoronaviruses and *Betacoronaviruses* are predominantly found in mammalian species, whereas *Gammacoronaviruses* and *Deltacoronaviruses* are commonly associated with avian hosts. However, it is noteworthy that *Gammacoronaviruses* have been documented to infect specific cetaceans, including beluga whales and bottlenose dolphins. Notably, recent outbreaks of human diseases, including SARS, MERS, and SARS-CoV-2, can be linked to the *Sarbecovirus* subgroup within *Betacoronavirus*. According to Kiritipal et al., MERS is classified under the *Merbecovirus* subgenera. *Alphacoronavirus* includes HCoV- 229E and HCoV-NL63, two of the four previously identified coronavirus strains known to cause mild common cold symptoms in humans. HCoV-OC43 and HCoV-HKU1, on the other hand, fall under another *Betacoronavirus* subgroup, *Embecovirus* (Figure 2.3) (Kirtipal et al., 2020; Singh & Yi, 2021). SARS-CoV-2 comprises of 4 primary protein structures: spike glycoprotein, envelope glycoprotein, membrane glycoprotein, and nucleocapsid protein, also with various accessory proteins (Astuti, 2020; Singh & Yi, 2021; Rossi et al., 2020; Hasöksüz et al., 2020; Hu et al., 2021). Prior to the rise of SARS-CoV, the construction of CoVs' phylogenetic trees typically used the Polymerase (Pol) or Nucleocapsid (N) gene. This approach originally classified SARS-CoV as a GammaCoV member. Further investigation into the amino-terminal domain of the SARS-CoV spike protein uncovered a structural similarity, with 19 out of 20 cysteine residues resembling those in the

BetaCoV group. In contrast, only five conserved residues aligned with the AlphaCoV and *GammaCoV* groups (Kirtipal et al., 2020).

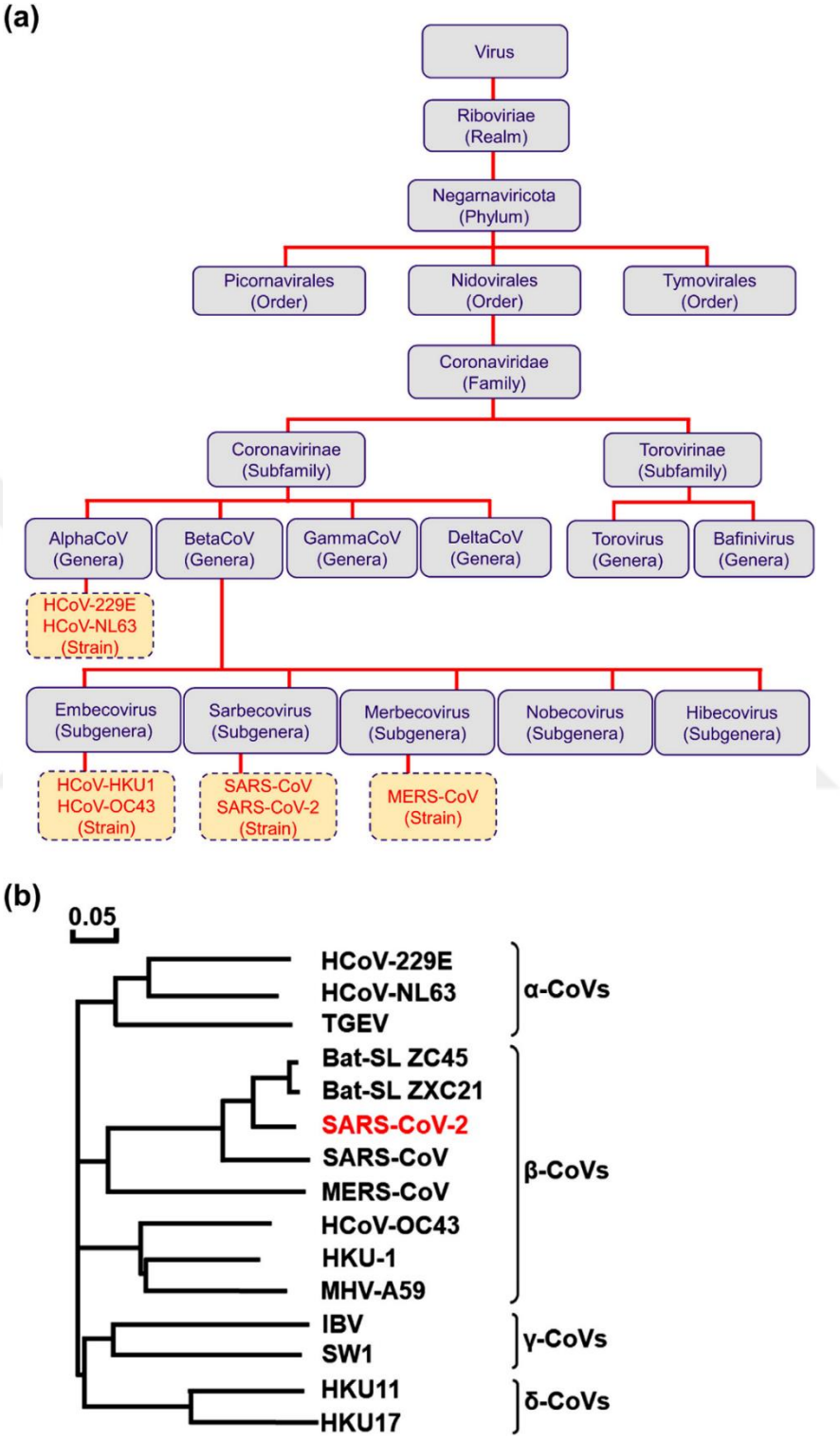

Figure 2.3. Classification of Coronaviruses: a) Taxonomic categorization of the seven identified HCoVs. b) Phylogenetic tree analysis of CoVs built using the Spike gene. (Kirtipal et al., 2020).

2.1.3 Diagnosis

Patients who infected through SARS-CoV-2 may manifest different symptoms, including but not limited to fever, fatigue, dry cough, and respiratory distress, coupled with possible nasal congestion or a runny nose. While mildly affected individuals might not display apparent symptoms, those exhibiting severe signs may suffer from breathlessness, moist rales within their lungs, diminished breathing sounds, and dullness upon percussion, along with increased or decreased tactile vocal fremitus (Wu, Liu, & Yang, 2020). Typically, COVID-19 presents as an acute viral infection of the respiratory tract. Consequently, medical professionals should entertain a broad spectrum of potential differential diagnoses associated with typical viral pneumonia, Examples of respiratory infections encompass influenza, *parainfluenza*, *adenovirus* infection, respiratory syncytial virus infection, *metapneumovirus* infection, as well as atypical pathogens like *Mycoplasma pneumoniae and Chlamydophila pneumoniae* infections (Wu et al., 2020). There are several ways to diagnosis COVID-19, and every one of them comes with its limitations.

In the battle against COVID-19, a pivotal measure involves the expeditious and precise detection of SARS-CoV-2, primarily accomplished through the application of real-time reverse transcription-polymerase chain reaction (RT-PCR) (Kevadiya et al., 2021). Currently, this method is considered the standard for detecting SARS-CoV-2 (Chau et al., 2020; Hu et al., 2021; Kevadiya et al., 2021; Wu, Liu, & Yang, 2020; Yüce et al., 2021) due to its capacity to directly analyze the genomic components of the virus.

RT-PCR, a specialized form of PCR, was developed specifically for the detection of (genomic) RNA. It is characterized by its rapidity and high efficiency, capable of producing results within hours while concurrently processing numerous samples (Yüce et al., 2021). The presence of SARS-CoV-2 can be identified in diverse respiratory samples, including throat swabs, posterior oropharyngeal saliva, and nasopharyngeal swabs (Chau et al., 2020), as well as in sputum and bronchial fluid. It is noteworthy that the viral load is generally more concentrated in specimens obtained from the lower respiratory tract (Wu, Liu, & Yang, 2020).

2.1.4 Therapy

Treatment of COVID-19 cannot be accomplished with only one drug, as the disease is characterized by different phases, requiring different therapeutic approaches. The viral replication phase is the very first phase, where drugs inhibiting viral replication can be effective. The inflammatory phase is the second phase, where the immune system's excessive response causes damage to the individual. Therefore, drugs that reduce the immune system response can be useful. Without understanding the different phases, administering drugs can worsen the disease. Drug development for COVID-19 can be classified into four main categories: repurposing or repositioning drugs, monoclonal antibody, convalescent plasma therapy, (Seungtaek, 2022) and vaccines.

2.2 SARS-CoV-2 Epidemiology

In December 2019, an emergent strain of coronavirus was identified in Wuhan, Hubei Province, China. Initially designated as 2019-nCoV, this pathogen is the etiological agent responsible for the ongoing COVID-19 outbreak, marked by an increasing prevalence of pneumonia cases (Wu et al., 2020). On February 11th, 2020, the World Health Organization (WHO) officially designated the illness caused by this novel coronavirus as coronavirus disease 2019. Then the virus named by the International Committee on Taxonomy of Viruses (ICTV) as severe acute respiratory syndrome coronavirus 2 (Wong, 2020). The propagation of COVID-19 cases rapidly intensified on a global scale, and the WHO in March 2020, officially announced the disease a pandemic (Salzberger et al., 2020; Wong, 2020).

2.2.1 Symptoms

Infections with SARS-CoV-2 can display a broad range of clinical outcomes, ranging from no symptoms to mild or severe disease culminating in multiple organ dysfunction (Salzberger et al., 2020). Fever, cough, and dyspnea are the utmost commonly reported clinical indicators, with an incidence of 83%, 82%, and 31% respectively among affected patients (Ciotti et al., 2020). The risk of severe disease manifestation can be enhanced by some factors such as advanced age, hypertension, pre-

existing cardiac or pulmonary disease, and immunodeficiency. Between different demographic cohorts, hospitalization rates fluctuate between 4 and 7%. Approximately one-quarter of hospitalized patients necessitate intensive care, with a substantial proportion requiring organ support therapy, 75% necessitating mechanical ventilation and 25% requiring renal replacement therapy (Salzberger et al., 2020).

2.2.2 Age distribution

The age group with the highest number of cases in most countries is between 20 and 59 years old. During all outbreaks, there have been relatively few infected children in the age group of 0-9 years old (Figure 2.4) (Salzberger et al., 2020).

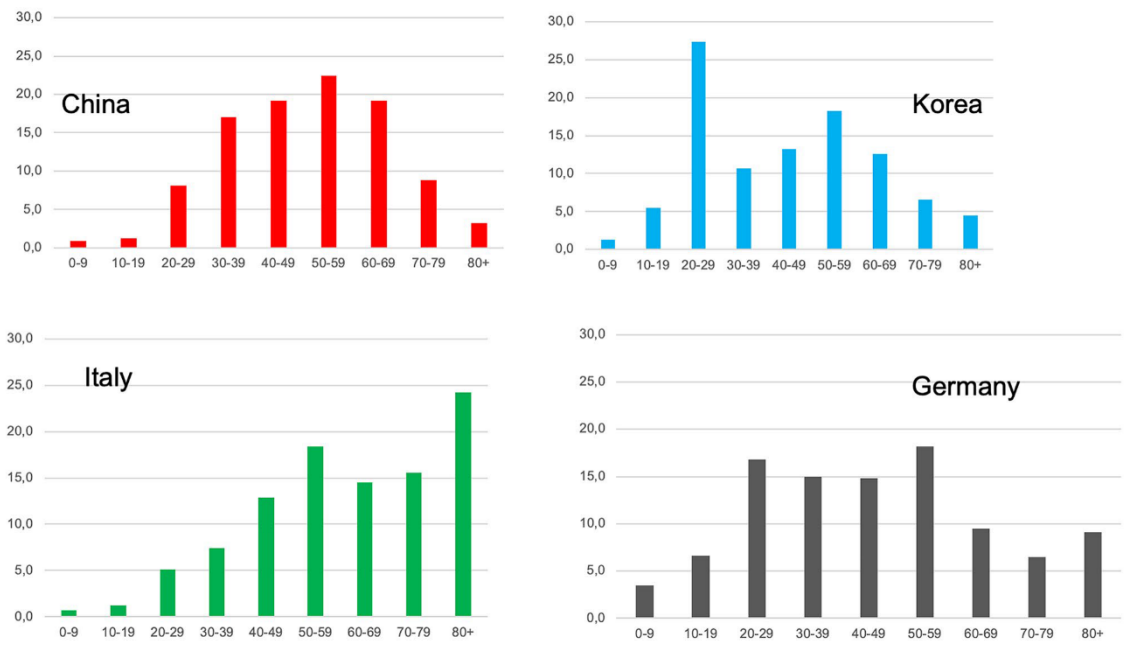

Figure 2.4. Age distribution of cases in China, Korea, Italy and Germany (Salzberger et al., 2020).

2.2.3 Transmission

As with SARS-CoV, it is possible that the virus may be transmitted through the oral-fecal route (Ciotti et al., 2020; Salzberger et al., 2020). In a study in 2020, Fuk- Woo et al. documented a family cluster of five patients, providing confirmation on person-to-person transmission of COVID-19 (Wu, Liu, & Yang, 2020). There is a high probability

that person-to-person transmission of the virus occurs through droplets and physical contact (Wu, Liu, & Yang, 2020; Wu et al., 2020; Salzberger et al., 2020). Health authorities have detected indications of human-to-human transmission of the virus that has continued over four "generations", indicating that the virus has spread from a non-human source to a person, who subsequently infected another individual, who in turn infected yet another person (Wu, Liu, & Yang, 2020). Individuals who show no symptoms of infection can also transmit SARS-CoV-2 (Wu, Liu, & Yang, 2020; Salzberger et al., 2020).

2.2.4 Incubation Period and Serial Interval

The incubation period of SARS-CoV-2 has demonstrated variability across different studies and geographical locations. An investigation into the very first 425 confirmed cases in Wuhan in January 22, 2020, identified an average incubation period of 5.2 days. In contrast, another study reported a median incubation period of 5.1 days, in which 181 cases were involved. The serial interval, or the average duration from the time an individual is infected to the point they infect others, was estimated to average 7.5, 4.0, and 5.81 days in three separate studies (Wong, 2020).

2.2.5 Reproduction Number

The reproductive number, a critical threshold parameter in disease transmission research, is a ratio (Wang et al., 2023). The basic reproductive number, or R_0, denotes the average number of people to whom an infected person can transmit the disease in a wholly susceptible population. A comprehensive understanding of R_0 is pivotal to fully appreciate the transmissibility of SARS-CoV-2. An R_0 value exceeding 1 suggests exponential disease spread, while a value less than 1 suggests a slow, diminishing rate of infection spread. For instance, an R_0 value of 2 indicates that one infected individual can potentially infect two others (Wong, 2020).

An early study into the coronavirus outbreak utilized likelihood and model analysis to project a basic reproductive number as high as 6.47, indicating a high infectiousness of COVID-19 (Wang et al., 2023). In their study, Liu et al. (Liu et al.,

2020) scrutinized the R_0 value of COVID-19 by examining 12 relevant studies. The R_0 was estimated to range from 1.4 to 6.49 (Wong, 2020).

2.3 SARS-CoV-2 Associated Systemic Diseases

The emergence of SARS-CoV-2 presents a substantial threat to human health, not only inducing adverse effects on the respiratory system leading to pneumonia or acute respiratory distress syndrome (ARDS) but also impacting various physiological systems (Koźlik et al., 2022). SARS-CoV-2 has the capacity to inflict damage on the cardiac muscle, giving rise to myocarditis (Hafiane, 2020). Moreover, heart failure serves as a significant predisposing factor for the progression of severe disease and increased mortality rates associated with COVID-19, and may also manifest as a complication of the disease (Szpukal et al., 2023). SARS-CoV-2 can have impact on gastrointestinal tract. ACE2 is found in various organs, for example the nasopharynx, esophagus, stomach, intestines, liver, and pancreas (Khreefa et al., 2023). Around 50% of those who were infect with SARS-CoV-2 have had detectable viral RNA in their stool (Szpukal et al., 2023; Khreefa et al., 2023), it confirms that the virus is present in the intestines of infected individuals and is capable of replication in this area. Literature frequently reports gastrointestinal symptoms among patients with SARS-CoV-2 infections, including diarrhea (Khreefa et al., 2023), abdominal pain, abdominal distension, anorexia, loss of appetite, nausea, and vomiting. The frequency of these symptoms varies from 2% to approximately 79% (Szpukal et al., 2023). In certain cases, the virus has the potential to penetrate the CNS, precipitating neurological afflictions (Zhang & Jin, 20023). Some common symptoms include headaches (Abboud et al., 2020; Zhang & Jin, 2023; Czarnowska et al., 2023), dizziness (Abboud et al., 2020; Zhang & Jin, 2023), fatigue (Abboud et al., 2020; Czarnowska et al., 2023), loss of smell or hyposmia (Zhang & Jin, 2023; Czarnowska et al., 2023; Abboud et al., 2020; Jha et al., 2021), hypogeusia, visual dysfunction, encephalopathy (Abboud et al., 2020; Zhang & Jin, 2023; Czarnowska et al., 2023), stroke, sleep disorder, ageusia, coma, seizures (Czarnowska et al., 2023), skeletal muscle symptoms, impaired consciousness, and acute cerebrovascular disease (Jha et al., 2021; Czarnowska et al., 2023). Along with systems, the renal system (Maghool et al.,

2021; Akbarialiabad et al., 2021) and endocrine systems (Lundholm et al., 2020; Ogarek et al., 2022) have also been reported to be affected by SARS-CoV-2.

2.4 Covid-19 Etiology

A comprehensive examination of the entire SARS-CoV-2 genome reveals a genetic resemblance of more than 70% with the SARS-CoV viruss (Lamers & Haagmans, 2022; Hussain et al., 2022) and over 95% similarity with a coronavirus found in bats (Hussain et al., 2022). Coronavirus is a type of virus that has a single strand of ribonucleic acid and is enveloped. It gets its name from the appearance of its surface spikes, which resemble the solar corona, and these spikes are about 9-12 nanometers in length (Rauf et al., 2020). The size of Coronaviruses can vary between 60 and 140 nanometers in diameter (Hussain et al., 2022; Lamers & Haagmans, 2022). The coronaviral genome encodes four primary structural proteins on its envelope (Rauf et al., 2020; Szpukal et al., 2023). These components are recognized as the spike, envelope, membrane, and nucleocapsid proteins (Szpukal et al., 2023). The spike (S) protein assumes a crucial role in mediating viral entry into the host cell by binding to the angiotensin-converting enzyme 2 (ACE2) receptor and facilitating the fusion between the viral envelope and host cell membranes (Rauf et al., 2020; Szpukal et al., 2023).

According to available information, COVID-19 may have originated in bats and then transmitted to humans, potentially through pangolins (Ciotti et al., 2020; Rauf et al., 2020; Devaux et al., 2020) or other wild animals that were sold at the Huanan seafood market. The virus then appears to have spread from person-to-person.

2.5 Pathogenesis of COVID-19

Our understanding of the pathological phases of COVID-19 remains partial at this point (Li et al., 2022). However, we can leverage our knowledge of how SARS-CoV infection develops to make predictions about the pathogenesis of COVID-19 (Woodby et al., 2021). In earlier research, it was suggested that SARS could be divided into three stages: viral replication, immune hyperactivity, and pulmonary destruction. More recently, a new framework has been put forward to describe the clinical phases of COVID-19, which include the viremia phase, acute phase, and recovery phase (Li et al.,

2022). It is thought that the transmission of COVID-19 happens when respiratory droplets and/or aerosol particles are inhaled, when people come into contact with contaminated surfaces (fomites), or when people have close contact with each other (Woodby et al., 2021; Jin et al., 2020). It is theorized that the primary replication of the virus occurs within the mucosal epithelium of the upper respiratory tract, encompassing the nasal cavity and pharynx. Following this, the virus undergoes subsequent multiplication within the mucosa of the lower respiratory tract and gastrointestinal tract (Jin et al., 2020). The S protein, a crucial determinant for the virus's entry into host cells, is composed of two segments: S1 and S2 subunits (Lamers & Haagmans, 2022; Woodby et al., 2021). Specifically, the S1 subunit binds to ACE2, while the S2 subunit anchors the spike protein to the cellular membrane (Jackson et al., 2022; Lamers & Haagmans, 2022).

The glycoprotein, the envelope spike protein, latches onto a specific receptor, ACE2, in the situation of SARS-CoV-2 (Lamers & Haagmans, 2022). ACE2 protein is prevalent on diverse human epithelial surfaces, including the upper and lower parts of respiratory tract, gastrointestinal tract, and endovascular epithelia (Howard-Jones et al., 2022). Moreover, the ACE2 receptor is widely distributed in various organs including the nasal mucosa, bronchus, lungs, heart, esophagus, kidneys, stomach, bladder, and ileum. This widespread expression makes these tissues susceptible to invasion by SARS-CoV-2 (Jin et al., 2020). Upon successful anchoring of the spike protein to the ACE2 receptor on the targeted cell, it undergoes cleavage at the S2' site by the transmembrane serine protease (TMPRSS2), thereby initiating the activation of the S2 subunit trimers. This activation event facilitates the fusion of the viral and host lipid bilayers, thereby enabling the release of the viral ribonucleoprotein complex into the cell (Lamers & Haagmans, 2022). Subsequent to this event, the viral genome is unleashed into the cytoplasm, kick-starting the replication process within the host cells (Li et al., 2022). Following entry into the host cell, the positive-sense genome of SARS-CoV-2 promptly initiates the synthesis of viral proteins, including replicase proteins that form replication organelles derived from the membranes of the endoplasmic reticulum (Lamers & Haagmans, 2022). The interval between initial infection and the onset of symptoms spans from 2 to 14 days, with respiratory symptoms typically manifesting 3 to 7 days post-exposure (Woodby et al., 2021).

Once the virus infiltrates the host cells, it duplicates and assembles itself before being released outside to target other cells, leading to the direct harm and deterioration of parenchymal cells, like the alveolar epithelial cells. Concurrently, a substantial release of pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules occurs, eliciting the innate immune response. This prompts the infiltration of inflammatory cells, the release of elevated levels of cytokines, chemokines, proteases, and free radicals, culminating in the development of acute respiratory distress syndrome (ARDS), sepsis, and multiple organ dysfunction syndrome (Li et al., 2022).

ARDS is a severe lung condition that hinders the adequate flow of oxygen to the lungs and its circulation throughout the body, contributing to the mortality associated with various respiratory diseases and acute lung injuries. Examinations of over 40 candidate genes, such as ACE2, interleukin 10 (IL-10), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF), among others, have been conducted to investigate their potential associations with the onset or outcome of acute respiratory distress syndrome (ARDS) (Jin et al., 2020).

Upon gaining entry into the pulmonary region, the SARS-CoV-2 pathogen can penetrate type II pneumocytes. The virus exhibits rapid proliferation by effectively circumventing the host immune response, thereby leading to a lag in the activation of native pulmonary macrophages. This circumstance precipitates extensive local inflammation coupled with heightened vascular permeability, which, in turn, draws in monocytes/macrophages and neutrophils, culminating in fluid accumulation within the alveoli. Moreover, upon binding with ACE2, SARS-CoV-2 hinders the conversion of the proinflammatory angiotensin-II to the anti-inflammatory angiotensin (1-7), thus amplifying the inflammation and subsequent damage induced by the virus. The amassed fluid acts as a barrier to air entry into the lungs, resulting in pneumonia, dyspnea, lung damage, and eventually, fatality (Woodby et al., 2021). Older individuals (65 years and above) diagnosed with COVID-19 have consistently exhibited lymphopenia, neutrophilia, elevated inflammation markers, and coagulation indicators when compared to younger and middle-aged counterparts. In instances of severe COVID-19 infection, a greater proportion of proinflammatory macrophages and neutrophils have been identified in the bronchoalveolar lavage fluid (BALF) relative to milder cases (Harrison et al.,

2020). The prolonged and heightened generation of proinflammatory cytokines, termed a cytokine storm, has the potential to cause substantial tissue damage (Woodby et al., 2021). This phenomenon has been observed in severely affected individuals with COVID-19 (Harrison et al., 2020). Variables that amplify the risk of severe pneumonia or death in association with COVID-19 comprise being of age 60 or over, smoking habits, and existing health conditions like diabetes mellitus, hypertension, cardiovascular disorders, chronic pulmonary disease, and cancer (Woodby et al., 2021). Older individuals infected with COVID-19 are vulnerable to more severe symptoms and outcomes, such as higher rates of ICU admission, ARDS development, elevated fever, and increased mortality (Harrison et al., 2020). (Figure 2.5) delineates the suggested mechanism underlying SARS-CoV-2 pathogenesis.

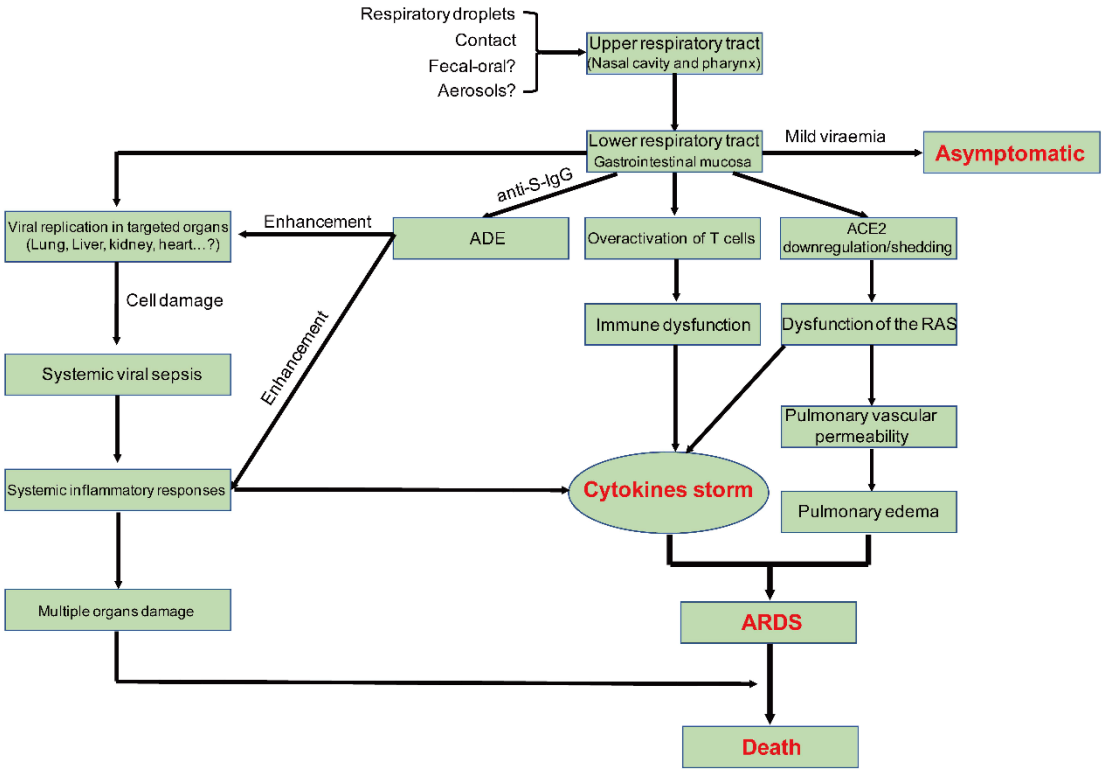

Figure 2.5. The proposed mechanism of SARS-CoV-2 pathogenesis (Jin et al., 2020).

2.6 RAAS (renin-angiotensin-aldosterone system)

Various physiological systems collaborate in an orchestrated manner to ensure the optimal functioning of the human organism. Paramount among these intricate systems is the renin-angiotensin-aldosterone system (RAAS), whose role is critical for human

survival (Patel et al., 2017). It participates indispensably in preserving the body's equilibrium during normal physical activity and is profoundly engaged in maintaining homeostasis beyond its conventional cardiovascular and renal confines (Yamamoto et al., 2021). The RAAS has been widely acknowledged for its significant role in regulating crucial physiological processes including sodium balance, body fluid volume, and arterial pressure (Colafella et al., 2019; Patel et al., 2017).

As implied by its nomenclature, the RAAS incorporates two essential constituents, renin and angiotensin (Patel et al., 2017). Renin is the gatekeeper of the RAAS cascade, the absence of which hampers the generation of angiotensins. Angiotensinogen, the forerunner of all angiotensins, undergoes transformation into angiotensin I by renin. Subsequently, angiotensin-converting enzyme (ACE) catalyzes the conversion of angiotensin I into the central effector peptide of the RAAS, angiotensin II. By stimulating the angiotensin II type 1 (AT1) receptor, angiotensin II orchestrates the classical actions of the RAAS, encompassing vasoconstriction, water and sodium retention, aldosterone synthesis, and the incitement of pro-inflammatory, growth, and remodeling processes (Colafella et al., 2019). Additionally, the RAAS carries out a multitude of beneficial functions, including the provision of anti- inflammatory, anticoagulant, and antifibrotic effects, while safeguarding endothelial and neural function. As such, an imbalance between the ACE1/ACE2 arms and disturbances in RAAS homeostasis may bear considerable significance, particularly in the early stages of COVID-19 (Yamamoto et al., 2021).

2.7 ACE gene

ACE1 is situated on the long arm (q) of chromosome 17, specifically in subregion 23.3 (Aziz & Islam, 2022; Lahtela et al., 2017; Verma et al., 2021), and comprises 26 exons (Verma et al., 2021). It plays a crucial role in regulating vascular balance and is implicated in the development of hypertension and atherosclerosis (Mostafa et al., 2016). With diverse biological functions, ACE1 can alleviate the detrimental effects of the renin-angiotensin-aldosterone system in various illnesses (Rezaiezadeh et al., 2022). As a component of the RAAS, ACE contributes to maintaining blood pressure homeostasis (Marei et al., 2023; Patel et al., 2017). Its function involves the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) (Marei et al., 2023; Calabrese et al., 2021; Lahtela et

al., 2017; Aladag et al., 2021), which is a potent vasoconstrictor and the primary effector molecule of the RAAS (Lahtela et al., 2017). Ang II has a half-life of around 30 seconds (Patel et al., 2017) and interacts with the angiotensin II type 1 receptor (AT1R) to induce strong vasoconstriction and activate pro-inflammatory, pro- apoptotic, and pro-fibrotic pathways in the lung and other organs (Marei et al., 2023) Functioning within the RAAS, ACE2 plays a crucial role in mitigating Ang II by facilitating its breakdown into Ang-1-7 (Marei et al., 2023; Calabrese et al., 2021; Verma et al., 2021; Aladag et al., 2021). This enzymatic process leads to vasodilation and triggers anti-inflammatory, anti-fibrotic, and anti-thrombotic effects through the Ang-1-7/Mas receptor pathway. Consequently, ACE2 naturally counteracts the impacts of Ang II/AT1R (Marei et al., 2023) (Figure 2.6).

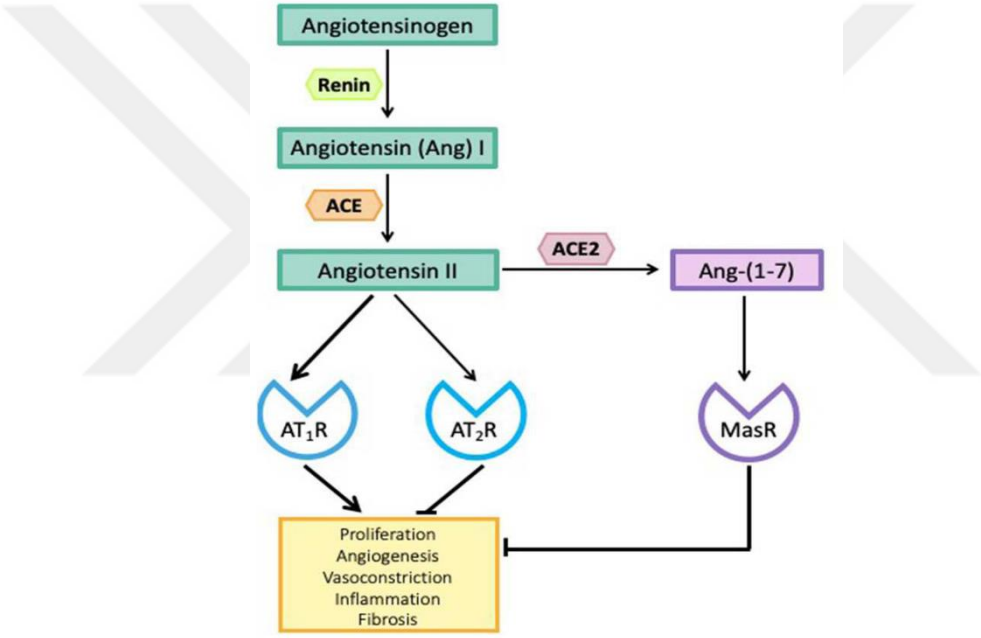

Figure 2.6. Mechanism of working angiotensinogen (Lumbers et al., 2020).

The scientific literature has recorded various genetic alterations within the ACE gene (Calabrese et al., 2021). One of the most extensively researched human polymorphisms is the ACE insertion (I)/deletion (D) polymorphisms (Aladag et al., 2021; Marei et al., 2023). This polymorphism is characterized by the presence (insertion, allele I) or absence (deletion, allele D) of a 287-base pair (bp) Alu repeat sequence in intron 16 (rs1799752) (Calabrese et al., 2021). It presents in three genotypic forms: heterozygous ID and homozygous II and DD (Rezaiezadeh et al., 2022; Aziz & Islam, 2022). Accumulating evidence indicates that the ACE1 I/D polymorphism plays a pivotal role

in determining the plasma level of the ACE protein (Rezaiezadeh et al., 2022; Aladag et al., 2021). The occurrence of the ACE D allele gives rise to augmented ACE1 levels and diminished ACE2 levels, leading to an increase in angiotensin II. This surge contributes to the genesis of pulmonary edema by amplifying microvascular permeability, potentially exacerbating the clinical trajectory and prognosis in conditions like acute respiratory distress syndrome (ARDS) (Aladag et al., 2021; Marei et al., 2023).

ACE1 plays a regulatory role in modulating the expression and function of ACE2 by influencing Ang II levels. In individuals infected with SARS-CoV-2, a notable elevation in plasma Ang II levels has been observed compared to those in healthy subjects. Moreover, the concentration of Ang II exhibits a robust correlation with both viral load and pulmonary injury in individuals affected by SARS-CoV-2, where increased viral load is associated with heightened disease severity and mortality. These findings suggest that SARS-CoV-2 may act as a precipitating factor for the observed imbalance in the renin-angiotensin-aldosterone system (RAAS) in patients (Yamamoto et al., 2021). The available data indicate that SARS-CoV-2 disrupts the natural equilibrium of ACE1/ACE2, prompting activation of the Angiotensin II/Angiotensin II type 1 receptor (AT1R) pathway, potentially amplifying the detrimental impacts of COVID-19 (Figure 2.7). There exists substantial evidence supporting a notable association between COVID-19 and the ACE1-insertion/deletion (I/D) polymorphism (Akbari et al., 2022). A hypothesis posits that genetic variations within the ACE1 gene may impact the severity of COVID-19 by influencing the virus's entry mechanism into host cells (Rezaiezadeh et al., 2022). Numerous studies have extensively investigated the potential correlation between ACE1 I/D polymorphism and susceptibility to severe COVID-19 infection. The majority of these inquiries have indicated that individuals with the ACE1 DD genotype are more predisposed to experiencing severe symptoms of COVID-19 (Aladag et al., 2021; Marei et al., 2023; Calabrese et al., 2021; Najafi & Mahdavi, 2022; Rezaiezadeh et al., 2022; Aziz & Islam, 2022; Mateos et al., 2023) compared to those carrying the ID and II genotypes. Notably, one study suggested that the presence of the D allele, in both homozygous and heterozygous forms, could increase the risk of severe COVID-19 (Aladag et al., 2021). However, some investigations did not find the II genotype to be significantly associated with severe COVID-19 disease (Rezaiezadeh et al., 2022; Yamamoto et al., 2021), and in certain instances, there might even be an inverse

relationship (Calabrese et al., 2021). Among the considerable research conducted on the association between ACE1 I/D polymorphism and the severity of COVID-19, only two studies have explored this relationship, one by Çelik et al. (Çelik et al., 2021) and another by Möhlendick et al. (Möhlendick et al., 2021), both of which did not identify a significant correlation. Conversely, Delanghe et al. (Delanghe et al., 2020) did not observe any association between DD genotype frequency and COVID-19 mortality. These findings suggest a relationship between ACE1 expression levels and genotype, with the DD genotype exhibiting the highest levels of ACE1 in serum/tissue, the ID genotype displaying moderate levels, and the II genotype demonstrating the lowest levels (Gemmati et al., 2020; Calabrese et al., 2021).

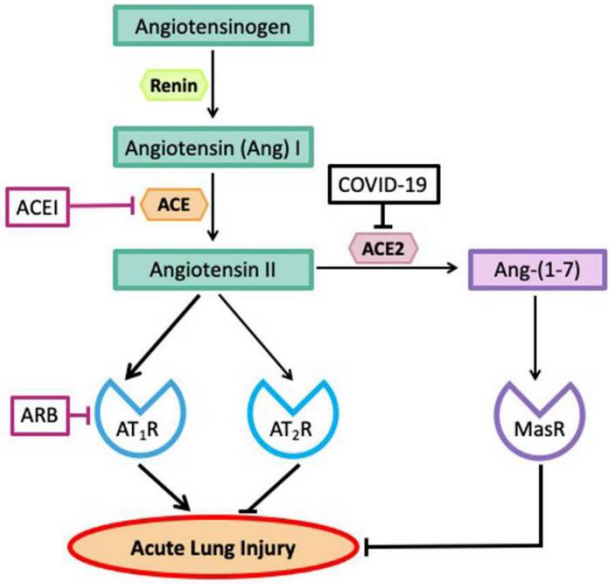

Figure 2.7. SARS-COV-2 disrupts the natural equilibrium of ACE1/ACE2 (Lumbers et al., 2020).

Numerous studies have examined the I/D polymorphism in the Turkish population. Within a cohort of 1063 healthy volunteers, the prevalence of the D allele of the ACE gene was determined to be 60.1% (Berdeli et al., 2009). Another investigation revealed a 29.5% prevalence of the DD genotype among healthy individuals in Turkey, a rate comparable to that observed in the majority of European populations (Tokgözoğlu et al., 1997). Recent studies posit ACE1 I/D polymorphism as a notable geographical variant and a potential genetic marker indicative of susceptibility and pathogenicity in the context of COVID-19 (Faridzadeh et al., 2022). Additionally, the D allele of the ACE1 I/D

polymorphism has been implicated in the progression of SARS-CoV-1, sharing a 70% similarity with SARS-CoV-2 (Itoyama et al., 2004; Lamers & Haagmans, 2022). The current investigation aims to elucidate whether a correlation exists between the severity of the disease and ACE gene polymorphism, particularly the D allele, which has a high prevalence in the Turkish population.

3. MATERIAL AND METHODS

3.1 Study setting and sampling

The ACE I/D polymorphism was genotyped in a total of 141 Participants, including 100 COVID-19 patients (Study group, n = 100) (Table 3.1) who were tested positive for the presence of SARS-COV-2 virus and applied to Tokat Government Hospital, and they were divided into three groups according to their symptoms, Mild, Inpatient, and Intensive Care. Additionally, 41 healthy participants as a control group participated (Control group, n = 41). The research was conducted in the Medical Biology and Genetics laboratories in Tokat Gaziosmanpasa University. For each of the participants, 3 ml of blood was taken and stored in anticoagulant tube, EDTA (Ethylenediamine tetra acetic acid), waiting for DNA isolation.

Approval from the Ethics Committee for Clinical Research at Tokat Gaziosmanpasa University's Faculty of Medicine was obtained with decision number 23-KAEK-005 for the current study. The project was financially supported by Tokat Gaziosmanpasa University's Scientific Research Center under project number 2023/59.

Table 3.1. Our study sample size for different groups

Symptoms	Number of samples
Mild	42
Intensive Care	43
Inpatient	15
Control	41

3.2 Chemical Materials, Kits and Kit contents

GeneAll® ExgeneTM Blood SV mini (105-101/105-152)

1. Proteinase K

2. RNase A

3. Buffer BL

4. Ethanol

5. Buffer BW

6. Buffer TW

7. Buffer AE

3.3 Devices used in the study

1. -20 ° C freezer (Arcelik, 2052DY, Turkey)

2. -20 ° C freezer (Profilo)

3. Power Supply (Biorad, 1645070, USA)

4. Microwave (Arcelik, MD554, Turkey)

5. PCR Thermal Cycler (Analytik Jena‘s Biometra, Germany)

6. Centrifuge (Hettich, D78532, Germany)

7. Vortex (Velp Scientifica, F20220176, Italy)

8. Kern ABJ 220-4NM Analitik Terazi 220 gr /0,0001 gr

9. Gel Imaging Device (Prizmalab)

3.4 DNA Isolation

In DNA isolation for the blood samples, GeneAll® ExgeneTM Blood SV mini (105-101/105-152) was used according to the manufacturers protocol:

I. Blood Lysate Protocol

Blood Lysate Protocol was meticulously implemented for DNA isolation from blood samples, ensuring the extraction of high-quality genetic material.

1. 20 µl of Proteinase K solution was add into the bottom of each 1.5 ml microcentrifuge tubes.
2. 200 µl of blood samples transferred into the tubes.
3. 20 µl of RNase A solution was add to the samples.

4. Mix the blood and enzymes by pipetting 2-3 times, then incubate the mixture at room temperature for 2 minutes.
5. 200 µl of Buffer BL (lysis buffer) was add to the tube. Vortex the tube to mix thoroughly.
6. Samples were incubated at 56˚C for 10 min inside water bath.
7. Add 200 µl of absolute ethanol to the samples, then vortex the samples thoroughly to ensure proper mixing.

II. Purification Protocol

The Purification Protocol employed stringent procedures to refine the isolated DNA, removing contaminants and enhancing its quality for accurate downstream analyses.

1. Transfer the mixture to Column Type G, centrifuge above 8,000 rpm for 1 minute, and replace the collection tubes with new ones.
2. 600 µl of Buffer BW (Washing Buffer) added to the samples.
3. Centrifuge the samples above 8,000 rpm for 1 minute, and subsequently replace the collection tubes with new ones.
4. 700 µl of Buffer TW (Tris-Wash Buffer) applied to the samples.
5. Samples were centrifuged above 8,000 rpm for 1 min.
6. Discard the pass-throughs and reinsert the mini columns back into the collection tube.
7. Samples were centrifuged at full speed for 1 min to remove residual wash buffer.
8. The mini columns were placed in a fresh 1.5 ml microcentrifuge tubes.
9. 200 µl of Buffer AE (Elution Buffer) was added to the samples, then incubated for 1 min at room temperature.
10. Centrifuge at full speed for 1 min, and DNA was ready.

3.5 Chemical and material preparation

3.5.1 Primer preparation

We diluted 100 µl of pre-prepared primers from Sentromer and vortexed for one second. We then transferred 5 µl of the forward primer and 4 µl of the reverse primer into separate tubes. Finally, we added 100 µl of distilled water to each tube and centrifuged

for 5 seconds. Gel electrophoresis tests were conducted to verify the presence of both primers, and was ready for further use in PCR procedure. (Figure 3.1).

3.5.2 TBE preparation

We used TBE (Tris-Borate-EDTA) for preparing Agarose Gel Electrophoresis (AGE) and also to store the AGE in it till further use.

The preparation was done by adding 100 ml of TBE and 900 ml of distilled water into 1000 ml conical flask.

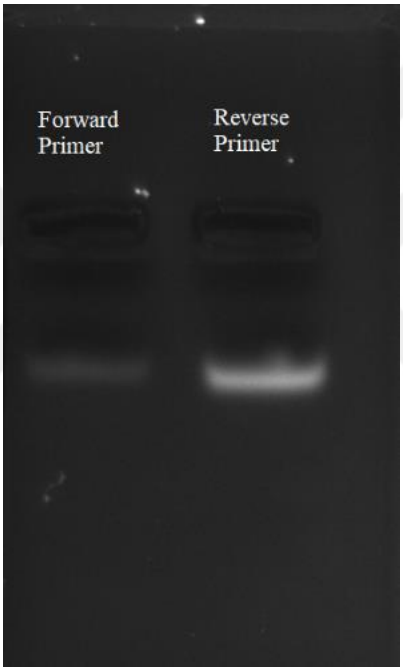

Figure 3.1. Forward and reverse primers in gel electrophoresis

3.5.3 Gel preparation

In Gel preparation 2 g (2%) of Agarose and 100 ml of TBE used, we put both of them inside a 300 ml conical flask, then putting it inside microwave in 360°C for 3 minutes, swirl it during the microwave, till all the agarose dissolves and we would have a clear agarose. After microwave we let the solution to cool down for a minute then adding 8.5 µl ethidium bromide then swirl it so the it will distribute equally and pouring it in the electrophoresis tray to cool down further and firm.

3.5.4 dNTP preparation

We need dNTP (Deoxynucleoside triphosphate) in PCR, which serve as the building blocks for synthesizing new DNA strands. dNTP supplies nucleotides to the unzipped DNA strand, utilizing the template of one side. This process results in the division of a single DNA strand into two, and the amplification continues exponentially as long as the necessary reagents persist, until reaching the final hold stage. For the preparation of it we added 200 µl of distilled water with 50 µl of each dGTP, dCTP, dTTP, and dATP for a tube and mixing it using Centrifuge for 5 seconds.

3.6 Polymerase Chain Reaction (PCR)

For the PCR, MiniAmp™ Plus Thermal Cycler was used and the reaction conducted in a 25 µl reaction volume. The sets of primers utilized in both polymerase chain reactions are presented in (Table 3.2).

Table 3.2. List of Primer sets.

Primers	Sequences (5′ to 3′)
ACE Forward	CTGGAGACACTCCCATCCTTTCT
ACE Reverse	GATGTGGCCATCACATTCGTCAGAT

We prepared a mix for each of the samples, which contained 16 µl of dH_2O, 2.5 µl PCR buffer, 1.5 µl $MgCl_2$, 0.3 µl dNTP, 0.8 µl of each forward primer and reverse primer and 0.1 µl Taq DNA polymerase, and then added to a tube which it has 3 µl of DNA sample.

The amplification protocol entailed an initial denaturation at 94°C for 5 minutes, followed by 33 cycles each comprising denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes. This was concluded by a final extension phase at 72°C for 5 minutes (Table 3.3) (Rigat et al. 1992). 5 µl of the PCR products were mixed with 5 µl bromophenol blue and then the samples were visualized on 2% agarose gel.

Table 3.3. PCR program applied for DNA synthesis (33 cycle)

PCR steps		Temperature (°C)	Duration
Initial Denaturation		94°C	5 minutes
33 cycles	Denaturation	94°C	1 minute
	Primer Annealing	62°C	1 minute
	Extension	72°C	2 minutes
Final Extension		72°C	5 minutes
Cooling		4°C	∞

The 490 base pairs product was indicative of the insert (I), while the 190 base pair product was representative of the deletion (D) (Figure 3.2).

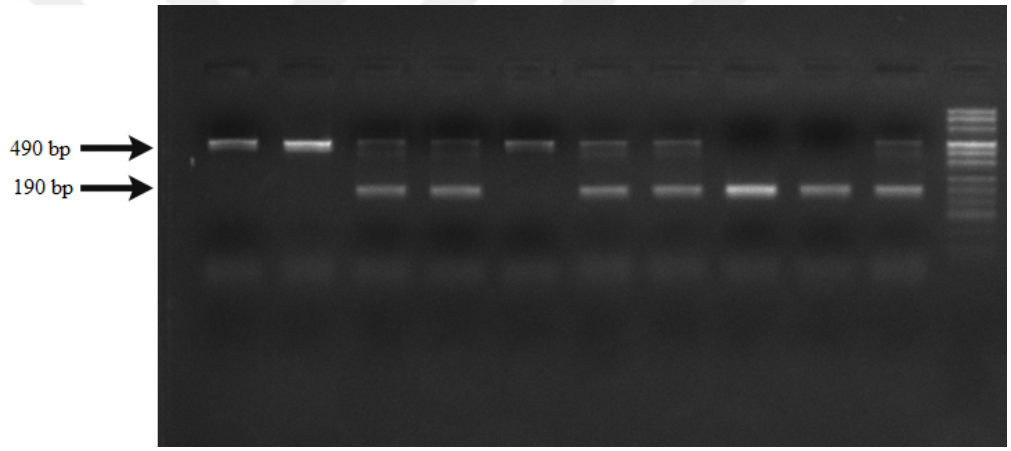

Figure 3.2. PCR products on agarose gel electrophoresis. 490 bp: Insertion, 190 bp: Deletion. Marker: PUC mix

3.7 Statistical Analysis

The data underwent statistical analysis through the utilization of SPSS Inc.'s version 26. Descriptive statistics, such as mean ± standard deviation and frequency, were employed for data summarization. Categorical variables were compared utilizing the Chi-square test. The exploration of differences in genotype and laboratory parameters was conducted through this statistical approach among patients exhibiting mild, inpatient, or intensive care clinical symptoms. Also, one-way analysis of variance (ANOVA) was employed. A significance level of $p < 0.05$ was deemed statistically significant.

4. RESULTS

In these 141 participants that were recruited in our study, 41(29%) of them were in control group and 100 (71%) of them were patients. Gender distribution is different between the two groups, in patient group 71 (71%) were Female and 29 (29%) were Male (Figure 4.1), In control group 18 (44%) were Female and 23 (56%) were Male, and the mean age of patients was 49.5 years, ranging from 17 to 94 years old (Table 4.1). The analysis reveals a statistically significant relationship between age and gender when comparing patients and the control group, with a p-value below 0.05.

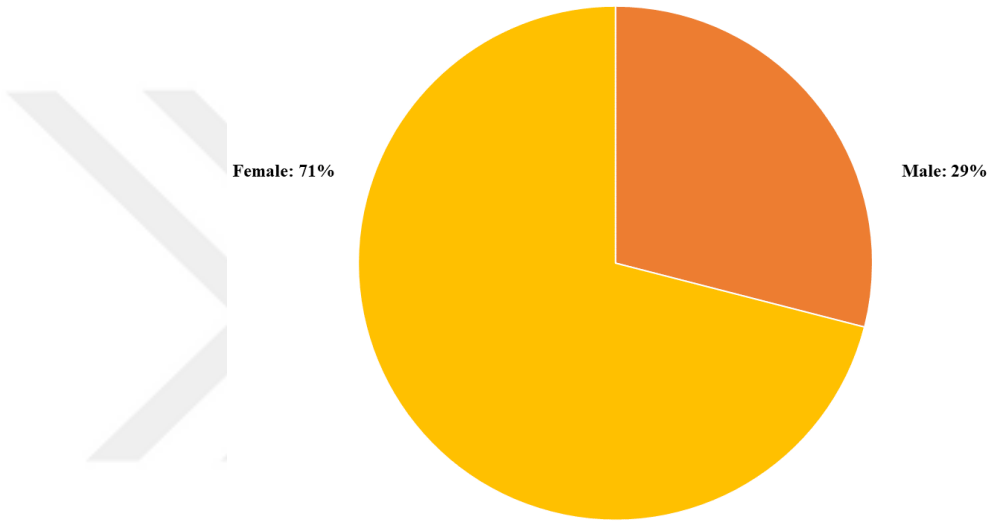

Figure 4.1. Gender distribution in patient group.

Table 4.1. Demographic data of patient and control group.

		Patient	**Control**	***P***
Mean age ±SD		49.5±21.5	37.8±10.7	0.000036
Gender	Male	29 (29%)	23 (56%)	0.0045
	Female	71 (71%)	18 (44%)	

4.1 ACE rs1799752 I/D polymorphism in COVID-19

As is shown in (Figure 4.2), the products of 490 base pairs were aligned with the Insertion (I) and the 190 base pair products were associated with the deletion (D). The

specimens displaying two bands at 490 bp and 190 bp, including samples 1, 2, 4, and 6, represent the heterozygous genotype ID. Conversely, the samples demonstrating only one band, such as samples 5, 7, 8, and 10 (representing II genotype) or 3 and 9 (representing DD genotype), are indicative of the homozygous genotype. The Marker (PUC Mix) is denoted by M.

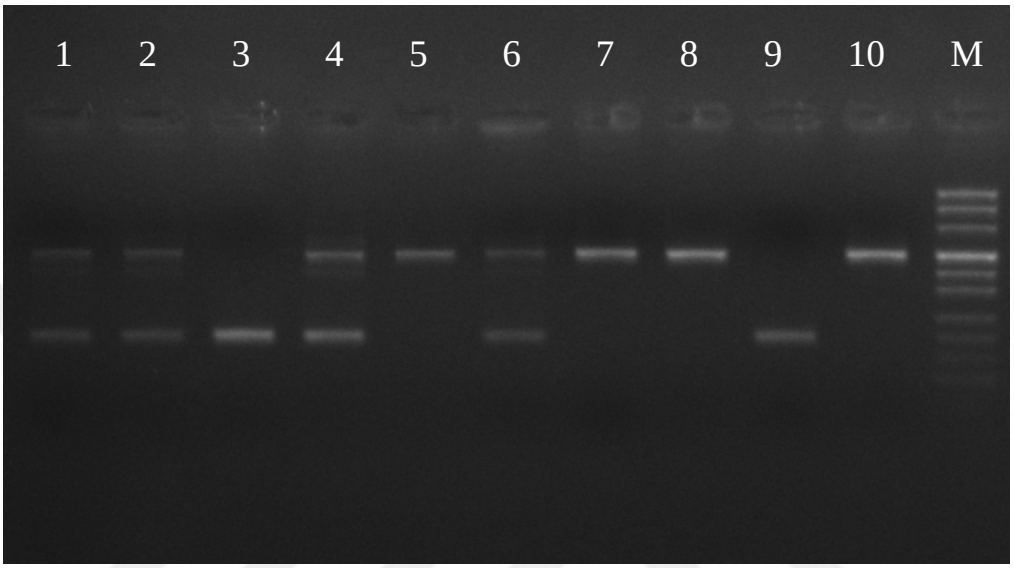

Figure 4.2. PCR results.

4.2 Genotype Comparison

We performed a genotype comparison between different groups of our study (Table 4.2). After comparing the control group with the total patients and then separately with patients classified as mild, in intensive care, and inpatient, the resulting p-values were 0.973, 0.945, 0.917, and 0.632, respectively. In all cases, the p-value was greater than 0.05. The same comparison was done between mild cases with intensive care and inpatient patients and the result of p-value was 0.760 and 0.494 respectively, $p > 0.05$. The last comparison was between intensive care and inpatient cases and the p-value was 0.724, also, $p > 0.05$.

The p-value of our study was bigger that 0.05 so the study was non-significant, it means that there wasn't any correlation between the severity of patients with the different genotype polymorphisms. This result was already seen in some other study before by

Çelik *et al.* (Çelik et al., 2021), Möhlendick *et al.* (Möhlendick et al., 2021), and Delanghe *et al.* (Delanghe et al., 2020a).

Table 4.2. Genotype comparison between different participate groups

Groups	II (%)	ID (%)	DD (%)	*P*
Control	7 (17%)	16 (39%)	18 (44%)	0.973
Patients	16 (16%)	41 (41%)	43 (43%)	
Control	7 (17%)	16 (39%)	18 (44%)	0.945
Mild	8 (19%)	15 (35.7%)	19 (45.3%)	
Control	7 (17%)	16 (39%)	18 (44%)	0.917
Intensive care	6 (14%)	18 (41.8%)	19 (44.2%)	
Control	7 (17%)	16 (39%)	18 (44%)	0.632
Inpatient	2 (13.3%)	8 (53.3%)	5 (33.4%)	
Mild	8 (19%)	15 (35.7%)	19 (45.3%)	0.760
Intensive care	6 (14%)	18 (41.8%)	19 (44.2%)	
Mild	8 (19%)	15 (35.7%)	19 (45.3%)	0.494
Inpatient	2 (13.3%)	8 (53.3%)	5 (33.4%)	
Intensive care	6 (14%)	18 (41.8%)	19 (44.2%)	0.724
Inpatient	2 (13.3%)	8 (53.3%)	5 (33.4%)	

4.3 Allele Comparison

We also performed allele comparison between different groups of our study (Table 4.3), in comparing control group with the total patients and then with mild, intensive care and inpatient cases, the p-value result was like that, 0.989, 0.966, 0.818, 0.741 respectively. The result of p-value between mild with intensive care and inpatient respectively was 0.784 and 0.764. Also, we got 0.616 in comparing intensive care cases with inpatient cases. The p-value in all allele comparisons was bigger than 0.05.

4.4 Analysis of I/D Allele Frequency

The frequency of homozygous and heterozygous polymorphism for ACE gene in control group were 17% II, 39% ID, and 44% DD. Allele frequency for I allele was 36.6% and for D allele was 63.4%. On the flip side, the prevalence of homozygous and heterozygous polymorphisms in COVID-19 patients was as follows: 16% II, 41% ID, and 43% DD. The observed frequency of the I allele was recorded at 36.5%, while the D allele had a frequency of 63.5% as demonstrated in (Table 4.3). The table illustrates the distribution of ACE I/D polymorphisms among the patient groups with COVID-19 and the control group. The difference in the prevalence of the D allele between the control and patient groups was slightly noticeable, with 63.4% in the control group compared to 63.5% in the patient group. However, this difference was not statistically significant, as confirmed by a p-value of 0.989.

Table 4.3. Allele comparison between different participate groups

Groups	**I (%)**	**D (%)**	***P***
Control	30 (36.6%)	52 (63.4%)	0.989
Patients	73 (36.5%)	127 (63.5%)	
Control	30 (36.6%)	52 (63.4%)	0.966
Mild	31 (36.9%)	53 (63.1%)	
Control	30 (36.6%)	52 (63.4%)	0.818
Intensive care	30 (34.9%)	56 (65.1%)	
Control	30 (36.6%)	52 (63.4%)	0.741
Inpatient	12 (40%)	18 (60%)	
Mild	31 (36.9%)	53 (63.1%)	0.784
Intensive care	30 (34.9%)	56 (65.1%)	
Mild	31 (36.9%)	53 (63.1%)	0.764
Inpatient	12 (40%)	18 (60%)	
Intensive care	30 (34.9%)	56 (65.1%)	0.616
Inpatient	12 (40%)	18 (60%)	

4.5 Genotype-Phenotype Correlation

The fundamental attributes of the COVID-19 patients included in this study are presented. There was no observed heterogeneity among these attributes spanning various genotypes. Hypertension emerged as the most commonly accompanying medical condition among the patients, with four patients each in the DD and ID groups diagnosed with hypertension, and one patient in the II group. Moreover, the variation between these groups did not yield statistical significance (Table 4.4).

Table 4.4. Comparison between clinical features according to genotype.

		DD	**ID**	**II**	***P***
Gender	M	12	14	3	0.504
	F	31	27	13	
Smoking	Yes	8	4	2	0.497
	No	35	37	14	
Alcohol		3	0	1	0.174
BMI		26.4	26.1	25.7	0.995
Chronic Disease		7	6	5	0.847

Age parameter is also associated to the severity of the patients. Also, the average age of mild patients are 32.3 years while in severe cases it's 68 years old. In a One-Way ANOVA test we didn't find any relationship between of each the genotypes with various laboratory parameters separately with the *p value* higher than significant level (Table 4.5).

Table 4.5. Mean and SD with One-way ANOVA comparison between Laboratory parameters of patient groups with different genotypes.

Lab Parameters	DD (Mean ± SD)	ID (Mean ± SD)	II (Mean ± SD)	*P*
Glucose mg/dL	118.62 ± 39.53	127.37 ± 40.13	122.46 ± 70.67	0.684
AST U/L	69.56 ± 162.36	44.91 ± 84.36	23.51 ± 20.78	0.378
ALT U/L	43.31 ± 82.64	35.91 ± 42.88	19.82 ± 12.97	0.425
TG mg/dL	223.84 ± 203.49	213.19 ± 116.40	162.45 ± 65.84	0.399
HDL mg/dL	40.57 ± 10.17	39.80 ± 9.59	41.47 ± 13.65	0.857
VLDL	48.04 ± 39.74	44.01 ± 23.30	33.74 ± 14.23	0.285
LDL mg/dL	81.66 ± 27.85	82.17 ± 31.01	88.73 ± 25.09	0.685
BUN mg/dL	22.24 ± 13.99	27.46 ± 22.58	22.2 ±17.69	0.385
CRP mg/dL	40.32 ± 49.33	51.79 ± 57.88	48.52 ± 63.59	0.629
Ferritin ng/mL	1182.42 ± 5590.44	436.09 ± 632.07	409.59 ± 540.94	0.604

Also, in (Table 4.6) we performed Pairwise Comparisons, to compare two different groups of our study with Laboratory Parameters such as, Glucose, AST, ALT, TG, HDL, VLDL, LDL, BUN, CRP and Ferritin. In this comparison, both Glucose and AST were statistically significantly while comparing Control group with Intensive Care group and also in comparing Mild with Intensive Care. ALT found to be statistically significant only while comparing Mild with Intensive Care, while HDL was only found to be significant while comparing Control with Patients and Control with Intensive Care. In the other hand, LDL was within significant level in comparing Control with Patients, Control with Mild, Control with Intensive Care and Mild with Intensive Care. For BUN and CRP, they were both statistically significant between Control and Patients, Control and Intensive Care, Mild and Intensive Care, also between Inpatients with Intensive Care. TG, VLDL and Ferritin show no significant level in comparing the groups with *p-value* greater than 0.05.

Table 4.6. Pairwise Comparisons between different groups with laboratory parameters

	P-value									
	Glucose	AST	ALT	TG	HDL	VLDL	LDL	BUN	CRP	Ferritin
Control/ Patients	0.143	0.950	0.999	0.999	0.002	0.934	<0.001	0.008	<0.001	0.999
Control/ Mild	0.999	0.999	0.999	0.360	0.104	0.051	0.032	0.999	0.999	0.999
Control/ Inpatients	0.999	0.999	0.999	0.999	0.988	0.999	0.229	0.999	0.197	0.999
Control/ IC*	<0.001	0.029	0.063	0.999	0.001	0.999	<0.001	<0.001	<0.001	0.475
Mild/ Inpatients	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.493	0.372	0.999
Mild/ IC*	<0.001	0.024	0.031	0.580	0.999	0.084	0.010	<0.001	<0.001	0.544
Inpatients/ IC*	0.122	0.999	0.999	0.999	0.999	0.999	0.228	0.008	<0.001	0.999

* Intensive care

5. DISCUSSION AND CONCLUSION

The pathogen instigating the respiratory ailment referred to as COVID-19 is identified as SARS-CoV-2 (Yamamoto et al., 2021), which exhibits a genetic resemblance of over 70% to SARS-CoV and an exceeding 95% similarity to the bat Coronavirus (Hussain et al., 2022; Verma et al., 2021). This virus has demonstrated worldwide transmission, and by the early days of May 2023, upwards of 764 million individuals have been infected by the disease, culminating in excess of 6.9 million fatalities (World Health Organization, accessed on May 2023). In March 2020, owing to its widespread dissemination and high level of severity, the World Health Organization characterized the COVID-19 outbreak as a pandemic (Yüce et al., 2021).

SARS-CoV-2 is capable of inciting severe ailments such as viral pneumonia, contributing to significant morbidity and mortality rates, and possesses the potential to initiate global pandemics. Based on data from WHO, approximately 450 million individuals worldwide are affected by pneumonia annually, resulting in close to 3 million deaths (Goren et al., 2022). SARS-CoV-2 is categorized as an enveloped virus possessing a positive-sense RNA genome (Rossi et al., 2020; Aziz & Islam, 2022; Albashir, 2021), and is a member of the genus of *Betacoronavirus* (Kirtipal et al., 2020; Hasöksüz et al., 2020; Singh & Yi, 2021). The primary mode of transmission is via the respiratory tract (Ciotti et al., 2020), but it also has the capacity to propagate through direct interpersonal contact (Wu, Liu, & Yang, 2020).

Initially discovered in nasopharyngeal secretions of common cold patients during the 1960s, human coronaviruses were only acknowledged as human pathogens after the occurrence of three notable outbreaks: severe acute respiratory syndrome (SARS) in China from 2002 to 2003, Middle East respiratory coronavirus (MERS-CoV) in Saudi Arabia in 2012, and the novel coronavirus SARS-CoV-2 in late 2019 (Mahmood et al., 2022). Human coronaviruses are RNA viruses enveloped in a membrane, with a diameter ranging from 60 to 140 nm (Hussain et al., 2022; Verma et al., 2021). SARS-CoV-2 is composed of four essential structural proteins: spike glycoprotein, small envelope glycoprotein, nucleocapsid protein, and membrane glycoprotein (Astuti, 2020; Singh & Yi, 2021; Rossi et al., 2020; Hasöksüz et al., 2020; Hu et al., 2021).

These viruses exhibit pathogenic capabilities across various human body systems, affecting the respiratory, gastrointestinal, hepatic, and central nervous systems (Rossi et al., 2020). The surface spike protein of SARS-CoV-2 plays a crucial role in facilitating virus entry into host cells by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, which is present on the membrane of host cells (Rauf et al., 2020; Szpukal et al., 2023). ACE2, an integral component of the renin-angiotensin-aldosterone system (RAAS) (Salamanna et al., 2020), is widely distributed across various organs, including the heart, kidney, testes, lungs, nasal epithelia, oral mucosa, and the nasopharynx (Salamanna et al., 2020; Möhlendick et al., 2021). Within the RAAS, which is critical for regulating physiological processes and maintaining homeostasis (Yamamoto et al., 2021), ACE2 and ACE are the primary enzymes, with SARS-CoV-2 demonstrating a distinct preference for ACE2 (Mahmood et al., 2022). This has spurred investigative endeavors to understand the implications of gene polymorphisms in ACE and ACE2 on the outcomes of COVID-19 since the inception of the pandemic (Najafi & Mahdavi, 2022).

In SARS-CoV-2-infected cells, the interaction with the RAAS can result in abnormal immune responses and blood clotting problems in individuals with COVID-19. The ACE converts Ang-I to Ang-II, which can cause vasoconstriction, inflammation, and blood clotting when it binds to the AT1 receptor. On the other hand, ACE2 converts Ang-II to Ang-1-7, which can lead to increased blood vessel dilation and decreased inflammation and blood clotting when it binds to the AT2 receptor. Therefore, in the development of hypertension and cardiovascular disease, both crucial comorbidities in COVID-19 patients, ACE1 and ACE2 play distinct roles. These conditions have been linked to increased disease severity and higher mortality rates (Mahmood et al., 2022). Although various factors such as male sex, age, obesity, diabetes, and ethnicity have been linked to increased risk of COVID-19 infection (Najafi & Mahdavi, 2022), our study have significant difference between age and severity of COVID-19.

Situated on the long arm of chromosome 17 (17q23.3), the ACE gene (Gene ID:1636) is comprised of 26 exons (Mahmood et al., 2022). This gene has been at the forefront of discussions since the pandemic's advent due to its variants and their correlation with COVID-19 susceptibility and severity (Aladag et al., 2021). The insertion/deletion of the ACE gene influences plasma protein concentration (Cintra et al.,

2018), identifying it as a critical genetic variant that could impact COVID-19 outcomes (Hubacek et al., 2021). Historical studies have shown a higher incidence of pneumonia in SARS-CoV-1 infection in individuals possessing the D allele (Aladag et al., 2021). The D allele is correlated with increased plasma protein levels, which subsequently induces a rise in angiotensin II levels and modulates steroid hormone synthesis (Cintra et al., 2018). The D allele has been associated with increased ACE activity, and genetic variations in the ACE2 receptor gene can impact circulating ACE2 receptor levels (Çelik et al., 2021). Another study reported a higher prevalence of the ACE D/D genotype in individuals with severe COVID-19, especially in those with concurrent hypertension (Calabrese et al., 2021).

Various inquiries posit an elevated susceptibility to severe outcomes in COVID-19 among individuals carrying the D allele or DD polymorphism. A study conducted in Iran, involving 120 COVID-19 patients, identified a discernible correlation between the ACE I/D polymorphism and the severity of COVID-19 symptoms. The ACE D allele demonstrated an association with an increased risk of disease severity, supported by a calculated p-value of 0.012 (Rezaei et al., 2022). In a broader study involving 251 participants, an investigation identified an association between the ACE1 DD genotype and the severity of COVID-19 in Iranian patients, potentially doubling the risk of developing the severe form of the disease. Additionally, the study results suggested that the II genotype did not significantly impact the mitigation of the severity of COVID-19 (Rezaiezadeh et al., 2022). Within the Turkish population, an examination of I/D allele frequencies in 112 COVID-19 patients disclosed a D allele frequency of 67%. Additionally, the study identified an association between ID genotype and increased infectious rates, particularly in cases of severe pneumonia (Rezaei et al., 2022). Remarkably, a study conducted in Italy, which included 68 patients, noted a DD ACE polymorphism in individuals experiencing severe symptoms were significantly higher prevalence of the (Calabrese et al., 2021). Meanwhile, a comprehensive investigation spanning 24 European and 2 non-European countries explored the impact of ACE gene polymorphisms on COVID-19 outcomes, revealing an association between the DD polymorphism and COVID-19-related deaths. The study suggests that individuals with the DD polymorphism may be susceptible to more aggressive forms of COVID-19 (Bellone et al., 2020). Moreover, a study encompassing 250 COVID-19 patients and 371

healthy controls in Northern Cyprus identified a notably increased frequency of ACE DD homozygotes genotype and D allele in COVID-19 patients compared to the control group, with a calculated p-value of 0.022 and less than 0.05, respectively (Çobanogullari et al., 2023).

Nevertheless, divergent viewpoints arise from certain investigations, challenging the premise of a link between DD polymorphism and the severity of COVID-19. A study focused on characterizing the distribution of the ACE1 D allele in Bosnia and Herzegovina, utilizing regression analysis, failed to discern any significant correlation between D allele frequency and the occurrence of COVID-19 infections, mortality rates, or case severity in the examined populations (Cenanovic et al., 2021). In a separate study by Çelik et al. (Çelik et al., 2021), involving 155 COVID-19 patients, it was concluded from the findings that the ACE I/D polymorphism is not associated with the severity of COVID-19 infection. Their results provide no substantiation for a direct association between the ACE gene polymorphism and the severity of COVID-19. Similarly, Möhlendick et al. (Möhlendick et al., 2021) conducted genotyping for ACE gene polymorphism in 297 positive and 253 negative SARS-CoV-2 cases, intending to explore the polymorphism's association with susceptibility to SARS-CoV-2 infection and the severity of COVID-19. In contrast to previous assertions, the study revealed no correlation between ACE polymorphism and infection risk or disease severity.

Upon analyzing the ACE D allele frequencies across 18 meticulously chosen European populations and subsequently comparing them with COVID-19 prevalence, mortality, and severity indicators, regression analysis uncovered a lack of any significant correlation between the D allele frequency and the incidence of infections, as well as the rates of fatality and severe cases within the examined populations (Cenanovic et al., 2021). In a comprehensive study involving 1252 individuals diagnosed with COVID-19, it was discovered that the DD genotype of the ACE variant is linked to an elevated risk for invasive mechanical ventilation (IMV) requirement, accompanied by a reduction in Serum ACE activity among patients with COVID-19 (Galindo et al., 2023). Noteworthy insights arise from the work of Delanghe et al., who conducted two distinct meta-analyses encompassing diverse participants. The initial study focused on 25 European countries (Delanghe et al., 2020a), while the subsequent investigation encompassing 33 countries

across Europe, North Africa, and the Middle East (Delanghe et al., 2020b), analyses revealed an inverse correlation between the prevalence of the D allele of the ACE I/D polymorphism and the incidence and fatality rates of COVID-19. Notably, these findings contradict data obtained from the eastern Asian population, where the frequency of the D allele is comparatively lower. The projected higher prevalence and mortality rate of COVID-19 in the Asian population, attributed to the low D allele frequency, contradicts empirical data indicating higher rates in Europe. The I/D ACE polymorphism demonstrates substantial geographical and ethnic variation (Çelik et al., 2021). Within the white population, the frequency of the DD genotype exhibits a considerable range, fluctuating from approximately 22 to 37%, while the frequency of the D allele stands at 56% (Berdeli et al., 2009). Notably, African American populations showcase a higher frequency of the D allele (89%) compared to Indian and Caucasian populations (69%). Conversely, certain European nations such as Spain, France, and Italy demonstrate D allele frequencies reaching up to 87%. In contrast, II allele frequency is higher in Asian countries relative to Europe (Aladag et al., 2021). In the Chinese population, the DD frequency is as low as 13–15% (Berdeli et al., 2009). These observations underscore significant geographical and ethnic variation in the I/D ACE polymorphism (Najafi & Mahdavi, 2022), emphasizing that findings in one ethnic group cannot be extrapolated to another, and differences and risks identified in one group may differ in another ethnic group (Berdeli et al., 2009).

Despite the widespread acknowledgment in the literature of a significant correlation between the severity of SARS-CoV-2 and the ACE I/D polymorphism, particularly the D allele, which is associated with increased susceptibility to COVID-19 and higher morbidity rates, like it seen in Rezaei et al., Calabrese et al., Bellone et al., and Çobanogullari et al. Despite that, our study aligns with the findings of Cenanovic et al., Möhlendick et al., and Delanghe et al., indicating no significant correlation between ACE polymorphism and the severity of SARS-CoV-2. Notably, this discrepancy exists despite the high frequency of the D polymorphism in the Turkish population (Berdeli et al., 2009).

The present investigation conducted on the Turkish population indicates a lack of association between ACE I/D polymorphism and the severity of symptoms in SARS-CoV-2 patients, as evidenced by nonsignificant P-values exceeding 0.05. To achieve a

comprehensive understanding of the impact of the ACE gene on symptom severity in COVID-19 patients, it is advisable to undertake more extensive research. Such investigations should encompass various variables, including environmental factors, duration of admission, and concurrent medical conditions, to comprehensively evaluate the severity of symptoms.

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