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The Role of Angiotensin-Converting Enzyme 1 and Angiotensin-Converting Enzyme 2 Genetic Polymorphism in Severe Acute Respiratory Syndrome Coronavirus-2 in Palestinian Population

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1445 / 2023

Dedication

To my mother and father

To all my family

To my teachers

To everyone who supported me in my graduate study

Lama Khaled Abu Saleh

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study or any part thereof has not been submitted for a higher degree to any other university or institution.

Signature: 

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Date: 19/08/2023

Title

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Abstract:

As a key enzyme of the renin-angiotensin system (RAS), angiotensin-converting enzyme 2 (ACE2), has been validated as a SARS-CoV-2 receptor, linking RAS to COVID-19. It is likely that functional ACE1/ACE2 gene polymorphism cause the imbalance of ACE1/ACE2 ratio, causing a RAS imbalance that may contribute to the COVID-19 infection complications by causing higher lung damage and disease with severe symptoms. Herein, we developed a new genotyping method using next generation sequencing (NGS) to study four single nucleotide polymorphisms (SNPs), three for ACE1, rs4343, rs4342, rs4341 and one for ACE 2, rs2285666 in one multiplex PCR tube. Bioinformatics analysis was done using free online galaxy program (https://usegalaxy.org.au/). The association of ACE 1 (rs4343, rs4342, rs4341) and ACE 2 (rs2285666) polymorphisms with COVID-19 infection in Palestine were investigated. A total of 130 samples were collected, including 50 negative controls without COVID-19 infection, 50 positive controls with COVID-19 infection but not hospitalized, and 30 patients with severe COVID-19 infection in the intensive care unit. Results showed that the genotype distribution of ACE2 (rs2285666) polymorphism was significantly different between COVID 19 patients and the control group (P-value = 0.049, X^2), while no statistical differences were observed between

the ACE1 mutations (rs4341, rs4342, rs4343) and the control group (P-value > 0.05, X^2). Individuals with ACE2 rs2285666 GG genotype were more prevalent in COVID-19 patients compared to control group (P-value = 0.049, X^2). Age and comorbidities such as hypertension and coronary artery disease were independent risk factors for COVID-19 disease (P-value < 0.05, X^2).

Symptoms of COVID-19 patients such as fatigue, headaches, runny noses, and loss of smell were significantly higher in the positive cases COVID-19 (P-value < 0.05, X^2), while dyspnea was more frequent in the ICU patients (P-value < 0.05, X^2), In this study, we support the hypothesis that wild genotypes of ACE2 rs2285666 GG are associated with COVID-19 infection.

العنوان: دور الإنزيم المحول للأنجيوتنسين 1 والإنزيم المحول للأنجيوتنسين 2 في متلازمة الجهاز التنفسي الحادة فيروس كورونا-2 قي المجتمع الفلسطيني.

اعداد : لما خالد أبوصالح.

بإشراف : د.سهير عريقات و د.عبد المجيد ناصر الدين.

الملخص

الإنزيم المحول للأنجيوتنسين 2 (ACE2) هو إنزيم رئيسي ومهم لنظام الرينين-أنجيوتنسين (RAS) ويعد مستقبلاً مهماً لسارس-كوفيد-2 (SARS-COV-2)، أي أن كوفيد-19 مرتبط بنظام الرينين-أنجيوتنسين. من المحتمل أن يؤدي تعدد التغيرات الجينية للإنزيم المحول للأنجيوتنسين 1 والإنزيم المحول للأنجيوتنسين 2 إلى اختلال نسبة الإنزيم المحول للأنجيوتنسين 1 والإنزيم المحول للأنجيوتنسين 2 ، مما يتسبب في اختلال توازن الانزيم المحول للأنجيوتنسين الذي قد يساهم في نتيجة الإصابة بكوفيد-19 عن طريق التسبب في تلف اوسع للرئة ومضاعفات اشد خطورة. الهدف من الدراسة هو انتاج طريقة حديثة تعتمد على فك الشيفرة الجينية بتقنيات الجيل الجديد وباستخدام التفاعل التسلسلي البوليميرازي بداخل أنبوب مخبري واحد لدراسة تعدد الاشكال المتعلقة بنّوَيد واحد ومن ثم ايجاد علاقة بين فيروس كوفيد-19 والتغيرات الجينية بمستوى الحمض النووي متعدد الاشكال المتعلقة بنّوَيد واحد للإنزيم المحول للأنجيوتنسين 1(rs4343, rs4342, rs4341) ACE1 والإنزيم المحول للأنجيوتنسين 2 (rs2285666) ACE 2 في المجتمع الفلسطيني. أجريت الدراسة على 130 عينة تم تقسيمها إلى 50 عينة من أشخاص غير مصابين بعدوى كوفيد -19 ، و 50 عينة من أشخاص يعانون من عدوى كوفيد -19 ولكن لم يتم إدخالها إلى المستشفى و 30 مريضا يعانون من عدوى كوفيد -19 الشديدة في وحدة العناية المركزة.

جاءت نتائج الدراسة كما يلي: كان توزيع النمط الجيني المتعدد الاشكال المتعلقة بنّوَيد واحد ل ACE2 (rs2285666) ذا فروق مصادقة احصائيا بين مرضى كوفيد 19 مقارنة بالمرضى الذين لا يعانون من كوفيد 19، في حين لم يلاحظ أي فروق إحصائية في توزيع الأنماط الجينية المتعدد الاشكال المتعلقة بنّوَيد واحد ل ACE1(rs4343, rs4342, rs4341) . الأفراد الذين يحملون الصورة الجينية GG لجين ال ACE2 (rs2285666) أكثر انتشارا في مرضى كوفيد -19 مقارنة بالأفراد الذين لا يعانون من كوفيد-19 (P=0.049). كان العمر والأمراض المزمنة مثل ارتفاع ضغط الدم ومرض الشريان التاجي

من عوامل الخطر المستقلة لمرض كوفيد -19. وأيضا كانت أعراض مرضى كوفيد -19 مثل التعب والصداع وسيلان الأنف وفقدان حاسة الشم أعلى بشكل ملحوظ في مرضى كوفيد -19 ($P < 0.05$)، بينما كان ضيق التنفس أكثر شيوعا في مرضى وحدة العناية المركزة ($P < 0.05$). في هذه الدراسة ، ندعم الفرضية القائلة بأن الأنماط الجينية الشائعة للإنزيم المحول للأنجيوتنسين 2 مرتبطة بعدوى كوفيد -19.

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List of Abbreviations

ACE1: Angiotensin-Converting Enzyme 1

ACE2: Angiotensin-Converting Enzyme 2

SNP: Single Nucleotide Polymorphism

RAS: Renin Angiotensin System

COVID-19: Coronavirus Disease 2019

ICU: Intensive Care Unit

ARDS: Acute Respiratory Distress System

CT: Computed Tomography

DNA: Deoxyribonucleic Acid

MERS: Middle East Respiratory Syndrome

NAAT: Nucleic Acid Amplification Test

NGS: Next Generation Sequencing

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

RT-PCR Reverse Transcriptase Polymerase Chain Reaction

RBD: Receptor Binding Domain

SARS-COV-2: Severe Acute Respiratory Syndrome Coronavirus 2

TMPRSS2: Transmembrane Protease, Serine 2

μl: Microliter

WHO: World Health Organization

NTD: N-terminal Domain

CTD: Carboxy-terminal Domain

HR1 and HR2: Heptad Repeats 1 and 2

TM: Transmembrane Anchor

IC: Intracellular

ANG1-7: Angiotensin 1-7

ANG II: Angiotensin 2

FPPR: Fusion Peptide Proximal Region

TLR: Toll Like Receptor

IFITMs: Interferon-induced transmembrane proteins

LYE6: Lymphocyte antigen 6 family member E

Chapter One

1. Introduction

1.1 Coronaviruses and SARS-CoV-2

Among the Coronaviruses (CoVs) is the Orthocoronavirinae subfamily, which belongs to the family Coronaviridae, Order Nidovirales. It is composed of four genera: Alphacoronavirus (α-CoV), Betacoronavirus (β-CoV), Gammacoronavirus (γ-CoV) and Deltacoronavirus (δ-CoV). CoV genomes have positive-sense enveloped, single-stranded RNA, and have a size ranging from 26 kb to 32 kb, making them the largest RNA genomes. CoVs are known to infect mammals as well as birds. The α- and β-CoVs infect mammals, while in contrast, δ- and γ-CoVs infect birds (Banerjee *et. al*., 2019). The β-CoVs spread in the form of the severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) viruses. When SARS broke out in China in 2002, it spread rapidly around the world, resulting in hundreds of deaths with 11% mortality rate (Song *et. al*., 2019). MERS first developed in Saudi Arabia in 2012 and spread to other countries, killing 37 percent of its human victims (Hui *et. al*., 2018). Both of these epidemics were likely caused by bat viruses that then infected humans via intermediate animal hosts, such as the civet (*Paguma larvata*) (Figure. 1) for SARS-CoV and the camel for MERS-CoV (Reusken *et. al*., 2013).

Figure 1: The civet (*Paguma larvata*).

Several patients in Wuhan City, Hubei Province, Central China, developed pneumonia with an unknown cause beginning in December 2019. Genome sequencing has demonstrated that this pneumonia, known as Coronavirus disease 2019 (COVID-19), is caused by a novel coronavirus, namely the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also referred to as the 2019 novel coronavirus (2019-nCoV). Together with SARS-CoV and MERS-CoV, this newly emerged SARS-CoV-2 belongs to the B lineage of coVs (Wong *et. al*., 2020).

1.2 Global, regional prevalence of coronavirus disease 2019 (COVID-19)

During late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which causes coronavirus disease 2019 (COVID-19) spread widely throughout the world after

emerging in Wuhan province in China. In March 2020, the WHO declared the coronavirus disease-19 (COVID-19) a global pandemic (WHO 2020). Since then, until July 12th, 2023, about 6.9 million people have died from this highly contagious and pathogenic disease while about 767 million cases have been confirmed. According to WHO COVID-19 reports in Palestine, including east Jerusalem (WHO 2023), by July 12th, 2023, A total of 703,228 confirmed COVID-19 cases have been recorded, resulting in 5,708 death (https://covid19.who.int/region/emro/country/ps).

It is important to conduct clinical research to figure out how SARS-COV-2 develops and how to manage it around the world after the COVID-19 pandemic which resulted in lost livelihoods, prolonged lockdowns, and severely affected the global economy. There has been an increase in the number of SARSCoV-2 variants spreading throughout the world. In almost all countries, there has been an increase in the number of SARSCoV-2 variants that are more pathogenic and more transmissible (WHO, 2020).

1.3 Symptoms and diagnosis of COVID-19

Similar to SARS-CoV-1 and MERS, COVID-19 has mild and self-limiting clinical manifestations 80% of the time. Less than 20% of cases aggravate secondary complications of acute respiratory distress syndrome (ARDS) or multiple organ failure. The risk of exacerbating ARDS is higher in people over 60 years old. An individual suffering from COVID-19 symptoms will usually display symptoms for 11-14 days after incubation, including fever, fatigue, coughing, dyspnea, myalgia, breathlessness, runny nose, nausea, joint pain, gastrointestinal symptom and loss of taste or smell. Additionally, patients with co-morbidities such as diabetes,

hypertension, acute kidney disease, cardiac problems, cerebrovascular disease, and liver dysfunction may be more prone to infection (Rastogi *et. al*., 2020).

There is a wide range of clinical symptoms associated with COVID-19, from asymptomatic to mildly symptomatic or may develop to acute respiratory damage and organ failure. It is therefore essential to accurately diagnose COVID-19 in order to manage it appropriately. Symptoms and history of the patient are the primary determinants of the diagnosis of COVID-19. Tests are then conducted to confirm these manifestations. COVID-19 is diagnosed using two types of tests: nucleic acid- based amplification tests reverse transcriptase (RT-PCR) to extract viral RNA from nasopharyngeal or oropharyngeal swabs by patients, and serology tests to detect antibodies. Computed tomography (CT) scans can also be used for monitoring and managing the patient's condition during the disease (Corman *et. al*., 2020).

1.4 SARS-CoV-2 genome

As illustrated in figure 2, SARS-CoV-2 enveloped virus is characterized by a positive single-stranded non-fragmented RNA genome containing 29.95–30.2 kb, and it is approximately 60–140 nm in diameter (Safiabadi *et. al*., 2021), with five genes ordered as 5'-replicase genes, spikes (S), envelopes (E), membranes (M), and nucleocapsids (N) -3’(Chen *et. al*., 2020). There are three non-structural proteins encoded by the replicase gene: the papain-like protease PLpro, the viral 3C-like protease (3CLpro), and the RNA-dependent RNA-polymerase RdRp (Figure. 2).

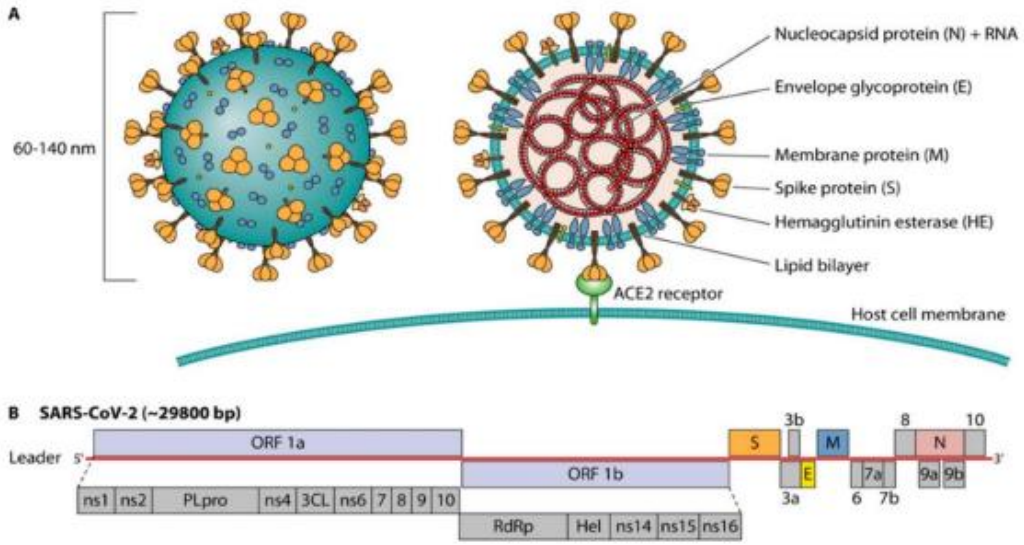

Figure 1: Structure of SARS-CoV-2 virus: A: is a schematic of SARS-CoV-2 virion and B: is a schematic of SARS-CoV-2 genome structure (Chen *et. al*., 2020).

SARS-COV-2 is composed of the following structural proteins:

1. Spike (S) glycoproteins - these proteins are the largest parts of the virus (175 kDa) and are responsible for entering host cells, such as receptor binding domain (RBD) (Sahu, Sreepadmanabh *et. al*., 2021).

2- Membrane/Matrix (M) proteins – determine the shape of the viral membrane.

3- Nucleocapsid (N) proteins – aid in the stability of RNA conformation, preventing damage to the genome (Sahu *et. al*., 2021).

1.4.1 SARS-CoV-2 spike protein

There are three parts of the spike protein as illustrated in figure 3, large ectodomain contains (S1 + S2). And other domains are the transmembrane anchor (TM) and short

intracellular tail. within the spike protein, there are two domains, the S1 domain located at the N-terminus, the glycosylated N-terminal domain (NTD), and the receptor binding domain (RBD) which binds to the host angiotensin-converting enzyme 2 (ACE2) receptor (Sahu *et. al*., 2021). A second subunit is the S2 domain, which contains two heptad repeats (HR1 and HR2) that are necessary for membrane fusion (Xia *et. al*., 2020). As shown in figure 2 Moreover, Spike protein contains 1,273 amino acids. Mutations in the receptor binding domain have received a great deal of attention due to its significance. In addition, mutations in other domains of the S protein, such as the NTD, can affect its structure and function (Liu *et. al*., 2020) (Figure. 3).

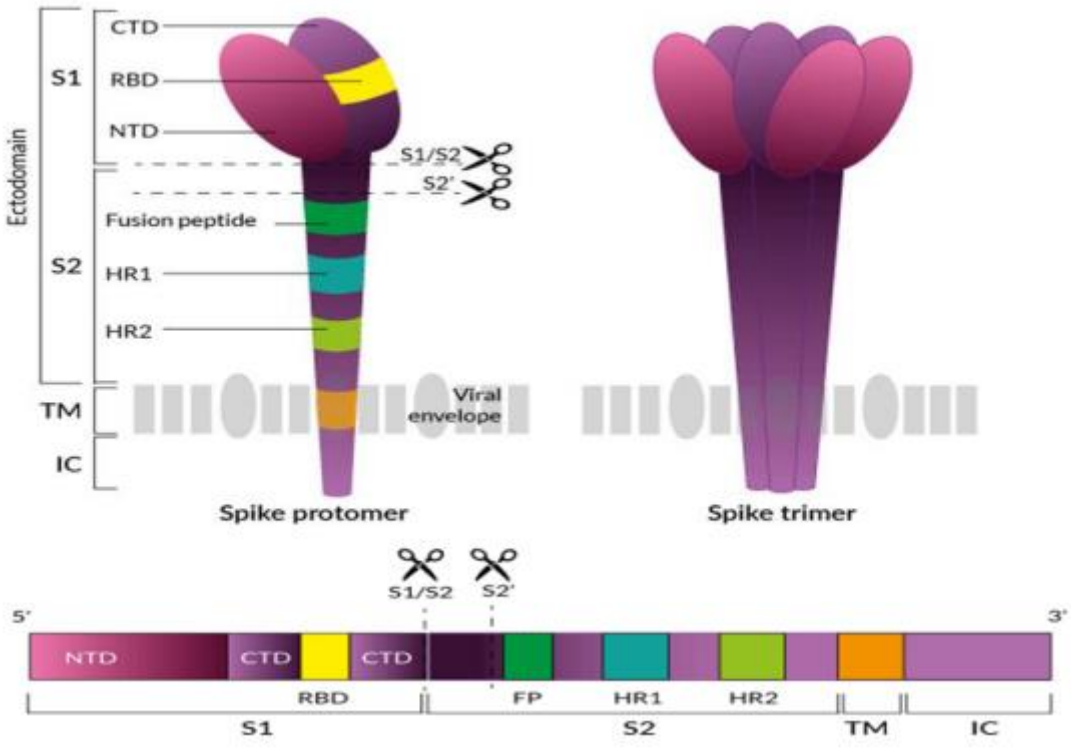

Figure 2: The three parts of the spike protein: Large ectodomain contains (S1 + S2). Transmembrane anchor (TM) and short intracellular tail (Liu *et. al*., 2020).

1.5 ACE2 - The SARS-CoV-2 receptor

SARS-CoV-2 possesses a transmembrane spike glycoprotein consisting of subunits S1 and S2 that are responsible for binding to the host cell receptor and facilitating fusion of the virus with the host cell membrane. In order for SARS-CoV-2 to enter the body, spike protein must bind to ACE2. As a result of SARS-CoV-2 binding to ACE2, the virus enters host cells through two routes: direct membrane fusion and endocytosis (Rangu *et. al*., 2022). There is a direct link between COVID-19 and the renin-angiotensin system (RAS) through angiotensin-converting enzyme 2 (ACE2) which is a critical component for the renin-angiotensin system (RAS).

ACE2 is a multifunctional protein (805 amino acids) that has multiple roles in disease and health. In the body, ACE2 is a carboxy-monopeptidase that converts angiotensin (ANG) II into angiotensin-1-7 (ANG-1-7) (Kai *et. al*., 2020). There are many organ systems involved in the distribution of ACE2 across tissues, including the heart, the vascular system, the digestive tract, the lungs, the kidneys, and the nervous system. The widespread expression of this gene might explain the pathological manifestations and multi-organ system disease manifestations seen in patients with severe clinical outcomes (Hashimoto *et. al*., 2012). However, SARS-CoV-2 and RAS are linked by ACE2 in which SARS-CoV-2 virus uses as the functional receptor for cell fusion and to incite respiratory infections (Wu *et. al*., 2021). ACE2 binds to ectodomain S1 of SARS-CoV-2 in order to initiate COVID-19-induced inflammation. A decrease in ACE2 levels will happen after membrane fusion and metabolism of Angiotensin II (ANG II) will be disrupted (Liu *et. al*., 2020). When Angiotensin II (ANG II) levels are elevated, inflammatory cytokines are released and local inflammation occurs (Liu *et. al*., 2020).

1.5.1 Renin Angiotensin System and COVID-19 Infection

Blood pressure, fluid balance, and electrolyte balance are regulated by the renin-angiotensin system. When blood pressure falls, A reduction in renal blood flow triggers the formation of renin in the cells of the juxtaglomerular cells in the kidneys and its release directly into the circulation. As a result, plasma renin converts angiotensinogen, released by the liver, into angiotensin I. Angiotensin I is then converted into angiotensin II by the angiotensin-converting enzyme (ACE), found mostly on vascular endothelial cells in the lungs. Angiotensin II is a potent vasoconstrictor that narrow or constrict blood vessels and does another function in cells to increase the blood pressure, then Angiotensin 2 will be cleaved to angiotensin 1-7 (Ang1-7) to vasodilate and restore homeostasis to the body by the Angiotensin converting enzyme 2 (ACE2). As we mentioned before, the SARS-CoV-2 virus is capable of entering cells through angiotensin-converting enzyme (ACE) 2, an enzyme within the renin-angiotensin system (RAS). In order to initiate COVID-19-induced inflammation, ACE2 binds to ectodomain S1 of SARS-CoV-2. As membrane fusion occurs and Angiotensin II (Ang II) metabolism is disrupted, ACE2 levels will decrease. As a result, angiotensin II (Ang II) levels will be elevated, inflammatory cytokines are released, and local inflammation occurs (Ferrara *et. al*., 2022).

1.6 SARS-CoV-2 entry process

In order for virus entry proteins to overcome the natural repulsion between the virus and cellular membrane, they must fold into an energetically stable state while undergoing a subsequent conformational transition. The S-protein therefore enters a metastable state, one prone to change into a lower-energy state before membrane fusion occurs. It is well known that

SARS-CoV and other coronaviruses proteins are cleaved by two proteolytic steps that follow ACE2 binding (Figure 4). The first proteolytic step related to endosomal entry takes place at the S1–S2 boundary, and the second proteolytic step related to cell surface entry is at the S2′ site of the S2 subunit. As for SARS-CoV (Belouzard *et. al*., 2012), the target cell cleaves both sites by proteases. For SARS-CoV-2, the S protein is cleaved by furin, which is a calcium-dependent protease that recognizes and cleaves the specific sequence motif R-X-R/K-R, where X may be any amino acid, and it is responsible for cleaving the S1–S2 boundary in the virus producer cell, but target-cell proteases are needed for cleavage of the S2′ site. To enter cells, both viruses depend on target-cell proteases, TMPRSS2 and cathepsin L are the major proteases that activate S proteins. A TMPRSS2-mediated activation of S proteins occurs at the plasma membrane due to the presence of TMPRSS2, whereas a cathepsin-mediated activation takes place at an endolysosome due to the presence of cathepsin (Hoffman *et. al*., 2020) (Figure. 4).

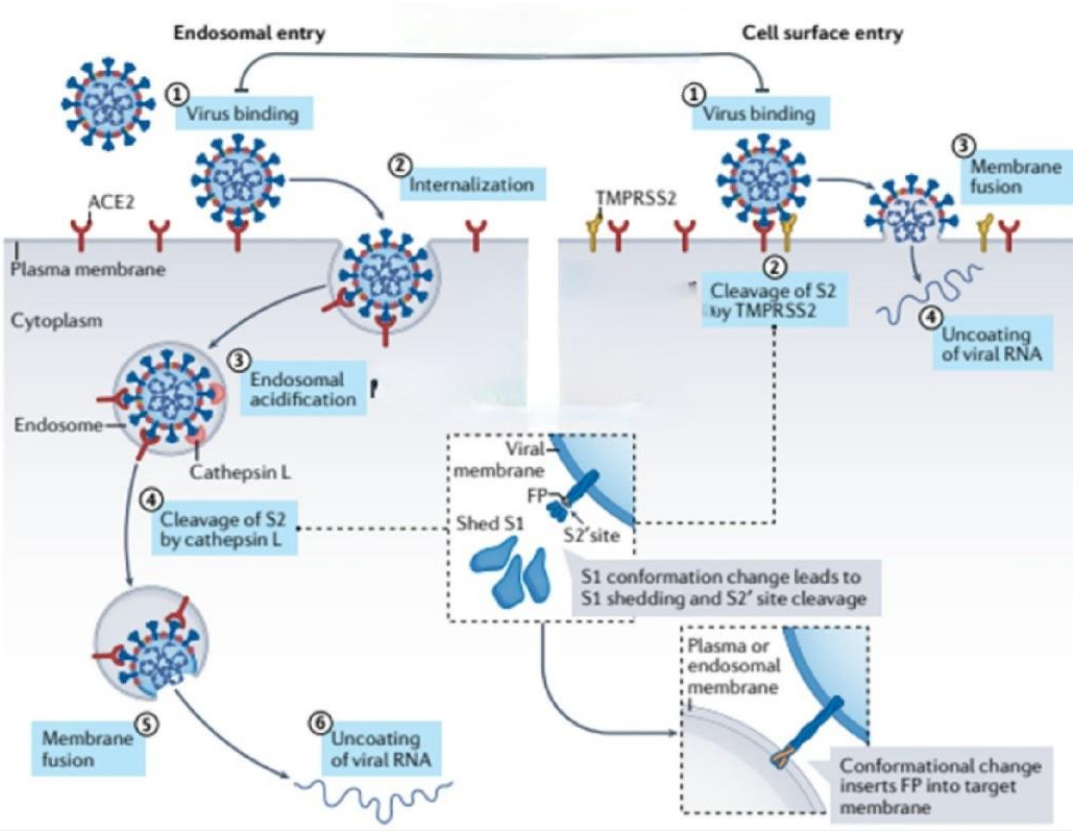

Figure 3. Two distinct SARS-CoV-2 entry pathways with two different proteolytic cleavage processes (Hoffman *et. al*., 2020).

1.6.1 Cleavage of the S protein S1–S2 boundary by furin

SARS-CoV-2 differs from other viruses with the presence of a multibasic furin cleavage site (Arg-Arg-Ala-Arg) at the S1–S2 junction. (Figure 5). Both cleavage events are required to initiate membrane fusion; cleavage of the S1–S2 boundary precedes cleavage of the S2′ site (Belouzard *et. al*., 2009). A human SARS-CoV-2 Wuhan-Hu-1 strain, sheds the S1 subunit easily from the S2 subunit and assumes a premature postfusion conformation of the S2 trimer, which is non-functional. The acquisition of a furin-cleavage site by SARS-CoV-2 may have been a recent event, based on this perplexing observation. SARSCoV-2 rather acquired a

different mutation, D614G, in order to stabilize the S protein and slow the shedding of S1 (Zhang *et. al*., 2020), whereas it would have been relatively straightforward for the virus to have eliminated the furin-cleavage site and rely on target-cell proteases. According to this observation, the destabilizing furin site is essential for virus fitness in human hosts. Recent research with co-housed ferrets has demonstrated that the furin site plays a key role in SARS-CoV-2 transmission (Peacock *et. al*., 2021).

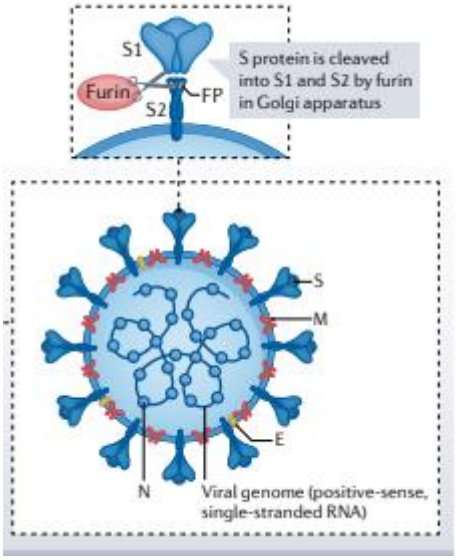

Figure 4: Spike protein cleavage by furin (Belouzard *et. al*., 2009).

1.6.2 Role of TMPRSS2 in cell surface viral entry

As referred to figure 4, the cleavage of the S1–S2 boundary is necessary for fully activating the fusion process, either by the cell surface TMPRSS2 or by endosome cathepsins. Type II transmembrane protein with serine 2 protease (TMPRSS2) is an enzyme that in humans is encoded by the TMPRSS2 gene. Its activity has not been well defined for its major physiological role and substrate specificity. Nevertheless, its role in respiratory virus infection is well

established, particularly for influenza viruses (Sakai *et. al*., 2016) as well as SARS coronaviruses (Glowacka *et. al*., 2011). There are three major types of cells in these tissues co-expressing TMPRSS2 and ACE2: Pneumocytes of type II, Ileal absorptive enterocytes, and nasal goblet secretory cells (Szabo *et. al*., 2008). ACE2 is expressed at high levels in nasal goblet cells but not in nasal ciliated cells (Ziegler *et. al*., 2020) or it is expressed at high levels by both cells (Sungnak *et. al*., 2020). Although ACE2 expression is limited in the lower airway, it is more prominent in the upper airway, and indeed many ACE2-expressing airway cells express transmembrane serine protease-2 (TMPRSS2) as well (Qi *et. al*., 2020). There are many serine proteases present in the lung that contribute to infection with many respiratory viruses. Transmembrane serine protease-2 (TMPRSS2) is the protease most frequently studied during coronavirus entry, but many other serine proteases play a role, such as human airway trypsin-like protease (HAT), TMPRSS4, TMPRSS11A, TMPRSS11E, matriptase, and neutrophil elastase (Laporte *et. al*., 2017). Further studies on their involvement in SARS-CoV-2 infection will shed light on the entry pathway and how to inhibit it. TMPRSS2 is utilized by both viruses, although SARS-CoV relies less on it than SARS-CoV-2 (Ozono *et. al*., 2021). A furin site may indicate whether the SARS-CoV S protein is a suitable substrate for TMPRSS2. However, it is possible that cathepsins are more effective at cleaving the SARS-CoV S-protein's S1–S2 junction (Zhu *et. al*., 2021). Accordingly, replacing the furin site in SARS-CoV-2 with that of the equivalent sequence of SARS-CoV, which has no multibasic site, made SARS-CoV-2 incapable of infecting TMPRSS2+ human airway cells (Hoffmann *et. al*., 2020).

1.6.3 Role of Cathepsins in endosomal viral entry

In spite of preferential activation by TMPRSS2, cathepsins particularly cathepsin L, can also cleave the S2′ site in SARS-CoV-2 (Figure 4). When the target cells do not express sufficient TMPRSS2 or when the viral–ACE2 complex encounters TMPRSS2, the virus is internalized in the late endolysosome by clathrin-mediated endocytosis, where the cathepsins cleave the S2′ site (Bayati *et. al*., 2021).

It is proposed that multiple tethers to ACE2 induce ACE2 endocytosis when SARS-CoV, SARS-CoV-2 or purified S protein are bound. Cathepsin L inhibitors partially inhibit pseudovirus entry in TMPRSS2+ cells, presumably due to multiple ligations to the S2′ site (Ou *et. al*., 2021). Cathepsins, known as non-specific proteases, function in the late endosomes and lysosomes as endopeptidases and exopeptidases to degrade proteins. Among the proteases, there are aspartic proteases (D and E), serine proteases (G), and cysteine proteases (B, C, K, L, S, and V). Cathepsins B, L, and S are the most important cysteine proteases that contribute to viral entry. Despite the importance of cathepsin B in the entry of Ebola viruses (Chandran *et. al*., 2005), cathepsin L is more important for entry of SARS-CoV and SARS-CoV-2. Since SARS-CoV-2 uses a different endosomal pathway than it does to infect target cells, endosomal acidification inhibitors such as hydroxychloroquine have a limited effect on how the virus infects them. In one investigation, whereas TMPRSS2 expression significantly reduced the potency of hydroxychloroquine, the potency was largely recovered in the presence of a TMPRSS2 inhibitor, pointing to a potential benefit of using a TMPRSS2 inhibitor in combination with hydroxychloroquine (Ou *et. al*., 2021).

1.6.4 Membrane fusion

Fusion-peptide proximal region (FPPR), 630 loop, carboxy-terminal domain (CTD2), and other components of the S fusion machinery have recently been identified in structural studies and appear to modulate S protein fusogenic structural changes (Figure 6). By keeping the receptor binding domain (RBDs) down, the FPPR and the 630 loop assist in preventing them from moving out of position and, consequently, from maintaining the down conformation.

As shown in Figure 7, ACE2 may be able to sample the up conformation as a result of intrinsic protein dynamics. FPPR shift may help expose the S2′ site for proteolytic cleavage if ACE2 captures the RBD-up conformation, which expels the 630 loop and the FPPR from their position in closed S trimer conformation. As the 630 loop departs from the hydrophobic surface of CTD2, the N-terminal segment of S2 will be released from S1, releasing S1 (Figure 6).

As discussed earlier, furin precleaves the S1–S2 boundary of the SARS-CoV-2 S protein. Upon dissociation of S1, a cascade of refolding events would occur in the metastable prefusion S2, resulting in the fusogenic transition (Figure 5). These transitions lead to the insertion of fusion peptides into the target cell membrane in conjunction with HR1 unfolding (Fan *et. al*., 2020). In heptad repeat 2 (HR2), the fold back creates a location where the fusion peptide and transmembrane segments are close together, causing the membranes to bend towards each other, thereby resulting in membrane fusion (Figure 7) (Shaik *et. al*., 2019).

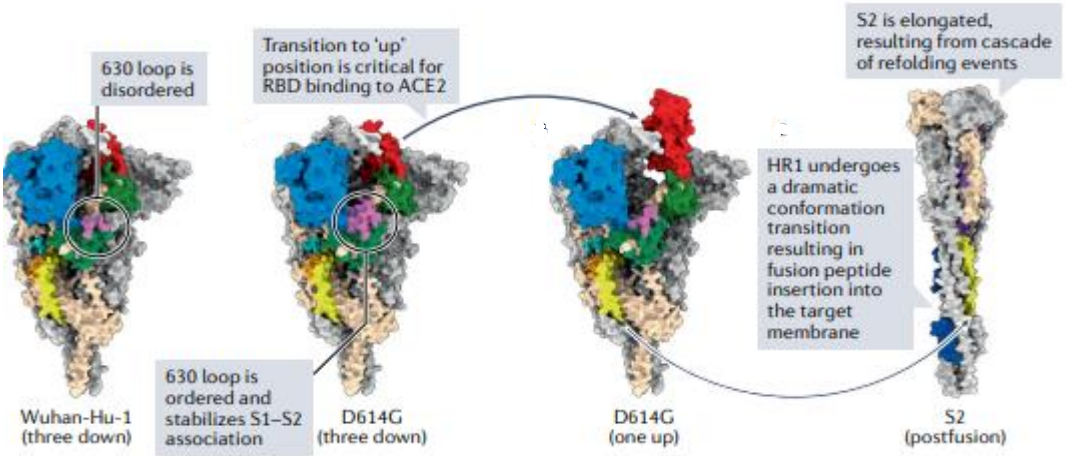

Figure 5: Structures of the S protein, Subdomains and interaction between the RBD (Fan *et. al*., 2020).

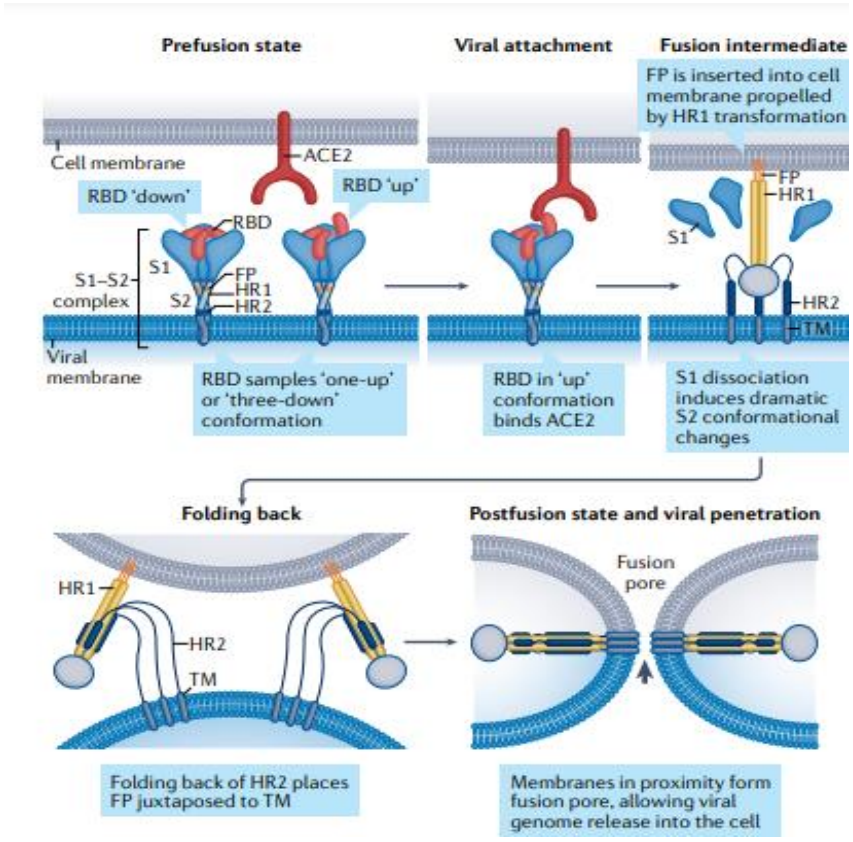

Figure 6: A model for membrane fusion induced by the SARS-CoV-2 S protein (Shaik *et. al*., 2019).

1.6.5 Cellular proteins restricting viral entry

As part of the antiviral immune response, toll-like receptors TLR3, TLR7, TLR8, and TLR9 recognize pathogen-associated molecular patterns and induce type I interferon production. A double-stranded RNA virus is detected by TLR3, and an unmethylated CpG in viral DNA is detected by TLR9. For Coronavirus, TLR7 and TLR8 bind single-stranded viral RNA rich in G/U. TLR7 and TLR8 are both expressed in lung tissue and have been shown to induce proinflammatory cytokines after exposure to SARS-CoV and SARS-CoV-2 RNA (Campbell *et. al*., 2021). They are both expressed in endosomes. However, TLR8 is primarily expressed in the lung and lymphoid tissues, while TLR7 is more highly expressed in the brain, skin, and lymphoid tissues.

It has been identified that a number of interferon-stimulated gene products are crucial to SARS-CoV-2 replication, but only a few of these genes are involved in the entry sequence, namely the interferon-induced transmembrane proteins (IFITMs) (Shi *et. al*., 2021) and lymphocyte antigen 6 family member E (LY6E) (Pfaender *et. al*., 2020).

In the human body, four members of the IFITM family (IFITM1, IFITM2, IFITM3, and IFITM5) are constitutively expressed, but they are highly stimulated by interferons type I and II. They have been identified as antiviral proteins against influenza A viruses and flaviviruses, and later against SARS-CoV (Huang *et. al*., 2011). In recent years, IFITM2 has been shown to restrict the entry of SARS-CoV-2. By an unclear mechanism, IFITM proteins prevent viruses from accessing cellular cytoplasm across endosomal membranes. However, if SARS-CoV were directed to enter cells only through the plasma membrane, it could bypass such a restriction. In the absence of the furin site in SARS-CoV-2, this restriction is amplified, and TMPRSS2

overexpression can compensate. Furthermore, it does not affect viruses whose entry occurs solely at the plasma membrane, indicating that the fusion site of the IFITM protein is crucial to its antiviral activity (Zhao *et. al*., 2020).

LY6E inhibits the infection of SARS-CoV, SAR-CoV-2, and MERS-CoV by inhibiting the S protein-mediated membrane fusion. It is important to clarify how LY6E regulates infection with SARS-CoV-2 and other viruses, unlike IFITM-mediated restriction, which was overcome by TMPRSS2 expression. Studies are needed to clarify how LY6E regulates infection with other viruses (Zhao *et. al*., 2020).

1.7 Study Objectives

The main objective of this study is to investigate the association between ACE1 (rs4343, rs4342, rs4341) and ACE 2 (rs2285666) polymorphisms and severity of COVID-19 infection among Palestinian patients.

Specific objectives:

1. To develop a new method for screening specific SNPs in ACE1 and ACE2 genes using next-generation sequencing technique.
2. To identify the genotypic distribution of ACE1 (rs4343, rs4342, rs4341) and ACE 2 (rs2285666) polymorphisms in the Palestinian population.
3. To investigate the association between ACE1 (rs4343, rs4342, rs4341) and ACE 2 9(rs2285666) polymorphisms with signs and symptoms in COVID-19 infected individuals.

1.8 -Literature review

1.8.1 Genetic polymorphism of ACE gene with COVID-19 infection

Genetic polymorphism of ACE1 and ACE2 have been associated with the severity of COVID-19, but their importance in the severity and prognosis of the disease remains unclear.

It has been documented that ACE polymorphisms play a pivotal role in the development of cardiovascular diseases as well as respiratory diseases; for example, there are ACE polymorphism variants that have been linked to the severity of SARS COV-2 (Alimoradi *et. al*., 2021). Additionally, it has been linked to poor clinical outcomes of acute respiratory distress syndrome (ARDS) among individuals.

On the other hand, a number of studies suggest that the ACE2 polymorphism (G8790A) may influence the susceptibility to SARS-CoV-2 infection by increasing the expression of the ACE2 receptor (Saengsiwaritt *et. al*., 2022).

Different studies about genetic polymorphism of ACE and ACE2 and their association with the severity of COVID-19 will be discussed in this literature review.

1.8.2 ACE-1 rs4343/ rs4341 polymorphisms and COVID-19 infection

In an Egyptian study the relationship between the ACE-1 rs4343, TMPRSS2 rs12329760, and ACE-2 rs908004 single nucleotide polymorphism (SNPs) and the COVID-19 disease infection was investigated. The results showed that GG genotype and wild-type ACE-2 rs908004 as well as mutant ACE-1 rs4343 were significantly more prevalent in severe COVID-

19 patients, thus making these SNPs useful as COVID-19 severity predictors. (Abdelgawad *et. al*., 2023).

In a Spanish pilot study by Iniguez, et al, ACE rs4341 and rs4343 polymorphisms were studied to see if there is an association of rising risk of COVID-19 in patients with hypertension, dyslipidemia, and diabetes. Researchers found that G alleles of the rs4341 and rs4343 alleles were associated with severe COVID-19 in hypertensive patients, regardless of sex ($p<0.05$). Additionally, they found that patients with dyslipidemia ($p<0.05$) and type 2 diabetes ($p< 0.01$) who carry both SNPs had higher mortality rates ($p< 0.05$) and more severe COVID-19. In other words, their results suggest that G-containing genotypes of rs4341 and rs4343 are associated with an increased risk of adverse COVID-19 prognosis. Therefore, ACE polymorphisms rs4341 and rs4343 could be used to predict the severity of COVID-19 in patients with hypertension, dyslipidemia or *Diabetes mellitus* (Iniguez *et. al*., 2021).

1.8.3 Association of ACE2 gene variants with SARS-CoV2 infection

In an earlier report, ACE2 variants were not associated with SARS-CoV susceptibility or outcomes with no sex differences (Chiu *et. al*., 2004).

According to Gemmati *et. al*., there is a higher incidence of COVID-19 infection among male with more severe manifestations. In addition, death rates from SARS-CoV-2 infection were 65 percent higher in male than in female, possibly because ACE2 is located on chromosome Xp22. 22.. When heterozygous female express higher expression levels of ACE2, they are more protected than male with hemizygous ACE2 expression (Gemmati *et. al*., 2020).

Researchers have discovered that there may be "population-specific" genetic variations in ACE2 that influence susceptibility to SARS-CoV-2 infection, based on analysis of the "1000 Genomes Project," which contains samples from almost all ethnicities (Simsek *et. al*., 2020). Furthermore, Cao *et. al*., found no mutation difference in ACE2 that could affect SARS-CoV-2/S-protein binding in their genetic analysis. However, they were criticized by some researchers for focusing merely on minor variations in a small population (Cao *et. al*., 2020).

According to Suryamohan *et. al*., several ACE2 variants could be predicted using data mining and structural predictions (i.e., "E23K, S19P, I21V, K26R, T27A, N64K, T92I, K26E, H378R, Q102P, and M383T) that can increase the host's sensitivity to SARS-CoV. It was predicted that "N33I, K31R, D38V, H34R, E35K, E37K, N51S, K68E, Y50F, F72V, G326E, G352V, Y83H, D355N and Q388L" variants would reduce the affinity between S-protein and ACE2, thus decreasing infection susceptibility (Suryamohan *et. al*., 2021). Interestingly, most of the previously predicted variants were centered in ACE2's extracellular catalytic domain (N-terminus) that interacts with S-proteins. Nevertheless, it was confirmed that these variants are found in the general population at rare allele frequencies without observable variations between populations or even by sex. ACE2 variants were examined by another Italian research group, whose results indicated that a mutation at c.1517 T>C (Val506Ala) had the greatest effect on protein stability, followed by a mutation at c.631G > A (Gly211Arg) and a mutation at c.77A > G. A high frequency of allele (Lys26Arg) as well as a mutation at c.1166C>A (Pro389His) and a mutation at c.1051C> G (Leu351-Val) were predicted to affect spike protein interaction (Benetti *et. al*., 2020). Further, Hou et al investigated 63 potentially deleterious ACE2 variants across eight populations that could influence genetic susceptibility to COVID-19 by comparing nearly 81,000 human genomes (Hou *et. al*., 2020).

1.8.4 ACE2 gene variants and COVID-19 outcome

ACE2 protein expression levels have been reported to be modified by several mutations, as reported previously in a murine model (Wysocki *et. al*., 2006), as well as by ACE2 deletion in a mice model, which was associated with increased levels of ANG II in the tissue and circulation and cardiovascular dysfunction (Yamamoto *et. al*., 2006). Moreover, Khayat *et. al*., found ten ACE2-related variants in coding, noncoding, and regulatory sites, which may explain the epidemiological differences associated with COVID-19. East Asian population had a higher prevalence (30% to 180% higher prevalence) of the variants associated with ACE2 upregulation (rs182366225 and rs2097723), whereas native populations from Amazon were found only to possess variants rs1027571965 and rs889263894 associated with ACE2 upregulation. The later population, however, was more likely to have "rs2285666" and "rs35803318" than the older population. Three relevant polymorphisms (rs147311723, rs142017934, and rs4646140) were found to be more common in Africans, of which “rs142017934” was associated with gene upregulation and was exclusively found in this population. There is, however, an allele (rs5934250) that appears to lower ACE2 expression in some tissues in Europeans and some Africans (Khayat *et. al*., 2020).

According to Wooster *et. al*., ACE2 variants "rs4240157, rs6632680, rs4830965, rs1476524, and rs2048683" have been linked with higher tissue-specific expression of ACE2, leading to hospitalization, according to Wooster *et. al*., the "rs1548474" variant was associated with low tissue expression and fewer complications (Wooster *et. al*., 2020).

According to a study conducted by Chaudhary *et. al*., rs2106809 is associated with variable levels of circulating ACE2, while the CC/CT genotype was associated with higher levels compared to the TT genotype. Thus, "quantification of soluble ACE2 (sACE2) in body fluids was suggested as a protective biomarker for rapid test screening (Chaudhary *et. al*., 2020).

ACE2 and genetic variants involved in the synthesis of proinflammatory cytokines/chemokines have also been implicated in differences in patients' response to COVID-19 in terms of hypercytokinemia/cytokine storm, which causes excessive proinflammatory cytokine production that is associated with multiple organ failures (Badawi *et. al*., 2020).

So, there are a number of studies that identified various single nucleotide polymorphisms (SNPs) on ACE2 that have a role in COVID-19 disease; however, only a few of these were tested clinically such as rs2106809 and rs2285666 (Srivastava *et. al*., 2020). G8790A (rs2285666). One of the functional SNPs identified on ACE2, is located on chromosome X, p22, intron 3 and is likely to affect ACE2 gene expression by altering mRNA splicing (Patel *et. al*., 2014). A2350G (rs4343) is another SNP that will be studied in this study, which is located on exon 17 of ACE1 in addition to rs4342, rs4341of ACE1. It may be postulated that COVID-19 is more prevalent among carriers of specific genotypes of this variant due to its effect on the activity and serum levels of the ACE-1 enzyme (Firouzabadi *et. al*., 2011).

Chapter Two

2. Materials and Methods

2.1 Study design and study samples

A case-control study was conducted on 130 participants who were recruited between April and May 2021. Frozen EDTA blood samples were collected at random from the in-house blood bank in the Medical Molecular Biology Research laboratory, at the faculty of medicine, Al-Quds University. Ethics approval was received from Al-Quds University's ethics committee under the reference number of (184/REC/2021). All samples have had the approval to be used in genetics studies of COVID-19. These samples were obtained from COVID-19 infected patients and from healthy individuals in different districts in Palestine. The samples were divided into three groups: Control group (those who had no clinical evidence of infection and tested negative by reverse transcriptase (RT-PCR) and living in the vicinity of a COVID 19 positive patient). Case group (those who had COVID 19 positive RT-PCR and clinically diagnosed with COVID-19, regardless of severity of symptoms). Intensive care unit (ICU) group (those who were hospitalized in the intensive care unit due to severe life-threatening COVID-19 infection in Ramallah Hospital). All individuals aged less than 18 years were excluded. Demographic information of participants such as sex, age, and address and clinical data such as symptoms and comorbidities were collected via a questionnaire.

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2.2 Study procedure

2.2.1 DNA extraction

Genomic DNAs were extracted from whole blood using NucleoSpin® Blood kit as described by the manufacturers. In brief, 25µl of proteinase K and 200µl lysis Buffer BQ1were added to 200µl blood sample to digest protein and for lyse blood samples respectively, then incubated for 10 minutes at 70°C. Then, for adjusting DNA binding conditions 210µl of ethanol was added and mixed by pipetting. To bind DNA for each preparation, one NucleoSpin® blood column was placed in a collection tube was taken and samples were loaded. Then centrifugation for 1 min at 11,000 x g was done. Collection tube containing flow-through was discarded and the column was placed into a new 2ml collection tube. A volume of 350 µL buffer BQ2 was added for wash silica membranes and then centrifuged at 11000rpm for 3 minutes. The flow-through was discarded and column was placed back into the collection tube. The column was transferred into a sterile 1.5 ml microcentrifuge tube. Finally, 50µl of preheated elution buffer (BE) was added to elute genomic DNA, then incubated for 1 minutes and centrifuged at 11000 rpm for 1 minute. At last, nucleic acid was quantified and assessed for its purity in two ratios: 260/280 and260/230. These numbers indicate the absorbance at the wavelengths 230, 260 and 280 nm. Concentration of DNA samples was measured by nanodrop spectrophotometer 1000 (ND-1000).

2.2.2Primer selection and PCR Amplification

To design new primers targeting the studied SNP, two regions were selected, one of human ACE1 (see below) including the target SNPS rs4341, rs4342, and 4343. Figure 8 shows the PCR target regions including the primers that were designed using primers 3 program [(Primer3 on Biowulf (nih.gov).]

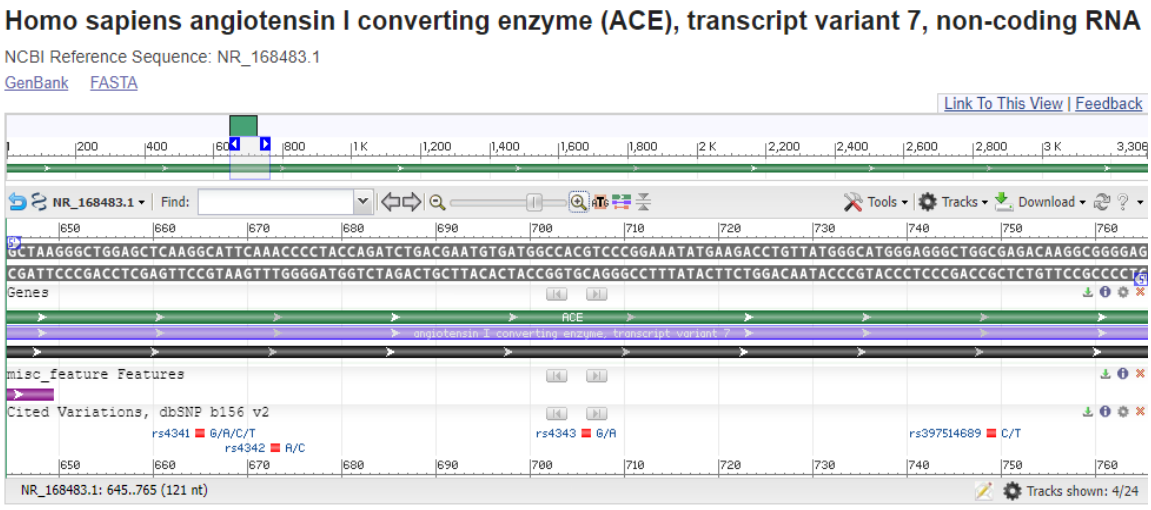


Figure 7: Primers and PCR target regions for ACE1 SNPS rs4341, rs4342, and rs4343**.**

> Homo sapiens angiotensin I converting enzyme (ACE), transcript variant 7, non-coding RNA

NCBI Reference Sequence: NR_168483.1

CGAGCCAGCTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGAT
CTGACGAATGTGATGGCCACGTCCCGGAAATATGAAGACCTGTTATGGGCATGGGAGGGCTGGCGAGACAAGGCGGGGAGAGCCATCC
TCCAGTTTTACCCGAAATACGTGGAACTCATCAA

PCR DNA MW: 210 bp+67 Illumina primers =277 in total

Primers in green, SNPS are in purple, other colors present the selected virtual probes for galaxy analysis.

Figure 9 shows the designed primers and the PCR target regions for the studied SNP for ACE2 SNP rs2285666. Primers were designed using primers 3 program (Primer3 on Biowulf (nih.gov).

> Homo sapiens angiotensin converting enzyme 2 (ACE2), RefSeqGene on chromosome X.

Reference Sequence: NG_012575.3_G8790A_ C/T reverse complement

TGAAACACACATATCTGCAATCATTTTTAAAATCTGAGAGAAAAGTAAATTTCATAATCACTACT
AAAAATTAGTAGCCTACCTGGTTCAAGTAATAAGCATTCTTGTGGATTATCTGGGTTACAAACTT
TTCCAGTACTGTAGATGGTGCTCATTGTATTTAGAATTGTGTTCAACTGCAAATTAAAGATAATA
AACAATGTTAACAATGGAAATTTTGCTGAAGA

PCR DNA MW: 227 bp+67 Illumina primers =294 in total

Figure 8: Primers and PCR target regions for ACE2 SNP rs2285666.

After designing the primers, Illumina adaptors were added to the genes flanking regions for sequencing purposes according to 16S Metagenomic Sequencing Library Preparation protocol (16S Sample Preparation Guide (illumina.com). Each forward primer was attached to the Illumina overhang adapter sequences at the 5' end (5'-CGTCGGCAGCGTCAGATGTGTATAAGAGACA-3') and each reverse primer was modified by adding the Illumina overhang adapter sequences at the 5' end of it (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-3). These adaptors used so they can attach the sequencer machine flow cell before the start of sequences process. The complete sequence of the combined primers was ordered from IDT Company.

The sequencing DNA library was prepared using two-PCR steps. The first one included the amplification of the two- target genes by a multiplex PCR using four primers. Amplification of ACE1 (rs4343, rs4342, rs4341) and ACE2 (rs2285666) were performed using primers in Table1. Multiplex PCR was performed using two primer pairs, which targeted the three SNPs

of ACE1 (rs4343, rs4342, rs4341) and one SNP of ACE2 (rs2285666). In all primers, Illumina adaptor sequences were added to the forward ends of the 5' ends (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG3') and reverse (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3). The reaction was carried out using 3µl of the extracted DNA in a final volume of 26µl, which contained 12.5µl PCRBIO HS Taq Mix Red (PCR Biosystems, Ltd.), 8.5µl double distilled water (dH2O) and 0.5µl of each primer (10pmol). The amplification conditions were as follows: initial denaturation at 95˚C for 5 minutes followed by cycles of 95˚C for 15 seconds, 60˚C for 15 seconds, and 72˚C for 40 seconds, with a final extension step of 72˚C for 6 minutes. The product was loaded on 2% agarose gel producing, two bands of DNA with a molecular size of 277bp for ACE1 and 294bp for ACE2.

Table 1: The primer sequences used in PCR for SNP detection.

Primer name	**Primer sequence (5′-3′)**	**Gene\SNP**
F2, R_rs4343_NGS	F- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CGAGCCAGCTCTGAAATTCT R- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTGATGAGTTCCACGTATTTCG	ACE1\rs4341 ACE1\rs4342 ACE2\rs4343
F, R**_G8790A _NGS**	F- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGT GAAACACACATATCTGCAATCA R- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCTTCAGCAAAATTTCCATTGTT	ACE2\rs2285666

F, forward; R, reverse

2.2.3 Gel Electrophoresis

Approximately 2g of agarose was added to 100 ml Tris-acetate-EDTA (TAE) buffer, then the mixture was boiled for 30 seconds, followed by the addition of 2 µl ethidium bromide. Following that, the mixture was poured into an agarose gel casting system (Bio-Rad, SUBCELL®GT). In each well, 5 µl of each PCR product was loaded. Samples were then run at 120 volts for 40 minutes. PCR products were visualized using gel documentation system (GelDoc).

2.2.4 Cleaning PCR product

The PCR products (25 µl) were cleaned by AMPure XP beads, Beckman Coulter (X1), and eluted in 25 µl elution buffer according to company protocol (https://www.protocols.io/view/magnetic-particle-based-dna-purification-e6nvwb27vmkj/v2).

In order to purify samples, AMPure XP beads (50 µl) were added to 25µl of each sample at room temperature and incubated for 10 minutes. On a magnetic plate, samples were left for two minutes until they became clear. After the supernatant was discarded, the pellets were washed two times with ethanol 80% (180 µl) and left for 10 minutes to dry. A final step involved adding 25 µl of elution buffer (EB) to each sample and incubating it at room temperature for two minutes. Then, the supernatant (25µl) was transferred to new collection tubes and kept frozen at -20C until further use.

2.2.5 The second PCR and sequencing library preparation

The purified products (7.5 µl) were subjected to a second PCR, (PCR conditions for barcoding were: 72°C for 3 minutes, 95°C for 30 seconds, 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes) (12 cycles) to include unique index sequences (N7XX and S5XX) for barcoding of each sample using Nextera XT Index Kit (Illumina, San Diego, CA, USA). Then, 5 µl from each barcoded sample was pooled together, mixed and spun down. Finally, 100 µl of pooled DNA was purified using X1 AMPure XP beads and quantified by Qubit® Fluorometer (Invitrogen) machine. The concentration of 4.0 nM was prepared from the pooled sample. At least 10,000 reads for each sample were targeted. Samples were deep-sequenced (Macrogen Company) with the Nextseq500 machine using the 150-cycle mid output kit (Illumina, Inc., USA) from the forward read direction. The obtained DNA sequences were then analyzed using the Galaxy program. (https://usegalaxy.eu/).

2.2.6 Bioinformatics analysis

Sequences as FASTQ format files were received from the company. The files were analyzed using the Galaxy program (Galaxy Version 0.7.17.1) (https://usegalaxy.eu/). Initially, the obtained sequences were run through fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc//) to check the quality of the generated reads. The sequences were then trimmed (https://usegalaxy.org/) using default parameters to retain the highest quality reads (reads above 100bp) and a minimum quality score of > 20, which represents an error rate of 1 in 100 (according to Illumina Nextseq machine sequencing error rate), with a corresponding call accuracy of 99%. The filtered data were captured by virtual specific SNPs probes (see

below). Based on the SNPs-specific probes, the number of sequences reads for each SNP was determined. Identical sequence pattern of 12-16 bp selection with specific virtual probes for each SNP was chose and uploaded as sequence in the workflow for the count of each one, see table 2 and variation was shown within the probe sequence as underline.

To identify the targeted polymorphisms, eight virtual probe sequences were used (six for ACE1 gene variants, two for ACE2 gene variants (Table2).

Table 2: Virtual probe sequences used in PCR and sequence analysis.

Gene	Probe name	Probe sequence	Targeted SNP
ACE1	rs4341G	GGCTGGAGCTCAAGGC	**G**
	rs4341A	GGCTGGAGCTCAAGAC	**A**
	rs4342A	CAAACCCCTACC	**A**
	rs4342C	CAACCCCCTACC	**C**
	rs4343G	TGATGGCCACGT	**G**
	rs4343A	TGATGGCCACAT	**A**
ACE2	rsG8790	CTAAAAATTAGTAGCC	**G**
	rsA8790	CTAAAAATTAGTAGCT	**A**

Sequences of the ACE2 were designed from the reverse complement for that C represent G and T represent A as shown in the table.

2.3 Statistical Analysis

IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY was used for significant correlations (P- value <0.05). Analyses of categorical and quantitative values were presented as mean ±standard deviation. For comparing categorical parameters between groups, Chi-Square test (χ^2) was used. The odds ratio (OR) and 95% confidence interval (CI) of multiple logistic regression analyses were calculated to estimate the association between genotypes and allele frequencies with the possibility and severity of COVID-19 disease and with the signs and symptoms of COVID-19 disease. Statistical significance was determined by P< 0.05 in all tests.

Chapter Three

3. Results

3.1 Characteristics of Study Participants

A total of 130 Palestinians were included in this study and divided into three groups; COVID-19 positive cases group (n=50), COVID-19 control group (n=50) and COVID -19 ICU group (n=30). Among the 130 participants in this study, 50% were male and 50% were female, showing a female- to -male ratio of 1:1. In each group, the median age in case group was (36.06±19.9) years, (32.14±13.6) in control group, and (56.33±17.0) years for ICU group. A significant difference in age existed among the ICU patients as they are the oldest.

The characteristics and comorbidities of each study group i.e., prevalence of smoking, *Diabetes mellitus* (DM), hypertension, and coronary artery disease (CAD) are shown in table 3. Sex, prevalence of diabetes and smoking showed no significant difference between controls versus COVID-19 patients (P-value > 0.05, X^2). However, severity of the COVID-19 disease was associated with hypertension, coronary artery disease and age and they were significantly higher in the ICU group (P-value=0.000, X^2).

Table 3: Demographic properties and co-morbidties of the studied groups.

Variables	Case(n=50)	Control (n=50)	ICU (n=30)	Total (n=130)	P<0.05
Sex, n (%)					0.091
Female	31 (23.8)	22 (16.9)	12 (9.2)	65 (50.0)	
Male	19 (14.6)	28 (21.5)	18 (13.8)	65 (50.0)	
Age	36.06±19.9	32.14±13.6	56.33±17.0	39.23±19.4	0.000
Smoking, n (%)	6 (4.6)	13 (10.0)	6 (4.6)	25 (19.2)	0.205
Diabetes, n (%)	4 (3.1)	4 (3.1)	6 (4.6)	14 (10.8)	0.177
Hypertension, n (%)	3 (2.3)	2 (1.5)	12 (9.2)	17 (13.1)	0.000
CAD, n(%)	3 (2.3)	2 (1.5)	10 (7.7)	15 (11.5)	0.000

ICU, Intensive care unit; P, P value, CAD; Coronary artery disease.

The clinical characteristics of COVID-19 patients in the case and ICU groups with signs and symptoms frequencies are illustrated in table 4. The frequency of fatigue, headaches, runny noses, and loss of smell were significantly higher in the positive cases group (P-value < 0.05, X^2) compared to ICU group. However, dyspnea was more frequent in the ICU group (P-value < 0.05, X^2) as shown in table 4.

Table 4: Clinical signs and symptoms of case and ICU patients.

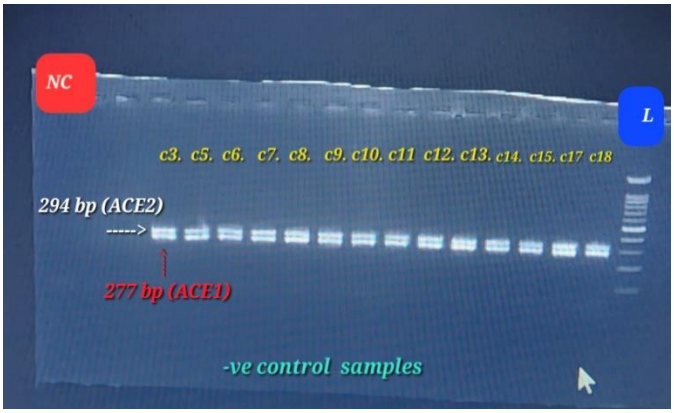
Variables	Case(n=50)	ICU (n=30)	Total (n=130)	P<0.05
Runny Nose, n (%)	28 (35.0)	7 (8.8)	35 (43.8)	0.004
Fatigue, n (%)	37 (46.3)	10 (12.5)	47 (58.8)	0.000
Headache, n (%)	36 (45.0)	9 (11.3)	45 (56.3)	0.000
Fever, n (%)	31 (38.8)	19 (23.8)	50 (62.5)	0.905
Loss of smell, n (%)	33 (41.3)	10 (12.5)	43 (53.8)	0.005
Muscle/body aches, n (%)	31(38.8)	20(25.0)	51(63.7)	0.674
Diarrhea, n (%)	18 (22.5)	5 (6.3)	23 (28.7)	0.064
Sore throat, n (%)	23 (28.7)	8 (10.0)	31(38.8)	0.086
Cough, n (%)	24 (30.0)	20 (25.0)	44 (55.0)	0.104
Dyspnea, n (%)	18 (22.5)	26 (32.5)	44 (55.0)	0.000

ICU, Intensive care unit; P, P value

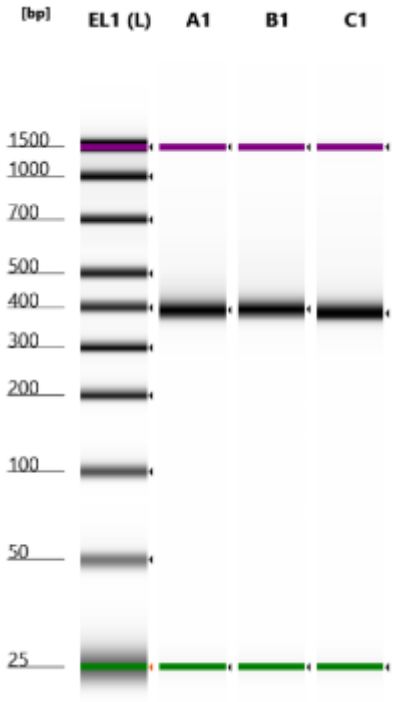
3.2 Amplification of ACE1 and ACE 2 target sequences and Gel electrophoresis

The multiplex PCR assay shows a successful PCR combination by adding 4 primers of two genes combinations, as you can see the 2 bands which shown on the gel below. So, the method is specific in which upon identical sequence pattern selection with specific virtual probes revealed by the sequences showed 100% with probe selection, in addition the sequence results was blasted and showed identical to specific gene and SNP results.

In the pictures below, PCR products revealed two bands with a size of 300-400 bp targeting the angiotensin converting enzyme genes (ACE1 and ACE2). Figure 9 shows the agarose gel electrophoresis and a picture of representative samples to visualize the PCR products.



(A)-



(B)-

Figure 9: (A) The PCR products of the angiotensin converting enzyme genes ACE1, and ACE 2 analyzed by agarose gel electrophoresis. Lane L refers to DNA ladder (100bp), NC: Negative control, C3-C18, represent negative control samples with the size of the upper and lower band were approximately 300bp for PCR products. (B) Three positive case representative samples (A1+B1+C1) (bands in black shows the size of the PCR products is around 300bp).

Figure 10 shows one example of the multiple sequence alignment of heterozygote control sample number 11 (LC11) using free online program (Multalin interface page (inra.fr)**.** We confirm the results by showing the multiple alignment against the reference gene. In another words, workflow results are confirmed, and the correct sequence were captured as seen below.

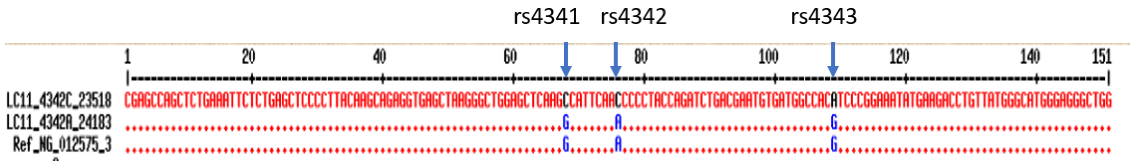


Figure 10: An example of the multiple sequence alignment of heterozygote negative control sample number 11 (LC11).

3.3 Determination of ACE1 and ACE2 genotypes

Table 4 below shows the ACE1 and ACE2 genotype determination for representative samples. The genotypes were determined based on the calculated ratio between the read counts for wild type and mutant alleles, for each allele in each individual sample. For example, for ACE1 rs4343 sample number 1, the number of reads for G allele is G to A ratio for sample number 1 equals 1, revealing that this patient is a heterozygous for ACE1 rs4343 polymorphism and thus has a

GA genotype. Consequently, if the ratio between the wild type and mutant allele is less than 1; it's homozygous for the minor allele AA, if it equals to 1; it's heterozygous (GA) and if its higher than 1; it's a homozygous for the wildtype allele (GG).

Table 5 :ACE1 and ACE2 genotype determination for a representative group of samples.

	rs4343		rs2285666		rs4342		rs4341	
Samples	**G/A**	**Genotype**	**G/A**	**Genotype**	**C\A**	**Genotype**	**C\G**	**Genotype**
1	1.0	GA	1.0	GA	0.97	CA	0.94	CG
4	1.0	GA	571.3	GG	0.99	CA	0.97	CG
10	18.2	GG	25.8	GG	0.02	AA	0.003	GG
13	105.0	GG	466.9	GG	0.02	AA	0.004	GG
14	183.3	GG	381.0	GG	0.02	AA	0.004	GG
15	2.5	GG	0.4	AA	0.01	AA	0.002	GG
16	1.1	GA	1.0	GA	0.96	CA	0.94	CG
18	1.0	GA	1.0	GA	0.98	CA	0.95	CG
19	1.0	GA	393.8	GG	1.02	CA	0.99	CG

3.4 Genotypes distribution of ACE1 (rs4341, rs4342, rs4343) and ACE2 (rs2285666) polymorphisms

The frequency and genotypes distribution of the ACE1 (rs4343, rs4342, rs4341) and ACE2 (rs2285666) among the three study groups are provided in table 6. The ACE2 (rs2285666) genotypes distribution was marginally different between the study groups (P-value= 0.049), while no significant differences were observed in the distribution of ACE1 (rs4343, rs4342, rs4341) genotypes (P-value > 0.05, X^2). As shown in table 6, for ACE2/rs2285666 polymorphism, the GG genotype (wild type) were more prevalent in case group compared to the other genotypes (P-value=0.049, X^2).

Table 6: Genotype distribution of ACE1 and ACE2 polymorphisms in COVID-19 patients (cases group and ICU group) and healthy controls.

Gene/SNPs	Genotype	Case(n)%	Control(n)%	ICU(n)%	P-Value
ACE1/rs4343	**GG**	24 (18.5)	17 (13.1)	15 (11.5)	0.128
	GA	23 (17.7)	27 (20.8)	9 (6.9)	
	AA	3 (2.3)	6 (4.6)	6 (4.6)	
ACE1/rs4342	**AA**	34 (26.2)	26 (20.0)	18 (13.8)	0.175
	CA	14(10.8)	23 (17.7)	9 (6.9)	
	CC	2 (1.5)	1 (0.8)	3 (2.3)	
ACE1/rs4341	**GG**	34 (26.2)	27 (20.8)	18 (13.8)	0.234
	GC	14 (10.8)	22 (16.9)	9 (6.9)	
	CC	2 (1.5)	1 (0.8)	3 (2.3)	
ACE2/rs2285666	**GG**	32 (24.6)	20 (15.4)	19 (14.6)	0.049
	GA	13 (10.0)	24 (18.5)	6 (4.6)	
	AA	5 (3.8)	6 (4.6)	5 (3.8)	

3.5 Association of ACE1 and ACE 2 polymorphisms with COVID-19 infection

Logistic regression analysis adjusted for age, sex, hypertension, and CAD was used to investigate the effect of ACE1 (rs4343, rs4342, rs4341) and ACE2 (rs2285666) polymorphisms on the susceptibility of COVID-19 infection. The case group and ICU group were compared with the control group, separately as a post-hoc test and the ICU group was compared to the control group (Table 7).

As shown in table 7, the case group and ICU patients who had GA genotypes of ACE2 rs2285666 were unlikely to have COVID-19 infection (AOR= 0.208, (95% CI: 0.07-0.5); P = 0.002) (AOR= 0.153, (95% CI: 0.03-0.6); P =0.013) respectively, GA SNP in ACE2 rs2285666 is protective. However, these correlations were not observed in patients with AA genotype

among case or ICU groups. In addition, no significant relationship between the ACE 1 polymorphisms (rs4341, rs4342, rs4343) and COVID-19 illness was observed (Table7)

Table 7: Association of ACE1 and ACE2 polymorphisms with COVID-19 infection**.**

Gene/SNP	positive Vs. Controls AOR (95% CI); P-value	ICU group Vs. Control AOR (95% CI); P-value
ACE1/rs4343		
GG	Ref.	Ref.
GA	0.55 (0.23-1.3); 0.182	0.648 (0.1 -2.2); 0.486
AA	0.29 (0.06-1.4); 0.12	1.47 (0.29-7.3); 0.636
ACE1/rs4342		
AA	Ref.	Ref.
CA	0.362 (0.02-4.5); 0.430	0.118 (0.009-1); 0.110
CC	0.779 (0.06-9.3); 0.844	0.120 (0.009-1.6); 0.111
ACE1/rs4341		
GG	Ref.	Ref.
GC	0.500 (0.21-1.1); 0.115	1.063 (0.3-3.4); 0.918
CC	1.324 (0.11-15.8); 0.825	8.624 (0.6-116.9); 0.105
ACE2/ rs2285666		
GG	Ref.	Ref.
GA	0.208 (0.07-0.5); 0.002	0.153 (0.03-0.6); 0.013
AA	0.518 (0.12-2.1); 0.358	0.234 (0.04-1.3); 0.103

AOR, adjusted-odds ration; CI, 95% confidence interval; Ref., Reference (Wildtype gene)

3.6 Association of ACE1 and ACE 2 polymorphisms with COVID-19 signs and symptoms

Logistic regression analysis adjusted for age, sex, hypertension, and CAD was used to investigate the effect of ACE1 (rs4341, rs4342, rs4343) and ACE2 rs2285666 polymorphisms on the signs and symptoms of COVID-19.

Table 8 shows that patients who have GA genotypes of ACE2 rs2285666 were unlikely to develop fatigue (AOR= 0.405 (95% CI: 0.168-0.973); P = 0.043), headache (AOR= 0.277 (95% CI: 0.110 -0.699); P = 0.007), loss of smell (AOR= 0.373 (95% CI: 0.155-0.901); P = 0.028) and dyspnea (AOR= 0.197 (95% CI: 0.063 -0.613); P = 0.005) In addition, patients who have AA genotypes of ACE2 rs2285666 were unlikely to developed dyspnea (AOR= 0.843 (95% CI: 0.183-0.040); P =0.029). Moreover, patients who have AA genotype for the ACE1 rs4343 polymorphism were unlikely to develop headache (AOR= 0.188 (95% CI: 0.036-0.982); P =0.048). No significant association was found for ACE1 (rs4343, rs4342, rs4341) with COVID 19 signs and symptom.

Table 8 : Association of ACE1 and ACE 2 polymorphisms with COVID-19 signs and symptoms.

	Gene/SNP	Alleles	P-value	AOR	CI		P-value	AOR	CI
Runny nose	ACE1/rs4343	GG	Ref.	Ref.	Ref.	Fatigue	Ref.	Ref.	Ref.
		GA	.932	.965	.423 - 2.200		.527	.777	.356 - 1.698
		AA	.108	.173	.021 - 1.465		.092	.291	.069 - 1.224
	ACE1/rs4342	AA	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		CA	.239	.593	.249 - 1.414		.114	.523	.234 - 1.169
		CC	.462	.431	.046 - 4.070		.534	.565	.093 - 3.420
	ACE1/rs4341	GG	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		CG	.281	.620	.260 – 1.479		.146	.550	.245 - 1.231
		CC	.472	.438	.046 - 4.145		.550	.578	.095 - 3.495
	ACE2/rs2285666	GG	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		GA	.055	.382	.143 - 1.021		.043	.405	.168 - 0.973
		AA	.523	1.50	.428 - 5.302		.969	1.02	.309 - 3.387
Headache	ACE1/rs4343	GG	Ref.	Ref.	Ref.	Dyspnea	Ref.	Ref.	Ref.
		GA	.188	.580	.257–1.306		.478	.727	.301 - 1.755
		AA	.048	.188	.036 -.982		.526	.641	.163 - 2.529
	ACE1/rs4342	AA	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		CA	.116	.516	.226 -1.177		.390	.681	.284 -1.635
		CC	.225	.241	.024 - 2.398		.839	.819	.120 -5.608
	ACE1/rs4341	GG	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		CG	.144	.540	.236 – 1.234		.452	.714	.297 – 1.717
		CC	.232	.246	.025 - 2.447		.854	.835	.122 -5.718
	ACE2/rs2285666	GG	Ref.	Ref.	Ref.		Ref.	Ref.	Ref.
		GA	.007	.277	.110 - 0.699		.005	.197	.063 -.613
		AA	.207	.395	.094 -1.670		.029	.183	.040 -.843

AOR, adjusted-odds ration; CI, 95% confidence interval; Ref., Reference (Wildtype gene).

Loss of smell	**ACE1/rs4343**	**GG**	Ref.	Ref.	Ref.
		GA	.552	1.26	.579 -2.775
		AA	.626	.722	.195 -2.672
	ACE1/rs4342	**AA**	Ref.	Ref.	Ref.
		CA	.698	1.16	.537 -2.531
		CC	.933	.925	.153 -5.594
	ACE1/rs4341	**GG**	Ref.	Ref.	Ref.
		CG	.605	1.22	.565 -2.667
		CC	.950	.944	.156 - 5.703
	ACE2/rs2285666	**GG**	Ref.	Ref.	Ref.
		GA	.028	.373	.155 -.901
		AA	.126	.341	.086 -1.351

AOR, adjusted-odds ration; CI, 95% confidence interval; Ref., Reference (Wildtype gene).

Chapter Four

4.1 Discussion

Symptoms of COVID-19 can range from mild to severe symptoms and include fatigue, headaches, runny noses, loss of smell fever, cough, difficulty of breathing and body aches. Some people may be asymptomatic or have very mild symptoms but can still spread the virus to others. There are several molecules that can serve as alternative receptors for SARS-CoV and SARS-CoV-2, apart from ACE2 such as C-type lectins, DC-SIGN, L-SIGN, TIM1 and AXL. By binding to phosphatidylserine on the viral membrane, they enhance the entry of a wide variety of enveloped viruses. Despite increasing viral entry, lectins and phosphatidylserine receptors are non-specific and cannot support efficient infection by SARS-CoV or SARS-CoV-2 without ACE2.

Genetic polymorphism of ACE1 and ACE2 have been associated with the severity of COVID-19. A number of studies suggest that the ACE2 polymorphism (rs2285666) may influence the susceptibility and severity to SARS-CoV-2 infection by increasing the expression of the ACE2 receptor (Saengsiwaritt *et. al*., 2022). An intronic SNP (G8790A, rs2285666) can affect ACE2 gene expression and protein levels, since it is located in an intronic position so, it is likely to affect ACE2 gene expression by altering mRNA splicing (Patel *et. al*., 2014).

The introduced NGS method was able to detect four SNPs in one tube. This will help all studies targeting these SNPs with lower costs and less laborious to use with no need for gel and stains.

Also, no need for enzymes for RFLP analysis as commonly used worldwide. This method is sensitive and specific as a low number of sequences will be sufficient for the genotyping process.

In this study, the distribution of ACE2 (rs2285666) genotypes had marginally significant differences among the three studied groups, case, ICU, and control groups (P=0.049, X^2). We noticed that the wildtype GG genotype of ACE2 (rs2285666) were more prevalent in positive case group (24.6%) compared to control group. Such differences were not observed in the ACE1 (rs4343, rs4342, rs4341) variants. Our results are in agreement with another study that investigated the association of ACE1 (rs4343) and ACE2 (rs2285666) with COVID-19 susceptibility in which COVID-19 infection risk was significantly increased in individuals carrying the GG genotype of ACE2 (rs2285666) and ACE1 (rs4343) (Alimoradi *et. al*., 2022).

Logistic regression analysis adjusted for possible confounding factors such as age, sex, CAD and hypertension revealed that the GA carriers of ACE2 rs2285666 among positive case group and ICU patients were less prone to COVID-19 infection (OR= 0.208 (95% CI: 0.07-0.5); P = 0.002) (OR= 0.153 (95% CI: 0.03-0.6); P =0.013) respectively, indicating a protective effect of the minor allele. However, the homozygous AA genotype was not associated with COVID 19 disease which could be attributed to the small sample size. A previous report found that age and comorbidities associated with ACE2 polymorphisms such as hypertension and coronary artery disease, could exacerbate COVID-19-induced-ACE2 deficiency and exacerbate its severity and mortality (Verdecchia *et. al*., 2020). Based on the polymorphisms of ACE1 I/D and ACE2 rs2285666, researchers in previous research determined that severe COVID-19 cases were associated with hypertension, high cholesterol but that the effect varies with hypertension severity (Gómez *et. al*., 2020). This supports the current study which revealed an association of hypertension and coronary artery disease (CAD) with the severity of the COVID-19 disease

as the prevalence of hypertension and CAD were significantly higher in the ICU group ($P < 0.05$, X^2). Moreover, our study revealed that the age was an independent risk factor for the severity of COVID 19, the ICU group was older than case and control groups. Similarly, a previous study have shown that age affects the severity and prevalence of COVID-19 (Alimoradi *et. al*., 2022). In this study, we noted that sex had no significant impact on the severity or prevalence of COVID-19 disease, contrary to previous studies finding that men are more prone to develop severe COVID-19 cases (Gómez *et. al*., 2020). In an earlier report, ACE2 variants were not associated with SARS-CoV susceptibility or outcomes with no sex differences (Chiu *et. al*., 2004).

A cross-sectional study that included 60,000 COVID-19 patients, reported that fever and breathing difficulties increased the likelihood of hospitalization and death. A running nose, sore throat, diarrhea, and headache, on the other hand, were associated with a lower risk of hospitalization and death; the researchers concluded that these symptoms provide protection against hospitalization and death (dos Reis *et. al*., 2022). In this study, dyspnea was more frequent in the ICU group ($P < 0.05$) whereas the frequency of fatigue, headaches, runny noses, and loss of smell were significantly higher in the positive cases group ($P < 0.05$). Our results showed that ACE2 rs2285666 GA carriers were unlikely to develop fatigue (OR= 0.405 (95% CI: 0.168-0.973); P = 0.043), headache (OR= 0.277 (95% CI: 0.110 -0.699); P = 0.007), loss of smell (OR= 0.373 (95% CI: 0.155-0.901); P = 0.028) and dyspnea (OR= 0.197 (95% CI: 0.063 -0.613); P = 0.005) indicating the association of the minor allele A with less severity of COVID19. In addition, we found that the homozygous rs2285666 (AA) patients were unlikely to develop dyspnea (OR= 0.843 (95% CI: 0.183-0.040); P =0.029). For the ACE1 rs4343

polymorphism, patients who have AA genotype were unlikely to develop headache (OR= 0.188 (95% CI: 0.036-0.982); P =0.048).

Our findings suggest that the ACE2 rs2285666 A-allele has a protective effect: If A-allele carriers produce more ACE2 than those with GG genotypes, they may be at least partially protected by the imbalance that happens between ACE1 and ACE2 when SARS-COV2 infect. It is also possible to deal better with the adverse effects of high levels of Ang II, which can cause severe lung and heart damage (Lumber *et. al*., 2020).

It is believed that ACE2 and ACE1 have different physiological functions. Because RAS system plays a crucial role in cardiovascular, respiratory, and diabetes pathogenesis, cross-models of ACE1 and ACE2 genotypes may aggravate COVID-19 by causing RAS imbalance by increasing the ratio of ACE1 to ACE2. Acute respiratory distress syndrome (ARDS) is associated with the imbalance of ACE2 activity in favor of ACE1 activity, leading to a RAS imbalance and greater lung damage. This may be due to the reduction of pulmonary Ang-(1–7) levels and elimination of its anti-inflammatory effects (Magalhaes *et. al*., 2018).

Lastly, researchers have discovered that there may be "population-specific" genetic variations in ACE2 that influence susceptibility to SARS-CoV2 infection, based on analysis of the "1000 Genomes Project," which contains samples from almost all ethnicities (Simsek *et. al*., 2020). Khayat *et. al*., 2020 found ten ACE2-related variants in coding, noncoding, and regulatory sites, which can explain the epidemiological differences associated with COVID-19.

4.2 Study limitations and recommendation

In terms of the study limitation, we should note that the number of enrolled subjects was relatively small, and we should consider that some people may have got vaccinated while others

did not. On the other hand, some people may have been infected with different variants of the virus such as Beta variant, Gamma variant, Delta variant, Lambda variant and Omicron. In this study, investigation revealed that the hypothesis of wild genotype GG of ACE2 were associated with COVID-19 infection in the Palestinian population. As a result of continuous monitoring, the Ministry of Health keeps track of infections caused by Coronavirus lineages. COVID-19 hospitalizations have increased slightly since the time of this message, most of them mild in nature. Additionally, there was a slight increase in the number of cases in critical condition and deaths linked to COVID-19. As a result of mutations, the ministry of health can also monitor the spread of viruses across the globe and make a guideline for it. Based on this, future research should examine this hypothesis in this research, with a larger sample size.

4.3 Conclusions

The genotype distribution of ACE2 (rs2285666) polymorphism was marginally significantly different among COVID 19 patients compared with the control group (P-value < 0.05, X2) , while no statistical differences were observed in the genotype's distribution of the ACE1 (rs4343, rs4342, rs4341) variants (P-value > 0.05, X2) (Table 6).

We showed novelty in detection ACE2 (rs2285666) polymorphism and ACE1 (rs4343, rs4342, rs4341) variants in one reaction tube. Individuals with ACE2 rs2285666 GG genotype were more prevalent in COVID-19 patients compared to control group (P-value =0.049, X^2) (Table 6).

Age and comorbidities such as hypertension and coronary artery disease were independent risk factors for COVID-19 disease (P-value < 0.05, X^2) (Table 3).

Symptoms of COVID-19 patients such as fatigue, headaches, runny noses, and loss of smell were significantly higher in the COVID-19 case group ($P < 0.05$), while dyspnea was more frequent in the ICU group (P-value < 0.05, X^2) (Table 4).

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