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العلاقة الجينية لبعض اشكال تشوه الجينات مع التوزيع المكاني ل **كوفيد-** ١٩ في محافظة بابل/ العراق .

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إشـــــراف

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Genetic Association of Some Genes Polymorphism with Covid-19 Special Distribution in Babylon Province, Iraq.

A Thesis

Submitted to the Council of the College of science for women-University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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الخلاصة

في ديسمبر ٢٠١٩ ، تسبب فيروس كورونا الجديد ، المسمى الآن بـ SARS-CoV-2 ، في سلسلة من أمراض الجهاز التنفسي غير النمطية الحادة في ووهان ، مقاطعة هوبي ، الصين. سمي المرض الناجم عن هذا الفيروس بـ COVID-19. الفيروس قابل للانتقال بين البشر وقد تسبب في حدوث جائحة في جميع أنحاء العالم. يستمر عدد الوفيات في الارتفاع واضطر عدد كبير من البلدان إلى التباعد الاجتماعي والإغلاق. فيروسات كورونا (CoVs) هي عائلة من فيروسات الحمض النووي الريبي التي تسبب المرض للإنسان والحيوانات الأخرى عن طريق دخول الجسم عن طريق مستقبلات *ACE2* التي توجد في أعضاء مختلفة مثل القلب والرئتين والكلى والجهاز الهضمي ، وهو مكمل في الشكل لشكل السنبلة. فيروسات كورونا هي فيروسات جسيمات كبيرة ، مغلفة ، كروية الشكل ولها طفرات تشكل إسقاطًا سطحيًا ، مع جينومات الحمض النووي الريبي ذات الإحساس الإيجابي أحادية الخيط. ينتمي SARS-CoV-2 إلى جنس Sarbecovirus من جنس Betacoronavirus ، وفقًا لتحليل النشوء والتطور.

تهدف هذه الدراسة إلى تقييم تأثير تعدد الأشكال لبعض الجينات مثل *MIF* و *Sp110* و *IL-10* و *IL-6* Interleukin-6 على القابلية للإصابة بـ covid-19 في محافظة بابل. إجمالي (١١٣) حالة في هذه الدراسة (٦٣) حالة ظهرت عليها أعراض سريرية لمرض كوفيد -١٩ مريض ، (٥٠) عينة دم تم جمعها من أشخاص أصحاء كمجموعة ضابطة في هذه الدراسة خلال (نوفمبر ٢٠٢١ إلى فبراير ٢٠٢٢) في مستشفى المرجان . كان هناك العديد من البيانات الديموغرافية المدرجة في هذه الدراسة مثل التوزيع العمري ، وتوزيع الجنس ، والتوزيع الجغرافي لمرضى كوفيد. من بين (٦٣) عينة من مرضى كوفيد -١٩ مشتبه بهم من مختلف الفئات العمرية (٢٠ إلى ٨٠ سنة).

كان هناك ارتفاع في الإصابة بفيروس كوفيد -١٩ بين الفئات العمرية مقارنة بالفئات العمرية الأخرى. تظهر نتائج هذه الدراسة أن كوفيد -١٩ موجود في جميع الفئات العمرية. على الرغم من أن الحقل يشير إلى ارتفاع معدل الإصابة بـ covid-19 في الفئة العمرية الصغيرة مقارنة بالفئات العمرية الأخرى ، إلا أن Covid-19 يظهر بشكل شائع في الفئات العمرية النشطة (٢٠-٨٠) عامًا. يوصي هذا الاستنتاج بأن فيروس كوفيد -١٩ يصيب الفئة العمرية النشطة لأن لها تأثيرًا مباشرًا على الأسرة والجمهور والاقتصاد الوطني . تعرض هذا الحمض النووي لتضخيم PCR باستخدام بادئات محددة تستهدف مناطق محددة في الحمض النووي ثم تم تسجيلها للكشف

عن تعدد أشكال النوكليوتيدات المفردة (SNPs) باستخدام نظام الطفرة الحرارية للتضخيم - تفاعل البوليميراز المتسلسل (*VDR*) (ARMS-PCR، *IL-6*، *IL-10*، *TNF*) ، نظام طفرة حرارية تضخيم رباعي التمهيدي (*ACEI* \ *D*) (ARMS، *SP110*، *MIF*) وتقنيات التسلسل. تم الكشف عن تعدد الأشكال لـ *Sp110*gene عند (Rs7580900) في كل من الحالتين والسيطرة. تم تحديد تعدد الأشكال الجيني لـ *Sp110* في المواضع *Sp110 Rs7580900*.

بالنسبة لـ rs7580900، كانت الأنماط الجينية (AA، AT، TT) (28.32، 16.81، 10.62٪ ، 21.24)، 9.73، 13.27) ،٪ أو حالات كوفيد -١٩ الرئوية ، تحكم صحي ، على التوالي. في هذه الدراسة ، كان النمط الجيني AA *Sp110 Rs7580900* هو الأكثر شيوعًا في كل من مجموعتي 28.32) covid-19٪) والسيطرة (٢١.٢٤٪). يشير هذا إلى تعدد أشكال النوكليوتيدات المفردة في جينات *Sp110 Rs7580900* غير المرتبطة بالتعرض لفيروس covid-19 في المرضى. مع قيمة P (≤ 0.05). بالنسبة لـ *IL-6* ، كانت الأنماط الجينية (CC، 5.31) (CG & GG، 38.94، (11.50٪ ، 15.04)، 23.01، 6.19) ، ٪ أو حالات كوفيد -١٩ الرئوية ، تحكم صحي ، على التوالي. كان النمط الجيني ٦ CG هو الأكثر شيوعًا في كل من مجموعتي 38.94) covid-19٪) والسيطرة (٢٣.٠١٪). بالنسبة لـ *IL-10* ، كانت الأنماط الجينية (GG، GA، 3.54) (AA، 51.33، (0.88٪ ، 7.96)، 20.35، 15.93) ،٪ أو حالات كوفيد -١٩ الرئوية ، التحكم الصحي ، على التوالي. كان النمط الجيني ١٠ GA هو الأكثر شيوعًا في كل من 51.33) covid-19٪) ومجموعة السيطرة (٢٠.٣٥٪). كان هناك ٤٦.٣ سنة بين متوسط أعمار المرضى. تضمنت هذه الدراسة ما مجموعه ٦٣ مشاركًا في COVID-19 كانوا جميعًا مسجلين في الدراسة. وكان ٦٧ في المائة من المرضى يعانون من أعراض طفيفة ، بينما يعاني المرضى الباقون من أعراض خطيرة. توفي مريض واحد فقط من بين مجموعة المرضى الشديدة .

Summary

In December 2019, a novel coronavirus, now named as SARS-CoV-2, caused a series of acute atypical respiratory diseases in Wuhan, Hubei Province, China. The disease caused by this virus was termed COVID-19. The virus is transmittable between humans and has caused pandemic worldwide. The number of death tolls continues to rise and a large number of countries have been forced to do social distancing and lockdown.Coronaviruses (CoVs) are a family of RNA viruses that cause disease in humans and other animals by getting inside the body by *ACE2* receptors that are located in different organs like the heart, lungs, kidneys, and digestive system, which is complementary in shape to the spike shape. Coronaviruses are large particles viruses, enveloped, spherical in shape and have spikes that form a surface projection, with large single-strand positive sense RNA genomes. SARS-CoV-2 belongs to subgenus Sarbecovirus of the genus Betacoronavirus, according to phylogenetic analysis.

This study aims to evaluate the polymorphism effect of some gene like*ACEI/D,ACET>C,GSTM1,GSTT1,Sp110,VDR,IL10,TNF*,macrophage migration inhibitory factor *MIF patients*, Interleukin-6 *IL-6* on susceptibility to covid-19 in Babylon province. A total of (113) cases in this study (63) cases have clinical symptoms of covid -19 patient , (50) blood samples were collected from healthy people as a control group in this study during (november 2021 to february 2022) at Al-Marjan Hospital. There were many demographic data included in the present study such as age distribution, sex distribution, and geographic distribution of covid patients. Among (63) samples from suspected covid -19 patients from different age groups (20 to 80 years old).

There was elevation in covid-19 infection among old age group in comparison with other age groups.The results of this study show that covid-19 is found in all age groups. Although the field indicates that there were high covid-19 infection rate in young age group in comparison to other age groups, covid-19 is commonly presented in active age groups (20-80) years old.This conclusion recommends that covid-19 infects the active age group because they have a direct influence of the family, public, and national economy.These DNA samples were subjected to PCR amplification using specific primers targeting specific regions in the DNA and then enrolled for detection of single nucleotide polymorphisms (SNPs) by using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR)*(VDR,IL-6,IL-10,TNF***)**, Tetra-primer amplification refractory mutation system (ARMS)***(****ACEI\D, SP110,MIF*) and sequencing techniques.

The polymorphism of *Sp110*gene at (*Rs7580900)* was detected in both cases and control. The genetic polymorphism of *Sp110* was determined at positions *Sp110 Rs7580900*. For rs7580900 the genotypes (AA, AT &TT) were (28.32, 16.81, 10.62) %, (21.24, 9.73, 13.27), % or pulmonary covid-19 cases, healthy control, respectively. In this study ,the *Sp110 Rs7580900* AA genotype was the most frequent in both covid-19 (28.32%) and control (21.24%) groups. This indicates the single nucleotide polymorphisms in the Sp110 Rs7580900 genes no associated with susceptibility to covid-19 in patients. with, P value (≤ 0.05). For *IL-6* the genotypes (CC,CG &GG) were (5.31, 38.94, 11.50) %, (15.04, 23.01, 6.19) , % or pulmonary covid-19 cases, healthy control, respectively. In this study ,the *IL-6* CG genotype was the most frequent in both covid-19 (38.94%) and control (23.01%) groups. For ***IL-10*****,** the genotypes (GG, GA &AA) were (3.54, 51.33, 0.88) %, (7.96, 20.35,

15.93) , % or pulmonary covid-19 cases, healthy control, respectively. In this study ,the ***IL-10*** GA genotype was the most frequent in both covid-19 (51.33%) and control (20.35%) groups. There were 46.3 years between the mean ages of the patients. This study included a total of 63 COVID-19 participants who were all enrolled in the study. Sixty-seven percent of patients had minor symptoms, while the remaining patients were suffering from serious symptoms. Only one patient died out of the entire group of severe patients.

بِسْمِ ِالله الرَّحْمنِ الرَّحِيمِ

﴿قَالُواْ سبحَانكَ لاَ علمَ لنَاَ إِلَّا مَا عَلَّمتَنَا إِنَكَ أَنتَ العَلِيمُ الحَكِيمُ﴾

صَدقَ ُالله العليُ العظيمُ

سُورةُ البقرة
الآية /٣٢

Supervisor Certification

I certify that this thesis entitles (**Genetic Association of Some Gene Polymorphism with Covid -19 Special Distribution in Babil Province, Iraq**) was prepared under our supervision at the department of Biology, College of Science for Women, University of Babylon as a partial requirement for the degree of Master in Biology.

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In view of the available recommendation, I forward this thesis for debate by the examining committee.

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Certification of Examining Committee

We, the member of examining committee, certify that we have read this thesis entitled (**Genetic Association of Some Gene Polymorphism with Covid -19 Special Distribution in Babil Province, Iraq**) and after examining the Master student (Zahraa Hamza Ali) in its contents in 2/ 4 / 2023 and that in our opinion it is adequate as a thesis for the degree of Master in Biology with degree (Excellent)

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Appendix

3.4.3.1. Isolation kit Components

Components	Amount	Components	Amount
Cell Lysis Solution	500 ml	Protein Precipitation Solution	125 ml
Nuclei Lysis Solution	250 ml	DNA Rehydration Solution	100 ml

3.4.3.2. The Protocol for DNA Separation and RNA

Procedure which favor prep kit recommend for DNA separation as reveled in bellow:

Step 1 – RBC Lysis

1. First of all , we collect fresh human bloodin an anticoagulant-treat collection tube
2. Then, 300µl fresh blood was transferred up to a 1.5ml micro centrifuge tube
3. An inversion mixture used for 3 x the sample of RBC lysis buffer, then incubated at room temperature for 10 minutes ,We centrifugethe tube at 3,000-x g for 5 minutes and thesupernatant completely removed
4. At the last of step 1 **,** the pellet was re-suspended with 100 µl of RBC Lysis Buffer

Step 2 – Cell Lysis

5. A vortex was used as mixture for 200µl of FABG buffer then, we incubate the tube for 10 minutes at room temperature or until the sample, lysate is clear.Duringincubation, the tube was inverted every 3 minutes.

Step 3 – Binding

6. In the beginning of step 3 , 200µl ethanol (96~100%) was added to the sample and vortex for 10 seconds
7. A FABG Column was added to a 2ml collection tube. We transferred the sample mixture carefully to FABG Column and then Centrifuged

for 5 minute at full speed (14,000 rpm or 10,000 x g) after that ,the 2ml collection tube was discarded ,the FABG Column was addedin a new 2ml Collection tube .

Step 4 – Washing

8. In step 4, we washed FABG Column with 400µl W1 Buffer and then Centrifuged for 30 seconds at full speed (14,000 rpm or 10,000-x g) and after that, the flow-through was discarded.

9. The FABG Column placed back in the 2ml Collection tube and then FABG Column washed with 600µl Wash Buffer (ethanol added). Centrifuged for 30 seconds at full speed (14,000 rpm or 10,000-x g) and discarded the flow-through.

10. FABG Column placed back to a 2ml collection tube. Centrifuged for an additional 3 min at full speed (14,000 rpm or 10,000-x g) to dry the column.

Step 5 – Elution

11. In this step, we addedthe dry FABG Columnto a new 1.5ml microcentrifuge tube.

12. We take100µl of Preheated Elution Buffer and added to the membrane center of FABG Column. Then; FAGB Column stranded for three~5 min or until the buffer is absorbed by the membrane.

13. the sample Centrifuged for 30 seconds at full speed (14,000 rpm or 10,000 x g) to elute the DNA

Step Final - Pure DNA

14. At last, the DNA fragment stored at 4ºC or -20ºC as the final step.

Chapter One

1.1 Introduction

Millions of people have been impacted by the 2019 coronavirus illness (COVID-19), which is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1), the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), and four seasonal coronaviruses that cause moderate infections are all members of the Coronaviridae family (Wang *et al*.,2020;Corman *et al*.,2018).

COVID-19 has a wide clinical spectrum, ranging from asymptomatic to mild, moderate, severe, and catastrophic disease. Fever, cough, headache, weariness, breathing difficulties, anosmia, and ageusia are the most common symptoms. A third group of people afflicted show no signs or symptoms,81 percent of symptomatic individuals have mild to moderate symptoms, 14 % have severe symptoms (dyspnea, hypoxia, lung involvement on imaging), and 5% develop respiratory failure, ARDS, shock, or multiorgan failure (Guo *et al*.,2021).

The case fatality rate in hospitalized patients varies between 1 and 10% and 17 percent worldwide, with variances according to denominator size, number of people examined, demography, ethnicity, health-care system functionality, and virus types Hopkins, (2021). A significant underestimating of asymptomatic infections was caused by undetected cases during the first wave (Picchiotti *et.al*.,2020). Immune dysregulation and a cytokine storm (CS) (Yang *et al*.,2021, Fajgenbaum and June 2020) with a sudden surge in proinflammatory cytokines and other inflammatory markers is a common hallmark of severe disease.

COVID-19 is largely a vascular, rather than a pure respiratory disease (Zhang *et al*.,2021), therefore this hyperinflammatory state generates coagulopathies, oxidative stress, organ damage, and mortality (Fajgenbaum and June,2020). A multisystem inflammatory syndrome is a rare occurrence in children (MIS-C). Adults can also get a MIS (MIS-A) (Yamada *et al*.,2021).

Older age, male sex, and comorbidities such as chronic lung disease, cardiovascular disease and hypertension, diabetes, obesity, and cancer (Velavan *et al*.,2020), (Phua *et al*.,2020), (Boutin *et al*.,2020), as well as ethnicity (Garg *etal*.,2020, Niedzwiedz *etal*.,2020). disease (including pneumonia and dyspnoea) that may necessitate hospitalization and be exacerbated by cytokine storm, acute respiratory distress syndrome (ARDS), multi-organ and respiratory failure, all of which can be fatal (Guo *et al*.,2021,Wang *et al*.,2020).

Unlike the SARS-CoV (-1)-caused SARS pandemic (2002/2003), which was the first pandemic of the twenty-first century, the SARS-CoV-2-caused COVID-19 pandemic was not halted by public health preventative efforts in its first season 2019/20. Instead, it mandated that vaccines be developed quickly. SARS-1 was never able to do this (Hopkins, 2021,Picchiotti *et al.*,2020).

Immune responses in the host and immune-related symptoms are highly varied. Between patients who have effective control of SARS-CoV-2, i.e., asymptomatic, and those who are unable to control the virus, i.e., impacted by severe COVID-19, host immune responses and immunological-related symptoms are tremendously diverse. This shows that in some circumstances, host immunological dysregulation plays a role in pathogenesis. However, it is unclear whether the emergence of a

severe form of the disease is governed by immunological hyperactivity or a failure to resolve the inflammatory response as a result of continued viral replication and immune dysregulation.In mild cases, the relationship between cytokine levels, nasopharyngeal viral load, and falling viral load implies that the immune response is linked to viral burden (Yang *et al*.,2021, Fajgenbaum and June,2020).

Vaccines are the most effective prophylactic tool against diseases that have ever been devised. They have resulted in the eradication of smallpox and a significant decrease in the occurrence of diseases like diphtheria, tetanus, and poliomyelitis. Nonetheless, the need for novel vaccinations has never been greater, as the current SARS-CoV-2 pandemic has proved. Novel vaccinations are also needed to combat "old" illnesses such as malaria and tuberculosis (Zhang *et al.*,2021, Yamada *et al*.,2021). which continue to cause millions of new infections and hundreds of thousands of deaths each year.

The most severe outcomes were recorded in cancer patients with blood, lung, or metastatic malignancies (Velavan *etal*.,2020). As highlighted by a preliminary report on New Yorkers admitted to ICU (Phua *et al*.,2020), these retrospective reports, which are of limited size and limited to patients hospitalized in Chinese hospitals, may not be fully transposable to Western healthcare systems.

Aim of the study:

1. Examine the polymorphisms of a few related genes and how their genotype and allele frequency relate to each type of infection. These genes include those for important cytokines like interleukin-6 (*IL-6*), tumour necrosis factor (*TNF-308 A/G*), interleukin-10 (*IL-10*), vitamin D receptors (*VDR*), (*SP 110*), macrophage migration inhibitory factor (*MIF*), glutathione S-transferase Mu 1(*GSTM1*), glutathione S-transferase

(*GST*) theta 1 (*GSTT1*), Angiotensin-converting enzyme Insertion/Deletion (*ACE I/D*) and Angiotensin-converting enzyme 2 (*ACE 2*), in a samples of patients affected by covid -19 infection in addition to healthy people as control.

2. Measuring the serum levels of a few cytokines, including *TNF-*, *IL-10*, and *IL-6* to assess variations in the aforementioned cytokines between patients with covid-19 and between patients and control.

Chapter Two :- Literature Review

2.1 Coronavirus SARS-CoV-2

Coronavirus, also known as COVID-19, is a virus that was initially discovered in Wuhan, China, in December 2019 and quickly spread around the world (Zhu *et.al*., 2020, Chan *et.al*., 2020).

SARS-CoV-2 is a Coronavirus that causes Respiratory Syndrome. COVID-19 is a highly infectious virus. As a result, it is critical to screen for contagious disease as soon as possible. To prevent the spread of the disease, doctors must diagnose and isolate patients and help them get the therapy they need faster. Detection of COVID-19 infection can be detected using medical imaging techniques such as CXR. CT scans, for example, have been reported to produce accurate results is commonly utilized in the illness screening process (Rubin *et.al*., 2020, Chowdhury *et.al.*, 2020).

2.2 Coronavirus Epidemiology

China has already stated that a new outbreak of the virus is likely in the future, emphasizing the importance of developing a health system improvement and preparedness plan in the wake of the SARS epidemic's conditions and harsh criticism of global institutions for delayed provision of and sharing of information (Dong *et.al*.,2020).

COVID-19 can also be found in the feces and urine of infected patients with diarrheal symptoms, in addition to aerosols and large respiratory droplets. The WHO reported 45,171 cases and 1115 deaths globally from COVID-19 as of February 12, 2020. In China, 99 percent of infections and 99.9% of COVID-19 deaths were reported, according to statistics. WHO is carrying out activities to reflect changes in the COVID-1929 WHO scenario evaluations as of February 12, 2020. Drug studies are

being conducted in Wuhan, China, to improve COVID-19 drugs (Cyranoski ., 2020).

The COVID-19 infection has been made public as a result of the viral's susceptibility to immune suppression and periodicity. It is also obvious, according to research, people between the ages of 25 and 89 should be spread. The majority of adults admitted to Tongji Hospital were between the ages of 35 and 55, with cases of young persons and children being reported to be less numerous(Wang *et.al*.,2020). The patients' median ages ranged from 15 to 89 years, with the majority (5 percent) of men reporting early transmission dynamics findings(Li *et.al*.,2020). COVID-19 has enlarged the spectrum of transmissibility and pandemic hazards relative to SARSCoV, since COVID-19 estimates that the reproductive effectiveness (R) is higher than the high quadratic species indicated.(Li *et.al*.,2020).

2.2.1 Pathogenicity of Corona Virus

Coronavirus disease 2019 (COVID-19) was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It quickly spread around the world. According to the COVID-19 open datasets of the center for systems science and engineering at Johns Hopkins University, there have been 457 million confirmed fatalities and 221 million confirmed cases of COVID-19 as of September 6, 2021. COVID-19 has a wide spectrum of symptoms, ranging from asymptomatic or flulike sickness with fever, dry cough, lethargy, headache, loss of taste and smell to severe pneumonia with ARDS and respiratory failure requiring mechanical ventilation (Zhang *et.al*.,2021,Guan *et.al*.,2020).

The COVID-19 infection has been made public due to the widespread distribution of a virus that is sensitive to immune suppression as well as periodicity. In the WHO study, about 82% of COVID-19 patients who had

COVID-19 had no signs and were recovered immediately. By Feb. 20, 18264 (24%) cases were recovered in China and in the extreme cases Guang-dong recovery and death rates were 26.4% and 13.4%, respectively. In moderate and extreme cases, median signs began to heal once for two and three to 6 weeks.

In addition, there have been four coronaviruses (HCOVs, HCoV 229E, NL63, OC43, HKU1), non-extreme Acute Air Syndroma (SARS)-like coVs just one week before initiating and developing extreme symptoms, like hypoxia (Kratzel *et.al*.,2020), known as CoVs. They are mild and endemic all over the world. During this two-year period, three zoonotic CoVs have appeared, generating a lot of media and public attention, notably pathogenic CoVs that have caused human disease and death in the last two years. Coronavirus Diseases-2019 (COVID-19) is the disease caused by the coronavirus SARS (SARS-CoV now called SARS-CoV-1) discovered in November 2002, the coronavirus of the Middle East (MERS) (SERS-CoV)(Zaki *et.al.*,2012) and SARS-CoV-2, b.

Coronavirus Diseases-2019 (COVID-19) is the disease caused by the use of SARS-CoV-2.19. For investigations outside mainland China, symptoms began once 22.2 days for recovery (95% self belief interval 18-83). Furthermore, the duration of beginning the symptoms varies from 20.2 days to 22.3 days (Zaki *et.al.*,2012 ,Jung *et.al*.,2020) (95% self-confidence range of 15.1-29.9). Even though the ages are a deterministic decisive factor in the severity of the symptoms, various aspects of the development of signs and symptoms including a record of underlying conditions or co-infection with various infections, like the flu virus or *Klebsiella* can speed up the development of the disease, resulting in negative disease prognostics (Dorigatti *et.al*.,2020). The results of Singapore findings indicate, however, that infected patients without any background history are further

strengthened by severe disease and want intensive treatment(Ng and Hiscox, 2020).

Table (2-1): Pathological features of the SARS-CoV-2 virus in the body systems of COVID-19 cases (Afewerky., 2020).

Body system	**Pathological feature**
respiratory	A damage of pneumocytes with hyaline membrane formation,interstitial lymphocyte infiltration,and multinucleated syncytial cells in the lung
cardiovascular	Mayocardial and blood vessels injury
Immune	Lymphopena and exacerbated inflamation
Digestive	Fecal sample positive for SARS-COV- 2 RNA
Urinary	Protienuria,elevated level of serum creatinine,blood urea nitrogen, tubular necrosis,luminal brush border sloughing, and vacuole degeneration
Reproductive	Decreased serum testosterone to luteinizing hormone ratio in male patient
Skeletal	Joint problem such as arthritis
Integumentary	Ischemic changes in the fingers and toes
Nervous	Viral encephalitis,acute toxic encephalitis,and acute cerebrovascular disease
Other	Abnormal levels of alanine aminotransferase and aspartate aminotransferase in serum bilirubin

2.2.2 COVID-19 Genome: Comparison of Genomic Sequences

COVID-19, SARS-CoV, and MERS-CoV all showed that 2019-CoV is more similar to SARS-CoV than to MERS-CoV. In areas with 1av polyprotein and glycoprotein or s-protein surface alone, the COVID-19 amino acid sequence differs from those of other coronaviruses.(Huang *et.al*.,2020).

The S-protein is made up of two subunits, one of which binds to the host receptor right away to help the virus infect the host. In SARS EuropeanCoV, the S-RNA protein's binding region is more compatible with COVID-19.Although some of the residues connect the receptor, they are all universal. Residues that are not similar. COVID-19 research could lead to the conclusion that the human COVID-19 receptor is angiotensin conversion enzyme 2 (*ACE2*)(Dong *et.al*.,2020) (Fig. 1).

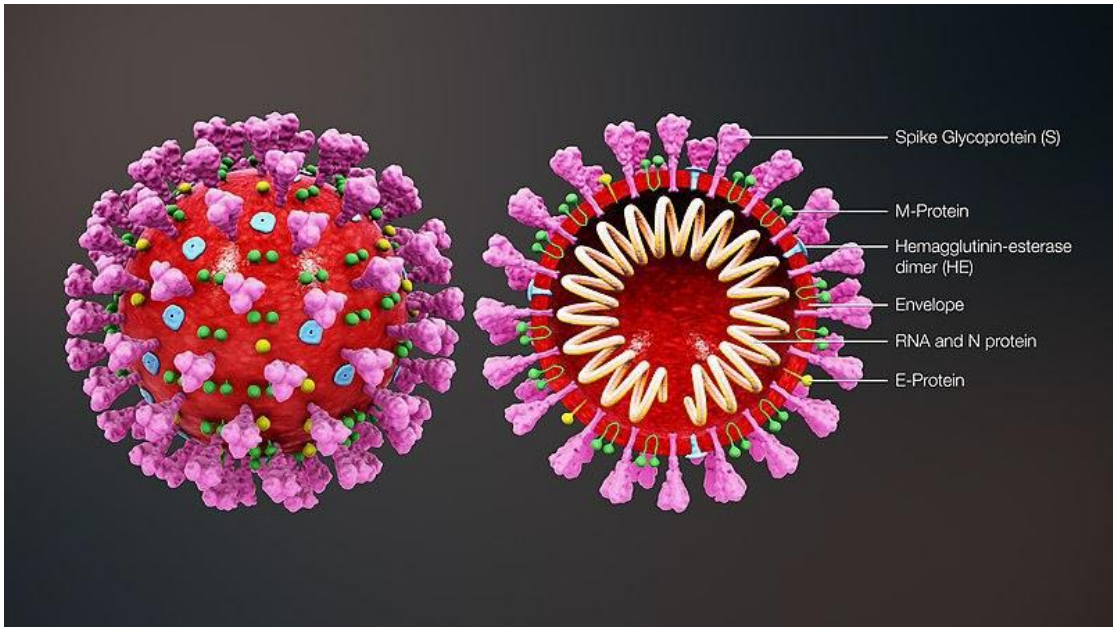

Figure (2-1): Schematic diagram of SARS-CoV-2 (Brian D.A. *et al*.; 2005).

In response to the current epidemic, the first clinical trials of an mRNA-based vaccine targeting the S protein SARS-CoV-2 began on March 16, 2020. Coronavirus protein, the most visible and protruding protein, is a key mediator in virus entrance. Because of their ability to neutralize host cell antibodies, full-scale S proteins (sub-compatible with the receptor domain binding domain S1) have been frequently used as vaccine antigen for developing SARS vaccines. However, research has shown that S protein-based vaccination does not provide complete safety and frequently raises protection concerns (De Wit *et.al*.,2016,Roper and Rehm.,2009).

2.2.3 Mechanism of Action Human Coronaviruses

Human coronaviruses have genes that encode viral proteins, nucleocapsides, and spikes in downstream sections of ORF1(Wan *et.al*.,2020). The spikes in glycoprotein on the coronavirus's exterior surface cause the virus to infect host cells. As a result, the virus can infect multiple hosts. The RBD (receptor-associating area) is constant between viruses. Other coronaviruses, including SARS-CoV and MERS-CoV, exopeptide a critical receptor in human cells (WHO., 2020). Mobile proteases such as HAT, cathepsins, and transmembrane protease serine 2) are involved in Coronavirus entrance. TMPRSS2 is a protein that divides and aggregates spikes.Shifts in penetration(Chu *et.al*.,2020,Mahallawi *et.al*.,2018). For HCoV-NL63 and SARS-coronavirus, four Dipeptidyl Peptidase (DPP4) and two angiotensin-converting enzymes (ACE2) are required as the major receptors. (Wang *et.al*.,2013,Yam *et.al.*,2003).

To retain van der Waals' (Xu *et.al*.,2020) capabilities, the SARS-CoV-2 spike protein possesses a three-dimensional structure in the RBD area. The 394 glutamine residue in SARS-RBD CoV-2's region binds to the human *ACE2* receptor via crucial lysine (Van Boheemen *et.al.*,2012, She

et.al.,2020). From replication attachment to pathogenicity, SARS-CoV-2 has a complex pathogenicity pathway (Figure. 2) Wan *et.al.*,2020).

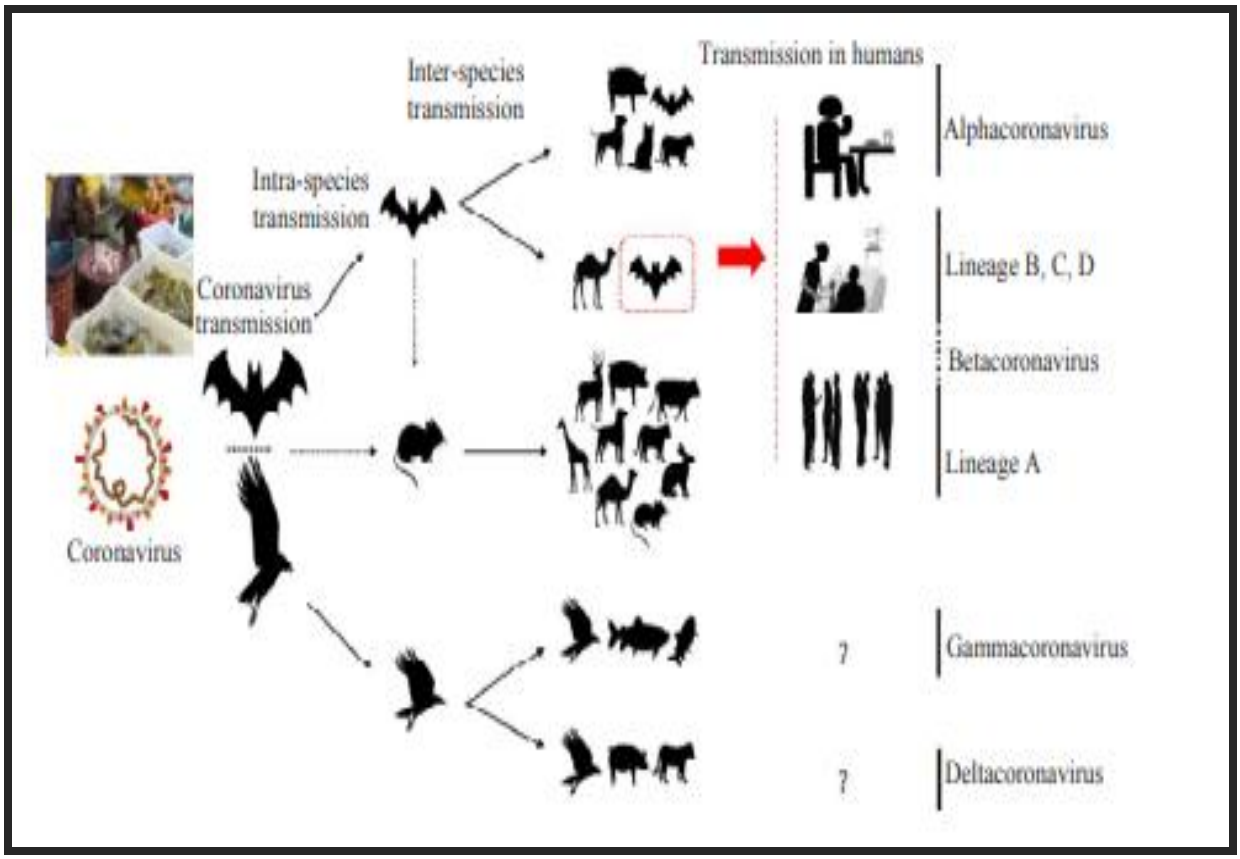

Figure (2-2): The key reservoirs and mode of transmission of coronaviruses(Wan *et.al.*,2020).

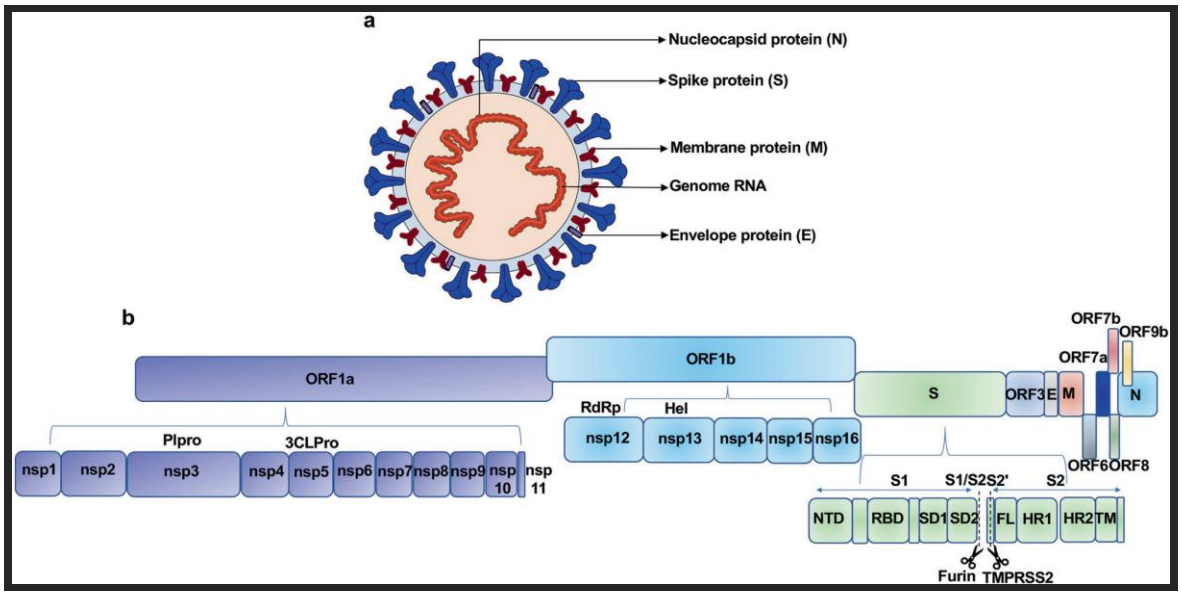

Figure (2-3) Schematic diagrams of the SARS-CoV-2 virus particle and genome. a Four structural proteins of SARS-CoV-2 include Spike protein (S), Membrane protein (M), Nucleocaspid protein (N), and Envelope protein (E). b The genome includes ORF1a-ORF1b-S-ORF3-E-M-ORF6-ORF7 (7a and 7b)-ORF8-ORF9b-N in order. Sixteen nonstructural proteins (nsp1–11, 12–16) are encoded by ORF1a and ORF1b, respectively, and six accessory proteins were delineated. Plpro papain like protease, 3CLPro 3C-like proteinase, RdRp RNA-dependent RNA polymerase, Hel Helicase, S encodes NTD N-terminal domain, RBD receptor-binding domain, SD1 subdomain 1, SD2 subdomain 2, FL fusion loop, HR1 heptad repeat 1, HR2 heptad repeat 2, TM transmembrane domain. Dotted line indicates S1/S2 and S2′ site cleavage by Furin and TMPRSS2.(Wu *et.al*.,2020).

2.2.4 COVID-19's Geographic Distribution

Since the first reports of instances from Wuhan, a city in China's Hubei Province, at the end of 2019, and the official announcement of 27 viral pneumonia cases, seven of which were serious, on December 31, 2019. Patients who applied to the hospital for etiologic studies had comparable viral medical histories, which increased the chance of an illness being transmitted from animals to humans (Dhama *et.al*.,2020).

The outbreak in China peaked in late January and early February 2020(WHO .,2020), according to a joint World Health Organization (WHO) China fact-finding expedition. The majority of instances have been reported in Hubei and nearby provinces, although there have been several cases reported in other provinces and towns across China, as well as in Many other nations, including Japan, Korea, Italy, and the United States, have reported outbreaks.(Mao *et.al.*,2020).

These cases were initially concentrated among Chinese travelers and those who had contact with Chinese travelers. However, ongoing local transmission has resulted in smaller outbreaks in some countries outside of China, including South Korea, Italy, Iran, and Japan, and infections in travelers from those countries have been identified elsewhere (Haynes *et.al.*,2020).

The demographic details about the cancer patients in various studies are tabulated in Table (2). A nationwide analysis in China found that cancer patients were of advanced age and more likely to be associated with a history of smoking, severe baseline computed tomography (CT) manifestation, and a higher risk of progressing to severe events (hazard ratio [HR]: 3.56, 95% confidence interval [CI] [1.65–7.69]; P < 0.0001)(Liang *et.al.*,2020).

In patients with severe episodes, a higher dose of corticosteroids was given for a longer period of time, but there was no statistically significant difference (dose [mg/kg/day]: 1.0 vs. 0.6, period [day]: 3.0 [2.0–4.8] vs. 5.0).When compared to nonsevere intensive care unit (ICU) cases (noninvasive: 13.3 percent vs. 0 percent, P 0.001), a considerable number of severe cases required mechanical breathing (noninvasive: 53.3 percent vs. 0 percent, P 0.001). For noninvasive ventilation, the median period of

mechanical ventilation was 2.5 days (1.0–5.0 days), and 2.5 days for invasive patients. (Singh *et.al*.,2020).

2.2.5 Transmission of COVID-19

The relationship with a seafood market that sold live animals was discovered during an epidemiologic study in Wuhan at the beginning of the outbreak. As the pandemic proceeded,however,person-to-person transmission became the primary mechanism of transmission (Cohen .,2020).

SARS-CoV-2 has affected health-care personnel in China and several other countries, as has other emerging high-threat viruses. Nosocomial transmission has not been a key amplifier of transmission in this outbreak in China, where infection prevention and control are taken diligently (Wang *et.al.*,2020). As in SARS and MERS epidemics in the past, human-to human transmission has accelerated the spread of the outbreak and case reports have also started from other states in China. The first non-Chinese case of the infection, which spread to the Chinese provinces and then to the Asian continent, was reported in Thailand on January 13th, 2020. The case reported being a Chinese tourist who has traveled to Thailand and had no epidemiologic connection with the marketplace (Hui *et.al.*,2020).

Other cases from overseas countries such as the USA and France have continued to be reported (Holshue *et.al.*,2020)Transmission rates from a person with a symptomatic infection have been reported to vary depending on geography and infection control strategies. SARS-CoV-2 is more common in households than SRAS-CoV and Middle East coronavirus respiratory illness. SARS-CoV-2 domestic transmission is particularly dangerous to older adults (up to 60 years old) (Jing *et.al.*,2020). In the United

States, 0.45 percent of 445 close contacts of 10 confirmed patients had a symptomatic subsequent episode (Burke .,2020).

2.2.5.1 Clinical Characteristics of COVID-19

The incubation period for COVID19 is expected to be 14 days after exposure, with the majority of cases occurring four to five days after exposure.(Chan *et.al.*,2020).

One modeling study estimated that symptoms would emerge in 2.5 percent of infected people in China based on data from 181 publically reported, confirmed cases with identified exposure. Within 2.2 days, 97.5 percent of infected people were infected, and within 11.5 days, 97.5 percent of infected people were infected (Lauer *et.al*.,2020). In this investigation, the median incubation duration was 5.1 days. The case fatality rate ranged from 5.8% in Wuhan to 0.7 percent throughout the rest of China, according to a joint of the World Health Organization (WHO) China fact-finding mission. (Mao *et.al*.,2020)

Patients with advanced age or underlying medical comorbidities (such as cardiovascular disease, diabetes mellitus, chronic lung disease, hypertension, and cancer) have been the majority of fatal cases (Sanyaolu *et.al*.,2020). The proportion of infections that are severe or deadly varies by geography. In Italy, for example, 16% of all COVID19 cases were detected, and 16% of all hospitalized patients were brought to the intensive care unit; the estimated case fatality rate was 5.8% in mid-March. In mid-March, however, the anticipated case fatality rate in South Korea was 0.9 % (Isam, Kamil and Banoon Shaima 2021). Clinical presentation of covid -19 in cancer patients (see in table (3) below) (Zarifkar, *et.al*.,2021).

Table (2-3):Clinical presentation of covid -19 in cancer patients (Zarifkar, *et.al*.,2021)

Features	4observation andcohortstudesMaetal.,2020(n=37)	Hrusake *et al*., 2020(n=9)	Zhang *et al*., 2020(n=67)	Yang *et al*., 2020(=3)
fever	75.7%	77.8%	79.1%	100%
cough	56.8%	ND	74.6%	33.3%
dyspnoea	32.4%	ND	65.7%	ND
Hypoxia/reducedSPO2	ND	ND	ND	33.3%
WBC	neutrophil	Neutrophiland lymphocyte	ND	in 33.3%
CRP	ND	ND		
Other inflammatory markers	IL-6 and LDH	ND	LDH	66.6% D-dimer
Imaging modality	ND	CT	CT	ND

CRP,c-reactive protein:CT,computed tomography:*IL-6*,inter leukin-6:LDH,lactate dehydrogenase:ND,no data available:WBC,white blood cells

2.2.5.2 Diagnosis of COVID19

Early detection of questionable cases, rapid isolation, and the implementation of infection control measures should be the focus of initial management. At this time, COVID19 should be explored primarily in patients who have had any of the following in the previous 14 days and have a fever and/or respiratory tract symptoms (e.g. cough, dyspnea). Although diarrhea was recorded in 20-25 percent of MERS-CoV or SARS-CoV patients, intestinal symptoms were only reported in a small percentage of COVID-19 patients. In a separate investigation of 99 patients, chest pain, confusion, and nausea-vomiting were reported in addition to the previously mentioned symptoms (Chen *et.al.*,2020).

In patients admitted to the intensive care unit, X-rays or thorax CT imaging revealed unilateral or bilateral involvement consistent with viral pneumonia, as well as bilateral numerous lobular and sub segmental consolidation areas.X-rays or thorax CT imaging of the patients demonstrated unilateral or bilateral lung involvement, consistent with viral pneumonia, similar to the previous results. Patients in the intensive care unit had bilateral numerous lobular and subsegmental consolidation areas (Yang *et.al.*,2020).The patients with underlying comorbidities had a more severe clinical course, as expected based on prior epidemic experience (Wu and McGoogan.,2020). The diagnosis of 2019 n-CoV infection, like SARS and MERS, is predicated on a detailed history of contact and travel, as well as precise laboratory testing. Molecular techniques, serology, and viral culture are the diagnostic tools. Molecular approaches such as RTPCR (reverse transcription) or real-time PCR, which use RNA from respiratory samples such as oropharyngeal swabs, sputum, nasopharyngeal aspirate, deep tracheal aspirate, or bronchoalveolar lavage, are the most popular diagnostic methods. Lower respiratory tract samples, in particular, can have much more viral load and genome fraction than upper respiratory tract samples (Zhu *et.al.*,2019).

The majority of immunoassays are based on enzyme-linked immunosorbent assays (ELISAs), which are commonly used to detect soluble components at low concentrations (ng to pg/mL) using absorbance measurements. ELISPOT and fluoro-immunospot (FLUOROSPOT) are ELISA-derived techniques that are used to investigate a cell's cytokine production capacity in response to a specific stimulus. Unlike ELISA, FLUOROSPOT allows for the simultaneous detection of up to four analytes utilizing fluorescent antibodies, allowing for the identification of poly-secreting cells. Despite their sensitivity, these methods are limited to a small number of analytes, and multiplying tests to increase soluble factor detection necessitates bigger sample volumes and takes time. (Anbarasu *et.al*.,2013)

Table (2-4): Shows top 10 countries highly affected by Covid-19 as of 8th May, 2020 .(Vinod, and Prabaharan.,2020).

Location	Confirmed	Cases per 1 million people	Recovered	Deaths
Worldwide	38,45,607	495	12,82,930	2,69,564
United States	12,90,222	3,915	1,74,709	76,600
Spain	2,22,857	4,732	1,31,148	26,299
Italy	2,15,858	3,583	96,276	29,958
UnitedKingdom	2,06,715	3,112	—	30,615
Russia	1,87,859	1,280	26,608	1,723
Germany	1,69,555	2,039	1,35,918	7,392
France	1,37,779	2,054	55,027	25,897
Brazil	1,36,519	646	55,350	9,265
Turkey	1,33,721	1,608	82,984	3,641
Iran	1,04,691	1,256	83,837	6,541

2.2.5.3 Treatment of COVID-19

Because of its transmission dynamics and polyphasic character of sickness, COVID-19 has placed a huge strain on the healthcare system. There is no indication now of the formation of herd immunity, therefore finding an effective vaccine will take some time. To reduce the impact of following local waves of COVID 19, treatment techniques based on powerful antiviral medicines against SARS-CoV-2 are required (Torneri *et.al.*,2020)

In February 2020, China International Exchange and Promotive Association for Medical and Health Care (CPAM) released a new 2019 coronavirus disease (COVID-19) guideline, which included recommendations on methodology, epidemiological characteristics, disease screening and prevention, diagnosis, treatment, and control, nosocomial infection prevention and control, and disease nursing. CPAM recommends lopinavir; ritonavir pill (dose unknown) by mouth twice daily in combination with nebulized alfa-interferon (5 million units in Sterile Water for Injection inhaled twice day) according to the American College of Physicians. This recommendation is based on insufficient evidence from retrospective cohort, historically controlled studies, case reports, and case series that suggest clinical benefit of lopinavir; ritonavir in the treatment of other coronavirus infections, such as the 2002 SARSCoV and the 2012 Middle East respiratory syndrome coronavirus (MERS-CoV) (.Yang *et.al.*,2020).

Interferon-beta (IFNB) was found to be the most effective interferon in studies testing the antiviral efficacy of types I and II interferons, lowering MERS-CoV replication in vitro(Holshue *et.al.*,2020).

According to a human MERSCoV case report from South Korea, virus clearance was achieved using a combination of Lopinavir/Ritonavir (LPV/RTV) (Anti-HIV medicines), pegylated interferon, and ribavirin

(Magro *et.al*.,2020). In 2016, 76 patients were enrolled in a randomized control experiment (MIRACLE Trial) to see if LPV/RTV-IFNb improved clinical outcomes in MERS-CoV patients. Although another antiviral medicine, remdesivir, appeared to be effective in the first case reported from the United States, additional controlled studies with more cases are needed (Sheahan *et.al*.,2020).

Remdesivir inhibited viral RNA transcription in the early stages of infection, according to in vitro studies. Remdesivir has been shown to have potent antiviral efficacy in epithelial cell cultures against SARSCoV, MERS-CoV, and similar zoonotic bat CoVs1. COVID-19 treatment recommendations have been produced by a group of Korean physicians with expertise treating SARS-CoV-2 infected patients. (Wang *et.al*.,2020,Yang *et.al*.,2020).

2.2.5.4 COVID19 Prevention

Coronavirus disease (COVID-19) is a serious and potentially fatal ailment caused by infection with the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), an airborne virus that expanded internationally following its initial epidemic in late 2019 in China (Yesudhas *et.al.*,2021). SARS-CoV-2 severity ranges from asymptomatic to moderate symptoms as fever, cough, ageusia, anosmia, and asthenia(Gao *et.al.*,2021), (Tan *et.al.*,2021), to the most severe illnesses like acute respiratory distress syndrome (ARDS) and multi-organ failure (Docea *et.al*.,2020). The case-fatality rate was as high as 15% (Filippini *et.al*.,2021). when the outbreak spread across Italy, which was the first country to be gravely and widely affected by the epidemic (Vinceti *et.al*.,2021). Several public health and preventive measures have been recommended to limit the risk of SARS-CoV-2 transmission, including mobility restrictions through lockdown and hand and respiratory cleanliness (Hemmer *et.al.*,2021, Vinceti *et.al*.,2020). Specific vaccinations have been produced in

recent months, followed by the launch of an amazing and highly effective vaccination campaign (Docea *et.al*.,2020 ,Wang.,2021). In the lack of a specific antiviral therapy, supportive care measures such as mechanical ventilation and a few pharmaceutical medications such as systemic corticosteroids remain the standard of care for COVID-19 patients (Tan *et.al.*,2021, DiCastelnuovo *et.al*.,2021, Gavriatopoulou *et.al*.,2021).

In the current circumstances, there is a lot of curiosity regarding prospective preventive or supportive medicines for SARS-CoV-2 infection and COVID-19, the associated condition. Supplementation with vitamins and minerals has been suggested to aid in the fight against the COVID-19 pandemic, and as a result, it has become more popular in some communities (Li *et.al*.,2020, . Jovic *et.al*.,2020,Corrao *et.al.*,2021, Petrelli *et.al.*,2021, Shakoor *et.al*.,2021, Schomburg .,2021, Taheri *et.al*.,2021). When it comes to trace elements, zinc and selenium are the two minerals that have sparked the most interest among researchers and the general public, evidence suggests that self-supplementation with these two minerals has increased significantly as a result of this perception in areas with a high COVID-19 prevalence (Aysin *et.al*.,2021) .

2.2.5.4.1 Cytokines Associated with Covid -19 Infection

The COVID-19 cytokine storm demonstrated the importance of inflammatory cytokines in disease etiology. High levels of *IL-6, TNFa, IFNg, IL-1b, IL-2,* and *IL-10* have been recorded on a regular basis (Pedersen and Ho.,2020,Wu and Yang.,2020). Their genes have mutations that influence gene expression and may be linked to the progression of COVID-19. The allele frequencies were primarily derived from AFND (two IL1B polymorphisms had data for less than four populations and were therefore excluded) and supplemented with information from the Ensembl database release 100 (Yates *et.al*.,2020). The number of nations having information on cytokine polymorphisms ranged from 16 to 54. Table (5) lists the cytokine polymorphisms used, along with the number of nations involved.

The allele frequencies were associated with CFR and DDR estimates (Spearman's correlation). (de Meira Leite *et.al*.,2021).

Table (2-5): Correlation of CFR and DDR with cytokine polymorphisms(de Meira Leite *et.al*.,2021)

Marker	Number of countries	Average allele frequency (frequency range)	CFR rs	p	DDR rs	p
IL6-174C	47	0.21(0.05)	- 0.099	0.51	0.54	<0.0001
IL6+565A	19	0.26(0-0.442)	0.16	0.49	0.43	0.06
TNFA-238A	40	0.07(0.007-0.225)	0.31	0.04	0.41	0.0081
TNFA-308A	54	0.11(0.017-0.267)	0.32	0.04	0.35	0.0079
TNFA-1031C	17	0.21(0.103-0.436)	- 0.17	0.51	- 0.1	0.68
TNFA-857T	21	0.21(0.0110-0.282)	- 0.8	0.71	0.24	0.30
TNFA-863A	17	0.15(0.065-0.331)	- 0.09	0.73	- 0.32	0.20
IFNG+874T	31	0.34(0.072-0.579)	- 0.22	0.23	0.46	0.0092
IFNG+5644T	16	0.55(0.225-0.584)	- 0.04	0.87	0.28	0.28
IL10-1082G	46	0.32(0.024-0.549)	0.11	0.46	0.58	<0.0001
IL10-592C	41	0.61(0.258-0.796)	- 0.02	0.89	0.60	<0.0001
IL10-819C	42	0.62(0.249-0.800)	0.03	0.87	0.59	<0.0001
IL1B+3962T	31	0.18(0.010-0.316)	- 0.06	0.72	0.55	0.0013
IL1B-511C	34	0.45(0.275-0.700)	- 0.25	0.14	0.34	0.05
IL2B-330G	38	0.31(0-0.567)	- 0.2	0.21	- 0.05	0.78
IL2B-166T	32	0.30(0.053-0.640)	0.13	0.45	0.15	0.38
IL12B+1188A	32	0.68(0.456-0.833)	- 0.04	0.80	0.62	<0.0001

Immune-based assays are frequently employed to assess vaccine reactions,and multiple studies have established connections between transcriptome and cytokine signatures to various vaccines since the rise of systems vaccinology. Various approaches, ranging from single-plex to multiplex analysis, are currently available to identify soluble vaccination

biomarkers. D. Furman and MM. Davis have reviewed several of them (Furman and Davis.,2015).

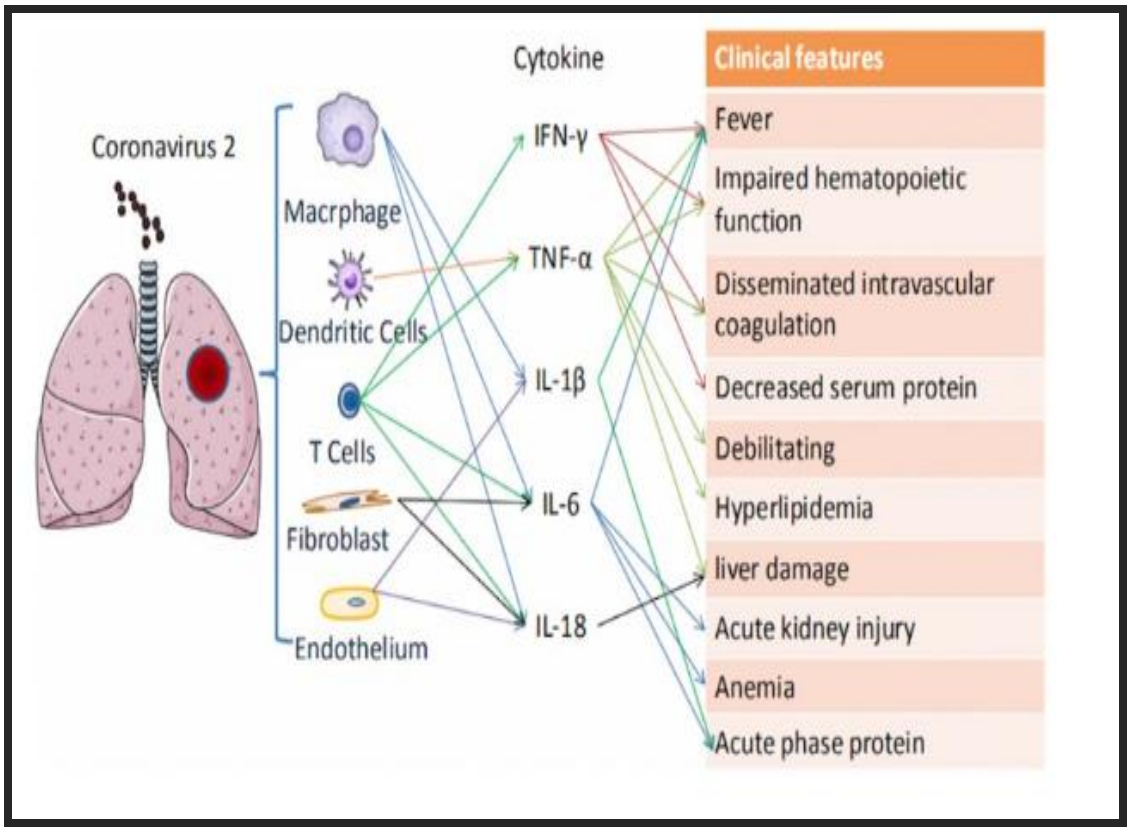

Figure (2-4) Schematic representation of clinical features versus pathogenic inflammatory cytokine response in SARS-CoV-2 infections (Sun et.al.,2020).

Table (2-6): Clinical conditions associated with cytokine storm. Abbreviations: HLH, hemophagocytic lymphohistiocytosis; MCD, multicentric Castleman disease; HHV-8, human herpesvirus 8; EBV, Epstein-Barr virus; SARS, severe acute respiratory syndrome; MERS, Middle East respiratorysyndrome; COVID-19, coronavirus disease 2019; CAR, chimeric antigen receptor.(Zanza, *et.al.*,2022).

Genetic/Idiopathic Diseases
Primary HLH
Autoinflammatory diseases
Idiopathic MCD
Secondary HLH-associated diseases
Autoimmune diseases
Malignancies
Intracellular pathogens
Infective diseases
Sepsis
HHV-8 infection (secondary MCD)
EBV infection
Coronaviruses (SARS, MERS, COVID-19)
Iatrogenic causes
Blinatumomab
CAR T cell therapy
TGN1412

Table (2-7): Main characteristics of cytokine profile during different coronavirus infections(Zhao *et.al.*,.2021). An enlightening role for cytokine storm in coronavirus infection. (*Clinical Immunology*, *222*, 108615).

Major cytokines	Origin	Major actions	COVID-19	MERS	SARS
TNF-α	Th, Monocyte, Macrophages, DC, NK, B, Mastocyte	Proinflammatory; activates cytotoxic T-lymphocytes	↑ [4]	↑ [41]	↑ Healthy group(n = 12):3.77(3.40)ng/L
					SARS group(n = 24):4.

					79(14.48)ng/L [40]
IL-6	Th-2, Monocyte, Macrophage, DC, BMSC	Differentiation of stem cells and lymphocytes; proliferation of T-lymphocytes; proinflammatory	↑↑ Mild group(*n* = 102):13.41 ± 1.84 ng/L Severe group(*n* = 21):37.77 ± 7.80 ng/L [37]	↑ [42]	↑ SARS group(*n* = 20):>3.1 ng/L [39]
IFN-α	Leukocyte	Antiviral properties; regulation MHC II		↑ [42]	
IFN-β	Th, B, Macrophage, Mastocyte	Proinflammatory or anti-inflammatory; promote tissue repairing			
IFN-γ	Th1, Tc1, NK	Antiviral properties; regulation of innate immunity; antiproliferative effects	↑ [4]	↑ [41,43]	↑ SARS group(*n* = 20):>15.6 ng/L [39]
IL-10	Th2, Monocyte, Macrophage	Cytokine inhibition; immunosuppression; anti-inflammatory	↑ [4]		

2.2.5.4.2 Cancer Incidence Among COVID-19 Patients

The number of cancer patients infected in COVID19 pandemic has been determined by eight investigations. Table (8) shows a summary of the findings. Single institutional studies from China, such as (Huang *et.al.*, 2020), (Chen *et.al.,* 2020), (Wang *et.al*.,2020) and (Wu et al. 2020), have documented cancer incidence among COVID19 positive cases.

Table (2-8): Incidence of cancer among COVID-19 positive Patients.(Singh, *et.al*.,2020).

Study	n	Malignancy	Incidence rate (%)
Huang et al.	41	1	2.43

Chen et al.	99	1	1.01
Wang et al.	138	10	7.24
Guan et al.	1099	10	0.90
Liang et al.	1590	18	1.1
Wu et al.	80	1	1.25
Liu et al.	137	2	1.45
Wu and McGoogan	20.982	107	0.5

2.2.5.4.3 The Prevalence of Various Malignancies

The most common site of cancer in (Zhang *et.al.*,2020) study was the lung (25 percent, n = 7) and it was found that symptoms of dyspnea appeared much earlier in cancer patients than in the general population (1.0 [0.0–3.5] vs. 8.0 [5.0–13.0] days) and other cancer patients (1.0 [0.0–3.5] vs. 5.0 [4.0–7.0] days. (Grasselli *et.al.*,2020)

In the study by Liang *et al.*,(2020) the lung was likewise the most common site (28 percent n = 5), but compared to other sites, individuals with lung cancer (1/5, 20 percent vs. 8/13, 62 percent) had a lower risk of serious outcomes (1/5, 20 percent vs. 8/13, 62 percent).

Table (2-9):Distribution of various cancer among the covid-19-positive patients(Singh *et.al.*,2020)

Types of cancer	Zhangetal.(n=28),n (%)	Liangetal.(n=18),n (%)	Yu et al.(n=12),n (%)
Lung cancer	7(25.0)	5(28)	7(58.3)

Esophaguscancer	4(14.3)	.	.
Breast cancer	3(10.7)	3(16.6)	1(8.3)
Larynx	2(7.1)	.	.
Liver cancer	2(7.1)	.	.
Prostatic cancer	2(7.1)	.	.
Cervical cancer	1(3.6)	.	.
Gastric cancer	1(3.6)	.	.
Colorectalcancer	2(7.1)	4(22.2)	2(16.7)
Adrenalneoplasm	.	1(5.5)	.

2.2.6 Cancer Patients at a High- risk Population

According to growing research, cancer patients are more likely than the general population to contract COVID19 and experience increased morbidity and mortality. When compared to the general population, cancer patients exhibited a twofold greater incidence of COVID19 infection in a study involving 1,524 cancer patients (Yu *et.al*.,2020).

As of February 11, 2020, the Chinese Center for Disease Control and Prevention had described the epidemiological characteristics of 72,314 COVID19 cases in mainland China. They found that 107 patients (0.5%) had cancer, with six of them dying. The case fatality rate was 5.6 percent, which is greater than the aggregate COVID19 (Zhonghua .,2019) reported case fatality rate of 2.3 %.Similarly, the WHO—China Joint Mission on COVID19 found that patients with preexisting malignancy had a

considerably higher case fatality rate (7.6%) than patients without comorbid diseases (1.4%) (WHO .,2020). In a study by Liang et al.(2020), cancer was linked to a higher incidence of serious events (i.e., admission to the intensive care unit, invasive ventilation, or death in 7 of 18 patients [39 %] with cancer vs. 124 of 1,572 patients [8%] without cancer; p =.0003) (Liang *et.al.*,2020). An Italian study analyzing the case fatality of COVID19 indicated that 72 (20.3 %) of 355 patients who died and received extensive chart review had current cancer (Onder *et.al*.,2020). These findings are preliminary and need to be confirmed in larger international cohorts, several factors, such as frequent hospital visits and admissions (YuJ *et.al.*,2020), immunocompromised state, advanced age, and poor functional status, could account for an increased risk of COVID19 infection and subsequent complications in cancer patients.

The COVID19 pandemic is expected to have an impact on medical goods supply chains. The US Food and Medicine Administration (FDA) published a statement on February 27, 2020, prior to the pandemic declaration, stating that one drug (which was not identified) is now in short supply due to COVID19. The FDA Drug Shortages list contains 26 oncology drugs as of March 21, 2020 (Food and Drug Administration.,2020). While prescription shortages may not appear immediately because many businesses stockpile ingredients or supplies to protect against unexpected stoppages, identifying and anticipating such shortages during a pandemic can be extremely difficult (Vox .,2020). For the successful implementation of drug shortage management methods, clear communication and transparency between stakeholders, suppliers, and health organizations is required. The American Society of Health-System Pharmacists recommended the following steps to overcome drug shortages: making contact with other sites or health systems, as large health systems can often survive drug shortages by shifting drug

inventory between sites; identifying alternative substitute therapies; and developing criteria for patient prioritization during drug shortages with a multidisciplinary team involving pharmacy,medical and nursing staff (Ventola.,2011).

2.2.7 Cancer Treatment with Active Anticancer Therapy Patients who are Infected or at risk of becoming Infected

During a pandemic, the potential for benefit from chemotherapy would remain same, but the chance of harm would rise to an unquantifiable degree. Patients who received chemotherapy or surgery in the month before their COVID19 diagnosis had a numerically higher risk of clinically severe events (three of four patients) than those who did not receive chemotherapy or surgery (six of 14 patients) (Liang *et.al*.,2020).

The consent process may shift during a pandemic because the risk-benefit ratio may change. Patients with cancer, for example, should be aware that anticancer medication may be more dangerous during a pandemic. In addition, rising demand and a probable personnel shortfall will limit hospital bed availability. Patients may certainly make an informed choice for a potentially less effective but less myelosuppressive medication in this situation. Furthermore, surgery carries the danger of nosocomial infection with the pandemic pathogen. (Battershill.,2006)

Adjuvant chemotherapy for stage III colorectal cancer can be safely delayed for up to 8 weeks, but more than 12 weeks is not suggested (Bos *et.al*.,2015). Delaying curative adjuvant chemotherapy can be explored within the acceptable period for each disease location (Liang *et.al*.,2020). During the COVID19 epidemic, recommendations for prioritizing, therapy, and triage of patients with breast cancer were recently published (American College of Surgeons.,2020). Cancer patients will be classed as either

confirmed infection or at high risk for COVID19 during a pandemic. Patients with proven COVID19 infection should be evaluated for the possibility of delaying anticancer therapy until they are medically cleared, according to WHO and CDC guidelines (World Health organization.,2020, Centers for Disease Control And Prevention.,2020). Patients who are receiving active anticancer therapy are nonetheless at high risk and should be on the lookout for COVID19 signs.

2.2.7.1 Testing for Covid-19 in Lung Cancer Patient

The majority of SARS-CoV-2 infected patients were asymptomatic, according to statistics from China and Italy, the first two nations with the highest incidence or a minor upper respiratory tract infection symptoms. However, around 14% e24% of individuals developed Pneumonitis need hospitalization as well as oxygen. Acute respiratory infections affected about 5% of patients. Acute respiratory distress syndrome (ARDS) or sepsis-related acute organ failure complication that necessitates admission to an intensive care unit. (Yang *et.al*.,2020).

The case fatality rate (CFR 14%) is much higher, defined as the number of deaths in COVID-19-positive patients divided by the number of patients who tested positive. Those who have an underlying concurrent condition, such as diabetes، Cardiovascular disease, diabetes, and chronic obstructive pulmonary disease are all examples of chronic diseases. This is a common occurrence. It has been observed in Chinese and Italian populations, but in the Caucasian population, it appears to be more apparent. (Wu & McGoogan. .,2020).

According to data from China and Italy, the first two countries with the highest incidence, the majority of patients infected by SARS-CoV-2 were asymptomatic or presented with mild upper respiratory tract symptoms. However, about 14%e24% of patients developed pneumonitis and required

hospitalization and oxygen support. About 5% of patients developed acute respiratory distress syndrome (ARDS) or sepsis-related acute organ dysfunction, requiring admission to intensive care units.(Onder *et.al*.,2020)

2.2.7.2 Vaccine Reluctance as a Threat to Global Health

High rates of acceptance and population coverage are required for successful immunization programs, as a result, the availability of safe and efficient vaccines is insufficient; vaccines must be broadly accepted by the general public and the medical profession in order to benefit the population. (Sanche *et.al*.,2020, DeRoo *et.al.*,2020).

A growing body of research suggests that some members of the American population are (DeRoo *et.al.*,2020, Brunson.,2020) hesitant to accept vaccination. (Brunson.,2020) Indeed, vaccine hesitancy, lack of trust in vaccination, and/or complacency about vaccination can lead to delays in vaccination acceptance or refusal despite access to vaccination services. (MacDonald.,2015). In 2019, the World Health Organization named, as one of the top ten threats to global health. Despite the fact that this classification came before the COVID-19 epidemic, the sociopolitical response to the pandemic in the United States and other nations is a relevant example of the threat.(World Health Organization.,2020).

Vaccination confidence is impacted by trust in vaccine safety and effectiveness, health-care professionals, public health and health-care delivery systems, and lawmakers who create vaccination regulations. (MacDonald.,2015).

The public's trust in immunization has eroded, according to experts. Individuals' health beliefs (e.g., perceived danger of vaccination, perceived risk and severity of disease, perceived necessity for the vaccine, and self-efficacy of vaccination) and assessments of the risks and benefits of

vaccination influence vaccination complacency. Surprisingly, the success of vaccination has contributed to this complacency by lowering illness severity and perceived risk (Larson *et.al.*,2011).

2.2.8 Angiotensin-Converting Enzymes (*ACE,ACE2*) Gene Variants and COVID-19 Outcome

SARS-CoV-2, the virus that caused the COVID-19 pandemic, is a novel coronavirus belonging to the subgenus Sarbecovirus (Gorbalenya *et al*., 2020). SARS-CoV-2 is an Angiotensin I Converting Enzyme 2 virus, just like SARS-CoV.(*ACE2*)-tropic virus, and the viral envelope's "spike" (S) protein As a result, it would bind to the mucosa of the nasopharynx and alveolar pneumocytes.(Yan *et al*., 2020; Shang *et al.,* 2020) have surface *ACE2* expression. (Hoffmann *et al.,* 2020). This disease's clinical spectrum‹COVID-19 encompasses a spectrum of symptoms ranging from mild to severe (Fu *et al*., 2004).

(Rivieccio *et.al*., 2020) It's been suggested that viral infection is to blame. It causes an exacerbation of the inflammatory response, resulting in severe consequences Lung injury that may necessitate ICU admission, mechanical ventilation, and other interventions raises the chance of mortality and multi-organ failure(Jose and Manuel,2020).

Ingraham *et al.*, 2020, found that the Renin-Angiotensin-Aldosterone system (RAAS) appears to play a key role in COVID-19 pathogenesis. The angiotensin-converting enzyme (*ACE* or *ACE1*) catalyzes the conversion of angiotensin II to angiotensin I. Angiotensin-II (Ang-II) is synthesized from Ang-I, and *ACE2* hydrolyzes it.Ang-II is transformed into Ang-1–7. The AT1-receptor is activated by Ang-II, which causes vasoconstriction. fibrosis, inflammation, and thrombosis, to name a few؛Ang-1–7, on the other hand, binds to the AT2-receptor and causes enhanced vasodilation.fibrosis,

inflammation, and thrombosis were all reduced. The *ACE* and *ACE2* are viewed as opposing forces in the equilibrium. assesses the likelihood of getting hypertension and cardiovascular disease. disease. In the lungs, *ACE2* triggers a protective response by lowering inflammation.(Imai *et al.*, 2005; Bao *et al.*, 2005) oedema, permeability, and pulmonary injury (Annoni *et al.*, 2019; Kuba *et al.*, 2005).

2.3 Association of *GSTM1* and *GSTT1* Gene Polymorphisms with COVID-19 Susceptibility

Glutathione Stransferases (GSTs) are a multifunctional isoenzyme superfamily that catalyzes glutathione conjugation with electrophilic chemicals, resulting in cellular detoxification of these substances. A number of endogenous and exogenous substances(Ginsberg *et.al*.,2009). Goods and Services Taxes (GSTs) play a vital role involvement in the detoxification of many carcinogens, drugs, and other substances against different forms of oxidative cellular damage(Hayes and Strange.,2000).

The GST enzyme is a type of enzyme that is found in reaction to stimuli, contributes to a variety of interindividual activities oxidative stress products clearance.(Dasari *et.al*.,2018).

2.4 Regulatory Domains Controlling High Intestinal Vitamin D Receptor Gene Expression Conserved in Mouse and Human

Vitamin D is a nutrient that is used to make 1,25 dihydroxyvitamin D (1,25(OH)2D), a hormone that regulates a number of biological processes that are important to human health (Norman.,2008). Following activation of the vitamin D receptor (*VDR*),a ligand-activated transcription factor, 1,25(OH)2D's activities are mediated by induction of gene transcription (TF) (Wagner and Hollis.,2022) . The regulation of intestinal calcium absorption is the best researched activity of vitamin D. (Xue and Fleet.,2009, Fleet.,2017). As a result, it's not surprising that the VDR protein was

discovered as a high-affinity 1,25(OH)2D-binding protein in the chick intestine (Brumbaugh and Haussler .,1974). *VDR* protein and gene expression have been found in a variety of tissues since then (Walters.,1992), although the intestinal epithelium has the highest *VDR* expression (Lee *et.al*.,2014, Cartwright *et.al*.,2018).

2.5 *MIF Gene* Structure and Expression

In 1989 (Weiser *et. al*., 1989) and 1994 (Paralkar and Wistow.,1994), the first studies reporting the sequencing of *MIF* cDNA and the *MIF* gene were published. In terms of sequencing, enzymatic activity, and gene structure, the D-DT gene is similar to the *MIF* gene, indicating that the two genes diverged from a common ancestor. *MIF* (22q11.23) is 35 % identical to D-DT (22q11.2), which is located 80 kb distant (Günther *et.al.*,2019). Three exons (107, 172, and 66 bp) are separated by two introns in the *MIF* gene (190 and 96 bp). The *MIF* gene does not have a TATA box, but it does have a CpG island with several CpG dinucleotides, according to sequence analysis. This CpG island is approximately 1.2 kb in length and begins 300 bp from the transcriptional start point (TSS). *MIF* is widely expressed as a single 0.8-kb mRNA strand in numerous tissues and organs, as is typical for genes with CpG islands. The activities of various cytokines, mitogens, microbial products, lipoproteins, and hormones, as well as glucose, ultraviolet B, and hypoxia, all boost *MIF* gene expression (Roger *et.al*.,2012, Günther *et.al*.,2019) .However, more research into the functional regulation of *MIF* expression and activity is required.

2.6 *IL-6* and *IL-10* as Predictors of Disease Severity in COVID-19 Patients:Results from Meta-Analysis and Regression

Clinical investigations with COVID-19 patient cohorts looked at whether *IL-6* alone (Grifoni *et.al*.,2020) or *IL-6* in combination with other cytokines such *IL-10, IL-2, IL-4, TNF-*, and *IFN-* could predict severe

disease (Han *et.al*.,2020). Except for the study by Elshazli et al who performed decision tree and Receiver Operating Characteristics (ROC) curve analysis to assess prognostic potential of multiple laboratory parameters including *IL-6* (Elshazli *et.al*.,2020), meta-analysis studies (Akbari *et.al*.,2020, Zeng *et.al*.,2020, Aziz *et.al*.,2020, Coomes and Haghbayan.,2020,Henry *et.al*.,2020) concluded elevated levels of cytokines in severe COVID-19 patients but did not attempt to establish their prognostic significance. Another study (Talayero *et.al*.,2020) used numerous clinical indicators and *IL-6* levels to try to develop a mortality risk model using 501 individuals.

2.6.1 Pharmacogenomics of Anti-*TNF* Treatment Response Marks a New Era of Tailored Rheumatoid Arthritis Therapy

Rheumatoid arthritis (RA) is the most common chronic inflammatory arthritis, although the specific mechanism behind it is unknown. *TNF*-targeting medicines have been demonstrated to be highly effective in the treatment of RA, indicating the critical role of this cytokine in this disease. Despite this, *TNF* inhibitor response varies, and around one-third of RA patients are non-responders, which can be explained by genetic variables. Although knowledge in the field of anti-*TNF* medication pharmacogenomics is expanding, it has yet to be utilized in clinical practice. Several genome-wide association studies have discovered a few single nucleotide polymorphisms linked to anti-*TNF* therapy response, most of which map to genes involved in T cell activity. Studies of RA patients' gene expression profiles have shown distinct gene signatures that could be used to generate new predictive tools. We examine the importance of *TNF* in RA and offer current pharmacogenomics research on anti-*TNF* treatment response in this article.(Wysocki and Paradowska-Gorycka., 2022).

Dedication

To the sake of Allah, my Creator and my Master.
To my great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life.
To my homeland Iraq.
To my great parents.

To my dear husband, To my dear son,

To MY dear brother, Dr. Khaled Al-Shibli

To my dear cousin, Dr. Mortada Ali Al-Saadi

To my dear cousin, Dr. Moaz Al-Salem..

To all my family, my friends and all people whom I love. To everyone who aided me in every possible way to make this work see the light ... I dedicate this work..

Zahraa,2023

Acknowledgment

At first of all, thanks to Allah the most gracious and the most merciful, who gave me the ability and desire to achieve this study.

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My special and sincere thanks to patients who i take the samples from and may Allah heal them and have mercy on those who died because of this epidemic.

My sincere thanks are also to my family, friends and to anyone who helped and supported me by his pray for success in my work.

Zahraa,2023

Chapter Three:- Materials & Methods

3.1. Materials

3.1.1 Instruments and Equipment for Laboratories.

Table (3-1) lists all the instruments and equipments required for this study.

Table (3-1): Instruments and equipment for laboratories

No.	items	Company	Country
1.	0.1-10µl with filter tips	Promega	USA
2.	1.5 µl Micro-centrifuge tube	Sterile	S. Korea
3.	100-100µl , 10-50 µl tips	Dolphin	Syria
4.	Biological safety cabinet	Labogene	Korea Denmark
5.	Distillator		Korea
6.	EDTA vacuum tube	Xinle	Germany
7.	Eppendrof centrifuge cooling centrifuge	eppendrof	
8.	flasks and beakers	Hirschman	Germany
9.	Gas burner	GFL	Germany
10.	Glass EDTA tubes 10 ml	AFCO	Jordan
11.	Incubator	Memmert	Germany
12.	Medical cotton	Kardelen	Turkey
13.	Medical injection Syringes	MEDECO	UAE
14.	Micro centrifuge	eppendrof	India Germany
15.	Micropipettes 5-50 ml, 100-1000 ml , 0.5 – 10 ml	Slamid eppendrof	USA
16.	Oven	Memmert	Germany
17.	Qiagen thermocycler	Qiagen	Germany
18.	Quantum vilber lourmat		France
19.	Refrigerator	Concord	Lebanon
20.	Sensitive electron balance	A & D	Japan
21.	Thermocycler system/Conventional	Bio epplied	USA
22.	Vortex	Gemmy	Taiwan
23.	Water bath	GFL	Germany

3.2 Chemicals and Biological Materials Required

Chemical and **Biological** materials are listed in table (3-2)

Table (3-2): Chemical and Biological materials

No.	Chemical material (molecular)	Company/country
1.	Agarose	Froggabio, Canada
2.	Nuclease-Free-Water	Bio basic, Canada
3.	DNA Ladder Marker 100bp	Promega, USA\Biosharp
4.	Ethanol Absolute 70%	BDH, UK
5.	Primers\Bioneer\Biosharp	Macrogen, Korea,China
6.	Tris borate TBE buffer (loading buffer) *10	Promega ,USA
7.	PCR pre mix (master mix)	Bioneer, Korea\Biosharp
8.	DNA loading dye	Promega/USA
9.	Red safe nucleic acid staining solution 1ml	BDH, England
10.	Protenase k	
11.	Distilled water,NFW(nuclease free water)	

3.3 Marketable kits

The GENEzol™ TriRNA Pure Kit is a phenol and guanidine isothiocyanate plus spin column system for convenient purification of high-quality total RNA from a variety of samples. Initially, samples are homogenized in GENEzol™ Reagent without chloroform phase separation or isopropanol RNA precipitation. Following sample homogenization, simply bind, wash and elute the high quality, total RNA in RNase-free Water and use in a variety of sensitive downstream applications.

The commercial kits that were utilized in the study are listed in table (3-3)

Table (3-3): Marketable kits used in the study.

No.	Type of kits	Company/Country
1.	Genezol TriRNA Pure Kit	Geneaid- Ltd
2.	gSYNC^{Tm} DNA Extraction Kit from fresh blood	Geneaid -Ltd
3.	Reverse Transcription Kit (with ds DNase)	Biosharp - Korea
4.	SYBR green qPCR Mix fluorenscence quantification pcr kit (BL698A)	Biosharp - Korea
5.	Universal SYBR qPCR Master Mix	Biosharp - Korea
6.	PCR pre-Mix 20 µl reaction	Bioneer- Korea

Table (3-4): gSYNC^{Tm} DNA Extraction KIT **(**Geneaid- Ltd**) from fresh blood:**

No	**Materials**
1.	Genomic Lysis/Binding Buffer
2.	Genomic Digestion Buffer
3.	Genomic Wash Buffer 1
4.	Genomic Wash Buffer 2
5.	Genomic Elution Buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA)
6.	RNase A (20 mg/mL) in 50 mM Tris-HCl, pH 8.0,10 mM EDTA

7.	Proteinase K (20 mg/mL) in storage buffer
8.	Spin Columns with Collection Tubes
9.	Collection Tubes (2.0 mL)

Table (3.5): DNA ladder 100-1500bp (Promega)

Materials
1-Ladder consist of 11 double-stranded DNA with size (100-1500bp). 2-Loading dye has a composition: [15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl (pH 7.5)50mm EDTA].

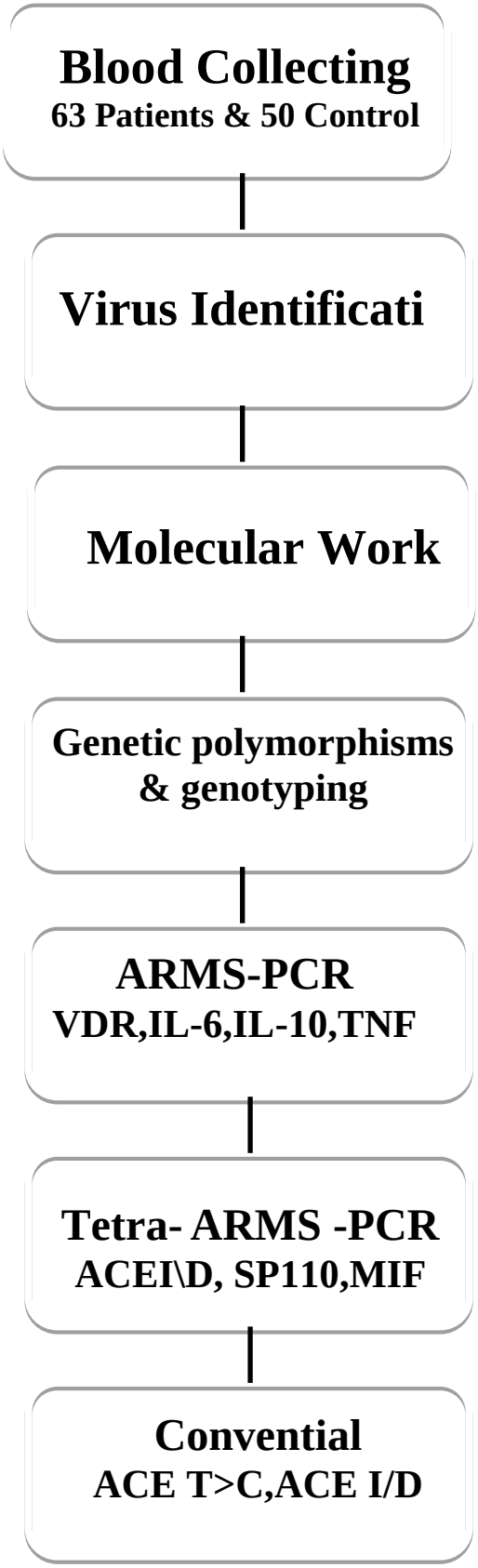
3.4 Methods

3.4.1 Study Subjects

The practical side of this study was done during the period from November 2021 to March 2022. 63 patients and 50 healthy individuals were collected.

3-4-1.1 Patients

In this study, individuals without Covid-19 had their lack of infection verified by PCR testing, while 63 individuals with Covid-19 had infection verified by testing and were admitted to the medical Merjan city. 19 male and 44 female patients, aged 20 to 80, were involved in the current study. They were selected after being given a diagnosis by serological and molecular tests. Every patient's blood and serum samples, as well as the controls, were collected according to an ethical standard that excluded certain criteria and included others.



Figure(3-1): project diagram

3.4.1.2 Blood Samples Collection

About five milliliters of venous blood were collected from each patient in this study. The blood was divided into two parts: one part (about two milliliters) was collected into EDTA containing tubes for genetic part. The second part of the blood was placed in gel tube for thirty minutes, then transferred to plain tube and serum was obtained by centrifugation at 3000 rpm for 15 min; after that the serum was collected and kept in the freezer (-20 ºC) until it was used for the immune assay.

3.4.2 Control Samples Collection

Venous blood samples were drawn from healthy , patients, males and females, with an age distribution that was similar to that of the patients.

3.4.3 Isolation of Genomic DNA

Human Genomic DNA is collected from the blood in EDTA tubes for molecular study, which collected in tubes of anti-coagulant EDTA and for frozen blood samples recommended using proteinase K were applied using for DNA purification; Geneaid, promega

3.4.4 The Estimation of DNA Concentration and Purity (Quantitative)

Human genomic DNA was extracted from whole blood samples of patients and controls using the Protocol for DNA Separation from Whole Blood Samples of Patients and Control Subjects. Human genomic DNA was extracted from entire blood samples of patients and control individuals using the gSYNC^{Tm} DNA Extraction Kit from fresh blood measure both DNA and RNA (Geneaid-Ltd).

The DNA concentration of samples was estimated by using the Nano drop by putting 2.5µl of the extracted DNA in the machine to detect

concentration in ng/µL and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ration for purifying DNA was between 1.7-1.9 (Sambrook and Russell, 2001).

3.4.5 DNA Electrophoresis(Qualitative DNA Estimation)

Agarose gel electrophoresis was embraced to affirm the nearness and uprightness of the separated DNA after genomic DNA extraction (Sambrook and Maniatis, 1989).

3.4.5.1 Gel Electrophoresis Reagents

- Powder of Agarose
- TBE Buffer with 1X concentration
- Loading dye
- Red safe
- DNA Ladder Marker

3.4.5.2 Protocol of Gel Electrophoresis

3.4.5.2.1 Tris Borate EDTA Buffer Preparation (1X TBE)

This solution was prepared by adding 900 ml Distill water to 100 ml 10X TBE (Promega/ Germany),forming 1 liter of(1x) TBE buffer (Sambrook and Russel, 2001).

3.4.5.2.2. Preparation of Agarose Gel

a) The amount of 1 X TBE (100 ml) was taken in a beaker.

b) Agarose powder (1.5 gm) was added to the buffer.

c) The solution was heated to boiling using a microwave oven for 2 minutes.

d) Red safe (1 µl) of (10mg/ml) was added to the agarose solution.

e) The agarose was stirred in order to be mixed to avoid making bubbles.

f) The solution was left to cool down at 50 – 60 C°.

3.4.6 PCR Technique

In this study, two types of molecular techniques were used that include RFLPand conventional PCR, to detect mutation genes by using ten primers as shown in table(3-5).The primers were supplied by Ligo(USA) Organization as a lyophilized result of various picomols fixations. Lyophilized preliminary was disintegrated in a free DNase/RNase water to give a final concentration of 100 pmol/µl and kept as a stock in -20ºC, to prepare 10µM concentration as work primer resuspended 10 pmol/µl in 90 µl of free DNase/RNase to reach a final concentration 10pmol/µl.

3.4.6.1 Primers Preparation

Using (10) primers to detect diagnostic as shown in the following table (3-5). The primers were supplied by Ligo /USA.

All primer pairs used in this study were spin down before opening the cap of primers tube. According to the instruction provided by primer manufacturer (Biosharp / Korea), a desired amount of nuclease free water was added to each primer to produce a 100 Pico-mole/microliter concentration of primer stock solution. The primer stock solution was resuspending by transferring 10 µl to a 1.5 ml Eppendorf tube that contain 90 µl of free nuclease water to yield 10 Pico-mole/microliter that is used in PCR amplification and the primer stock was stored at -20 C^0.

Table (3-6): Sequence of primers

No.	Gene	F/R	Primer	bp.	Ref.
1	VDR rs1544410	F	5′GGGAGACGTAGCAAAAGGAG3′	297 G: 192 + 05 A: 297	(Abdollahzadeh etal,.2021)
		R	5′CCATCTCTCAGGCTCCAAAG3′		

2	**GSTM1**	**F**	5′GAACTCCCTGAAAAGCTAAAGC 3′		**(Abbas etal,.2021)**
		R	5′GTTGGGCTCAAATATACGGTGG 3′		
3	**GSTT1**	**F**	5′TCCTTACTGGTCCTCACATCTC 3′		**(Abbas etal,.2021)**
		R	5′TCACCGGATCATGGCCAGCA 3′		
4	**ACE2 rs4646994 I/D**	**F**	5-CTGGAGACCACTCCCATCCTTTCT-3	190 G	**(Haruki etal,.2014)**
		R	5-GATGTGGCCATCACATTCGTCAGAT-3		
5	**ACE2 rs4240157 T > C**	**F0**	GCTGAGTTCTCAAAATAATGCCATAGAT	386	**(Mir etal,.2021)**
		R0	GCATTTCTTTCCAATCATTAAGAGTTCA		
		FI-T	GCCTCAGAACATTACAGAATCAACCT	244	
		RI-C	GAGGGTTGGTAAATAGTGTTCAGTGG	194	
6	**MIF-794CATT**	**F**	**5'-CTATCAGAGACCAAGGACAG-3'**	**169**	
		R	**5' -CCAGGCATATCAAGAGACAT-3'**		
7	**Sp110 Rs7580900**	**iF (A allele)**	**CCACCTTCTGTGATAATGAACATGGCA**	**225**	
		iR (T allele)	**GTTTGGAAGGCCCAGCGCA**	**184**	
		oF	**TTGGACTTCTGGCCTCCACACCT**	**363**	
		oR	**TGGGGATCGCCTATGCCATACA**		
8	**IL-6 (174 Promoter)**	**F**	**5´-CCC CTA GTT GTG TCT TGC C-3´**	**288**	**(Marzieh etal,.2016)**
		R	**5´-GCC TCA GAG ACA TCA CCA GTC C-3´**		
9	TNF- 308 A/G		**5-ATCTGGAGGAAGCGGTAGTG-3**	**222 bp**	
			5-AATAGGTTTTGAGGGCCATG-3		
10	IL-10 1082	**F**	**5-CTACTA AGG CTT CTT TGG GAG-3 5-ACT ACT AAG GCT TCT TTG GGA A-3**	**258 bp**	**(Samaneh etal,.2014)**
		R	**5-CAG TGC CAACTG AGA ATT TGG-3**		

A master premix of Bioneer was used, with components in table (3-7).

Table (3-7): PCR preMix 20 µl reaction (Bioshrpe and Bioneer)

Components	20 µl reaction
Top DNA polymerase (Taq)	1 U
Each: dNTPs (dATP, dGTP, dCTP, dTTP)	250µM
Tris-Hcl(pH 9.0)	10 mM
Kcl	30 mM
Mgcl2	1.5 mM
size	5
Template DNA	5-50ng
Primer	5-10pmol
Total	20**µ**l

Table (3-8): Master Mix components

Item	Concentration
DNA Taq polymerase	**1 U/ µl**
Each: dNTP (dATP, dCTP, dGTP, dTTP)	**250 Mm**
Tris-HCl (pH 9.0)	**10 mM**
KCl	**30 mM**
MgCl2	**1.5 mM**

3.4.6.2 Reverse Transcription Kit (with dsDNase)

Product introduction:

This product is an efficient, stable and fast reverse transcription system that can remove genomic DNA contamination. 5× RTMasterMix is a one-tube reverse transcription master mix, which contains a variety of reagents required for reverse transcription (H⁻RTase, RNaseInhibitor , dNTP Mixture, Buffer), just add template RNA primers and water to carry out the reaction. The reverse transcriptase used in the kit removes RNase H activity, and has stronger thermal stability, which can withstand 55 ℃ reaction and improve the reverse transcription of complex RNA templates. The heat-sensitive double-strand-specific nuclease is added to MasterMix, which can directly degrade and remove residual genomic DNA (gDNA) contamination in RNA samples during reverse

transcription. Oligo dT & Random Primer is provided in a single tube, if you need to use a specific primer, you can directly replace it.

Product composition:

Component 100T

5× RT MasterMix 400 µl

20× Oligo dT & Random Primer 100 µl

RNase free H 2 O 2×1 ml

Instructions:

1. Thaw the template RNA and reagents on ice, and mix each solution by flicking or vortexing gently before use. Briefly centrifuge to collect liquid remaining on the tube wall to the bottom of the tube.

2. Prepare the following reaction system on ice in an RNase free tube:

Component	Volume
Total RNA/mRNA	**0.1-2 µg**
5 x RT MasterMix	**4 µl**
20× Oligo dT & Random Primer or specific primer	**1 µl**
RNase free H2O make up	**20 µl**

3. Gently mix with a pipette, put it into the PCR machine and run the following program

- **Express procedure: 37˚C (15~30 min.) then 85 ˚C (5min.)**
- **Standard procedure: 25˚C (10min.), 55 ˚C (30~60min), 85 ˚C (5min.).**

Fast programs are usually the option for reverse transcription reactions; if the template has complex secondary structure or high GC regions, use the standard procedure.

4. The obtained cDNA product can be used for qPCR reaction immediately, or stored at -20℃ and used within half a year; For long-term storage, it is recommended to store at -80°C after

aliquoting. Repeated freezing and thawing of cDNA should be avoided.

Precautions:

1. Avoid RNase contamination.
2. To ensure successful reverse transcription, it is recommended to use high-quality RNA samples.
3. 5×RT MasterMix is very viscous, and it is easy to be adsorbed on the tube wall and outside the tip, resulting in loss. Please centrifuge before use.
4. Avoid the loss of adhesion to the outer wall of the tip.
5. For your safety and health, please wear a lab coat and disposable gloves for operation.

preservation method:

Avoid repeated freezing and thawing, and store at -20 ℃ for 1 year.

3.4.6.3 Universal SYBR qPCR Master Mix Universal Real-Time PCR Kit (BL697A)

Product number	product name	Specification
BL697A	Universal Fluorescence Quantitative PCR Kit	5 x 1ml

Product introduction:

This product uses Special reagents for Real Time PCR with SYBR Green I chimeric fluorescence method, which will hot-start Taq DNA polymerase, dNTP, SYBR Green I and other reagents are pre-mixed into a ready-to-use 2x Mix for rapid analysis of target cDNA Fast and highly specific quantitative detection. Hot-start Taq enzyme by antibody method, anti-Taq monoclonal antibody before high temperature heating Tightly bind to Taq enzyme, inhibit the polymerase activity of Taq, thereby inhibiting the formation of primers and templates at low temperature nonspecific amplification by DNA nonspecific hybridization or primer dimers. Antibodies will be included in the pre-denaturation step of the PCR reaction completely inactivated, it will not interfere with subsequent Taq polymerase reactions, greatly improving the

sensitivity and specificity of PCR reactions. 2x the concentration premixed reaction system minimizes human error, reduces the probability of contamination, saves experimental operation time, and is fast and simple. Convenience, wide versatility, high sensitivity, strong specificity and good stability.

Product components:

Product number	product name	Specification
1	2×Universal SYBR qPCR Mix	5 x 1ml
2	ROX Reference Dye (25mM)	200ul

Storage Conditions

After receiving this product, please store it at -20°C away from light immediately, it is valid for one year. When taken out from -20°C for use, thaw the frozen Universal SYBR qPCR Mix and ROX Reference Dye, then gently invert and mix until the solution is completely homogeneous before use. For frequent use over a period of time, it can be stored at 2 ~ 8 ℃ for 3 months. Avoid repeated freeze-thaw cycles.

Instructions for use

1. Preparation

Real Time PCR reaction system:

1. Melt all reagents 2× Universal SYBR qPCR Mix ROX Reference Dye, template, primers and RNase Free ddH 2 O at room temperature and mix thoroughly to avoid air bubbles. After a brief centrifugation, place on ice and prepare the reaction according to the following table.

Refer to the following table to prepare the reaction system:

Component	20ul system	50ul system
SYBR Green qPCR Mix	10μl	25 μl
Primer F (10 μM)*	0.5 μl	1μl
Primer R (10 μM)*	0.5 μl	1μl
cDNA template	1ul	1ul
ROX Reference Dye***	0.4 ul	1 ul
RNase-Free ddH $_2$ O	Make up to 20 μl	Make up to 50 μl

i. Generally speaking, the final primer concentration in the reaction system is 0.2 uM to obtain better amplification effect. When the reaction performance is

poor, the primer concentration can be adjusted within the range of 0.2~0.4 uM final concentration. In order to obtain the ideal qPCR effect, the length of the amplified fragment is recommended to be 80-200 bp.

ii. Template amount: 10 100 ng genomic DNA, or 1 10 ng cDNA as a reference, due to the different number of copies of the target gene contained in the template of different species, the template can be serially diluted to determine the optimal amount of template to use. In addition, when using Two Step RT PCR (the cDNA RT reaction solution for two-step reverse transcription reaction) as a template, do not add more than 10% of the total volume of the PCR reaction solution.

iii. The recommended final concentrations of ROX Reference Dye for different instruments are shown in the following table:

Instrument	Model ROX Amount 50 ul System	ROX Final Concentration
ABI PRISM 7000/7300/7700/7900HT/StepOne StepOne Plus etc.	1ul （0.6~1.0ul）	500nM （300~500nM）
ABI 7500/7500 Fast , QuantStudio® 3/5, QuantStudio 6/7 Flex, ViiA 7, Stratagene Mx3000P/ Mx3005P and Mx4000 etc.	0.1 ul （0.06~0.1 ul）	50nM （30~50nM）
Roche, Bio Rad, Eppendorf, etc.	No need to add	

2. Cap or seal the reaction tube/PCR plate and mix gently. Briefly centrifuge to ensure all components are on the bottom of the tube plate.

2. Carry out the Real Time PCR reaction

1. A two-step PCR reaction program is recommended for the reaction. two steps

Two-step reaction procedure

Step	Temperature	Duration	Cycles

Pre denaturation	95˚C	2 min	1
Denaturation	95˚C	15 sec.	40
Annealing extension data acquisition	60˚C	15-30 sec.	40
Melting Curve Analysis	**Set according to instrument recommended procedures**		

If the amplification effect is poor due to factors such as low template amount, or for amplicons exceeding 350 bp or high GC content, it is recommended to increase the extension time to 60 s or use the three-step method to improve the amplification efficiency.

Three-step reaction procedure

Step	Temperature	Duration	Cycles
Pre denaturation	95˚C	2 min	1
Denaturation	95˚C	15 sec.	40
Annealing	60˚C	15-30 sec.	40
Extended data collection	72˚C	30 sec.	40
Melting Curve Analysis	**Set according to instrument recommended procedures**		

- Note that This product uses antibody-modified HotStart DNA polymerase. If the template is pre-denatured before the PCR reaction, it is usually set to 95 °C, 2 min, and the time is appropriately extended to 5 minutes for complex or high GC templates.
- The above examples are conventional qPCR reaction systems and are for reference only. The actual reaction conditions vary due to the different structures of templates, primers, etc. It is necessary to set the optimal reaction conditions according to the characteristics of templates, primers and target fragments, and enlarge or reduce the reaction system according to the proportion.

3.4.7 Assay Procedures of Conventional PCR

The Protocol for technique Polymerase Chain Reaction (PCR). (See appendix chapter)

3.4.7.1 Detection of *VDR*

Genotype was determined using PCR amplification followed by restriction fragment length polymorphisms; RFLP assay (Kamen, and Tangpricha, 2010). Three fragments of VDR gene were amplified.

PCR optimization was done as a first step by using a gradient temperature. This is highly important to determine the optimum annealing temperature. The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume. PCR condition of gradient is shown in the following table (3-9).

Table (3-9): Gradient condition for *VDR*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	4	1
Denaturation	95	45s	40
Annealing Zones	57.2-58.4-61.5-62.3-64.6-66.4	40s	
Extension	72	1	
Final extension	72	7	1
Storage	4	∞	

After resolving of optimum annealing temperature for*VDR*gene by selecting the clearest and, which is 62.3C°, PCR mixture was 5µl DNA, 5µl master mix, 1.5 forward and reverses primer. PCR condition for *VDR* was performed as in the following tables (3-10). Results of PCR amplicons produce 800bp. site resulted in two fragments (650bp and 150bp).

The resulting 800bp PCR product is then digestedwith *BsmI* at 65 C for 18 h using 5 units of enzyme (BIOLAB, UK) per 20 ll reaction. The DNA fragments were separated using 2% agarosegel containing ethidium

bromide then, visualized under shortwaveUV light and compared to those of DNA ladder run at the same time.

Table (3-10): PCR condition for *VDR*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	94	4	1
Denaturation	94	45s	40
Annealing	62.3	40s	40
Extension	72	1	40
Final extension	72	7	1
Storage	4	∞	

3.4.7.2 Detection of *ACE I\D*

The analysis of I/D polymorphism that is located in 16th intron of *ACE* gene, was performed with PCR. The PCR was performed by using Primers (Moreiraetal,.2012 and Bayram et al,. 2011)

PCR optimization was done as a first step by using a gradient temperature. This is highly important to determine the optimum annealing temperature. The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume. PCR condition of gradient is shown in the following table (3-11).

Table (3-11): gradient condition for *ACE I\D*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	45 sec	35
Annealing Zones	55-57-59-61-63-65	30 sec	
Extension	72	1	
Final extension	72	5	
Storage	4	∞	

After the determination of optimum annealing temperature for ACE genes by selecting the clearest band which is 57 C°, PCR mixture was 5µl DNA, 5µl master mix, 1.5 forward and reverses primer. PCR conditions were performed as in the following table (3-12).

Table (3-12): PCR condition for *ACE I\D*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	45 sec	35
Annealing	57	30 sec	
Extension	72	1	
Final extension	72	5	
Storage	4	∞	

Tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) is reported as a prominent assay for SNP genotyping. However, there were published data that may question the reliability of this method on some occasions, in addition to a laborious and time-consuming procedure of the optimization step. (Mesrian Tanha *et al.*,2015)

The tetra-primer ARMS–PCR uses four primers in a single PCR to determine the genotype. In the beginning of the reaction, two non-allele-specific primers amplify the region that comprises the SNP. They are named outer primers, then. As the outer primers fragment is produced, it serves as a template to the two allele-specific primers (inner primers) which will produce the allele-specific fragments by placing the outer primers at different distances from the polymorphic nucleotide, the two allele specific fragments can be distinguished by their different sizes in an agarose gel (Rubio et al.,2008)

3.4.7.3. Detection of *MIF-794CATT* Genes

The gradient condition for ***MIF-794 CATT*** is like as shown in the following table (3-13). The gradient PCR reaction mixture included 4 µl template DNA, 10 µl master mix, and 3 µl of both forward and reverse primer in a total reaction volume of 20 µl.

Table (3-13): Gradient State for *MIF-794CATT* Genes

Steps	Temperature C°	Time/min.	Cycles
Step denaturation	94	5 m	1
Denaturation	94	45 s	35
Annealing Zones	57-59-61-63-65-76	15 s	
Extension	72	50 s	
Stage extension	72	7m	1
Storage	4	Hold	

MIF-794CATT genes were amplification by conventional PCR. As shown in the table (3-14) after the determination of optimum annealing temperature for MIF-794CATT by selecting the clearest band, which is (61 C°) PCR mixture was 4µl DNA, 10µl master mix, 3µl forward and reverse primer, PCR items were dissected on 2% agarose gel recolored with 5 µg/ml Red safe nucleic acid staining solution.

Table (3-14): PCR condition for *MIF-794CATT* Genes

Steps	Temp. °C	Time/Min.	Cycles
Step denaturation	94	5 min	1
Denaturation	94	45 sec	35
Annealing Zones	61	45 sec	
Extension	72	50 sec	
Stage extension	72	7 min	1
Storage	4	Hold	

3.4.7.4 Detection of *sp110 rs7580900* Genes

The gradient condition for ***sp110 rs7580900*** as shown in the following table (3-15). The gradient PCR reaction mixture included 5 µl template DNA, 15 µl master mix, and 5 µl of both forward and reverse primer in a total reaction volume of 30 µl.

Table (3-15): Gradient State *for sp110 rs7580900* Genes

Steps	Temperature C°	Time/min.	Cycles
Step denaturation	95	3 m	1
Denaturation	95	30 s	35
Annealing Zones	54.1-56.2-57.6-58.2	30 s	
Extension	72	30 s	

Stage extension	72	5m	1
Storage	4	Hold	

Sp110 rs7580900 genes were amplification by conventional PCR. As shown in the table (3-16). After selecting the clearest band, which is (57 C°), the optimal annealing temperature for *sp110 rs7580900* is determined. PCR products were dissected on a 2% agarose gel and recolored with 5 g/ml red safe nucleic acid staining solution. The PCR mixture included 5 µl of DNA, 15 µl of master mix, and 5 µl of forward and reverse primers.

Table (3-16): PCR condition for *sp110 rs7580900* Genes

Steps	Temperature C°	Time/min.	Cycles
Step denaturation	95	3 min	1
Denaturation	95	30 sec	35
Annealing	57.6	30 sec	
Extension	72	30 sec	
Stage extension	72	5 min	1
Storage	4	Hold	

3.4.7.5 Detection of *IL-6 (174* Promoter)

Genotype determination for three selected SNPs was performed by (SSP-PCR) method (Gao et al,. 2009). PCR mixture was 5µl DNA, 5µl master mix, 5µlforward and reverse primer. PCR conditions for *IL-6* were performed as in the following table **(**3-17).

Table (3-17): PCR condition for *IL-6 (174* Promoter)

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	4	1
Denaturation	95	20s	15
Annealing	58	40s	
Extension	72	40s	
Denaturation	95	20s	25
Annealing	54	50s	
Extension	72	50s	
Final extension	72	7	1
Storage	4	∞	

Genotype was screened by an approach based on Polymerase Chain Reaction-Restriction Fragment-Length Polymorphism (PCR–RFLP), and *SfaNI* restriction endonuclease was used.

3.4.7.6 Detection of *TNF- 308 A/G*

It was detected by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) (Li H.Q et al., 2005).

Gradient condition *ofTNF- 308 A/G*is explained in the following table (3-18). The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume.

Table (3-18): Gradient condition for *TNF- 308 A/G*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	35
Annealing Zones	55.4-56.2-59.3-61.6-63.4-63.3	0.5	
Extension	72	40s	
Final extension	72	7	1
Storage	4	∞	

Optimum result revealed with temperature 59.3°C. Then PCR amplification was done as illustrated in the following table (3-19) to get PCR 444bp product. The PCR products digested by the restriction enzymes *Nco*I(New England Biolabs) for A: 222bp; and for G: 206 bp+ 16bp, and digested products were resolved by electrophoresis in 2 % agarose gel and stained with ethidium bromide.

Table (3-19): PCR condition for *TNF- 308 A/G*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	35
Annealing	59.5	0.5	
Extension	72	40s	
Final extension	72	7	1
Storage	4	∞	

Electrophoresis was done on 2% Agarose gel using 1X TBE buffer system at 70V for 30 min and 100V for 30 min. Then they were analyzed in a gel documentation system.

3.4.7.7 Detection of *IL-10 1082*

Two SNPs of *IL-10* (*IL-10-592* and *IL-10-1082*) were detected and genotyped using polymerase chain reaction with (SSP-PCR)(Lu, Y.L et al., 2010).

PCR optimization was done as a first step by using a gradient temperature. This is highly important to determine the optimum annealing temperature. The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume. PCR condition of gradient is shown in the following table (3-20).

Table (3-20): Gradient condition for *IL-10 1082*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	35
Annealing Zones	55.4-56.2-59.3-61.6-63.4-63.3	0.5	
Extension	72	40s	
Final extension	72	7	1
Storage	4	∞	

After the determination of optimum annealing temperature for*IL-101082* genes by selecting the clearest band which is 61.6 C°, PCR mixture was 5µl DNA, 5µl master mix, 1.5 forward and reverses primer. PCR conditions were performed as in the following table (3-21).

Table (3-21): PCR condition for *IL-10 1082*

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	94	1	1
Denaturation	94	1	40
Annealing	61.6	1	
Extension	72	1	
Final extension	72	10	1
Storage	4		

3.5 Hardy-Weinberg Equilibrium

In the absence of perturbing events, the Hardy-Weinberg equilibrium says that genetic variation in a population will remain constant from generation to generation. The law assumes that genotype and allele frequencies will stay constant in a large population with no disruptive forces because genotype and allele frequencies are in equilibrium in a large population with no disruptive causes. Forces such as mutations, natural selection, nonrandom matching, genetic drift, and gene transfer will all disturb the Hardy-Weinberg equilibrium. By introducing novel alleles into a population, mutants, for example, upset the balance of allele frequencies. By causing gene frequency changes, natural selection and nonrandom matching, on the other hand, disrupt the Hardy-Weinberg equilibrium. This happens because the alleles in question either aid or hinder the reproductive viability of the species that bears them. Genetic drift, which happens when allele frequencies increase or fall by random and often happens in small samples, is another factor that might upset the equilibrium. Gene migration, which occurs when two species breed and new alleles are formed, can disrupt the Hardy-Weinberg equilibrium.The Hardy-Weinberg equilibrium will be disrupted by gene migration, which happens when two species breed and new alleles are introduced into the population.

The Hardy-Weinberg equilibrium seldom holds in fact since both of these destructive forces are natural in nature. As a result, the Hardy-Weinberg equilibrium represents an idealized state, and hereditary differences in existence can be calculated as deviations from it. (Graffelman and Weir 2017).

Chapter Four:- Results & Discussion

4.2 Molecular Study

4.2.1 Human DNA Extraction and PCR Products Detection

The extracted human DNA genome from whole blood of all samples of Covid-19 patients (63) and samples (50) of healthy control revealed a DNA concentration from (40-100 ng) with purity (1.7-1.9). These DNA samples were subjected to PCR amplification using specific primers targeting specific regions in the DNA and then enrolled for detection of single nucleotide polymorphisms (SNPs) by using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR)**(***VDR,IL-6,IL-10,TNF***),**Tetra-primer amplification refractory mutation system (ARMS)**(***ACEI\D, SP110,MIF*) and sequencing techniques.

4.2.2 *ACE I\D* Gene Polymorphism

Table (4-1) displays the three genotypes (II, ID & DD), (11.50, 38.05, 6.19) respectively, and the allele frequency of (I,D) was (54.76, 45.24) respectively for the patients and genotypes (II, ID &DD), (10.62, 8.85, 24.78), respectively, and allele frequency (I,D) was (34.00, 66.00) respectively for control people. Figure (4-1) displays the genotyping .

Table (4-1): Genotype frequency of polymorphisms of (Genotype *ACE2rs4646994 I/D*) gene associated with Covid-19 patients and control

Genotype	Patients(63)		Healthy (50)		Total (113)	
	No	%	No	%	No	%
II	13	11.50	12	10.62	25	**22.12**
ID	43	38.05	10	8.85	53	**46.90**
DD	7	6.19	28	24.78	35	**30.97**
TOTAL	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
I	69	54.76	34	34.00	103	**45.58**
D	57	45.24	66	66.00	123	**54.42**
TOTAL	**126**	**100.00**	**100**	**100.00**	**226**	**100.00**

Table (4-2): Genetic association of Genotype *ACE I\D* gene with disease.

(Genotype *ACE2rs4646994 I/D*)			
ALLELE	OOD RATIO	Significance level	CI 95%
II*ID	0.2519	0.0096	0.0887 to 0.7154
II*DD	4.3333	0.0118	1.3846 to 13.5615
ID*DD	17.2000	0.0001	5.8598 to 50.4867

As showed in the table above, there is significant between II*ID (0.0096), II*DD(0.0118), ID*DD(0.0001).These results agreed with (Calabrese *et.al*.,2021)demographic studies have found in the racial variance of *ACE I/D* genotype a potential explanation of the different prevalence and outcomes due to COVID-19. In fact, the higher frequency of the D allele seems to perfectly match with the higher mortality rates observed in the African American population, as compared to Indians and White people, and in the European populations (particularly Italian, Spanish, and French) as compared to the Asian ethnic group (Zheng and Cao 2020, Gemmati and Tisato 2020).

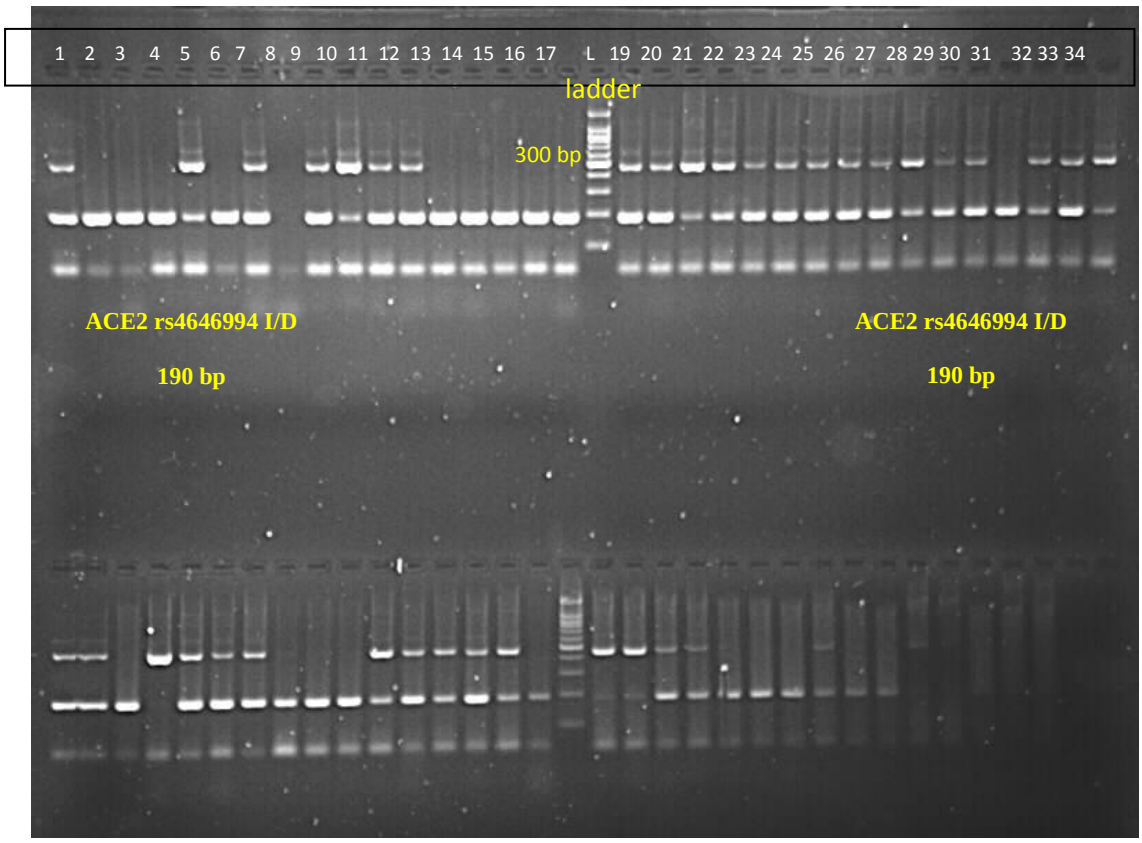


Figure (4-1): The Electrophoresis Pattern of *ACE2* *rs4646994 I/D* (190 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

Angiotensin-converting enzyme (*ACE*) plays an important role in rennin-angiotensin system (RAS) that regulates blood pressure, angiogenesis of ovarian endothelium, follicular growth, steroidogenesis and inflammation. The production of angiotensin II of the RAS can make follicle atresia in every period of the follicle development and promote the formation of polycystic ovary and hyperandrogenism (Bayram *et.al.*, 2011).*ACE2* is the primary cellular receptor for SARS-CoV-2(Ovsyannikova *et.al.*,2020) Severe acute respiratory syndrome corona virus-2 (SARS-CoV-2) has first emerged from China in December 2019 and causes coronavirus induced disease 19 (COVID-19). Since then, researchers in the world have been struggling to detect the possible pathogenesis of this disease. COVID-19 showed a wide range of clinical

behavior from asymptomatic to severe acute respiratory disease syndrome. However, the etiology of susceptibility to severe lung injury is not yet fully understood. Angiotensin-converting enzyme1 (*ACE1*) convert angiotensin I into Angiotensin II that was further metabolized by *ACE 2 (ACE2*). The binding *ACE2* receptor to SARS-CoV-2 facilitate its enter into the host cell. The interaction and imbalance between *ACE1* and *ACE2* play a crucial role in the pathogenesis of lung injury. One of aims of this study was to investigate the association of *ACE1 I/D* polymorphism with severity of Covid-19. We looked at 113 people; 50 healthy control, 63 people with Covid. Results for the *ACE2 rs4240157 I/D* polymorphism were obtained. Logistic regression was used to evaluate the distribution frequencies of variables across the study groups.COVID-19 severity was found to be associated with the *ACE2–ID* genotype through.

In the context of the co-dominant inheritance paradigm, multiple logistic regression II*DD Allele, 95% CI (1.3846 to 13.5615), Significance level, (0.0118), Odd Ratio (4.3333), and ID*DD Allele, 95% CI (0.0887 to 0.7154), Significance level, (0.0096), Odd Ratio (0.2519). (17.2000). assuming that the severity of covid-19 was associated with the *ACE2*-II*ID genotype. The *ACE2* polymorphism had no impact on the onset of disease. In conclusion, a negative COVID-19 result was associated with malignancy and the ACE1 genotype. These results indicated that *ACE1-I/D* may affect COVID-19 severity; however, this association was hypertensive status-specific. This study agreed with (Verma *et.al*.,2021) the study included RT-PCR confirmed 269 cases of Covid-19. All cases were genotyped for *ACE1 I/D* polymorphism using polymerase chain reaction and followed by statistical analysis (SPSS, version 15.0). found that *ACE1 DD* genotype, frequency of D allele, older age (≥46 years), unmarried status, and presence of diabetes and

hypertension were significantly higher in severe COVID-19 patient. *ACE1 ID* genotype was significantly independently associated with high socio-economic COVID-19 patients (OR: 2.48, 95% CI: 1.331–4.609). These data suggest that the *ACE1* genotype may impact the incidence and clinical outcome of COVID-19 and serve as a predictive marker for COVID-19 risk and severity. This study agreed with (Aladag *et.al.*,2021) the objective of this study is to evaluate the influence of the ACE gene insertion/deletion polymorphisms on the clinical outcomes and vulnerability of the COVID-19 immunoinflammatory disease. 112 participants were included in the research after receiving a COVID-19 diagnosis between May 1 and May 15, 2020. *ACE* gene allele frequencies were compared to the 300-person Turkish population that had been previously published.The most common genotype in the patients and control group was DI with 53% and II with 42%, respectively. The difference in the presence of the D allele between the patient and control groups was statistically significant (67% vs. 42%, respectively, p <0:0001). The mortality rate, time to defervescence, and the hospitalization duration were not different between the genotype groups. Genotype DI of *ACE I/D* polymorphism is associated with the infectious rate particularly severe pneumonia in this study conducted in the Turkish population. Therefore, *ACE D/I* polymorphism could affect the clinical course of COVID-19. *ACE2* receptor is expressed by lymphocytes and might be a direct goal of SARS-CoV-2 infection (Xu *et .al*., 2020), in addition an increase in pro-inflammatory cytokines, especially *IL-6*, in Covid-19 patients may possibly lead to much further decline in lymphocyte (Lin *et .al*.,2020).

4.2.3 *Sp110 Rs7580900* Gene Polymorphism

Table (4-3) and Figure (4-2) present the results of the Covid-19 genotyping of patients and the control group, respectively, and show the

three genotypes (AA, AT &TT), (21.24, 9.73, 13.27), and (59,41) for controls and (AA, AT &TT), (28.32, 16.81, 10.62), and allele frequencies of (A,T) of (65.87, 34.13) for patients.

Table (4-3) Genotype and allele frequency of *Sp110 Rs7580900* associated with Covid-19 patients and control

Genotype Sp110 Rs7580900	Patients (63)		Healthy (50)		Total (113)	
	No	%	No	%	No	%
AA	32	28.32	24	21.24	56	**49.56**
AT	19	16.81	11	9.73	30	**26.55**
TT	12	10.62	15	13.27	27	**23.89**
Total	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
A	83	65.87	59	59	142	**62.83**
T	43	34.13	41	41	84	**37.17**
Total	**126**	**100.00**	**100**	**100**	**226**	**100.00**

Table (4-4): Genetic association of Genotype *SP110* gene with disease.

Sp110 Rs7580900			
ALLELE	OODRATIO	Significance level	CI 95%
AA*AT	0.7719	0.5779	0.3101 to 1.9213
AA*TT	1.6667	0.2793	0.6606 to 4.2048
AT*TT	2.1591	0.1554	0.7466 to 6.2439

P VALUE≤ 0.05

As showed in the table above, there is significance between AA*AT (0.5779), AA*TT (0.2793), AT*TT (0.1554).the O.D between AA*AT (0.7719), AA*TT (1.6667), AT*TT (2.1591) and the P VALUE≤ 0.05 the result of table (4-4) show that SARS-CoV-2 gene expression revealed a high percentage. These results agreed with (Fox *et.al*.,2014) SP110 variants were associated with increased susceptibility to both pulmonary and extra-pulmonary TB in the Vietnamese. Genetic variants in SP110 may influence macrophage signaling responses and apoptosis during M. tuberculosis infection.

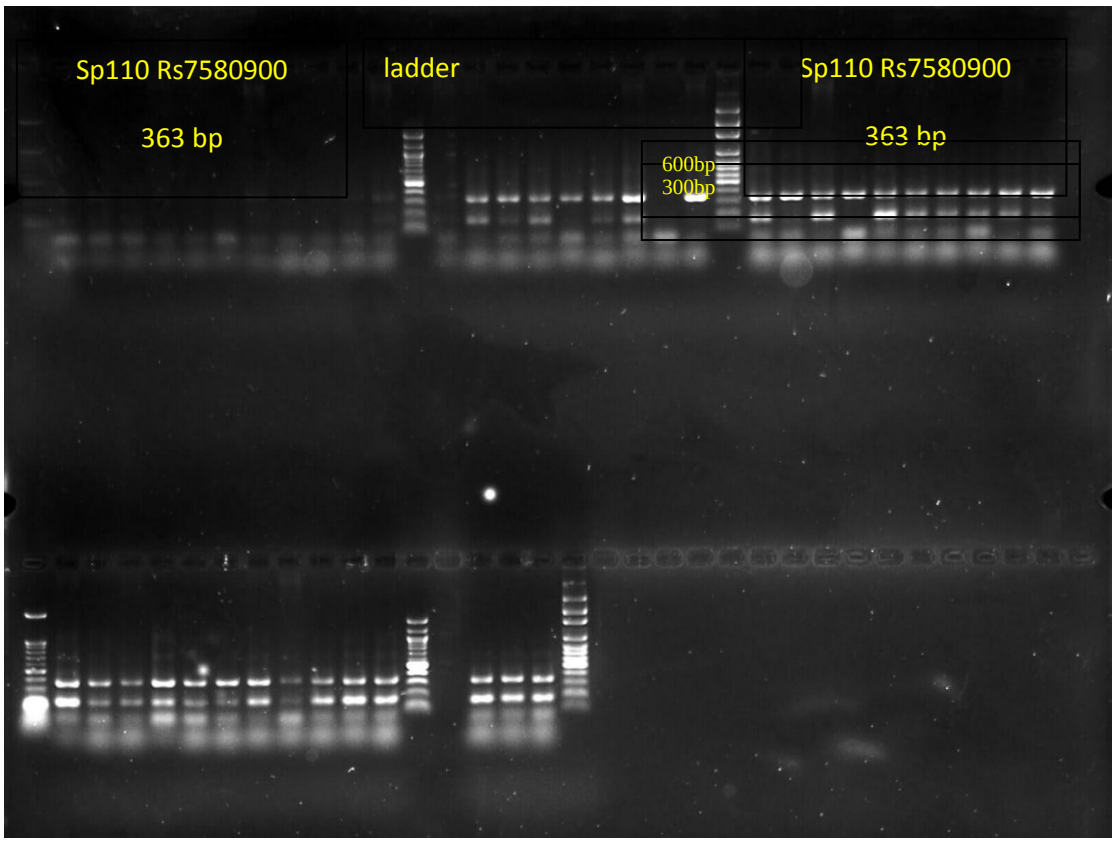


Figure (4-2): The Electrophoresis Pattern of *Sp110 Rs7580900* (363 bp) gene Polymorphisms. At 100 V.L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

SPll0 gene is a candidate gene for susceptibility of TB. It has been found that three single nucleotide polymorphisms (SNPs) of *SP110* are susceptible gene polymorphisms of TB in West African populations (Cai *et.al*.,2019) .50 healthy controls, 63 Covid patients, and a total of 113 persons were examined.Results were found for the *SP110* polymorphism. To assess the frequency distributions of variables among the study groups, logistic regression was utilized .The *SP110* AA*AT genotype (p = 0.05) and male gender (p 0.001) were related with severe COVID-19.COVID-19 severity was found to be associated with the *SP110* genotype through AA*AT Allele, 95% CI (0.3101 to 1.9213), Significance level, (0.5779) Odd Ratio (0.7719); AA*TT Allele, 95% CI (0.6606 to 4.2048), Significance level, (0.2793) Odd Ratio (1.6667); and

AT*TT Allele, 95% CI (0.3101 to 1.9213), Significance level, (0.2793) Odd Ratio (1.66 (2.1591). assuming that the severity of covid-19 was associated with the *SP110*-AA*AT genotype.This study agreed with (Cai *et.al*.,2019) the *SP110* nucleus protein may mediate the interaction between the host and pathogen by participating in the transcription activation of macrophages to intracellular pathogens. This study agreed with (Cekerevac *et.al*.,2021)SARS-CoV-2 virus causes infection which led to a global pandemic in 2020 with the development of severe acute respiratory syndrome. This study aimed at examining its possible role in predicting severity and intrahospital mortality of COVID-19, alongside with other laboratory and biochemical procedures, clinical signs, symptoms, and comorbidity. This study, approved by the Ethical Committee of Clinical Center Kragujevac, was designed as an observational prospective cross-sectional clinical study which was conducted on 127 patients with diagnosed respiratory COVID-19 viral infection from April to August 2020. This study agreed with (Yuki, Fujiogi, and Koutsogiannaki 2020) inflammation was thought to play an important role in the disease's development. There are no published studies linking this gene to Covid-19.

4.2.4 *IL-6* Gene Polymorphism

The three genotypes (CC,CG &GG), respectively (5.31, 38.94, 11.50), and the allele frequency of (C,G) were (44.44, 55.56) for patients and genotypes (CC,CG &GG), respectively (15.04, 23.01, 6.19) and the allele frequency of (C,G) was (60.00, 40.00) for controls. Tables (4-5), figure (4-3): exhibit the genotyping results for patients with Covid-19 and controls .

Table (4-5): Genotype frequency of polymorphisms of (*IL-6*) gene associated with Covid-19 patients and control						
Genotype	**patients** (63)		Healthy (50)		Total (113)	
	No	%	No	%	No	%
CC	6	5.31	17	15.04	23	**20.35**
CG	44	38.94	26	23.01	70	**61.95**
GG	13	11.50	7	6.19	20	**17.70**
TOTAL	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
C	56	44.44	60	60.00	116	**51.33**
G	70	55.56	40	40.00	110	**48.67**
TOTAL	**126**	**100.00**	**100**	**100.00**	**226**	**100.00**

Table (4-6): Genetic association of Genotype *IL-6* gene with disease.

polymorphisms of (*IL-6*) gene			
ALLELE	OODRATIO	Significance level	CI 95%
CC*CG	0.2086	0.0034	0.0730 to 0.5956
CC*GG	0.1900	0.0128	0.0514 to 0.7028
GG*CG	1.0974	0.8608	0.3883 to 3.1015

As showed in the above table, there is significance between CC*CG (0.0034), CC*GG (0.0128), GG*CG (0.8608).These results agreed with the findings of the WHO, which indicated Population diversities of *IL-6* gene polymorphisms at rs1800796/ rs1800795 loci showed that the populations of India, Mexico, Turkey, Brazil, Russia, Italy, South Africa, Netherland, Greece frequently have the GG genotype while the populations of China, Spain , Sweden, Poland, Germany, and the UK frequently have GC genotype .Only the Japanese population frequently showed the CC genotype for rs1800796 polymorphism(WHO, 2020). These results agreed with the findings of (Falahi *et.al.*, 2022) indicated there were no appreciable variations in the genotype or allele distribution of a few selected SNPs in the promoter region of the *IL-6* gene between patients with severe COVID-19 and patients with mild COVID-19.Another local Study by Iman S.H.(2022) explained that G allele may represent a significant risk factor for COVID-19 in the Iraqi population

and there is an association between *IL-6*-174 G/C polymorphism and COVID-19 patients.

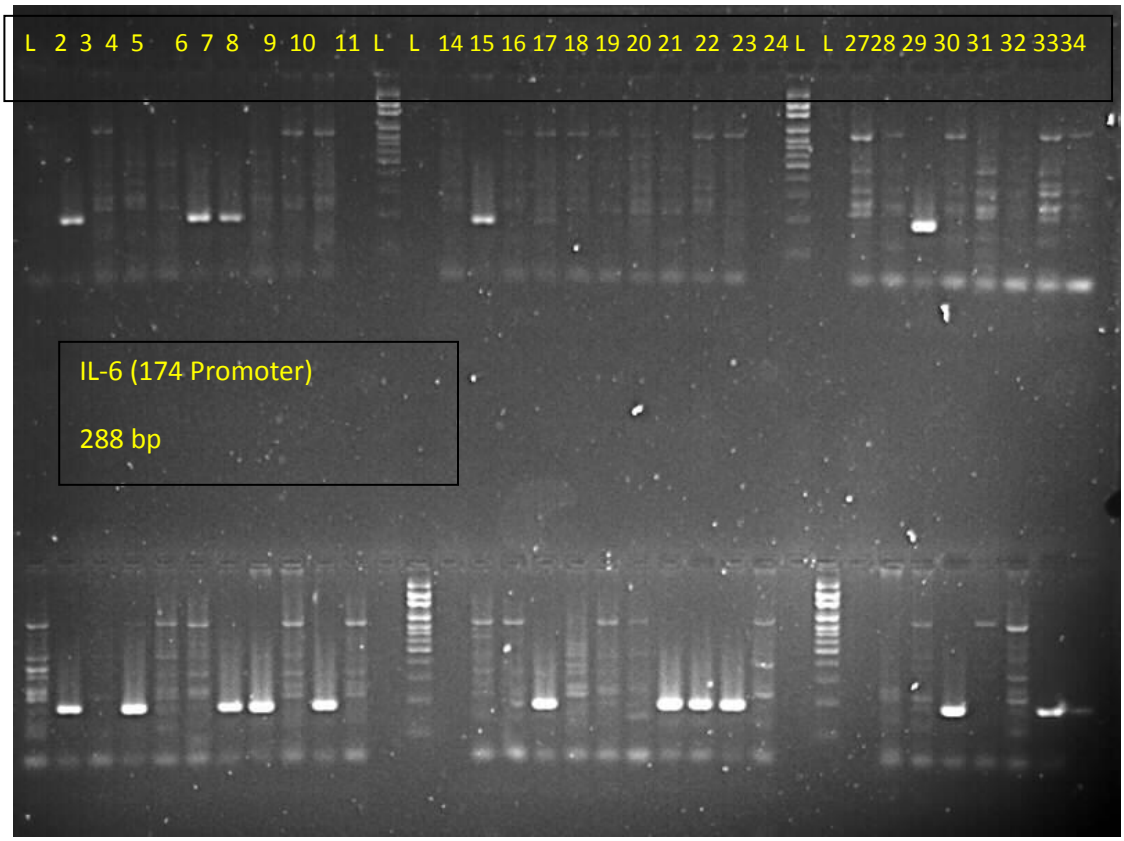


Figure (4-3): The Electrophoresis Pattern of *IL6* (288 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

With the sustained spread of COVID-19 in most societies, a suitable prognostic test that can predict progression of patients to severe state of disease with reasonable accuracy need hour for efficient management and care. Early research on COVID-19 indicated the role of pro-inflammatory cytokines such as *IL-6* and *TNF-α* released by activated mast cells in respiratory tract submucosa aggravating the inflammatory state and pathogenesis (Conti *et.al.*,2020, Kritas *et.al.*,2020, Ross and Conti 2020). *IL-6* can promote T-cell population expansion and activation and B-cell differentiation, regulate the acute phase response and release of a large number of cytokines. SARS-CoV2 activates the innate and adaptive

immune systems, resulting in the release of a large number of cytokines, including *IL-6*.These results in a systemic inflammatory response called cytokine release syndrome (CRS) in a large number of patients with severe COVID-19, which is an important reason of death (Zhang and Li 2020).Elevated levels of *IL-6*, a pro-inflammatory molecule, is known to down-modulate NK cell activity and are also found to be associated with reduction in granzyme and perforin levels causing impairment of lytic activities (Cifaldi *et.al*.,2015). In COVID-19 patients, exacerbation symptoms such as increased body temperature, elevation in inflammation markers like CRP and serum ferritin and progressed chest computed tomography images were associated with increased *IL-6* levels which showed a downturn during recovery (Liu *et.al*.,2020). This association of *IL-6* with pulmonary conditions were reported earlier in patients with pneumonia (de Brito *et.al*.,2016) or severe pneumonitis caused by radiation therapy (Chen *et.al*.,2001).Since the outbreak of coronavirus disease 2019 (COVID-19) in Wuhan, China, it has rapidly spread across many other countries. While the majority of patients were considered mild, critically ill patients involving respiratory failure and multiple organ dysfunction syndrome are not uncommon, which could result death. Hypothesized that cytokine storm is associated with severe outcome (Han *et.al*.,2020).looked at 113 people; 50 healthy controls, 63 people with Covid, and a total of 113 people. Results for the *IL-6* polymorphism were obtained. Logistic regression was used to evaluate the distribution frequencies of variables across the study groups. The *IL-6* GG*GA genotype (p = 0.049) and male gender (p 0.001) were related with severe COVID-19.COVID-19 severity was found to be associated with the *IL-6* genotype through. In the context of the co-dominant inheritance paradigm, multiple logistic regression GG*CG, CC*GG, and CC*GG alleles all have 95% confidence intervals of 0.0730 to 0.5956, 0.0034 for significance and 0.2086 for odds ratio, respectively (1.0974). assuming

that the severity of covid-19 was associated with the *IL6*-CC*CG genotype. This study agreed with (Han *et.al*.,2020) enrolled 102 COVID-19 patients who were admitted to Renmin Hospital (Wuhan, China). All patients were classified into moderate, severe and critical groups according to their symptoms. 45 control samples of healthy volunteers were also included.This study agreed with (Michot *et.al.*,2020) COVID-19 with hyperinflammatory pulmonary symptoms is associated with a cytokine storm involving interleukins and chemokine dysregulation.This study agreed with (Batur and Hekim 2020) a very recent meta-analysis reported a relation between the *IL6* gene polymorphism and predisposition as well as disease severity of pneumonia, suggested the carrier status of *IL6* allele with higher *IL-6* production and pneumonia severity. This study agreed with (Ulhaq and Soraya 2020).These severe COVID-19 cases are marked with excess cytokine production and a higher mortality rate that an elevated level of interleukin-6 (*IL-6*) and C-reactive protein (CRP) are strongly associated with COVID-19 progression. This study agreed with (Szulc-Kielbik, Kielbik, Nowak, and Klink, 2021) elevated concentrations of *IL-6* are observed in the serum and ascites of ovarian cancer patients. Thus, its level is discussed in the literature as a potential biomarker that can help to discriminate malignant and nonmalignant ovarian tumors and allow for the prediction of the chemotherapy response.

4.2.5 *IL-10* gene polymorphism

Figures 4-4 show the three genotypes (GG, GA &AA), (3.54, 51.33, 0.88) respectively, and allele frequency of (G,A) was (52.38, 47.62) respectively for the patients and genotypes (GG, GA &AA), (7.96, 20.35, 15.93) respectively, and allele frequency (G,A) was (41.00, 59.00) respectively for control people. Table (4-7) displays the genotyping results for patients with Covid-19 and control people.

Table (4-7) Genotype frequency of polymorphisms of *(IL-10)* gene associated with Covid-19 patients and control						
Genotype	Control (63)		Healthy (50)		**Total (113)**	
	No	%	No	%	No	%
GG	4	3.54	9	7.96	13	**11.50**
GA	58	51.33	23	20.35	81	**71.68**
AA	1	0.88	18	15.93	19	**16.81**
TOTAL	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
G	66	52.38	41	41.00	107	**47.35**
A	60	47.62	59	59.00	119	**52.65**
TOTAL	**126**	**100.00**	**100**	**100.00**	**226**	**100.00**

Table (4-8): Genetic association of Genotype *IL-10* gene with disease.

polymorphisms of (*IL-10*) gene			
ALLELE	OODRATIO	Significance level	CI 95%
GG*GA	0.1762	0.0075	0.0493 to 0.6295
GG*AA	8.0000	0.0806	0.7761 to 82.4596
GA*AA	45.3913	0.0003	5.7231 to 360.0120

As showed in the above table, there is significance between GG*GA (0.0075), GG*AA (0.0806), GA*AA (0.0003).These results agreed with (Neumann *et.al*.,2020) Production of *IL-10* is a hallmark of activated regulatory T cells that reside in tissues such as the lung. This population, normally rare in healthy individuals, rose up to ~ 10% of the regulatory T-cell pool in severe COVID-19 patients. The murine analogue to this population has a potent ability to limit inflammation and tissue damage triggered by microbial and environmental interactions at mucosal surfaces. (Bedoya *et.al*.,2013). In the case of viral infections of the lung, *IL-10* restrains the development of *IL-17*-producing cells that damage the tissue, (Chaudhry *et.al*.,2011,McKinstry *et.al*.,2009) inhibits the innate inflammatory response to viral particles, and is likely beneficial in reducing the production of cytokines such as *IL-6* that have been implicated in COVID-19 morbidity.(Chang, Kunkel and Chang (2009), Coomes and

Haghbayan (2020). Increase of this suppressive regulatory T-cell subset could be a direct response to the progressing lung inflammation in COVID-19 patients, comprising a feedback inhibition circuit to prevent runaway inflammation and death.(Rojas, Avia, Martín, and Sevilla (2017) Potentially, elevated *IL-10* could provide a blood-based biomarker for cases progressing to more severe lung damage. A more intriguing possibility is that individuals with higher *IL-10*-producing regulatory T cells exhibit defective adaptive immunity. *IL-10*$^{+}$ regulatory T cells are symptomatic of many unresolved viral infections and are associated with long-term persistence. In respiratory infections, *IL-10* potently suppresses anti-viral responses(Sun, Torres, and Metzger (2010) and weakens the immune reaction to superinfection with bacteria.(Chaudhry ***et.al***.,2011,van der Sluijs *et.al.*,2004) Since secondary infection leading to pneumonia is a major cause of death in influenza, and perhaps for some COVID-19 patients as well,(Cox, Loman, Bogaert and O'Grady (2020), Zhou *et.al*.,2020) excessive *IL-10* production by regulatory T cells may be a key factor in COVID-19 outcomes.

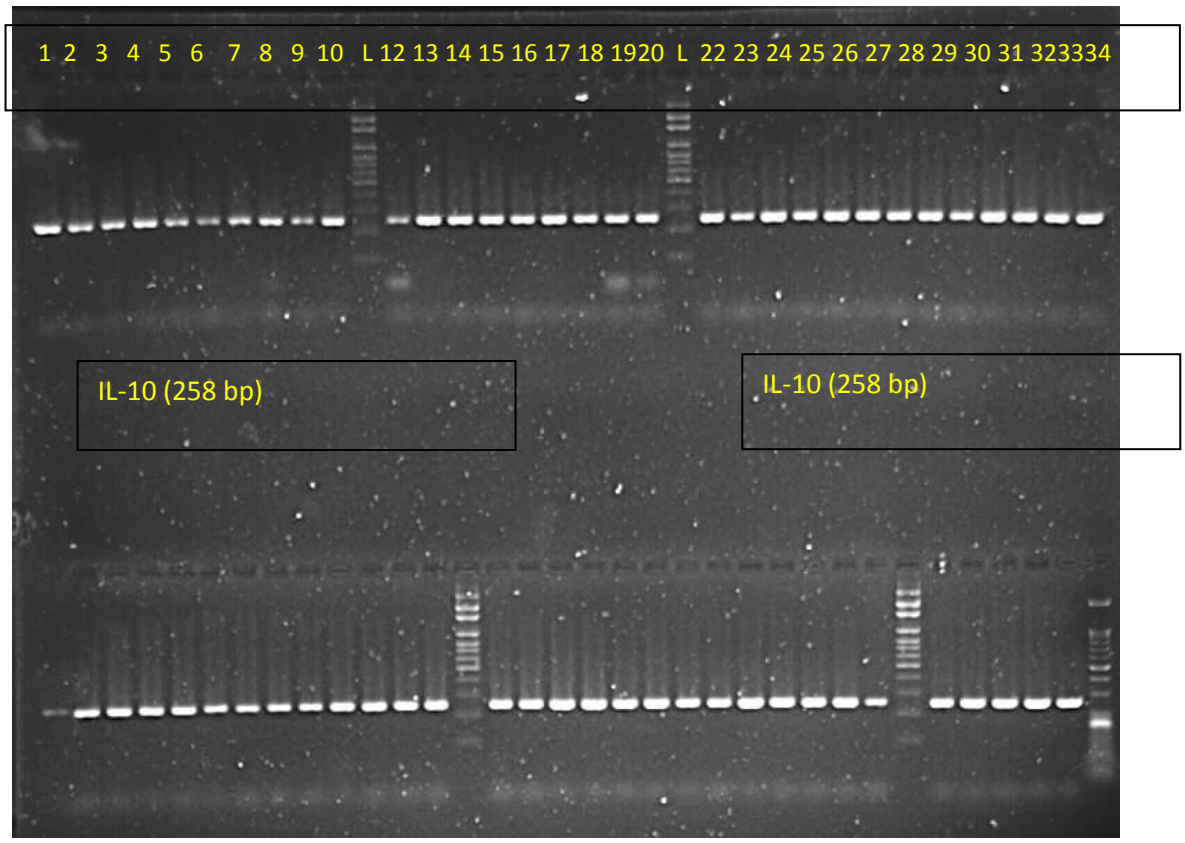


Figure (4-4): The Electrophoresis Pattern of *IL10* (258 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

IL-10 is an anti-inflammatory cytokine that was found elevated in severe COVID-19 patients (Huang *et.al*.,2020, Qin *et.al*.,2020, Chen *et.al*.,2019). Levels of *IL-6, IL-10* and *TNF-α* were also found to be indicators of T-cell exhaustion in COVID-19 patients (Diao *et.al*.,2020). *IL-10* is a multifunctional cytokine whose primary function is to limit the inflammatory response. *IL-10* is also known to introduce anergy or non-responsiveness of T-cells in anti-tumour cell response (Moore, de Waal Malefyt, Coffman, and O'Garra, 2001) as well as in viral infection (Maris, Chappell, and Jacob, 2007). *IL-10* is one of the complex group of mediators participating in the pathogenesis of COVID-19. In influenza infection, *IL-10* is highly abundant, especially during the adaptive immune response.58 Serum *IL-10* levels with *IL-6* were found to be

significantly higher in critical COVID-19 patients, than in moderate and severe patients. The levels of *IL-10* were also reported to be positively correlated with CRP amount, and *IL-6* and *IL-10* were found to be predictive of disease severity(Han *et.al.*,2020).Blockade of *IL-10* using antibody against *IL-10* or its receptor or genetic removal of *IL-10* resulted in elimination of infection by virus (Brooks et.al.,2006,Ejrnaes *et.al*.,2006) or bacterial pathogen (Biswas *et.al*.,2007). Thus, the elevated levels of *IL-10* in severe COVID-19 patients were initially ascribed to a negative feedback mechanism through their anti-inflammatory activities (Zhao *et.al*.,2020).The potential of *IL-10* to protect lung tissue from immune-mediated damage but also to impede antimicrobial defense, and consider possible cellular sources of *IL-10* (Lindner, Velásquez, Thiel, and Kirschning, 2021).our looked at 113 people; 50 healthy controls, 63 people with Covid, and a total of 113 people.

Results for the *IL-10* polymorphism were obtained. Logistic regression was used to evaluate the distribution frequencies of variables across the study groups. *The IL-10* GG*GA genotype (p = 0.049) and male gender (p 0.001) were related with severe COVID-19.COVID-19 severity was found to be associated with the *ACE2–CT* genotype through the co-dominant inheritance model and multivariate logistic regression GG*GA Allele, 95% CI (0.0493 to 0.6295), Significance level, 0.0075, Odd Ratio, 0.1762, GG*AA Allele, 95% CI (0.7761 to 82.4596), Significance level, 0.0806, Odd Ratio, 8.0000, and GA*AA Allele (45.3913). assuming that the severity of covid-19 was related to the genotype *IL10*-GG*GA. This study agreed with (lu *et.al.*,2021) that *IL-10* is a potential target for reducing COVID-19 mortality. As mentioned, severe/critically ill COVID-19 patients present dramatically elevated serum *IL-10* concentrations that correlate with disease severity .Accordingly, recent studies also demonstrate immune activation and inflammation in COVID-19 patients which supports the hypothesis that

IL-10 may play a proinflammatory and immune-activating role in COVID-19 pathogenesis. This study agreed with (Chen *et.al*., 2020) increased cytokine levels of *IL-10* to correlate with COVID-19 disease severity. This study differed with (Zhu *et.al*.,2021) the levels of *IL-10* of male were noticeably higher than those of female.

4.2.6 *ACE2 rs4240157 T > C* Gene Polymorphism

Patients with Covid-19 and control people the genotyping findings for patients and control people with Covid-19 are shown in table (4-9) and figures (4-5) that are refering to the three genotypes (CC,CT &TT),(1.77, 47.79, 6.19) respectively and allele frequency of (C,G) was (46.03, 53.97) respectively for the patients and genotypes (CC, CT &TT),(7.08, 10.62, 26.55) respectively and allele frequency (C,G) was (28.00, 72.00) respectively for control people.

Table (4-9): Genotype frequency of polymorphisms of (Genotype *ACE2 rs4240157 T > C*) gene associated with Covid-19 patients and control

Genotype	patients (82)		Healthy (50)		Total (113)	
	No	%	No	%	No	%
CC	2	1.77	8	7.08	10	**8.85**
CT	54	47.79	12	10.62	66	**58.41**
TT	7	6.19	30	26.55	37	**32.74**
TOTAL	**63**	**55.75**	**50**	**44.25**	**113**	**100.00**
Allele Frequency						
C	58	46.03	28	28.00	86	**38.05**
G	68	53.97	72	72.00	140	**61.95**
TOTAL	**126**	**100.00**	**100**	**100.00**	**226**	**100.00**

Table(4-10):Genetic association of Genotype *ACE2 rs4240157 T > C* gene with disease.

(Genotype *ACE2 rs4240157*)			
ALLELE	OODRATIO	Significance level	CI 95%
CC*CT	0.0556	0.0007	0.0104 to 0.2954
CC*TT	1.0714	0.9386	0.1854 to 6.1927
CT*TT	19.2857	0.0001	6.8611 to 54.2101

As showed in the above table, there is significance between CC*CT (0.0007), CC*TT (0.9386), CT*TT (0.0001).these results agreed with (Mir *et.al.*,2021) ACE2–CT genotype was strong associated with SARS-CoV-2 severity with an OR 2.18 (95% CI) (1.92–3.99), $p < 0.010$ and also *ACE2*–CC genotype was linked with COVID-19 severity with an OR 2.66 (95% CI) (1.53–4.62), $p < 0.005$. A significant correlation of *ACE2*-*T > C* genotypes was reported with gender ($p < 0.04$), T2D ($p < 0.035$). *ACE2*-CC genotype was strongly associated with increased COVID-19 mortality OR 3.66 (95%) CI = (1.34 to 9.97), $p < 0.011$ and also *ACE2*-C allele was associated with COVID-19 mortality OR 2, 01 (1.1761–3.45), $p < 0.010$. Conclusions: It is concluded that *ACE*-DD genotype and D allele was strongly associated with increased COVID-19 patient severity. In addition, *ACE I/D* polymorphism were strongly associated with advanced age, diabetes and ischemic heart disease in COVID-19 patients whereas *ACE-II* genotype was a protective factor against the development of severe COVID-19. *ACE2*-DD genotype was strongly associated with increased COVID-19 mortality.Additionally,*ACE2*–CCand CT genotypes were strongly associated with COVID-19 severity.

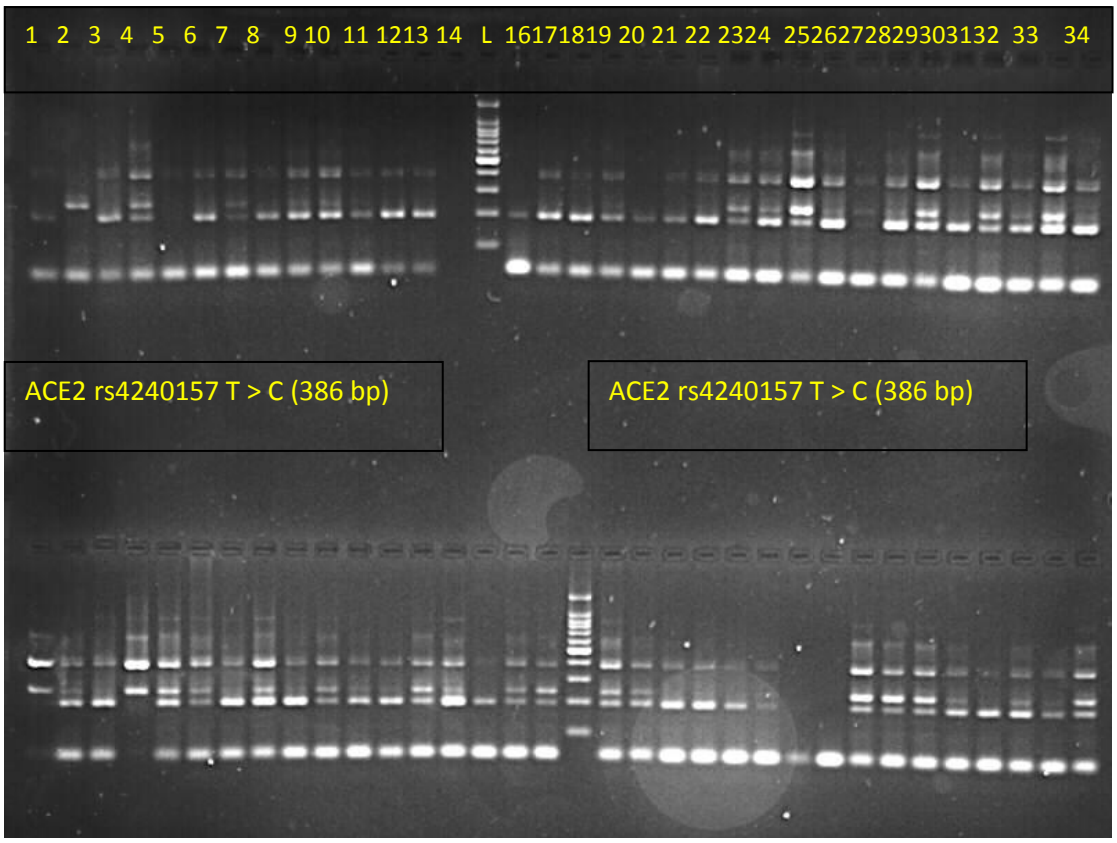


Figure (4-5): The Electrophoresis Pattern of ACE2 rs4240157 T > C (386 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

Pathogenesis of COVID-19 has been linked to the Angiotensin system. To begin, *ACE2* serves as a cellular receptor for SARS-CoV-2, suggesting that a person's vulnerability to infection may be controlled by how much of the *ACE2* gene is expressed. It is also possible that the severity of COVID-19 is related to the equilibrium between *ACE1* and *ACE2* activity, which has been linked to the aetiology of respiratory disorders. The outcome of COVID-19 may also be influenced by functional *ACE1/ACE2* gene polymorphisms, which have previously been linked to an increased risk of cardiovascular and pulmonary illnesses research at 113 people; 50 healthy controls, 63 people with Covid, and a total of 113 people. Results for the *ACE2 rs4240157 T > C* polymorphism were obtained. Logistic regression was used to evaluate

the distribution frequencies of variables across the study groups. The *ACE1*-CC*CT genotype (p = 0.049) and male gender (p 0.001) were related with severe COVID-19.COVID-19 severity was found to be associated with the *ACE2–CT* genotype through Using multivariate logistic regression and the co-dominant inheritance model CC*CT Allele, 95% CI (0.0104 to 0.2954), Significance level (0.0007), Odd Ratio (0.0556); CC*TT Allele, 95% CI (0.1854 to 6.1927), Significance level (0.9386), Odd Ratio (1.0714); and CT*TT Allele (19.2857). In the event that the severity of covid-19 was related to the *ACE2*-CC*CT genotype, this was the case. However, the *ACE2* polymorphism had no effect on the development of illness. In conclusion, male gender, malignancy, and the *ACE1* genotype were linked to a negative result of COVID-19. Our results indicated that *ACE1-C/T* may affect COVID-19 severity; however, this association was hypertensive status-specific. This finding needs to be confirmed in additional large samples. This study agreed with (Chaudhry *et.al*., 2020) that decreasing *ACE2* expression would result in decreased susceptibility to the virus by decreasing available binding sites for SARS-CoV-2 and restricting viral entry into the cells.

4.2.7 *MIF-794CATT, TNF-308 A/G and VDR* Gene Polymorphism

The genotyping findings for patients with Covid-19 and control people are shown in table (4-11) and figures (4-6) which refer to the ***MIF-794CATT*** genotypes male (6.30) respectively and female was (13.39) respectively for the patients and genotypes male (7.27) respectively and female was (12.73) respectively for control people and the ***TNF-308 A/G*** genotypes male (7.09) respectively and female was (9.84) respectively for the patients and genotypes male (10.91) , female was (8.18) respectively for control people and the ***VDR*** genotypes male (8.66) and female was (11.02) respectively for the patients and genotypes male (13.64) and female was (9.09) respectively for control and the

GSTM1 genotypes male (6.30) and female was (14.96) respectively for the patients and genotypes male (7.27) and female was (10.00) respectively for control subjects and the ***GSTT1*** genotypes male (6.30) and female was (16.14) respectively for the patients and genotypes male (9.09) and female was (11.82) respectively for control people.

Table (4-11) The correlation between *MIF-794CATT, TNF-308 A/G* and *VDR* genes polymorphism associated with Covid-19 patients and Control

Gene	Patients (63)				Total		Healthy (50)			Total		
	Male		Female				Male		Female			
	No	%	No	%	No	%	No	%	No	%	No	%
MIF-794CATT	16	6.30	34	13.39	**50**	**19.69**	8	7.27	14	12.73	**22**	**20.00**
TNF-308 A/G	18	7.09	25	9.84	**43**	**16.93**	12	10.91	9	8.18	**21**	**19.09**
VDR	22	8.66	28	11.02	**50**	**19.69**	15	13.64	10	9.09	**25**	**22.73**
GSTM1	16	6.30	38	14.96	**54**	**21.26**	8	7.27	11	10.00	**19**	**17.27**
GSTT1	16	6.30	41	16.14	**57**	**22.44**	10	9.09	13	11.82	**23**	**20.91**
Total	**88**	**34.65**	**166**	**65.35**	**254**	**100.00**	**53**	**48.18**	**57**	**51.82**	**110**	**100.00**

Table (4-12): Genetic association of Genotype *MIF-794CATT, TNF-308 A/G and VDR* gene with disease.

MIF-794CATT, TNF-308 A/G and *VDR*			
GENES	OODRATIO	Significancelevel	CI 95%
MIF-794CATT * TNF308A/G	0.6536	0.3260	0.2797 to 1.5271
MIF-794CATT * VDR	0.5989	0.2179	0.2650 to 1.3537
MIF-794CATT * GSTM1	1.1176	0.7936	0.4858 to 2.5714
MIF-794CATT * GSTT1	1.2059	0.6580	0.5265 to 2.7621
TNF-308 A/G * VDR	0.9164	0.8354	0.4020 to 2.0889
TNF-308 A/G * GSTM1	1.7100	0.2115	0.7370 to 3.9673
TNF-308 A/G * GSTT1	1.8450	0.1516	0.7987 to 4.2617
GSTM1 * GSTT1	1.0789	0.8562	0.4745 to 2.4536

As shown in the table above, there is significance between *MIF-794CATT * TNF-308 A/G* (0.3260), *MIF-794CATT * VDR* (0.2179), *MIF-794CATT * GSTM1* (0.7936), *MIF-794CATT * GSTT1*(0.6580), *TNF-308 A/G * VDR*(0.8354),*TNF-308 A/G * GSTM1*(0.2115), *TNF-308 A/G * GSTT1*(0.1516), *GSTM1 * GSTT1*(0.8562).These results agreed with (Nain *et.al.*,2021)the increased level of inflammatory chemokines

such as TNF-α has been observed in COVID-19 patients (Guo *et.al*.,2020). These results agreed with(Fishchuk *et.al*.,2021). It is found that a significant increase in the rate of the CC genotype and C allele (38.7 vs. 12.0% and 0.6 vs. 0.4%, respectively) of the *IL-6* gene in all patients of the study in comparison with population frequencies. There was a significantly higher rate of heterozygous genotypes TC and GA of the *VDR* gene in group of died patients. The rs1800629 variant of the *TNF-α* gene is associated with the need for respiratory support and its longer duration in patients with COVID-19.

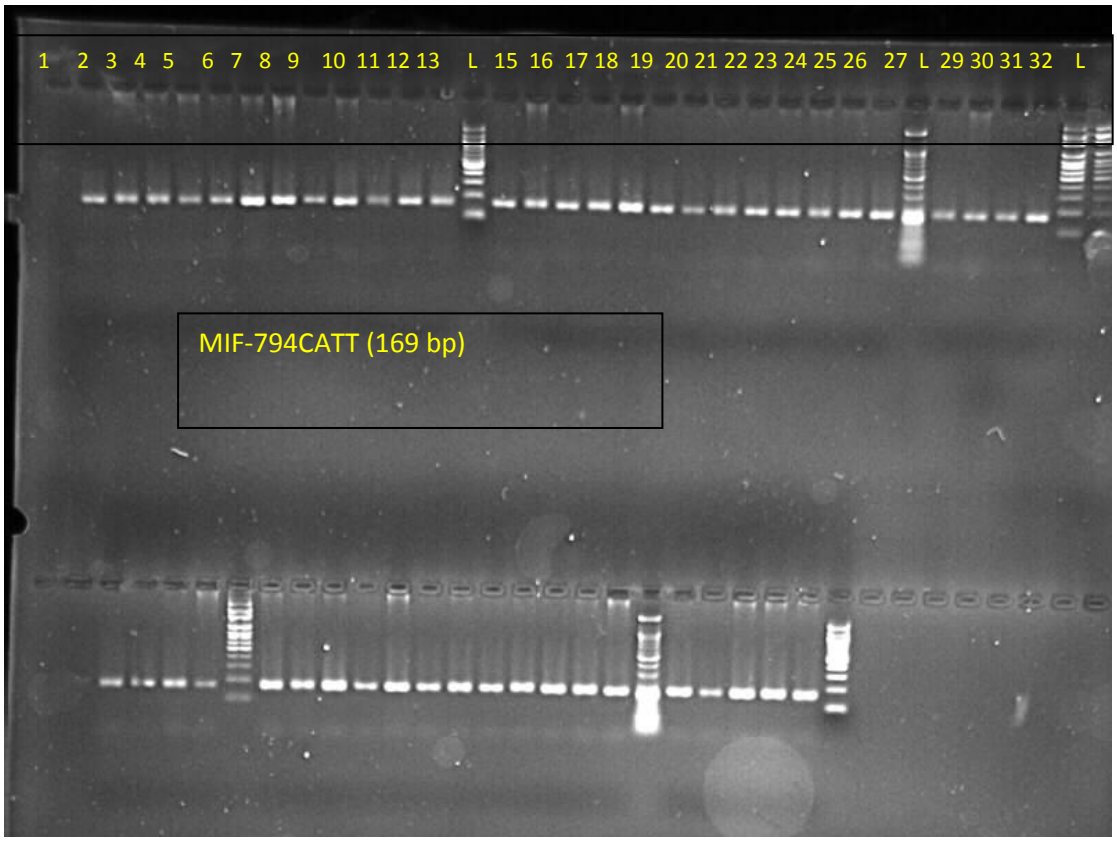


Figure (4-6): The Electrophoresis Pattern of *MIF-794CATT* (169 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

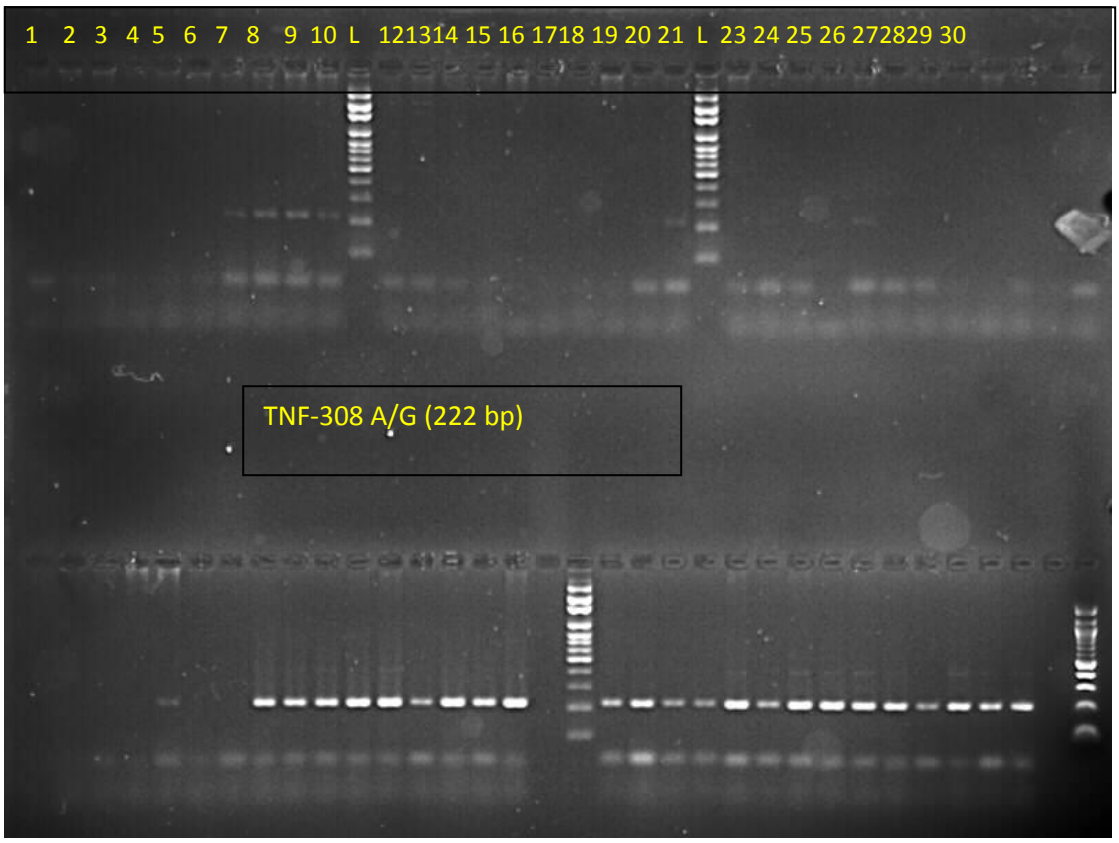


Figure (4-7): The Electrophoresis Pattern of *TNF-308 A/G* (222 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

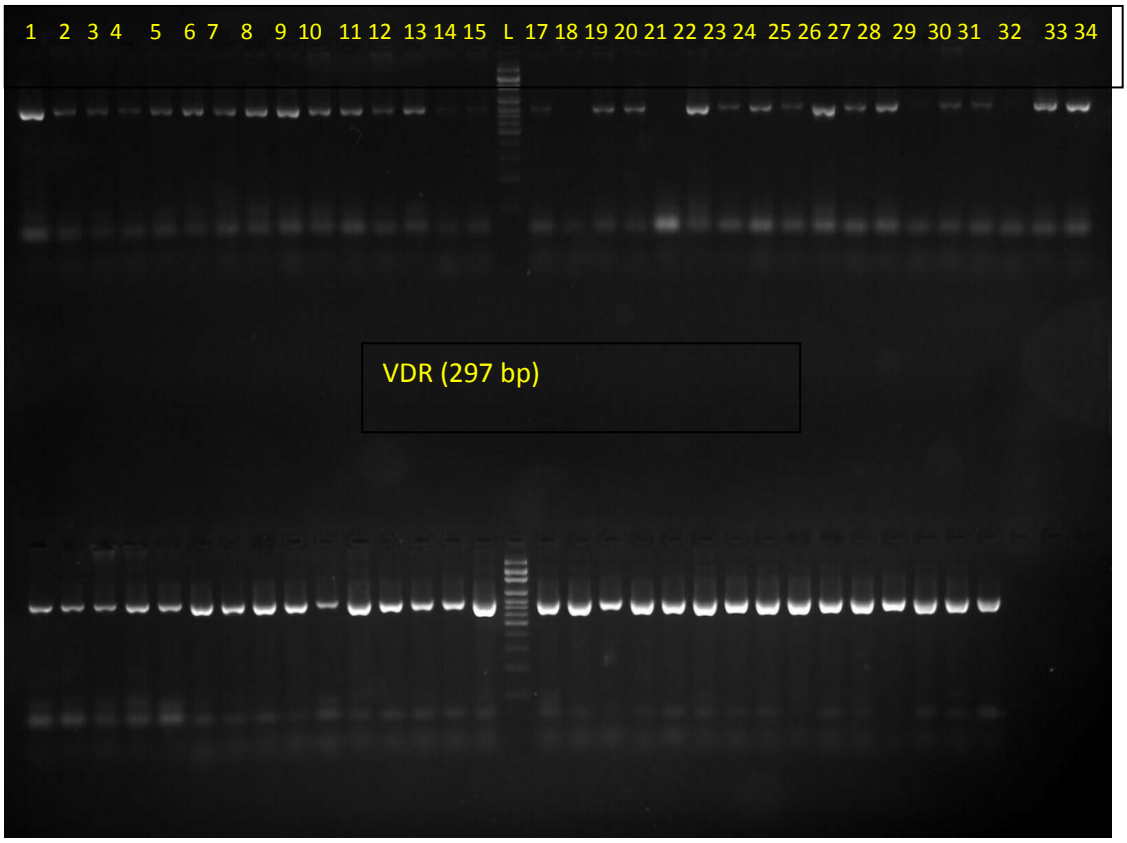


Figure (4-8): The Electrophoresis Pattern of *VDR* (297 bp) gene Polymorphisms. At 100 V. L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain for 1hr.

Macrophage migration inhibitory factor (*MIF*) is a proinflammatory cytokine encoded within a functionally polymorphic genetic locus. *MIF* was initially recognized as a cytokine generated by activated T cells, but in recent days it has been identified as a multipotent key cytokine secreted by many other cell types involved in immune response and physiological processes (Sumaiya,Langford,Natarajaseenivasan, and Shanmughapriya, 2021). Looked at 113 people; 50 healthy controls, 63 people with Covid, and a total of 113 people. Results for the ***MIF-794CATT*** polymorphism were obtained. Logistic regression was used to evaluate the distribution frequencies of variables across the study groups. COVID-19 severity was found to be associated with the ***MIF-794CATT*** genotype in the context of the co-dominant inheritance paradigm,

multiple logistic regression *MIF-794CATT * VDR* Allele, *MIF-794CATT * GSTT1*, and *MIF-794CATT * TNF-308 A/G* Allele, 95% CI (0.2797 to 1.5271), Significance level, (0.3260) Odd Ratio (0.6536), and *MIF-794CATT * GSTT1* (1.2059). If the *MIF-794CATT * TNF-308 A/G* genotype was associated with the severity of covid-19, this was the case. There might be relationships between these single nucleotide polymorphisms (SNPs) and cytokine mediated inflammation, which may affect the outcome of the disease (Chen *et. al.,* 2010;Kim *et. al*.,2003; Niro *et.al.,* 2005 and Hohler *et.al.,* 1998). Failure to secrete adequate amounts of *TNF-α* could possibly prevent viral clearance and lead to chronic infection as noted by saada *et. al*.,2015; which agrees with our study (Saadia *et. al*.,2015). This study agreed with (Conroy, Mawhinney and Donnelly 2010) that increasing body of evidence implicating the key pro-inflammatory cytokine *MIF* in specific biological activities related directly to cancer growth or contributing towards a microenvironment favouring cancer progression. This study studies agreed with(Conroy, Mawhinney and Donnelly 2010) *MIF*'s unique biological activities have the potential to contribute to an in vivo microenvironment favouring tumour growth and invasiveness. These functional activities include: tumour suppressor downregulation, potent induction of angiogenesis and enhanced tumour growth, proliferation and invasiveness. This study agreed with (Sumaiya,Langford, Natarajaseenivasan, and Shanmughapriya, 2021) *MIF* being an immune modulator accelerates detrimental inflammation, promotes cancer metastasis and progression. This study agreed with(Robinson et.al.,2020)Elevated levels of tumor necrosis factor (*TNF*), a key pro-inflammatory cytokine, have been shown to be associated with increased COVID-19 mortality. This study differed with (Abdollahzadeh *et.a*l.,2021) Our results didn't show any significant associations between studied *VDR* gene *SNPs* and the aforementioned demographic/clinical features as well as comorbidities in

both asymptomatic and in the mild/moderate COVID-19 patients (P values >0.05).Glutathione-S-transferase (*GST*) is an important enzyme that catalyzes the conjugation of glutathione (*GSH*) with electrophiles to protect the cell from oxidative damage and participates in the antioxidant defense mechanism in the lungs (Abbas *et.al.*,2021). This study differed with (Abbas *et.al*.,2021) *GSTM1/GSTT1* polymorphism was not shown to have a significant association with the severity of the COVID-19 ($p > 0.05$).

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Abbreviations	Terms
ACE 2	Angiotensin-converting enzyme 2
ACE I/D	Angiotensin-converting enzyme Insertion/Deletion
ARDS	Acute Respiratory Distress Syndrome
BP	Base pair
C	Cytosine
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CFR	Cost for Fright
CHD	Coronary Heart Disease
CI	confidence interval
Conc.	Concentration
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus disease 2019
CPAM	Congenital Pulmonary Airway Malformation
CRP	C-reactive protein
CS	cytokine storm
CT	Computed Tomography
CXR	chest x-ray
DDR	Double Data Rate
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DPP4	four Dipeptidyl Peptidase
EDTA	ethylene diamin tetraacetic acid
ELISAs	enzyme-linked immunosorbent assays
F primer	Forword
FDA	The US Food and Medicine Administration
GSTM1	glutathione S-transferase Mu 1
GSTT1	glutathione S-transferase (GST) theta 1
HCoV- 229E	Human coronavirus 229E
HCoV-HKU1	Human coronavirus HKU1

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HCoV-NL63	Human coronavirus NL63
HCoV-OC43	Human coronavirus OC43
HR	hazard ratio
ICU	intensive care unit
IFNB	Interferon-beta
IL-10	interleukin-10
IL-6	interleukin-6
LPV/RTV	Lopinavir/Ritonavir
MERS-CoV	the Middle East Respiratory Syndrome Coronavirus
MIF	Macrophage migration inhibitory factor
MIS-A	multisystem inflammatory syndrome in adults
MIS-C	multisystem inflammatory syndrome in children
OD	optical density
PCR	Polymerase chain reaction
PCR- RFLP	Polymerase chain reaction -restriction fragment- length polymorphism
qPCR	quantitative real-timePCR
R primer	Revers
RA	Rheumatoid arthritis
RAAS	Renin-Angiotensin-Aldosterone system
RBD	receptor-associating area
RNA	Ribonucleic acid
RT-PCR	Real Time- Polymerase chain reaction
S	spike
SARS	Severe acute respiratory syndrome coronavirus
SARS-CoV-1	The Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SNP	Single-nucleotide polymorphism
SP 110	nuclear body protein
SSP-PCR	Sequence-specific primer-polymerase chain reaction
TNF	tumor necrosis factor
USA	United States of America
VDR	Vitamin D receptors
WHO	World health organization

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Conclusion and Recommendations

Conclusion:

1- Works that can be applied in the future, and which we could not do, such as skewness.

2- Studying the efficiency of vaccines after a period and for the same genes to know its efficiency and its preventive role from future infections.

3- Significant results were seen in *SP110* gene polymorphism between covid patients and vaccinated persons at ($P \leq 0.05$).Tis may be role of *SP110* in mediating SARS-CoV-2 infection as a novel route for SARS-CoV-2 entry.

4- Significant results were seen in *FGA* gene polymorphism between covid patients and vaccinated persons at ($P \leq 0.05$). This may be the reason for blood clots and elevated level D-dimer in covid patients .

5- Male gender, malignancy, and the *ACE1* genotype were linked to a negative result of COVID-19. The research results indicated that *ACE1-C/T* may affect COVID-19 severity.

6- The selection of other genes that have an immunological and physiological relationship with viral infections.

Recommendations

1- Examining more gene polymorphisms related to cell immunity, receptors, and mutation to demonstrate their function in the development of COVID-19.

2- Estimating the contribution of additional cytokine markers to COVID-19 development.

3- Analyze the vast array of Covid patients with various diseases, locations, and Sars-Cov-2 variants.

4-Study the Spike protein expression and characterization before and after vaccination.

5-Estimating the vaccinated person comparing with Pfizer ,AstraZeneca and Sino pharm vaccine .

6-Further study to monitor the more critical cases , mortality rate and duration after onset of infection and relationship of proteins .

7-Fellow up study on vaccinlate people after the 3ed dose of vaccine to reveale the effect of vaccine and the status of immune system .

8-Study the rate and active of cell (immune cell) flow cytometry techniques.

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