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Genetic Polymorphism of Angiotensin Converting Enzyme-2 level and Receptors in Relation to Severity of Iraqi COVID-19 Pandemic.

A Thesis

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Dedication

To God, Who is Always There and Help Me Every Time in Anywhere?

To the Prophet of Mercy.... Mohammed and His Good Family [Al-Tahyreen] Peace be on Them

To My Mother and to My Father.

To My Beloved Family.

To My Dear Husband Arkan

To My Children.

I give you this humble work.

SUZAN

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Thanks for All

SUZAN

Supervisor's Certification

We certify that this M.Sc. thesis titled:-

Genetic Polymorphism of Angiotensin Converting Enzyme-2 level and Receptors in Relation to Severity of Iraqi COVID-19 Pandemic.

Was prepared under our supervision in laboratories at department of Chemistry and Biochemistry- College of Medicine/ University of Kerbala as a partial requirement for the Master Degree in Clinical Chemistry

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

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Summary

Coronavirus (Covid-19) is clustered under the viral family group that causes disease in mammals and birds. A pandemic novel coronavirus was named as ‘‘Corona Virus Disease 2019’’ by World Health Organization (WHO) in Geneva, Switzerland. This is the deadly third-generation virus in Corona family preceded by Middle East Respiratory Syndrome (MERS) in 2012 and Severe Acute Respiratory Syndrome (SARS) in 2003. The incidence of SARS-CoV-2 infection is seen most often in adult male patients with the median age of the patients was between 34 and 59 years. SARS-CoV-2 is also more likely to infect people with chronic comorbidities such as cardiovascular and, hypertension and diabetes. The highest proportion of severe cases occurs in adults ≥60 years of age.

Angiotensin converting enzyme-2 (ACE-2) is a protein on the surface of many cell types. It is an enzyme that generates small proteins – by cutting up the larger protein angiotensinogen – that then go on to regulate functions in the cell. Using the spike-like protein on its surface, the SARS-CoV-2 virus binds to ACE2 – like a key being inserted into a lock – prior to entry and infection of cells. Hence, ACE2 acts as a cellular doorway – a receptor – for the virus that causes covid-19. ACE2 acts as the receptor for the SARS-CoV-2 virus and allows it to infect the cell.

The quantity of receptors determine whether someone gets more or less sick, because the SARS-CoV-2 virus requires ACE2 to infect cells but the precise relationship between ACE2 levels, viral infectivity and severity of infection .

The aim of the presented project is to study the association between various biomarkers including angiotensin-converting enzyme and its receptor gene polymorphism with severity of covid-19 infection in Iraqi pandemic of Kerbala province.

This study was conducted on 176 subjects which diagnosed by physician and were divided into three groups: 59 of them were with severe infected covid-19, 54 of them were with moderate infected covid-19 and 63 of them were apparently

healthy control who's attended the hospital for checkup. Severe and moderate patients were collected from Al-Hussein Teaching Hospital, Al-Hussein Medical City, Kerbala Health Directorates, Kerbala – Iraq during April, 2020- March, 2021 with matched age ranged between (23-88) years. Genomic DNA was extracted from blood then genotyping of ACE-2 receptor by using special primers was investigated using polymerase chain reaction.

The anthropometric and biomarkers determined included: Age, Body mass index, Ferritin, Lactate dehydrogenase (LDH) Level , C-reactive protein (CRP), Aniotensin converting enzyme-2 (ACE-2) Level , Alanine aminotransferase (ALT) Level , Aspartate aminotransferase (AST) Level and Alkaline phosphatase (ALP) level .

The genotype of ACE -2 obtained in all of 176 samples of the presented study (severe, moderate and control) indicate that three types of allele were observed:

1. **ACE-1 deletion/deletion polymorphisms (D/D)**
2. **ACE-1 insertion/deletion polymorphisms (I/D)**
3. **ACE-1 insertion/insertion polymorphism (I/I))**

Every patients carry (D/D) they were severely infection and increased in ACE- 2 enzyme, but the patients that carry (I/D), (I/I) they were moderate infection with covid -19. The ACE-2 gene polymorphism and ACE-2 activity levels were associated with the severity of covid-19. The three selected groups were found to be different with respect to Ferritin, LDH, CRP, ACE, ALT, AST and ALK .The mean values of the variables Ferritin ,LDH,CRP,ACE,ALT,AST and ALK are significantly higher in the sever and moderate compared to that of healthy control .

In conclusion, the data observed indicated that if those control groups will be infected with covid-19, they considered as severe cases due to that the D-allele is associated with higher ACE-2 expresion ; therefore, patients that carry D-allele,

especially those with the D/D genotype, are at higher risk of pulmonary morbidity and/or mortality .

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List of Abbreviations

Abbreviations	Description
ACE-2	Angiotensin Converting Enzyme-2
Ala	Alanine
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANG I	Angiotensin-I
ANG II	Angiotensin-II
APA	Aminopeptidase A
APN	Aminopeptidase N
ARB	Angiotensin receptor blockers
ARDS	Acute respiratory stress syndrom
ARMS	Amplification refractory mutation system
Asp	Aspartate
AST	Aspartate aminotransferase
AT1R	Angiotensin type 1 receptor
AT2	Alveolar type 2
AT2R	Angiotensin type 2 receptor
ATP	Adenosine tri phosphate
BMI	Body Mass Index
bp	Base pair
cDNA	Complementary DNA
CKD	Chronic kidney disease
3-Clpro	3-Chemotrypsin-like protease
CTAP	Corona virus Treatment Acceleration program
CTSB	Cysteine proteases cathepsin -B
CTSL	Cysteine proteases cathepsin - L
DD	Deletion/Deletion
DNA	Deoxyribonucleic acid
DPP_4	Dipeptidyl peptidase
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked Immuno sorbant assay
ET.Br.	Ethidium bromide
EUA	Emergency use Authorization
FDA	Food and drug administration
Glu	Glutamic acid
Gly	Glysine
HCOV	Human Corona virus
Hcovs	Human Corona virus
HDL	High density lipoprotein
His	Histidine

HRP	Horse radish peroxidase
ICU	Intensive Care unite
ID	Insertion /Deletion
IFN	Interferon
II	Insertion /Insertion
IL	Interleukin
ISGS	Interferon-stimulated genes
LDH	Lactate dehydrogenase
LDL	Low density lipo protein
Leu	Leusine
MCP1	Monocyte chemo attractant protein -1
MDH	Malate dehydrogenase
MERS	Middle East Respiratory Syndrom
MIP1A	Macrophage inflamatory proteins 1A
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate
nCOV	Nonel Corona virus
NRP1	Neuropilin -1
ORFS	Open reading frames
PaO_2 / FiO_2	Ratio of arterial oxygen partial pressure to fractional inspired oxygen
PCR	Polymerase chain reaction
PHEIC	Public Health Emergency of international concern
PPIa	Poly protein 1a
qPCR	Quantitative polymerase chain reaction
RAS	Renin angiotensin system
RBD	Receptor Binding Domain
RSV	Respiratory Syncytial Virus
RT-PCR	Real time polymerase chain reaction
S ACE	Somatic angiotensin -1 converting enzyme
SARS	Severe acute respiratory syndrome
SARS-COV-2	Severe acute respiratory syndrome-Corona virus
Ser	Serine
SNP	Single nucleotide polymorphisms
SPO_2	Saturation of peripheral oxygen
SSRNA	Sense single-strand RNA
TBE	Tris-borate-EDTA
T2DM	Type 2 Diabetes mellitus
TG	Triglyceride
TMPRSS2	Trans membrane serine protease 2
WBC	White blood cell
WHO	World Health organization

Chapter ONE

Introduction and Review of Literature

1. Introduction

On December 31, 2019, the China Health Authority alerted the World Health Organization (WHO) to several cases of pneumonia of unknown etiology in Wuhan City in Hubei Province in central China. The cases had been reported since December 8, 2019, and many patients worked at or lived around the local Huanan Seafood Wholesale Market although other early cases had no exposure to this market **(Lu, *et al .,* 2020).** On January 7, a novel corona virus, originally abbreviated as 2019-nCoV by WHO, was identified from the throat swab sample of a patient **(Hui, *et al .,* 2020)**. This pathogen was later renamed as severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) by the corona virus study group **(Gorbalenya, *et al* .,2020)**, and the disease was named corona virus disease 2019 (Covid-19) by the WHO.

Among patients admitted to hospitals, the mortality rate ranged between 11% and 15% **(Huang,C. *et al* .,2020 ; Chen, .*et al .,* 2020).** Covid-19 is moderately infectious with a relatively high mortality rate, but the information available in public reports and published literature is rapidly increasing.

Angiotensin converting enzyme 2 receptor (ACE2) is a protein on the surface of many cell types : lungs , heart ,Kidneys ,blood vessels and gastrointestinal tract. It is an enzyme that generates small proteins – by cutting up the larger protein angiotensinogen – that then go on to regulate functions in the cell. Using the spike-like protein on its surface, the SARS-CoV-2 virus binds to ACE2 –like a key being inserted into a lock – prior to entry and infection of cells. Hence, ACE2 acts as a cellular doorway – a receptor – for the virus that causes Covid-19. ACE2 acts as the receptor for the SARS-CoV-2 virus and allows it to infect the cell**(Li, *et al.,*2003).**

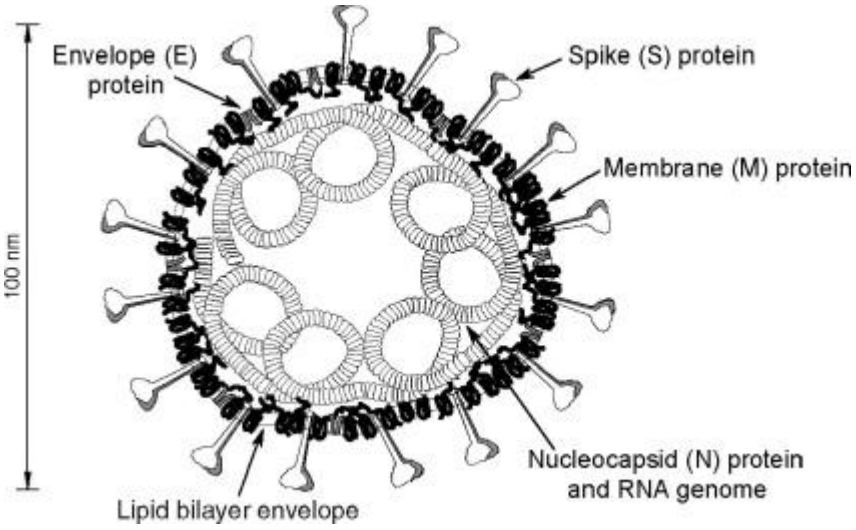
The aim of this review is to summarize the to study the association between various biomarkers including Angiotensin-converting enzyme and its receptor gene polymorphism with severity of Covid-19 infection in Iraqi pandemic of Kerbala Province .

1.1. Corona Viruses

Coronaviruses (Covid-19) are a family of viruses that cause illness such as respiratory diseases or gastrointestinal diseases. It is clustered under the viral family group that causes disease in mammals and birds. A pandemic novel coronavirus was named as „„Corona Virus Disease 2019"" (2019-nC) **(Kucharski, & Althaus, 2015 ; Zhu, *et al.*,2020).** This is the deadly third-generation virus in corona family preceded by Middle East Respiratory Syndrome (MERS) in 201oV) by World Health Organization (WHO) in Geneva, Switzerl and **A**2 and Severe Acute Respiratory Syndrome (SARS) in 2003. After Rhinoviruses, coronaviruses are ranked as the main cause of the commonplace cold without triggering any sickness **(Brennan, *et al.* ,2014).** In 1937, the primary coronavirus was located in bats, rarely affecting humans and mostly circulating among animals like bats, camels, and cats. Later, they mutated to contaminate rats, cattle, pigs, mice cats, dogs, horses, and turkeys.The first human coronavirus, known in the sixties, infected the nasalcavities of humans resulting in common cough and cold. Symptoms in different species includes: higher breathing tract ailment in chickens, diarrhea in cows and pigs **(Smits, *et al.* ,2010),** dry cough tiredness, fever, sore throat, aches and pains, shortness of breath, runny nose and diarrhea in humans **(Hughes, *et al.* ,2009).**

1.1.1. History of Corona Viruses

A novel corona virus (nCoV) is a new strain that has not been identified in humans previously. Once scientists determine exactly what corona virus it is, they give it a name (as in the case of Covid-19, the virus causing it is SARS-CoV-2). Corona viruses got their name from the way that they look under a microscope. The virus consists of a core of genetic material surrounded by an envelope with protein spikes. This gives it the appearance of a crown. The word corona means "crown" in Latin. Corona viruses are zoonotic disease, meaning that the viruses are transmitted between animals and humans. It has been determined that MERS-CoV was transmitted from dromedary camels to humans and SARS-CoV from civet cats to humans. The source of the SARS-CoV-2 (Covid-19) is yet to be determined, but investigations are ongoing to identify the zoonotic source to the outbreak (**Liu, Y. C., *et al.* ,2020**).



Fig(1.1) Schematic of the coronavirus virion, with the minimal set of structural proteins **(Paul S.Masters ,*et al.*,2006).**

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1.1.2. Causes of Covid-19

The role of the Huanan Seafood Wholesale Market in propagating disease is unclear. Many initial Covid-19 cases were linked to this market suggesting that SARS-CoV-2 was transmitted from animals to humans **(Li, P. , *et al.* ,2020).** However, a genomic study has provided evidence that the virus was introduced from another, yet unknown location, into the market where it spread more rapidly, although human-to-human transmission may have occurred earlier (**Yu, *et al.*, 2020)**. Clusters of infected family members and medical workers have confirmed the presence of person-to-person transmission **(Chan, J. F. W., *et. al.,* 2020).**

After Jan. 1st 2020, less than 10% of patients had market exposure and more than 70% patients had no exposure to the market, Person-to-person transmission is thought to occur among close contacts mainly via respiratory droplets produced when an infected person coughs or sneezes. Fomites may be a large source of transmission, as SARS-CoV has been found to persist on surfaces up to 96 h and other coronaviruses for up to 9 days **(Kampf, *et al.* ,2020).**Whether or not there is asymptomatic transmission of disease is controversial.

1.1.3. Classification and Origin of Corona Viruses

SARS-CoV-2 is a member of the family Corona viridae and order Nidovirales. The family consists of two subfamilies, Corona virinae and Torovirinae and members of the subfamily coronavirinae are subdivided into four genera **(Adams and Carstens *,* 2012**; **Perlman and Netland, 2009) .**

A. Alpha coronavirus contains the human coronavirus (HCoV)-229E and HCoV-NL63.

B. Beta coronavirus includes HCoV-OC43, Severe Acute Respiratory Syndrome human coronavirus (SARS-HCoV), HCoV-HKU1, and Middle Eastern respiratory syndrome coronavirus (MERS-CoV).

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C. Gamma coronavirus includes viruses of whales and birds.

D. Delta coronavirus includes viruses isolated from pigs and birds.

SARS-CoV-2 belongs to Beta corona virus together with two highly pathogenic viruses, SARS-CoV and MERS-CoV. SARS-CoV-2 is an enveloped and positive-sense single-stranded RNA (+ss RNA) virus **(Englund,*et al.*, 2019).** SARS-CoV-2 is considered a novel human-infecting Beta corona virus **.** Phylo genetic analysis of the SARS-CoV-2 genome indicates that the virus is closely related (with 88% identity) to two bat-derived SARS-like corona viruses collected in 2018 in eastern China (bat-SL-CoVZC45 and bat-SL-CoVZXC21) and genetically distinct from SARS-Covid-19V (with about 79% similarity) and MERS-Covid-19 **(Lu, R., *et al.* ,2020)**. Using the genome sequences of SARS-CoV-2, RaTG13, and SARS-Covid-19, a further study found that the virus is more related to Bat Covid-19 RaTG13, a bat corona virus that was previously detected in Rhinolophus affinis from Yunnan Province, with 96.2% overall genome sequence identity **.** A study found that no evidence of recombination events detected in the genome of SARS-CoV-2 from other viruses originating from bats such as Bat Covid-19 V RaTG13, SARS-Covid-19 and SARSr-CoVs. Altogether, these findings suggest that bats might be the original host of this virus **(Lu, R., Zhao , *et al.* ,2020).**

1.1.4. Biochemical Properties of Corona Virus

SARS-CoV-2 is part of corona virinae subfamily with one of the largest positive-sense single-stranded RNA genomes~30 kilo bases and over 10 open reading frames (ORFs). Two polypeptides, poly protein 1a (pp1a) and pp1ab, are synthesized through ribosomal frame shift between ORF1a and ORF1b during translation **(Ferreira, J. C., & Rabeh, W. M. , 2020) .**

In addition to the papain-like protease, the 3-chymotrypsin-like protease

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(3CLpro), also known as the main protease, is important for the post translational processing of SARS-CoV-2 polypeptides and the production of 16 non-structural proteins **(Cui, *et al.* ,2019).** This proteins play fundamental roles in replication, transcription, and virus recombination during an infection, where inhibiting the proteases will block the release of the nsps and inhibit the maturation and infectivity of SARS-CoV-19**.** As a result, 3CLpro of SARS-CoV-2 is an attractive target for the design of broad- spectrum of antivirals against Covid-19 **(Cascella, *et al* ,2020) .**

Among the corona viridae family, the 3CLpro substrate‟s binding pocket is highly conserved with glutamine and leucine/methionine required at P1 and P2-positions, respectively, which correspond to the first and second residues before the cleavage site on the polypeptide substrate **(Ferreira, J. C., & Rabeh, W. M. , 2020) .**

The 3CLpro cleaves the SARS-CoV-2 polyproteins at 11 sites “Leu Gln ↓Ser Ala Gly,” which marks the cleavage site**.** Multiple crystal structures of 3CLpro have been deposited in the Protein Data Bank, including the recently determined structure in complex with α-keto amide inhibitors **(Zhang, L. *et al.* ,2020).** The 3CLpro from Beta coronaviruses have identical structural folds, where the active site is highly conserved. The monomer is split into three domains, with domains I (residues 10–

96) and II (residues 102–180) having a five-stranded antiparallel β-barrel structure with a chymotrypsin-like folding scaffold (Fig. 1-1a).

On the other hand, the C- terminal domain III (residues 200–303) has a five α-helices cluster that is connected to domain II by a long loop (residues 181–199). Domain III of 3CLpro from SARS- CoV was identified to be important in the dimerization and formation of an active 3CLpro protease.

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The active site of 3CLpro is at the interface between domains I and II, and different from the Ser–His–Asp triad of chymotrypsin, 3CLpro of SARS-CoV-2 has a catalytic Cys–His dyad (Fig. 1-1b) (**Jin, *et al.* , 2020).** His41 and Cys145 are part of domains I and II, respectively, and they are 3.6 Å apart, which is an optimum distance to initiate hydrogen bonding interactions (Fig. 1-1b).**(Kim, *et al.,2012)***

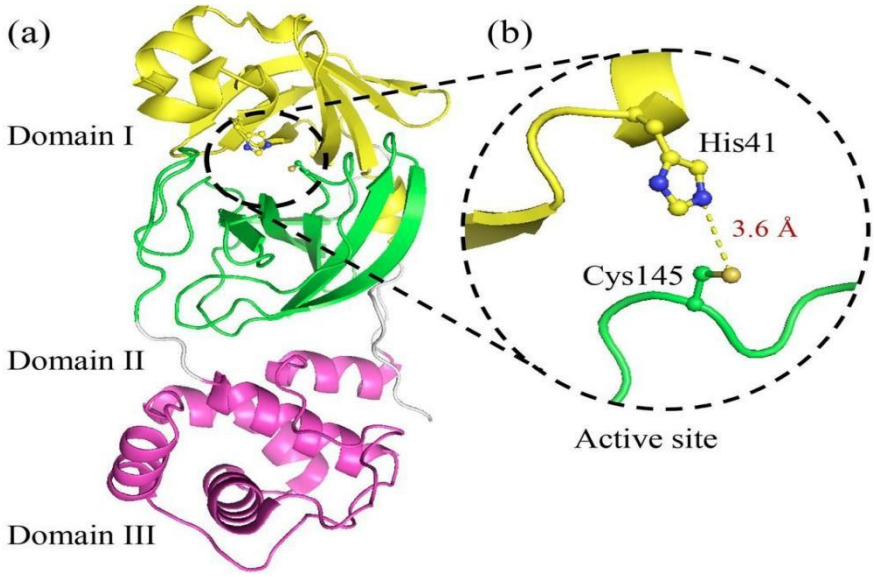

Fig. (1-2): Biochemical and biophysical characterization of the main protease, 3-chymotrypsin-like protease (3CLpro) from the novel coronavirus SARS-CoV**.(Ye . *et al.* ,2016)**

1.1.5. Life Cycle of Corona Viruses

SARS-CoV-2, like its other cousins SARS-CoV and Middle East Respiratory Syndrome (MERS)-CoV, bind to the ACE2 for entering the cells (Fig. 1). In this line, Zhou et al. performed virus infectivity studies. They used two groups of ACE2 expressing and non-expressing HeLa cells from humans, Chinese horseshoe bats, civet, pig, and mouse. As they reported, SARS-CoV-2 used all, but mouse ACE2, as an entry receptor in the ACE2-expressing cells; however, it was unable to enter into the ACE2 non-expressing cells. Interestingly, SARS-CoV-2 did not use aminopeptidase N (APN) and dipeptidyl peptidase 4 (DPPIV), the other coronavirus receptor (**Zhou ,*et al.*, 2020a)**. Although SARS-CoV-2, SARS-CoV-1, and MERS-CoV have genetic sequence homology, they have some distant sequencing. SARS-CoV-2 S-protein is suggested to have a strong binding affinity to human ACE2. SARS-CoV-2 and SARS-COV-1 share 73.5% identity in the alignment of RBD sequences of spike glycoprotein. Xu et al. assessed the binding free energy of SARS-CoV-2 S-protein in comparison with that of SARS-COV-1 S-protein. They estimated the free energy required for binding of SARS-CoV-2 S-protein to the ACE2 to be about −50.6 kcal/mol, which was significantly lower than that between SARS-CoV S-protein and ACE2 (−78.6 kcal/mol). This relatively higher affinity of SARS-CoV-2 S-protein to the ACE2 can be an ideal target for vaccine design and antiviral drug discovery (**Xu ,*et al.*, 2020b).**

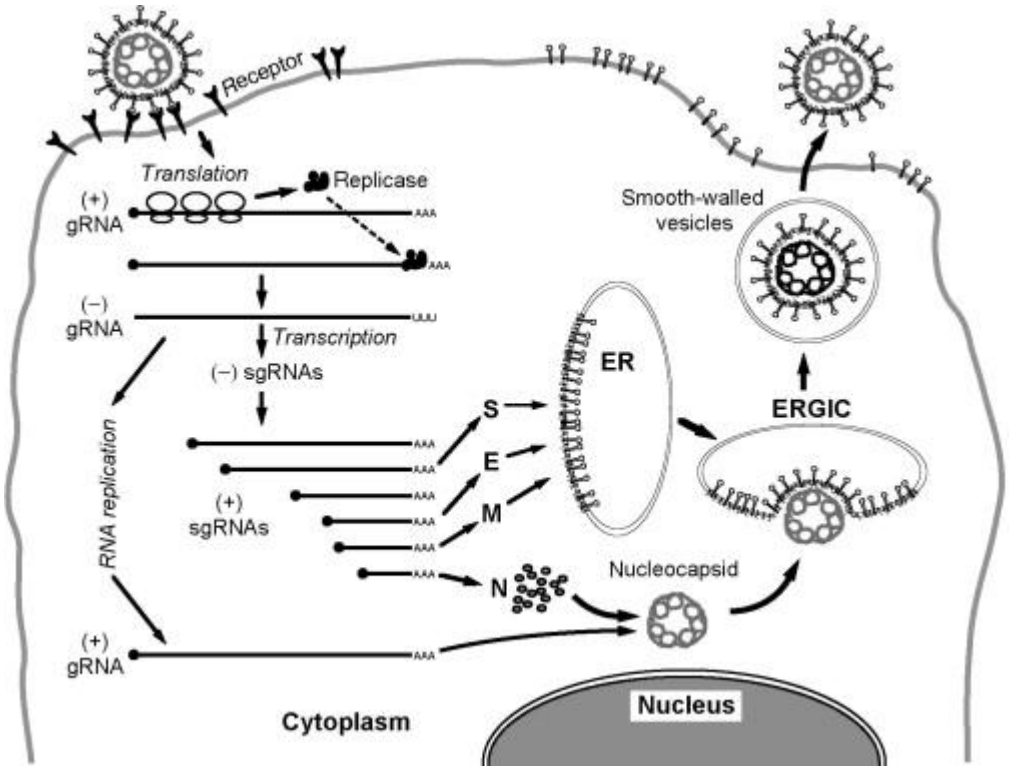

Fig.(1.3) The coronavirus life cycle (Paul S.Masters ,*et al.*,2006).

As for other coronaviruses, SARS-CoV-2 possesses a spike (S) glycoprotein, which binds to the cell membrane protein ACE2 to enter human cells. The virus-ACE2 binding results in the release of the viral genome in the host cells. The coronavirus S-protein has two functional units, S1 and S2. During infection, S-protein is a trimeric class I viral fusion protein, which is cleaved into these two subunits **(Liu ,*et al.*, 2020a**). SARS-CoV-2 binds to the host receptors by its S1 unit. S1 contains two domains: the N-terminal domain and the C-terminal RBD domain. RBD domain enables coronaviruses to directly bind to the peptidase domain (PD) of the human receptor. S2 subunit is suggested to play a role in membrane fusion **(Li, *et al.*,2012**).

Single-cell RNA sequencing (ScRNA) datasets provide evidence that the tissues of the lung, upper respiratory tract, ileum, heart, and kidney express ACE2, and this

expression might explain the role of these organs in the pathogenesis of COVID-19 (**Zou ,*et al.*, 2020**). Also, the observation of the high expression of ACE2 in the oral cavity, especially on the surface of epithelial cells of the tongue, suggests the oral cavity a favorable site of SARS-CoV-2 transmission **(Xu, *et al.*, 2020a**).

1.1.6. Diagnosis of Covid-19

Rapid and accurate detection of COVID-19 is crucial to control outbreaks in the community and in hospitals **(To, K. K. W.,*et al.* ,2020)**. Current diagnostic tests for coronavirus include reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR (rRT-PCR), and reverse transcription loop-mediated isothermal amplification(RT-LAMP**(Bhadra, *et al.* ,2015 ;Chan, *et al.* ,2015)** . RT-LAMP has similar sensitivity to rRT-PCR, is highly specific and is used to detect MERS-CoV **(Huang, *et al* .,2018 ;Lee, *et al* ., 2017)**. According to current diagnostic criteria founded by the China National Health Commission, laboratory examinations, including nasopharyngeal and oropharyngeal swab tests, have become a standard assessment for diagnosis of COVID-19 infection. To identify patients earlier, two one-step quantitative RT-PCR (qRT-PCR) assays were developed to detect two different regions (ORF1b and N) of the SARS-CoV-2 genome **(Chu,*et al.* ,2020).** Three novel RT-PCR assays targeting the RNA-dependent RNA polymerase (RdRp)/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 were developed. Among the three novel assays, the COVID-19-RdRp/Hel assay had the lowest limit of detection in vitro; highly sensitive and specific assays may help to improve the laboratory diagnosis of COVID-19 **(Chan,*et al.*,2020)**. The SARS-CoV E gene assay was more sensitive than the RdRp gene assay combined with the one-

step RT-PCR system **(Konrad, *et al.*,2020)** . The E gene PCR was sufficient to diagnose a SARS-CoV-2 infection but the RdRp protocol was recommended to confirm a positive result . The RT-PCR results usually become positive after several days (2-8 days) **(Huang, P., *et al* .,2020).**

COVID-19 infection should be diagnosed with typical chest computerized tomography (CT) characteristics despite negative RT-PCR results **(Xie,*et al* .,2020)** . Typical CT findings included bilateral pulmonary parenchymal ground-glass and consolidative pulmonary opacities, sometimes with a rounded morphology and peripheral lung distribution **(Xie,*et al* .,2020).** Notably, lung cavitation, discrete pulmonary nodules, pleural effusions, and lymphadenopathy were absent **(Chung, M.,*et al* .,2020)**. Lung abnormalities on chest CT scan were most severe approximately 10 days after the initial onset of symptoms **(Pan, *et al.*,2020)**. Chest CT scans can be used to assess the severity of COVID-19. COVID-19 also manifests with chest CT imaging abnormalities in asymptomatic patients, with rapid evolution from focal unilateral to diffuse bilateral ground-glass opacities that progressed to or co-existed with consolidations within 1-3 weeks. Combining assessment of imaging features with clinical and laboratory findings could facilitate early diagnosis of COVID-19 pneumonia **(Shi, H., *et al* .,2020).**

1.1.7. Hematological Changes in Covid-19

As already reviewed by Terpos et al. **(Terpos, *et al.*,2020)** such abnormalities have been reported by several authors and are associated, in different parts of the world, the need for ICU admission and SARS development. Still according to these authors, during the first days of the disease, when patients manifest non-specific symptoms, the leukocyte count and the absolute value of lymphocytes are normal or slightly reduced. Posteriorly, around the 7th to the 14th day of infection, the disease begins to affect organs with greater SARS-CoV-2 cell receptor expression, the angiotensin-converting enzyme 2 (ACE2) **(Zhou, P,*et al.*,2020)** . particularly a significant reduction in the lymphocytes number. This finding was more evident in those who suffered death compared to those who survived. The latter showed their nadir for lymphocytopenia around the 7th day of symptoms, with subsequent recovery **(Zhou, F.,*et al.*, 2020).**

According Terpos et al. **(Terpos, *et al.*,2020),** possible explanations for significant reduction in lymphocytes count include: (a) direct infection in these cells, causing their lysis by SARS-CoV-2, since lymphocytes have ACE2 receptors on the surface; (b) possible lymphocyte apoptosis caused by the systemic inflammatory process with consequent large cytokines production; (c) atrophy of lymphoid organs, such as the spleen, impairing lymphocyte turnover and (d) lactic acidosis inhibiting lymphocyte proliferation, which is more evident in cancer patients, a risk group for COVID-19 complications.Regarding the lymphocytes morphological aspect, as reported by Fan et al. **(Fan, *et al.*,2020).** and Chng et al. **(Chng, *et al.*,2005)**, in most patients with low lymphocyte count, these were shown to be reactive with lymphoplasmocytoid characteristics. In addition to the lymphocytes morphological abnormalities, Zini et al. **(Zini, *et al.*,2020),** after blood film microscopic observation of 40 patients with

COVID-19 on admission, before administering antiviral and anti-inflammatory treatment, observed the presence of marked morphological abnormalities of the neutrophil lineage. These changes have included presence of numerous dark and agglomerated granulations (similar to toxic granulations) and peripheral basophilic and agranular areas in the cytoplasm, and grossly grouped chromatin in the nucleus; pseudo-Pelger-Huet neutrophils segmented or not; apoptotic neutrophils; immature and dysplastic granulocytes, especially small myelocytes and metamielocytes, as well as promyelocytes. Platelet morphology also showed peculiar and frequent anomalies, consisting mainly in the presence of giant platelets, usually hyperchromatic, vacuolized, with some showing pseudopods, not only in patients with thrombocytosis but also in those with thrombocytopenia. The authors have suggested that these abnormalities may indicate a severe, transient and reversible myelopoiesis disorder, especially in accelerated and disordered granulopoiesis, in patients with severe symptomatic COVID-19. This disorder may be related to the cytokine "storm" and hyperinflammation, that appear in the progression o COVID-19 pneumonia, possibly as secondary hemophagocytic lymphohistiocytosis, leading to organ failure**(Qin, *et al.*,2020; Zini, *et al.*,2020 ; Mehta, *et al.*,2020; Siddiqi, & Mehra, 2020) .**

During the COVID-19 course, changes in hemostasis tests have also been reported, such as prolonged prothrombin and activated partial thromboplastin times and increased D-dimer levels. In cases of worsening COVID-19, D-dimer levels become rised, with formation of microthrombi in peripheral blood vessels and recurrent coagulation disorders **(Huang, C., *et al.*,2020 ;Jin, Y. H.*et al.*,2020).**

1.1.8. Testing for Coronavirus, SARS-CoV-2

For patients who meet diagnostic criteria for SARS-CoV-2 testing the CDC recommends collection of specimens from the upper respiratory tract (naso pharyngeal and or pharyngeal swab) and, if possible, the lower respiratory tract (sputum, tracheal aspirate, or bronco alveolar lavage) **(Patel and Jernigan, *et al* ., 2020)**. In each country, the tests are performed by laboratories designated by the government. Viral RNA can be detected by polymerase chain reaction (PCR, or quantitative PCR, qPCR, sometimes referred to as "real-time PCR" and RT-PCR, causing confusion with another term, "reverse transcriptase PCR") **(Drosten , *et al.*, 2002; Mackay *et al.*, 2002;Espy, *et al.*, 2006)**(Figure 1-3). In this test, the virus"s single-stranded RNA is converted to its complementary DNA by reverse transcriptase; specific regions of the DNA, marked by so-called primers, are then amplified. This is done by synthesizing new DNA strands from deoxynucleoside triphosphates using DNA polymerase. Occasional false negatives have been reported.

A primer is attached to the 3 prime end of a single strand of viral RNA. Deoxynucleoside triphosphates are added stepwise creating a DNA copy of the viral RNA. The single strand of DNA is separated and double-stranded complementaryDNA (cDNA) is prepared copies of which are synthesized using primers and DNA polymerase. Step 6 can be repeated many times, doubling the numbers of DNA molecules created each time; 30 steps **(Corman, *et al.*,2020).**

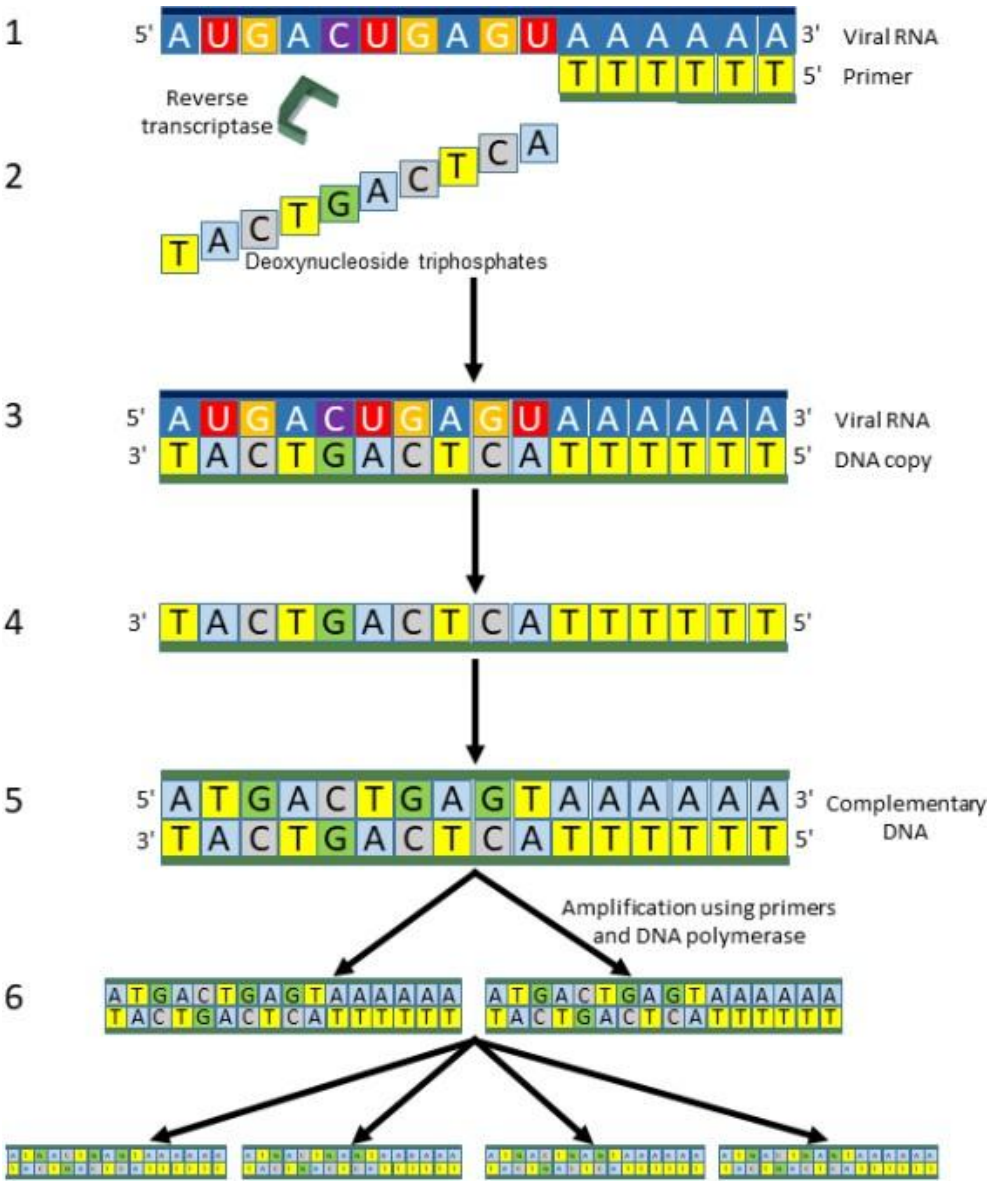

Fig. (1-4): Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (Sharifkashani S, *et al.*,2020).

1.1.9. Pathogenesis of Corona diseases

Like most other members of the corona virus family, Beta corona virus exhibit high species specificity, but subtle genetic changes can significantly alter their tissue tropism, host range, and pathogenicity. A striking example of the adaptability of these viruses is the emergence of deadly zoonotic diseases in human history caused by SARS-CoV **(Peiris, *et al.* , 2004)**, and MERS-CoV **(Zaki, *et al.*, 2012).**

In both viruses, bats served as the natural reservoir and humans were the terminal host, with the palm civet and dromedary camel the intermediary host for SARS-CoV and MERS- CoV, respectively **(Guan, *et. al.* , 2003 ; Alagaili, *et al.*,2014)**. Intermediate hosts clearly play a critical role in cross species transmission as they can facilitate increased contact between a virus and a new host and enable further adaptation necessary for an effective replication in the new host **(Brennan, *et. al. ,* 2014)**. Because of the pandemic potential of SARS-CoV-2, careful surveillance is immensely important to monitor its future host adaptation, viral evolution, infectivity, transmissibility, and pathogenicity.

The host range of a virus is governed by multiple molecular interactions, including receptor interaction. The envelope spike (S) protein receptor binding domain of SARS-CoV-2 was shown structurally similar to that of SARS-CoV, despite amino acid variation at some key residues (**Lu, R., *et. al.* ,2020)**. Further extensive structural analysis strongly suggests that SARS-CoV-2 may use host receptor angiotensin- converting enzyme 2 (ACE2) to enter the cells **(Wan, *et al.*, 2020)**,the same receptor facilitating SARS-CoV to infect the airway epithelium and alveolar type 2 (AT2) pneumocytes, pulmonary cells that synthesize pulmonary surfactant **(Li, W., *et al* .,2003)**. In general, the spike protein of corona virus is divided into the S1 and S2 domain, in which S1 is responsible for receptor binding

and S2 domain is responsible for cell membrane fusion(**Lu, R., *et. al.* , 2020)**. The S1 domain of SARS-CoV and SARS- CoV-2 share around 50 conserved amino acids, whereas most of the bat-derived viruses showed more variation **(Wan, *et. al.*, 2020)**. In addition, identification of several key residues (Gln493 and Asn501) that govern the binding of SARS-CoV-2 receptor binding domain with ACE2 further support that SARS-CoV-2 has acquired capacity for person-to-person transmission **(Wan, *et al.*, 2020)** Although, the spike protein sequence of receptor binding SARS-CoV-2 is more similar to that of SARS- CoV, at the whole genome level SARS-CoV-2 is more closely related to bat-SL- CoVZC45 and bat-SL-CoVZXC21 (**Lu, R., *et. al.* 2020)**.

However, receptor recognition is not the only determinant of species specificity. Immediately after binding to their receptive receptor, SARS-CoV-2 enters host cells where they encounter the innate immune response. In order to productively infect the new host, SARS-CoV-2 must be able to inhibit or evade host innate immune signalling. However, it is largely unknown how SARS-CoV-2 manages to evade immune response and drive pathogenesis. Given that Covid-19 and SARS have similar clinical features**(Huang, C.,*et al.*,2020)** , SARS-CoV-2 may have a similar pathogenesis mechanism as SARS-CoV. In response to SARS-CoV infections, the type I interferon (IFN) system induces the expression of IFN-stimulated genes (ISGs) to inhibit viral replication. To overcome this antiviral activity, SARS-CoV encodes at least 8 viral antagonists that modulate induction of IFN and cytokines and evade ISG effector function **(Totura, & Baric, 2012)**.

1.1.10. Complications of Covid-19

The lungs are the primary site of injury by SARS-CoV-2 infection, which causes Covid-19. The virus reaches the lungs after entry in the nose or mouth. Angiotensin -2 (ANG II)drives lung injury **(Jia, *et al.*, 2005)**.If there is a decrease in ACE2 activity (because the virus is binding to it), then ACE-2 can not break down the ANG II protein, which means there is more of it to cause inflammation and damage in the body. The virus also impacts other tissues that express ACE-2, including the heart, where damage and inflammation (myocarditis) can occur. The kidneys, liver and digestive tract can also be injured. Blood vessels may also be a site for damage **(Zhang, X., *et al.*,2020).**

Some patients with co-morbidities have increased susceptibility to morbidity and mortality from Covid-19. The pulmonary model and cardiac injury implies a role for crosstalk between ACE2-expressing cells infected by SARS viruses and other cell types, especially inflammatory cells (e.g. macrophages and neutrophils) and fibroblasts **(Flaherty, *et al.*, 2020).**Multiple clinical conditions associated with elevated vulnerability to Covid-19 such as:-

1.1.10.1. Chronic Lung Injury Disease

Lung injury (e.g. from fibrotic disease, radiation) is associated with increased local inflammatory signaling, predisposing the epithelium to Ang II-promoted injury. In these settings, epithelial cells and fibroblasts can have elevated pro-inflammatory Ang-mediated responses. The addition of SARS-CoV-2 further increases this imbalance, thereby enhancing lung injury **(Luks, Freer,*et al.*,2020).**

1.1.10.2. Cardiac Hypertrophy and Remodeling.

Ang II regulates cardiac remodeling in multiple settings, including hypertension. The elevation in Ang II signaling, derived in part from cardiac RAS, increases effects of Ang II in the heart . Patients with cardiac pathologies associated with remodeling are thus particularly susceptible to the imbalance in the RAS pathway caused by myocardial SARS-CoV-2 infection. Decreased cardiac function, especially in patients with left heart failure, may also increase the likelihood of pulmonary edema, accompanying pulmonary infection and complications.**(Forrester, *et.al.* 2018).**

1.1.10.3. Diabetes, Obesity, Metabolic Syndrome and Chronic Inflammatory Diseases

Advances in the understanding of the immune system and chronic inflammation have led to the concept of inflammageing", whereby aging is associated with the advent of chronic inflammation and the presence of inflammation associated illnesses, including Type 2 diabetes **(Ferrucci and Fabbri, 2018 ; Fulop, *et.al.* 2018)**. Chronic inflammation is also predicted to rise with obesity , a risk factor for Covid-19 morbidity. Inflammation is a key mechanism by which elevated Ang II signaling and ACE1/ACE2 imbalance causes injury. Certain patients with Type 2 diabetes and obesity also have hypertension and hypercholesterolaemia; together, these features characterize the metabolic syndrome. The metabolic syndrome is associated with chronic inflammation, which may be a causative feature of this syndrome **(Monteiro & Azevedo , 2010).** Increased RAS activity appears to be a pathogenic factor in metabolic syndrome **(Skov, *et. al.*, 2014).** Patients with the metabolic syndrome are thus pre-sensitized" to RAS-mediated effects .

1.1.11.Angiotensin Converting Enzyme Polymorphisms.

Polymorphism of angiotensin converting enzyme (ACE) leads to various mutations. ACE1 insertion/deletion polymorphisms (I/D) have been widely studied. The D-allele is associated with higher ACE1 activity. Patients with the D-allele, especially those with the D/D genotype, are at higher risk of morbidity and mortality from ARDS **(Adamzik, *et. al.*, 2007)** and certain cardiac, pulmonary and inflammatory conditions (**Gard, 2010)**. The ACE1/ACE-2 imbalance hypothesis predicts that patients with the D-allele of ACE1, in particular the D/D genotype, will have elevated severity of Covid-19, as was seen in patients with SARS-1 **(Itoyama, *et. al.* ,2004).** In addition, other genetic variants in ACE1 or ACE-2 activity or components involved in the actions of the peptides that they generate might also contribute to differences in severity of SARS-Cov-2 infections; assessment of expression of such genetic variants might aid in personalized therapeutic approaches **.** Therefore, Elderly individuals are at particular risk, since many of the co-morbidities are age-associated. Those with health conditions such as immune deficiencies, diabetes or cardiac disease will likely be at greater risk for more severe Covid-19 infections and ACE1/Ang II-mediated pathology. By contrast, children (who lack co-morbidities associated with ACE1/ACE-2 imbalance) are predicted to have less morbidity and mortality from Covid-19 **(Anand, *et al.*,2021).**

1.1.12. Covid-19 and Vitamin Supplements with Mineral

Virus infections are marked by impairment of the immune system with a subsequent insufficient micronutrient reserve, when several substances, such as vitamins (including vitamins A, B6, B12, C, D, E, and float) and other elements (i.e., zinc, iron, selenium, magnesium, and copper), are deficient **(Jovic, *et. al.* ,2020)**, the intake of different substances such as essential fatty acids, linoleic acids,

essential amino acids, and the vitamins and minerals mentioned above can improve the immune response, especially where immunity can also be conditioned by deficiencies as in the case of viral infections **(Martineau, *et. al.*, 2020)**. Adequate nutritional intake, combined with the integration of different functional foods, helps maintain optimal levels in the human body by improving various aspects of the immune system **(Adam , *et. al.* 2020)**.

Vitamin D insufficiency occurs in approximately 50% of the world's population. In a cross-sectional study it was shown that the high prevalence of vitamin D deficiency represents an important concern for public health because hypo-vitamin D is an independent risk factor for total mortality in the overall population **(Jovic, *et. al.*, 2020)**. Indeed, the adverse effects of vitamin D deficiency are widespread. In particular, playing a pivotal role in several important functions, reduced levels of vitamin D are strictly related to the development and progression of several chronic diseases such as cardiovascular disease, type 2 diabetes, cancer, and depression; moreover, its deficiency may be related to a worsening of bone health and inadequate immune function.

Finally, it is important to note that vitamin D deficiency is often linked to an increased risk of respiratory infections: The latter point could be crucial in viral infections such as Covid-19 **(d'Arqom, *et. al.*, 2020)**. For these reasons, in the scenario of a pandemic infection, although there is still no evidence in the literature that demonstrates with certainty the role of vitamin D in preventing the onset of Covid-19, the use of supplements based on vitamin D has often been discussed as it is believed to play an important role in the prevention of viral infections.

1.2.Enzyme Levels

1.2.1. Aminotransferases

Two clinical important enzyme , alanine aminotransferase (ALT) is an enzyme produced by liver cells and aspartate aminotransferase (AST). ALT is one of liver function test enzymes. It plays a crucial role in aminotransferase reaction of amino acids in the presence of pyridoxal-5‟-phosphate as coenzyme. ALT is normally found inside liver cells. However, when the liver is damaged or inflamed, ALT can be released into bloodstream. This causes serum an elevation of ALT levels **(Stavreva Veselinovska, *et al.*,2016).**

Aspartate aminotransferase (AST) is an enzyme that‟s present in various tissues of your body. An enzyme is a protein that helps trigger chemical reactions that your body needs to function. AST is found in the highest concentrations in your liver, muscles, heart, kidney, brain and red blood cells. A small amount of AST is typically in your bloodstream. Higher-than-normal amounts of this enzyme in your blood may be a sign of a health problem. Abnormal levels can be associated with liver injury. AST levels increase when there‟s damage to the tissues and cells where the enzyme is found. The normal range for AST is higher from birth to age 3 compared to the normal ranges for older children and adults. The AST test measures the amount of AST in your blood that has been released from injured tissue **(Brancaccio, *et al.*,2010).**

1.2.2. Angiotensin-Converting Enzyme

Angiotensin-converting enzyme (EC 3.4.15.1), or ACE, is a central component of the renin–angiotensin system (RAS), which controls blood pressure by regulating the volume of fluids in the body. It converts the hormone angiotensin I to the active vasoconstrictor angiotensin II. Therefore, ACE indirectly increases blood pressure by causing blood vessels to constrict **(Hikmet*,et al.*,2020).**

1.2.3. Lactate Dehydrogenase

Lactate dehydrogenase (LDH), also known as lactic acid dehydrogenase, is found in blood or sometimes in other body fluids. It is found in almost all the body's tissues, including those in the blood, heart, kidneys, brain, and lungs. When these tissues are damaged, they release LDH into the bloodstream or other body fluids. If serum LDH activity level or in body fluid levels is high, it may mean certain tissues in your body have been damaged by disease or injury **(Odinga,*et al.*2020).**

1.2.4. Ferritin

Ferritin is a protein that stores the iron inside the cells that need on requirement to make healthy red blood cells. Red blood cells carry oxygen from your lungs to the rest of your body. Iron is also important for healthy muscles, bone marrow, and organ function. Too little or too much iron in the system can cause serious health problems if not treated **(Vargas-Vargas & Cortés-Rojo , 2020).**

1.2.5. C-Reactive Protein

C-reactive protein (CRP) is a protein produced by the liver. It's sent into bloodstream in response to inflammation. Inflammation in the body is the way of protecting the tissues against injury or infection. It can cause pain, redness, and swelling in the injured or affected area. Some autoimmune disorders and chronic

diseases can also cause inflammation **(Polidoro, *et al.*,2020).**

1.3..Molecular Studies

Despite early predictions and rapid progress in research, the introduction of personal genomics into clinical practice has been slow. Several factors contribute to this translational gap between knowledge and clinical application. The evidence available to support genetic test use is often limited, and implementation of new testing programs can be challenging. In addition, the heterogeneity of genomic risk information points to the need for strategies to select and deliver the information.

most appropriate for particular clinical needs. Accomplishing these tasks also requires recognition that some expectation for personal genomics are unrealistic , notably expectation concerning the clinical utility of genomic risk assessment for common complex diseases. Efforts are needed to improve the body of evidence addressing clinical outcome for genomics, apply implementation science to personal genomics ,and develop realistic goal for genomic risk assessment. In addition, translational research should emphasize the broader benefits of genomic knowledge, including applications of genomic research that provide clinical benefit outside the context of personal genomic risk **(Burke & Korngiebel , *et al* , 2015).**

1.3.1. Genetic Polymorphism in Covid-19

Many risk factors have been described for this coronavirus such as elderly age, male gender, race, obesity, hypertension, diabetes and geographic region **(Kim, *et. al. ,*2019).** In addition to these elements, genetic factors also play a major role in Covid19 infection. Inter individual inherited differences in susceptibility to SARS-CoV-2 infection is linked to the presence of genetic polymorphisms (variants) in many genes especially in those that code for the host receptors involved in viral entry process. These DNA changes are transmissible from one generation to another,

detectable in at least 1% of individuals in a population and could explain the differences between individuals in the susceptibility to some multi genic, complex diseases like Covid19 **(Kaltoum, A. B. O. , 2021).** Two main approaches can be used in genetic epidemiology to establish a link between genetic variations and the risk of developing a disease: Genetic linkage analysis and association studies (candidate gene and genome-wide association studies). (Various results showed that many different genes are associated with a higher risk for Covid-19, notably those coding for the receptors ACE-2 (angiotensin I converting enzyme -2), TMPRSS2 (trans- membrane protease, serine 2) and CD26 **(Suh, *et al.* ,2021).**

1.3.2. Angiotensin Converting Enzyme-2 (ACE-2)

Angiotensin-converting enzyme was identified in 1956 (EC 3.4.15.1), or ACE, is a central component of the renin–angiotensin system (RAS), which controls blood pressure by regulating the volume of fluids in the body. It converts the hormone angiotensin I to the active vasoconstrictor angiotensin II. Therefore, ACE indirectly increases blood pressure by causing blood vessels to constrict. ACE inhibitors are widely used as pharmaceutical drugs for treatment of cardiovascular diseases **(Hikmet, *et .al.*, 2020)**. It is located mainly in the capillaries of the lungs but can also be found in endothelial and kidney epithelial cells **(Chamsi- Pasha, *et. al.* ,2014).** Other less known functions of ACE are degradation of bradykinin, substance P and amyloid beta-protein. ACE hydrolyzes peptides by the removal of a dipeptide from the C-terminus. Likewise it converts the inactive decapeptide angiotensin I to the octapeptide angiotensin II by removing the dipeptide His-Leu **(Guang, et al.,2012).**

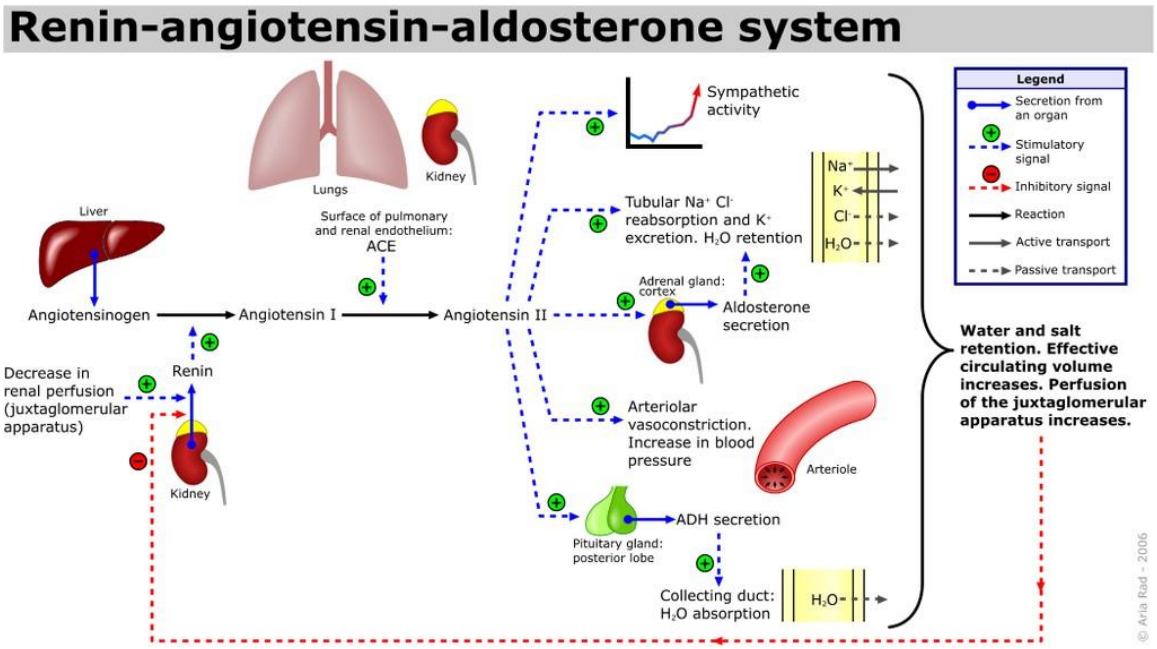

Fig. (1-5): Schematic diagram of the renin–angiotensin–aldosterone system.(Warner, F. J.,*et al.*,2004).

Angiotensin II is a potent vasoconstrictor in a substrate concentration-dependent manner. Angiotensin II binds to the type 1 angiotensin II receptor (AT1), which sets off a number of actions that result in vasoconstriction and therefore increased blood pressure. ACE is also part of the kinin-kallikrein system where it degrades bradykinin, a potent vasodilator, and other vasoactive peptides.**(Qiu , *et. al.* 2013).**

1.3.3. Biochemical Characteristics

Angiotensin-converting enzyme-2 is a zinc-containing metallo enzyme located on the surface of intestinal enterocytes, renal tubular cells and other cells **(Hikmet, *et. al.* ,2020)**. ACE-2 protein contains an N-terminal peptidase M2 domain and a C-terminal collectrin renal amino acid transporter domain . ACE2 is a single-pass type I membrane protein, with its enzymatically active domain exposed on the surface of cells in the intestines and other tissues **(Hikmet, *et. al.* ,2020)**.The extracellular domain of ACE2 is cleaved from the transmembrane domain by another enzyme known as sheddase, and the resulting soluble protein is released into the bloodstream and ultimately excreted in the urine.

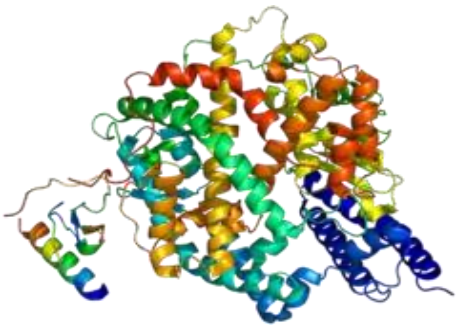

Fig. (1-6): Available structures for ACE-2
(Ahmad, *et al.*,2021)

1.3.3.1. Angiotensin Converting Enzyme-2 Receptor

Angiotensin converting enzyme 2 receptor (ACE 2) is a protein on the surface of many cell types. It is an enzyme that generates small proteins – by cutting up the larger protein angiotensinogen – that then go on to regulate functions in the cell. Using the spike-like protein on its surface, the SARS-CoV-2 virus binds to ACE-2 like a key being inserted into a lock – prior to entry and infection of cells. Hence, ACE-2 acts as a cellular doorway – a receptor – for the virus that causes Covid-19. ACE-2 acts as the receptor for the SARS-CoV-2 virus and allows it to infect the cell. ACE-2 is present in many cell types and tissues including the lungs, heart, blood vessels, kidneys, liver and gastrointestinal tract. It is present in epithelial cells, which line certain tissues and create protective barriers **(Sharma, *et al.*,2020).**

Efforts are needed to improve the body of evidence addressing clinical outcome for genomics ,apply implementation science to personal genomics ,and develop realistic goal for genomic risk assessment. In addition, translational research should emphasize the broader benefits of genomic Knowledge, including applications of genomic research that provide clinical benefit outside the context of personal genomic risk **(Burke and Korngiebel, 2015)**.

1.3.3.2. Angiotensin Converting Enzyme-2 Receptor Gene Polymorphism

The ACE gene, ACE, encodes two isozymes. The somatic isozyme is expressed in many tissues, mainly in the lung, including vascular endothelial cells, epithelial kidney cells, and testicular Leydig cells, whereas the germinal is expressed only in sperm. Brain tissue has ACE enzyme, which takes part in local RAS and converts Aβ42 (which aggregates into plaques) to Aβ40 (which is thought to be less toxic) forms of beta amyloid. The latter is predominantly a function of N domain portion on the ACE enzyme. ACE inhibitors that cross the blood–brain barrier and have preferentially selected N-terminal activity may therefore cause accumulation of Aβ42 and progression of dementia.

The angiotensin converting enzyme gene has more than 160 polymorphisms described as of 1990 **(Rigat ,*et. al.* 1990)**. Studies have shown that different genotypes of angiotensin converting enzyme can lead to varying influence on athletic performance **(Shenoy, *et. al.* 2010).**

The rs1799752 I/D polymorphism consists of either an insertion (I) or absence (D) of a 287 base pair alanine sequence in intron 16 of the gene. The DD genotype is associated with higher plasma levels of the ACE protein, the ID genotype with intermediate levels, and II with lower levels. During physical exercise, due to higher levels of the ACE for D-allele carriers, hence higher capacity to produce angiotensin II, the blood pressure will increase sooner than for I-allele carriers. This results in a lower maximal heart rate and lower maximum oxygen uptake (VO2max). Therefore, D-allele carriers have a 10% increased risk of cardiovascular diseases. Furthermore, the D-allele is associated with a greater increase in left ventricular growth in response to training compared to the I-allele **(Buford, *et.al.* 2014 ;sharkawy, *et al.*,2014).**

On the other hand, I-allele carriers usually show an increased maximal heart rate due to lower ACE levels, higher maximum oxygen uptake and therefore show an enhanced endurance performance . The I allele is found with increased frequency in elite distance runners, rowers and cyclists. Short distance swimmers show an increased frequency of the D-allele, since the discipline relies more on strength than endurance **(Yang, *et. al.* 2015).**

Aim of the Study

1. To study the effect of Covid-19 on the levels of some biomarker such as liver enzyme, ferritin, LDH, CRP and ACE-2 levels.
2. Study the gene polymorphism of ACE-2 receptor in covid-19 patients.
3. To study the association between various biomarkers including angiotensin-converting enzyme and its receptor gene polymorphism with severity of Covid- 19 infection in Iraqi pandemic of Kerbala Province.

Chapter Two

Subjects , Materials and Methods

2. Subjects, Materials and Methods

2.1. Subjects

2.1.1. Patients

This study was conducted on 176 subjects who diagnosed by physician and were divided into three groups: 59 of them were infected with severe covid-19, 54 of them were infected with moderate covid-19 and 63 of them were checked and obtained as apparently healthy control. Severe and moderate patients were collected from Al-Hayat tertiary center at Al-Hussein Medical City, Kerbala Health Directorates, Kerbala – Iraq during April, 2020- June, 2021 with matched age ranged between (23-88) years and they are classified as shown in Figure (2-1).

They are diagnosed by quantitative by RT-PCR and chest X-ray or CT scan at the 7-12 day from symptoms on set, with age ranged between (23-88) years and it consisted of three categories Covid-19 patients were collected at admission and the disease severity was assessed using Murray scores **(Murray, Matthay, *et al.* 1988)**.The patients were considered to have severe / moderate Covid-19 depending upon fever, respiratory manifestations and radiological indicative of pneumonia. Patients were considered to have severe Covid-19 if any of the following changes was present:

(i) Respiratory distraction (≥ 30 / min)

(ii) Resting oxygen saturated $\leq 90\%$ or

(iii) Arterial oxygen (PaO_2) / fraction of inspired oxygen ≤ 300 mmHg .or

(iv) Respiratory failure requiring mechanical ventilation and require intensive care unit. Moreover, patient dead considered as Non-survived.

The current investigation was approved by local medical ethics and all participants, information consent before the onset of study. The patients were

registered and handed over a file for recording their data such as name, age, gender, weight, height.

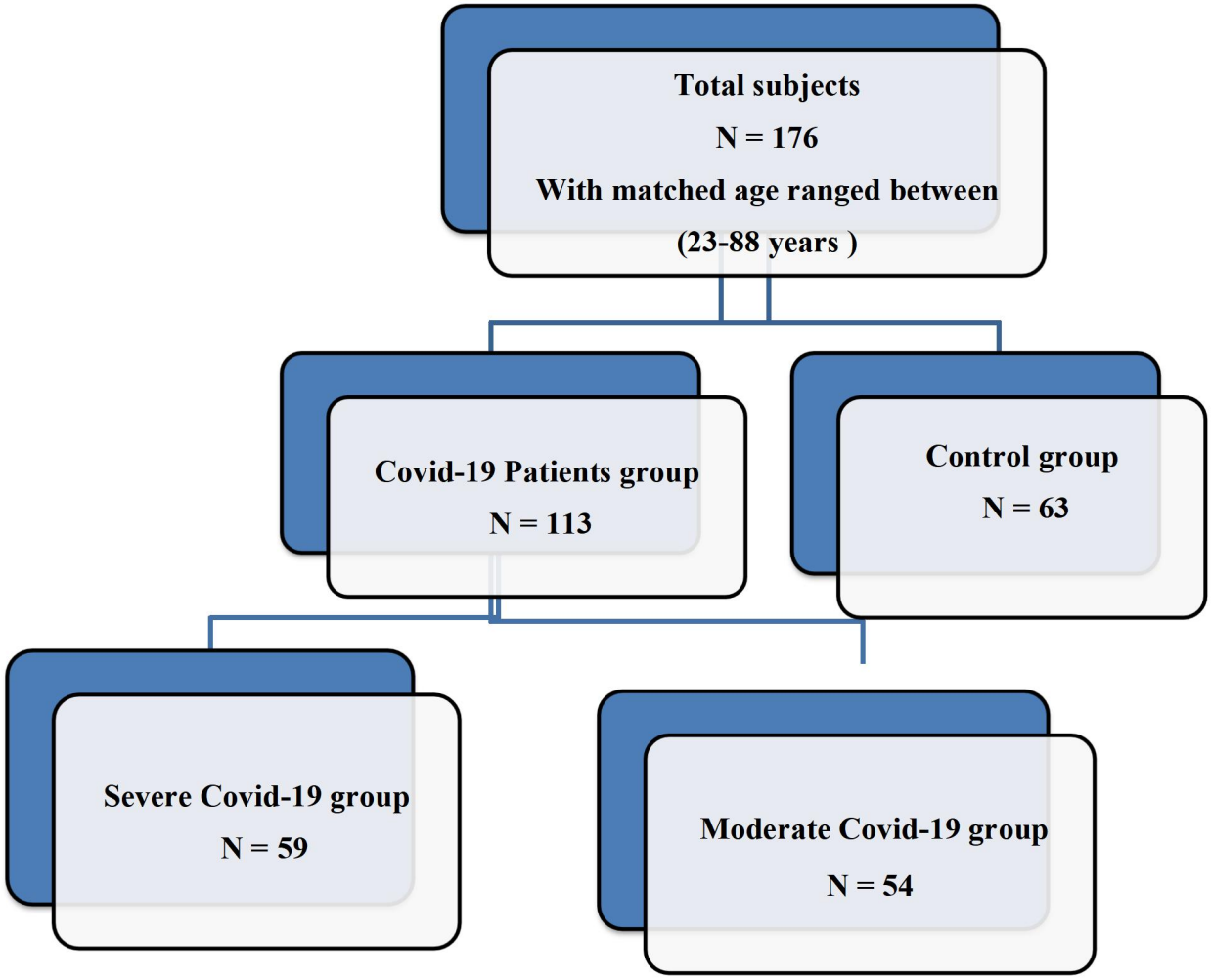

Fig. (2-1): Scheme of subjects groups

Exclusion criteria:

The study excluded female and children infected with Covid-19, patients with any chronic or immune diseases like type 1 diabetes mellitus,gestational diabetes mellitus infection and inflammation, and receiving long term oral corticosteroid, anti-IL-6 or anti-TNF therapy and patients had history of vasculitis connective tissue disease. Patients suffering from cancer and kidney diseases of, smoker, systematic immune disease, and also thyroid gland diseases were also excluded.

2.1.2. Control

Sixty three apparently healthy subjects were selected as control group. Their age was matched to that of patients. None of them was anemic or has an obvious systemic disease or any chronic diseases.

2.1.3. Blood Specimens Collection

Five milliliters of venous blood were drawn from each the patients and control group by medical syringes. The first part (3 ml) was put into two gel tubes and left at room temperature for nearly thirty minutes for clotting, then centrifuged at 3000 rpm for 10 minutes to separate serum which was divided into two tubes, the first one was used to determine ferritin, C-reactive protein concentration (CRP) and activity levels of each of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) , and the second tube was stored at -20 ^{o}C until using for determination of Angiotensin - Converting Enzyme-2 (ACE-2) levels by ELISA Technique. The second part (2 ml) of blood was collected in EDTA tube was stored by freezing at -20 ^{o}C until use for DNA extraction and then performing various molecular analyses concerning ACE-2 receptor gene polymorphism by polymerase chain reaction and related techniques.

2.2. Materials

2.2.1. Instruments and Tools

Various instruments and laboratory tool are used to perform this study as shown in table (2-1).

No.	Instruments	Company	Country
1	Different glassware	Different sources	China
2	ELISA micro plate reader	Biotek	USA
3	Refrigerator	Nikai	Japan
4	Incubator	Kubota	Kubota
5	Deep freeze	Nikai	Japan
6	Roche Cobas 6000 system	Roche	German
7	Centrifuge	Fisher Scientific,	USA
8	Gel Imaging System	Major Science,	Taiwan
9	Micro spin Centrifuge	My Fugene,	China
10	Microwave Oven	GOSONIC	Chaina
11	OWL Electrophoresis System	Thermo,	USA
12	Quantus Fluorometer	Promega,	USA
13	Thermal Cycler (Veriti)	Thermo Fisher Scientific	USA
14	Vortex mixer	Quality Lab System,	England

Table (2-1): Instruments and tools used in this study

2.2.2. Chemicals and Kits

Various biochemical and kits were used in this study. Table (2-2) shown the kits used with their sources.

Table (2-2): Chemicals and kits used in this study with their suppliers

No.	Chemicals and kits	Company	Country
1	ACE-2 ELISA Kit	Elabscience	USA
2	C- reactive protein Kit	Melsin	china
3	PrestoTM Mini genomic DNA kit	Geneaid	Taiwan
4	Agarose, Ethidium Bromide Solution (10 mg/ml), Tag Green Master Mix, Nuclease Free Water, TAE 40X, Quantifluor dsDNA System	Promega,	USA
5	Absolute Ethanol	ROMIL pure chemistry,	UK

2.2.3. Primers

Table (2-3) indicates the sequence of primers used in molecular analysis of this study.

Table (2-3) Primers used in molecular study

Primer Name	Sequence	Annealing Temp. (°C)	Product Size (bp)
ACE-rs4646994- F	5`-CTGGAGACCACTCCCATCCTTTCT-3`	58	190
ACE-rs4646994-R	5`-GATGTGGCCATCACATTCGTCAGAT-3`	58	490

2.3. Methods

2.3.1. Measurement of Body Mass Index

Body mass index (BMI) was used to define obesity. The range of BMI (18.5-24.99 kg/m^2) that set it WHO but it does not accurately indicate the degree of fatness. In the present study, WHO classification will be used for adults as shown in Table (2-4) **(Batsis , *et al.* ,2016 ; McDougall and Mace, 2018):**

The body mass index was measure by dividing weight in kilograms by length of individual in square meter: **BMI = (weight in kg) / (height in meters^2).**

Table (2-4): Estimation of body mass index

Weight status	BMI (kg/m²)
Under weight	< 18.5 kg/m^2
Normal weight	18.5 to 24.9 kg/m^2
Over weight	25.0 to 29.9 kg/m^2
Obese	≥ 30.0 kg/m^2

2.3.2. Determination of Serum Angiotensin-Converting Enzyme-2 Level:

Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human ACE. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human ACE and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain

Human ACE, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ±2 nm. The OD value is proportional to the concentration of Human ACE. I was calculate the concentration of Human ACE in the samples by comparing the OD of the samples to the standard curve.

Other supplies required

1. Micro-plate reader with 450nm wavelength filter High-precision transfer pipette, EP tubes and disposable pipette tips Incubator capable of maintaining 37°C.
2. De ionized or distilled water
3. Absorbent paper loading slot

The method of work

1. The samples used was serum.
2. Reagent preparation
3. All reagents were kept at room temperature (18-25 °C) before use, take out the necessary strips and reagents for present experiment.
4. 30 ml of concentrated wash buffer was diluted with 720 ml of de-ionized or distilled water to prepared 750 ml of wash buffer, if crystals have formed in the concentrate, it was warmed in a 40 °C water bath and then it was mixed gently until the crystals have completely dissolved.
5. Standard working solution: To centrifuged the standard at 10,000× g for 1 min. 1.0 ml of reference standard and sample diluent were added , then it stand for 10 min and invert it gently several times. After it dissolves fully, it thoroughly was mixed with a pipette. This reconstitution produces a working solution of 5000 pg/ml (or adds 1.0 ml of reference standard and sample

diluent, it stand for 1-2 min and then mixed it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then I was made serial dilutions as needed. The recommended dilution gradient is as follows: 5000, 2500, 1250, 625, 312.5, 156.25 and 78.13.0 pg/ml.

Dilution method: 7 EP tubes was taken ; 500 µl of Reference Standard and Sample Diluent were added to each tube. Pipette 500 µl of the 5000 pg/ml working solution to the first tube and mix up to produce a 2500 pg/ml working solution. Pipette 500 µl of the solution from the former tube into the latter one according to this step. The illustration below is for reference, the last tube is regarded as a blank.

6. Biotinylated detection Ab working solution: the required slightly amount before the experiment (100 µl / well)was calculated. In preparation, more than calculated should be prepared. the Concentrated Biotinylated Detection Ab was centerifuged at 800 × g for 1 min, then the 100 × Concentrated Biotinylated Detection Ab to 1× working solution was diluted with Biotinylated Detection Ab Diluent(Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).
7. 100 × concentrated HRP Conjugate to 1 × working solution was diluted with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99). 5000, 2500, 1250, 625, 312.5, 156.25, 78.130.

Procedure

1. Wells for diluted standard, blank and sample were determined . 100µl each dilution of standard, blank and sample were added into the appropriate wells. the plate was covered with the sealer provided in the kit.Then incubated for 90 min at 37 °C solutions should be added to the bottom of the micro ELISA

plate well, avoided touching the inside wall and causing foaming as much as possible.

2. The liquid was decanted from each well, and not wash. Immediately 100 µl of Biotinylated Detection Ab working solution was added to each well. the plate was covered with a new sealer.then incubated for 1 hour at 37°C.
3. The solution from each well was decanted 350µl of wash buffer was added to each well. Soak for 1-2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. this wash step was repeated 3 times.
4. 100 µl of HRP Conjugate working solution was added to each well. the plate was cover with a new sealer. Then incubate for 30 min at 37 °C.
5. The solution was decanted from each well, the wash process was repeated for 5 times as conducted in step 3
6. A 90 µl of Substrate Reagent was added to each well. the plate was covered with a new sealer. Then incubated for about 15 min at 37 °C. t the plate was protected from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min. the Micro plate Reader was preheated for about 15 min before OD measurement.
7. 50 µl of Stop Solution was added to each well. Note: adding the stop solution should be done in the same order as the substrate solution.

Interpretation of test result

Determine the optical density of each well at once with a micro-plate reader set to 450 nm.

Reference range

Men: 1.3-3.0 ng/ml ; Women: 1.3-3.0 ng/ml ; Working range :0.78-50 ng/ ml

2.3.3. Determination of Serum Ferritin Level

Principle

The method for measurement of Ferritin on the cobas e601 is a sandwich principle with a total duration time of 18 minutes. The 1st incubation uses 10 µL of sample, a ferritin-specific antibody and a labeled ferritin-specific antibody to form a sandwich complex. The 2nd incubation occurs after the addition of microparticles that cause the complex to bind to the solid phase. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed. Application of a voltage to the electrode then induces chemiluminescence emission which is measured by a photomultiplier, then the results are determined via a calibration curve.

Procedure

1. Allow Calibrators, QC and patient samples to reach ambient temperature.
2. Ensure that the amount of reagents, diluent, and wash solutions are adequate for the amount of samples to be run. You may place more than one bottle of reagent at a time on the analyzer; however, avoid using more than one lot number of reagent for a single run.
3. Make sure the analyzer and/or tests required are not masked.
4. Check to see if calibration is required for the tests that will be run.
5. Surelly all previously programmed samples were cleared from the Data Review screen after backing up the data.
6. The required maintenancewas performed on the e601 system.
7. The test result was read on the display of the Cobas test instrument.

Interpretation of test result

An instrument for Cobas tests calculates the test result automatically and displays the ferritin concentration of the test sample in terms of ng/ml. The cut-off

Reference range

Men: 30 - 400 ng/ml ; Women: 13 - 150 ng/ml ; Working range: 5 - 2000 ng/ml

2.3.4. Determination of C-Reactive Protein Level

Principle

CRP testing on the COBAS C311 is an immune-turbid metric assay for the in vitro quantitative determination of CRP in human serum and plasma on Roche/Hitachi Cobas c systems. Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The aggregates are determined turbid metrically.

Reagent and material

COBAS C311

R1 TRIS a) buffer with bovine serum albumin; preservatives

R2 Latex particles coated with anti-CRP (mouse) in glycine buffer; immune globulins (mouse); preservative

a)TRIS = Tris(hydroxymethyl)-aminomethane

R1 is in position B and R2 is in position C.

Procedure

The gel tube containing serum was entered to the equipment and analyzed automatically.

Calculation

The concentration of the serum CRP is measured automatically by using Cobas integra 400 plus analyzer .

Normal Range : Less than 10 mg/l ; **High:** greater than 10 mg/l.

2.3.5. Determination of Serum Lactate Dehydrogenase Level

Principle

Lactate dehydrogenase (LDH) catalyzes the conversion of L-lactate to pyruvate; NAD is reduced to NADH in the process. The initial rate of NADH formation is directly proportional to the catalytic LDH activity and is determined by measuring the increase in absorbance at 340 nm.

$$\text{L- lactate} + NAD^{+} \xleftarrow{\text{LDH}} \text{Pyruvate} + NADH + H^{+}$$

Reagents- working solutions

R1:- N- methyl glucamine: 400 mmol/l, pH 9.4 (37 °C); lithium lactate: 62 mmol/l; stabilizers and preservatives

R2 :- NAD: 62 mmol/l ; stabilizers and preservatives precautions .

Procedure:

The gel tube containing serum was entered to the equipment and analyzed automatically.

Calculations

The concentration of the serum LDH is measured automatically by using Cobas integra 400 plus analyzer.

Reference Range

Serum men: 0-300 IU/l ; women : 0-275 I U/l ; working range : 10-1000 U/l

2.3.6. Determination of Serum Alanine Aminotransferase Level

Principle :

Alanine aminotransferase (ALT) catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and NAD^+. The rate of the NADH oxidation is directly proportional to the catalytic ALT activity. It is determined by measuring the change in absorbance per unit time (ΔA/min).

Reagents- working solutions

Alanine Aminotransferase acc. to IFCC without pyridoxal phosphate activation, 500 tests – the reagent cassette is labeled as ALTL.

R1 is in position B and R2 is in position C.

R1 - TRIS buffer: 224 mmol/l, pH 7.3 (37o C); L-alanine: 1120 mmol/l; albumin (bovine): 0.25%; LDH (microorganisms): ≥45 μkat/l; stabilizers; preservative

R2 - 2-Oxoglutarate: 94 mmol/l; NADH: ≥1.7 mmol/l; additives; preservative Diluent NaCl 9%, 50 ml – the diluent cassette is labeled as NaCl.

Procedure:

The gel tube containing serum was entered to the equipment and analyzed automatically for ALT activity determination.

Calculations

The concentration of the serum ALT is measured automatically by the analyzer.

Reference Range:

Serum men: 5-45 U/l ; women : 5-40 U/l ; working rang : 5-7000 U/l

2.3.7. Determination of Serum Aspartate Aminotransferase Level

Principle :

Aspartate aminotransferase (AST) in the sample catalyzes the transfer of an amino group between L-aspartate and 2-oxoglutarate to form oxaloacetate and L-glutamate. The oxaloacetate then reacts with NADH, in the presence of malate dehydrogenase (MDH), to form NAD^+. The rate of the NADH oxidation is directly proportional to the catalytic AST activity. It is determined by measuring the change in absorbance per unit time (ΔA/min).

Reagents- working solutions :

Aspartate Aminotransferase acc. to IFCC without pyridoxal phosphate activation, 500 tests – the reagent cassette is labeled as ASTL. R1 is in position A; R2 is in positions B and C. R1 - TRIS buffer: 264 mmol/l, pH 7.8 (37 °C); L-aspartate: 792 mmol/L; MDH (microorganism): ≥24 µkat/l; LDH (microorganisms): ≥48 µkat/l; albumin (bovine): 0.25%; preservative R2 - NADH: ≥1.7 mmol/l; 2-oxoglutarate: 94 mmol/l; preservative Diluent NaCl 9%, 50 ml – the diluent cassette is labeled as NACL.

Procedure:

The gel tube containing serum was entered in machine for analyzed automatically.

Calculations

The concentration of the serum AST is measured automatically by using Cobas integra 400 plus analyzer.

Reference Range:

Serum men : 0-37 U/l ; women : 0-31 U/l ; working range 3-700 U/l

2.3.8. Determination of Serum Alkaline Phosphatase Level

Principle:

The Cobas C311 provides a colorimetric assay in accordance with a standardized method. In the presence of magnesium and zinc ions, p-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol.

p-Nitrophenyl phosphate + H_2O ----------► Phosphate + p-Nitrophenol

The p-nitrophenol released is directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance.

Reagents- working solutions :

R1- 2-amino-2-methyl-1-propanol: 1.724 mol/l, pH 10.44 (30 °C); magnesium acetate: 3.83 mmol/l; zinc sulfate: 0.766 mmol/l; N-(2-hydroxyethyl)-ethylenediamine triacetic acid: 3.83 mmol/l.

R2- p-nitrophenyl phosphate: 132.8 mmol/l, pH 8.50 (25 °C); preservatives R1 is in position B and R2 is in position C.

Procedure:

The gel tube containing serum was entered to the equipment and analyzed automatically.

Calculations

The concentration of the serum ALP is measured by using Cobas integra 400 plus analyzer .

Reference Range

Adults men : 40-129 U/l ; women :35-104 U/l ; orking range : 5-1200 U/l

2.4. Molecular Analysis of Angiotensin-Converting Enzyme-2

2.4.1. DNA Extraction

DNA was extracted from whole blood that collected from patients and control groups by using the Presto TM Mini gDNA Kit (Geneaid).

Principle

The DNA extraction kit uses proteinase K and chemotropic salt to lyse cells and degrade protein ,allowing DNA to bind to the glass fiber matrix of the spin column .contaminants are removed using awash buffer and purified genomic DNA is eluted by a low salt elution buffer. The purified DNA (approximately 20-30 kb) is suitable for use in polymerase chain reaction (PCR) or other enzymatic reactions.

Genomic DNA Extraction Protocol

1. Absolute ethanol was added to wash Buffer than mix by shaking for a few seconds. The bottle was closed tightly after each use to avoid ethanol evaporation. Then add deionized water pH (7.0 – 7.8) to proteinase K then vortex to ensure it is completely dissolved.
2. Check the box on the bottle. Once it is dissolved completely, centrifuge for a few seconds to spin down the mixture. For extended periods, the deionized water and proteinase K mixture should be stored at 4 °C. Use only fresh deionized water and proteinase K quickly cause acidification.
3. Blood sample was thoroughly mixed for 15 minutes in roll mixer at room temperature.
4. Two hundred µl of whole blood was transferred to a 1.5 ml microcentrifuge tube. Twenty µl of proteinase K was added then mixed by pipetting and incubated at 60 ° C for 5 minutes.
5. Cell lysis and protein digestion. Two hundred µl of GSB buffer was added then mixed by shaking vigorously and incubated at 60 ° C for 5 minutes , the tube was inverted every 2 minutes.

NOTE: It is essential that the sample and GSB buffer are mixed thoroughly to yield homogenous solution. Inverting the sample occasionally during incubation will facilitate proteinase K digestion and cell lysis. Using an auto shaking system is more convenient when incubation samples. During incubation, the required volume of elution buffer (200 µl /sample) was transferred to 1.5 ml micro centrifuge tube and heated to 60 ° C (for step 5 DNA elution).

DNA Binding

1. For DNA binding, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. If precipitate appears, break it up as much as possible with a pipette.
2. A GS column was placed in a 2 ml collection tube. And transferred all of the mixture (including any insoluble precipitate) to the GS column. Then centrifuged at 14000-16,000 x g for 1 minute. Following centrifugation, if the mixture did not flow through the GS column membrane , the centrifuge time would increase until it passes completely. The 2 ml collection tube containing the flow- through was discarded then transferred the GS column to anew 2 ml collection tube. NOTE: It is important that the lysate and ethanol are mixed thoroughly to yield homogeneous solution.
3. **Washing**
   - **a.** For washing, 400 µl of W1 buffer was added to the GD column and centrifuged at 14-16,000 x g for 30 seconds then the flow-through was discarded.
   - **b.** The GD column placed back in the 2 ml collection tube, and 600 µl of Wash buffer (make sure ethanol was added) was added to the GD column
   - **c.** Centrifugation at 14000-16,000 x g for 30 seconds was performed, then the flow- through was discarded, and the GD column was placed back in the 2ml collection tube. And centrifuged for 3 minutes at 14000-16000 x g to dry the column matrix.

     NOTE: Additional centrifugation at 14000-16,000 x g for 5 minutes incubation at 60 °C for 5 minutes will completely dry the GS column to avoid any residual ethanol carryover and ensure the most effective downstream applications.

4. Elution

a. The dried GD column was transferred into a clean 1.5ml micro centrifuge tube.

b. Aliquot of 100 µl pre-heated elution buffer 1 was added into the center of the column matrix. After waiting at least 3minutes (to allow Elution Buffer to be completely absorbed). Centrifugation at (14000-16,000 x g) for 30 seconds was performed to elute the purified DNA.

2.4.2. Polymerase Chain Reaction

Polymerase chain reaction (PCR) can amplify a small amount of template DNA or RNA into large quantities in a few hours. This is performed by mixing the DNA with primers on side of the DNA (forward and reverse), free nucleotides (dNTPs for DNA, NTPs for RNA), *Taq* polymerase (of the species Thermus aquaticus, a thermophile whose polymerase is able to withstand extremely high temperatures) and buffer. The temperature is then alternated between hot and cold to denature and re-anneal the DNA, with the polymerase adding new complementary strands each time **(Sambrook and Russell, 2001 ; Carr and Moore, 2012;Butt, J., *et al.*,2018).** There are three steps to any PCR that are cycled about 25-35 times as shown in Figure 2-2a and Figure (2-2b), which include:

1. **Denaturation**: It is the uncoiling of double stranded DNA into two single strands.
2. **Annealing**: This step occurs at 55-65°C. A pair of short (17-26) oligonucleotide sequences (primers) anneal to the ends of the template strands of the DNA and begin the reaction.
3. **Extension**: This step occurs at 72-74°C and entails the extension of the primers to form a new strand that is complementary to the template strand.

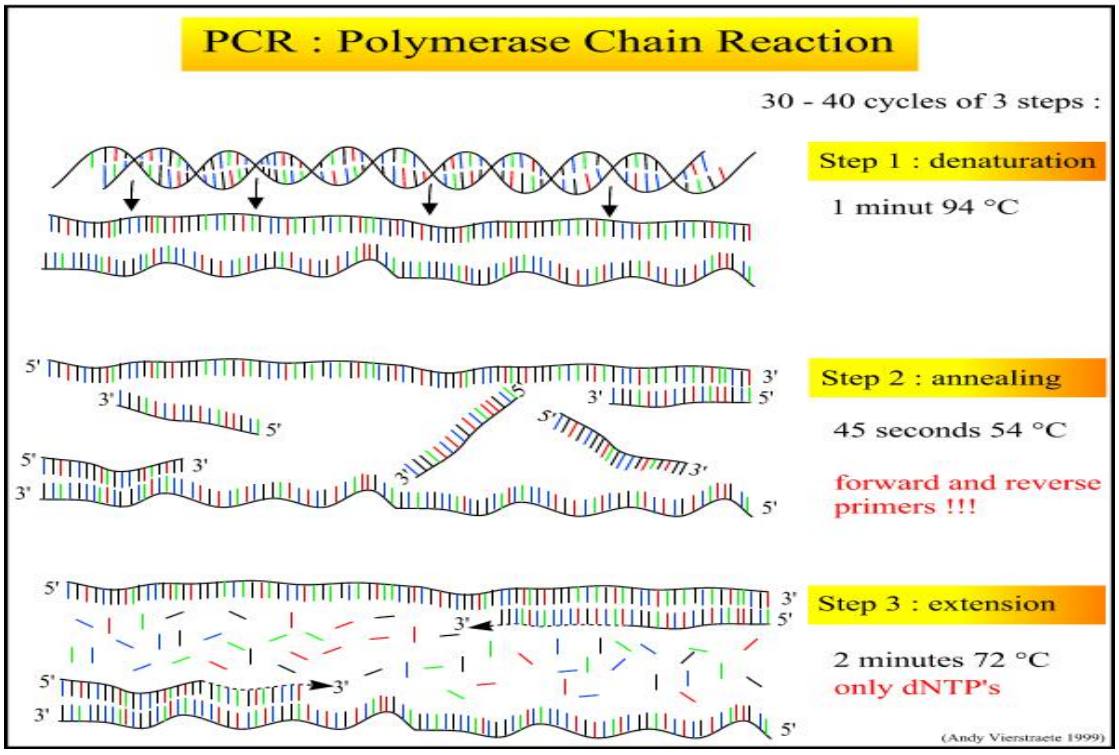

Fig. (2-2): PCR mechanism and experimental steps (Theis, T., Skurray,et al. , 2007).

2.4.3. Estimation of DNA concentration and purity

The concentration and purity of extracted DNA were estimated by Quantus Fluorometer in order to detect the quality of samples for downstream application. For 1µl of DNA, 199 µl of diluted Quantifluor dye was mixed. After 5 min incubation at room temperature, DNA concentration values were detected as indicated in table 2-5.

Table 2-5: DNA concentration and purity obtained

DNA Properties	Mean ± SD
DNA concentration (µg/ ml)	**35.67± 20.26**
DNA purity	**1.90 ± 0.20**

Primer preparation

Macrogen Company supplied these primers in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 °C) to 90 µl of nuclease-free water to obtain a working primer solution 10 pmol/µl.

Primer Name	Vol. of nuclease-free water (µl)	Concentration (pmol/µl)
ACE-rs4646994-F	**300**	**100**
ACE-rs4646994-R	**300**	**100**

The reaction setup and thermal cycling protocol was shown below:

No. of reaction	60	r x n	Annealing temperature of primers	58
Reaction Volume /run	20	µl	Length of PCR product (bp)	190,490
Safety Margin	5	%	No. of PCR Cycles	30

The PCR component is shown in table (2-6):

Table (2-6): Components of PCR of the presented work.

Master mix components	Stock	Unit	Final	Unit	Volume	Volume
					1 sample	176 samples
Master Mix	2	X	1	X	10	1760
Forward primer	10	µM	1	µM	1	176
Reverse primer	10	µM	1	µM	1	176
Nuclease Free Water					6	1056
DNA		ng/µl		ng/µl	2	
Total volume					20	
Aliquot per single rxn	18 µl of Master mix per tube and add 2 µl of Template					

2.4.4. Program of Polymerase Chain Reaction

The following table indicates the program of PCR performed.

Steps	°C	M : S	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	1

°C : Temperature ; M : Minute ; S : Second

Table (2-7): Program of Polymerase Chain Reaction

2.4.5. Electrophoresis

This technique is used to check whether the methods like PCR have worked properly or to check whether our plasmid has the right gene inserted. Electrophoresis involves running a current through a gel loaded with the molecules of interest. The movement of the samples is directed based on the charge that the molecule carries. Based on the size and charge, the molecules will travel through the gel at different speeds, allowing them to be separated from one another as shown in figure 2-3. Since , all the DNA molecules possess same amount of charge per mass , the gel electrophoresis separates them on the basis of size only **(Biotech, 2018).** Electrophoresis through agarose gels is the standard method used to separate, identify, and purify DNA fragments **(Liang, C., *et al* .,2018).**

The agarose gel electrophoresis was done according to Harisha method **(Sofi, M. S.,*et al* . , 2013).** The percentage of agarose used depends on the size of fragments to be resolved, where an agarose gels percentage are normally in the range of 0.5 % to 2 %; the ethidum bromide staining was done according to Robinson and Lafleche method **(Farouk, M., *et al.,*2015).**

Materials

1. Agarose powder.
2. 10 X of TBE (Tris-Borate EDTA buffer).
3. Ethidium bromide (10 mg/ml) [EtBr].
4. Electrophoresis equipment s and power supply.

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

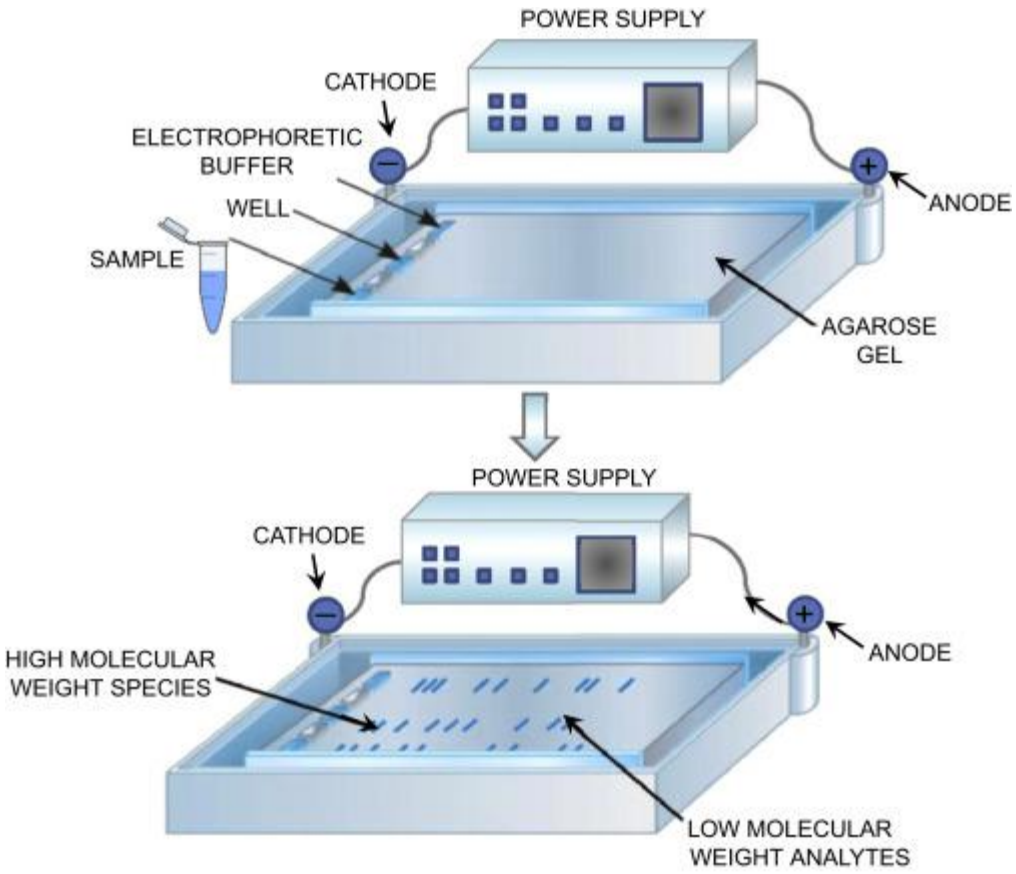

Fig. (2-3): Agarose Gel Electrophoresis for DNA samples (Biotech, 2018).

Preparation of agarose gel:

1. 1.5 grams of agarose were weight and placed into a conical flask, and then 100 ml of 1X (TBE) buffer was prepared and added .this gel was used to detect the band of PCR product.
2. The solution was heated to boiling (using a microwave) until all the gel particles were dissolved. and the solution was clear and then solution was allowed to cool to about 55 ºC before pouring.

3. One µl of ethidium bromide was added to the dissolved agarose and mixed. Ethidium bromide stains DNA by intercalating between the bases of DNA . So attention will be taken to not intercalate with human DNA.
4. Gel chamber were sealed with tape.
5. The comb was placed in the gel chamber about 1 inch from one end of the tray.
6. The gel solution was casted into the chamber and allowed to solidify for about 20 minutes at room temperature .
7. Carefully the dams and the comb were removed , and then the chamber is placed in a horizontal electrophoresis system in about 350 ml of 1X TBE (electrophoresis buffer).when small (10 wells) or large (20 wells) electrophoresis tray used respectively .
8. The gel is covered completely with the buffer to prevent overheating of the gel.
9. At this step, the samples had been loaded (5 µl) on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring one.
10. The cathode (black electrode) was connected to the well side of the unit as the migration is toward the anode (red electrode) in the other side.
11. Electrophoresis was done at 100 volts, for 75 minutes or until dye markers have migrated an appropriate distance, depending on the size of the DNA to be visualized.
12. Ladder 100 bp bands is used as standard for comparison with bands that resulted for allelic gene migration through gel electrophoresis.
13. For DNA detection, 2 µl of the loading dye is mixed with sample (5 µl) before putting it into the wells while PCR product don't need loading dye

addition. The micro pipette is used to place the samples into adjacent wells carefully by steady hand.

2.4.6. Photo Documentation

Agarose gel is placed above the UV trans illuminator device and exposed to UV light and the photos were captured using digital camera and visualized by PC connected to the trans illuminator. The UV transilluminator device was covered with protective shield to avoid the risk of exposure to UV light when light is on.

2.5. Statistical Analysis

Data (represented as Mean ± SD) were analyzed by using the Statistical Package for the Social Sciences (SPSS) (version 23). Independent t- test was used to evaluate significant differences between healthy and patients groups to mention the statistical relationship (association) between any two variables in present study.

The levels of significance of 5% ($p \leq 0.05$) and 1% ($p \leq 0.01$) were obtained to represent the strength of evidence in support of significant differences between variables.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Demographic Characteristics of Covid-19 patients Groups

Most data on Covid-19 have been classified in to moderate and severe **(Wu and McGoogan, 2020)**. Case fatality rates are difficult to assess with certainty, but could be as high as 1% **(Wu, *et al.*, 2020)**, which is much greater than seasonal influenza at about 1%.

The current study case control study included 176 participants (59 of them were severe infected with Covid-19, 54 participants were moderate infected with Covid-19 and the remaining 63 participants were apparently healthy control individuals). Some of anthropometric parameters including body mass index and age of the selected groups were illustrated in Table (3-1).

Table (3-1): Comparison of age and body mass index between moderate and severe Covid-19 groups as compared with control group.

Parameter	Moderate Covid-19 N = 54 Mean ± SD	Severe Covid-19 N = 59 Mean ± SD	*P .Value	Control group N = 63 Mean ± SD	**P .Value
Age,Year	52.06 ± 14.35	59.1 ± 12.64	<0.001	40.03 ± 11.86	<0.001
BMI, kg/m^2	32.59 ± 5.52	33.07 ± 5.72	0.495	33.31 ± 5.83	0.817

BMI: Body mass index; N: Number ; Significant P < 0.05; SD: Standard Deviation

*** Moderate VS.Control , ** Severe VS. Control**

The comparison of some anthropometric characteristics indicated in table (3-1) show significant differences between each of severe and moderate Covid-19 group as compared with control group with respect to the age ($P<0.01$), while BMI data show non-significant results ($P = 0.817$ and $P = 0.495$) respectively. The reason may be due to person ages and its relation with immunity decreases therefore chronic diseases increased due to lack of movement and lack of exercise **(Zhou, *et al.*, 2020 ; Liang , *et al.*, 2020)**.

Older people are more likely to progress to severe disease than middle aged adults people **(Liu, *et al.*, 2020)**. Covid-19 affects the lungs, can compromise the heart and may produce an increase in blood glucose levels; therefore, complications such as disseminated intravascular coagulation or deep vein thrombosis are risks **(Liu, *et al.*, 2020).**

Regarding to aging immunity, there are several other factors related to aging that could be reasons for higher mortality and morbidity in the elderly. The average number of comorbid conditions steadily increased with age and elderly Covid-19 patients had a significantly higher performance score than young and middle-aged patients **(Kim, *et al .,* 2020)**. Another well-recognized feature of aging immunity is chronic subclinical systemic inflammation. Inflammation is a key pathogenic mechanism in Covid-19; hence it has been estimated to contribute to the poorer outcome in elderly patients with Covid-19 **(Bonafè, *et al.* 2020)**. In older age, the cardiovascular disease, diabetes mellitus, chronic respiratory disease, hypertension and cancer were associated with increased the risk of death **(Wu and McGoogan, 2020).** Other reports indicated that older age and underlying comorbidities such as hypertension, diabetes and cardiovascular disease are risk factors of fatal outcome in adult patients with confirmed SARS-COV-2 infection **(Zhou.*et al.*, 2020)**. This can be explain that people above 60 years tend to chronic disease, exercise less, loss of muscle bulk, and gain weight

of fat as they age, sporadic studies tend to die after infection (**Zhou, *et al.*, 2020 ; Liang , *et al.*, 2020)**.

The age-related expression of ACE-2 has been shown previously. (**Bunyavanich, *et al.* ,2020)**compared the expression of ACE-2 in the nasal epithelium cells, one of the first sites of Covid-19 infection, of children and the other in adults. It has been shown that younger children have lower expressions of ACE-2 compared to older children and adults. Considering the impact of ACE-2 on the SARS-Covid-2 entrance, lower expressions of ACE-2 may justify the less infection rates of children . Mas receptor (MasR) and ATR2, which are the anti-inflammatory components of RAS, show an age-related expression as well. Indeed, possible higher expressions of MasR and ATR2 in children can decrease the inflammatory effect of SARS-COVID-19, leading to fewer complications **(Meftahi,*et al.* , 2020).**

3.2. Clinical and Biochemical Characteristics of Study Subjects

The biochemical and clinical feature of the moderate Covid-19 as compared with apparently healthy group were illustrated in table (3-2). The three groups studied were found to be different with respect to ferritin, CRP levels, LDH, ACE-2, ALT, AST and ALP levels.

The mean ± SD values of the variables ferritin, LDH, CRP, ACE-2, ALT, AST and ALP are significantly higher in the severe and moderate Covid-19 as compared to that found in healthy control, table (3-2). This table shows significant differences between moderate and control groups with respect to each of ferritin, LDH, CRP, ALT and AST, while ACE-2 levels was non-significant.

The elevation of ferritin may be due to that it is a key mediator of immune dys-regulation, especially under extreme hyper-ferritinemia, via direct immune-suppressive and pro-inflammatory effects, contributing to the cytokine storm. It has been reported that fatal outcomes by Covid-19 are accompanied by cytokine

storm syndrome, thereby it has been suggested that disease severity is dependent of the cytokine storm syndrome **(Vargas-Vargas, & Cortés-Rojo, *et al* ., 2020).**

Table (3-2): The data obtained of various biochemical parameters in sera of moderate and severe Covid-19 as compared with healthy control group.

Parameter	Moderate Covid-19 N = 54 Mean ± SD	Severe Covid -19 N = 59 Mean ± SD	*P. value	Healthy Control N = 63 Mean ± SD	**P value
Ferritin, ng/ml	601.82 ± 168.52	765.04 ± 259.37	**<0.001**	75.65 ± 39.5	**<0.001**
LDH , U/l	387.73 ± 129.64	432.9 ± 144.86	**<0.001**	135.79 ± 38.52	**<0.001**
CRP, mg/dl	9.14 ± 3.58	15.02 ± 6.53	**<0.001**	0.35 ± 0.15	**<0.001**
ACE-2 , ng/ml	3.83 ± 0.82	4.74 ± 0.85	0.721	3.03 ± 0.82	**<0.001**
ALT , U/l	61.92 ± 27.52	101.11 ± 34.69	**<0.001**	27.63 ± 9.26	**<0.001**
AST , U/l	40.86 ± 13.71	51.6 ± 17.43	**<0.001**	22.69 ± 8.65	**<0.001**
ALP , U/l	90.11 ± 32.02	109.48 ± 36.14	**0.006**	74.9 ± 23.82	**<0.001**

LDH: Lactate dehydrogenase; CRP: C-reactive protein; ACE-2: Angiotensin- converting enzyme; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; S.D: Standard Deviation ; P = Prober value.

*** Moderate VS.Control , ** Severe VS. Control**

Many individuals with diabetes exhibit elevated serum ferritin levels, and it is known that they face a higher probability to experience serious complications from Covid-19. On this basis, we briefly review evidence supporting the hypothesis that ferritin levels might be a crucial factor influencing the severity of Covid-19 **(Vargas-Vargas, & Cortés-Rojo, *et al.*, 2020).**

Notably, ferritin is not only the result of excessive inflammation, but also plays a pathogenic role in the inflammation process through its bind with the T-cell immunoglobulin and mucin domain 2 (TIM-2) by promoting the expression of multiple pro-inflammatory mediators **(Carcillo &Kernan, *et al.,* 2017).**

The cytokine storm and the exaggerated host immune response (i.e, ferritin) participate in the development of ARDS, which is the leading cause of mortality if progresses to respiratory failure **(Wu, *et al.*, 2020).** Hyper-ferritinemia caused by the excessive inflammation due to the infection is associated with the admission to the intensive care unit and high mortality, and represents an indication to recognize high-risk patients to guide the therapeutic intervention to control inflammation **(Carcillo & Kernan, *et al.,* 2017).** Serum ferritin, a feature of hemophagocytic lymphohistiocytosis, which is a known complication of viral infection, is closely related to poor recovery of Covid-19 patients, and those with impaired lung lesion are more likely to have increased ferritin levels **(Zhou, *et al.,* 2020).**

The ferritin levels in our study was significantly increase in severe more than that found in moderate group as compared with control and it was agreement with other report which indicate that Covid-19 patients with high levels of ferritin have greater proportions of severe and deceased cases (P = 0.0016) **(Cheng, *et al.,* 2020). .** Similarly, Sun *et al* revealed that severe patients and discharged patients have greater proportions of increased level of ferritin than non-severe patients and hospitalized patients (100% vs. 50%, 92.3% vs. 37.9% respectively, P < 0.001) and suggested that serum ferritin is a potential risk factor of poor prognosis in Covid-19 patients **(Sun, *et al.,* 2020).** Ferritin levels greater than 800 g/l were found in 100% of patients with severe disease and 30% of those with non-severe disease **(Gómez-Pastora, *et. al.,* 2020).** Ferritin and IL-6 showed higher levels in non-survivors throughout the clinical course, and increased with disease deterioration (**Velavan, *et al.*, 2020).** Another meta-analysis also recommended

serum ferritin as a candidate variable for risk stratification models that may serve as clinical predictors of severe and fatal Covid-19 **(Henry, *et al.,* 2020).**

Among inflammatory biomarkers, CRP levels was also increased significantly at the early stage of the patient studied, and is a positive correlation with the severity of Covid-19 as mentioned by other studies which described and has good diagnostic accuracy in early predicting severe Covid-19 **(Liu, Y., *et al.*, 2020).** The present findings suggest that serum CRP levels could be used as an essential indicator of the progression and the severity of Covid-19. Also, suggests that patients with higher CRP levels should be carefully monitored throughout their disease course **(Zeng, *et al.*, 2020).** The obtained level of CRP in this studies was increased in severe and moderate group without significant values when compared between them, but in others study CRP level could also be used in monitoring the progression and improvement of patients with Covid-19 **.** Tan *et al.* and other studies concluded that CRP was associated with disease progression and predicted early severe Covid-19 **(Tan, *et. al.*, 2020).** CRP is a well-known biomarker of inflammation and is found elevated in 60.7% of patients with Covid-19. More severe cases demonstrated a more evident elevation in CRP levels as compared to non-severe cases (81.5% vs 56.4%, respectively) **(Shi, *et. al.*, 2020).**

In response to infections, the liver synthesizes significant quantities of acute-phase proteins (APPs), such as CRP. This acute inflammatory protein is a highly sensitive biomarker for inflammation, tissue damage, and infection. It has been shown that CRP levels are correlated with levels of inflammation. CRP levels can promote phagocytosis and activate the complement system. In other words, CRP binds to microorganisms and promotes their removal through phagocytosis **(Povoa, *et al.* 2009)**.

As shown previously, this biomarker may be raised by viral or bacterial infections. The current study revealed significantly higher CRP levels in severe

cases than in non-severe patients suggesting that the CRP level may be a biomarker of disease severity and progression in patients with Covid-19. Other reported indicate that more severe cases infected with Covid-19 expressed significantly higher levels of CRP than non-severe patients **(Liu, Y., *et al.*, 2020)**, observed higher CRP levels in severe Covid-19 patients than in non-severe cases, suggesting that this biomarker can be monitored to evaluate disease progression (**Sahu, *et al.*, 2020)** performed a meta-analysis to assess CRP levels as a potential biomarker of the Covid-19 prognosis. Their results indicated that CRP concentrations remain high in expired patients and could be a promising biomarker for assessing mortality **(Liu,Y., *et al.*, 2020)**.

The most common patterns of liver enzyme abnormalities in patients with SARS-CoV-2 include elevated aminotransferases, with aspartate amino-transferase (AST) and alanine aminotransferase (ALT) typically 1–2 times more than the normal upper limit. There are many potential contributing etiologies to elevated liver enzymes in patients with SARS-CoV-2 including direct liver injury, associated inflammatory responses, congestive hepatopathy, hepatic ischemia, drug-induced liver injury (DILI), and muscle breakdown **(Moon & Barritt, 2021).** The alteration of liver enzyme activity levels are a common in sera of Covid-19 studied which related to liver dysfunction and could be the result of secondary liver damage due to the administration of hepatotoxic drugs, systemic inflammatory response, respiratory distress syndrome induced by increased levels of liver dysfunction biomarkers have been associated with severe Covid-19 and worse prognosis **(Lippi, *et al.*, 2020).**

The elevation in LDH level was also observed in sera of Covid-19 patients studied and may be due to that it is one of interest biomarkers, especially since elevated LDH levels have been associated with worse outcomes in patients with other viral infections. Early data in Covid-19 patients has suggested significant differences in LDH levels between patients without severe disease **(Henry, B.**

M., *et al.*,2020). Severe infections may cause cytokine-mediated tissue damage and LDH enzyme release. Since LDH3 isoenzyme is present in lung tissue, patients with severe Covid-19 infections can be expected to release greater amounts of LDH3 in the circulation, as a severe form of interstitial pneumonia, often evolving into acute respiratory distress syndrome, is the hallmark of the disease.

Multiple studies have found LDH level act as a predictor of worse outcomes in hospitalized patients. Many of the prognosticators and therapies currently being studied for Covid-19 are based on experience with the previous coronavirus outbreak severe Acute Respiratory Syndrome (SARS), or with other viral respiratory infections. LDH levels were also found to be elevated in patients with Middle East Respiratory Syndrome (MERS). Elevated LDH levels seem to reflect that the multiple organ injury and failure may play a more prominent role in this pathology in influencing the clinical outcomes in patients with Covid-19 **(Henry, B. M., , *et al.*, 2020).**

The LDH levels could be considered for inclusion in future risk stratification models for Covid-19 severity and mortality. Larger studies are needed to confirm these findings **(Lippi, *et al.*, 2020).**

Chen *et al.* reported that CRP, ferritin, LDH, and ALT were significantly higher in severe cases as compared with mild cases **(Chen, *et al.*, 2020).**

In the present study, we noticed significantly evaluate the inflammatory markers in different categories of Covid-19 (Severe and moderate). Increased CRP and ferritin levels were also associated with secondary bacterial infection and poor clinical outcomes. In the beginning, the laboratory findings depicted the cytopathic effect of SARS-CoV-2, leading to lung damage, apparent from pathological examination, with disease progression **(Xu, *et al.*, 2020).**

Notably, increasing serum CRP levels corresponded to disease progression, serving as an early predictor for Covid-19 complication, prior to indications of critical findings with CT scan **(Tan, *et al.*, 2020)**. Serum ferritin rises proportionately in reaction to the inflammatory process.

Particularly, this study indicated inflammatory events due to cytokine storms, high CRP, and markedly raised serum ferritin levels among critically ill patients contributing to worsened disease state, clearly highlighting the management of lung damage as a crucial step. Apart from this, our findings suggest that the expression of IL-6 and CRP accounts for timely diagnosis of patients having severe disease, keeping in view the substantial burden of healthcare in individual hospitals. Subsequently, hyperferritinemic cases were among the male and elderly age group, correlating towards more severe disease than those with normal serum ferritin levels.

Previous study, ferritin functions as iron binding and storage and is indirectly associated with immunity and inflammatory responses of the body **(Lalueza, *et al.*, 2020).** The link between CRP and Covid-19 has been emphasized by a study at a tertiary care hospital in Wuhan, where most of the patients in the severe category exhibited increased parameters in comparison with the moderate group.

3.3. Angiotensin-Converting Enzyme-2 and Covid-19

The mean **±** SD values of angiotensin-converting enzyme-2 (ACE-2) activity levels was higher in the severe and moderate Covid-19 as compared to that found in healthy control (3.83 ± 0.82, 4.74 ± 0.85 and 3.03 ± 0.82 ng/ml) respectively as shown in table (3-2) which show a non-significant differences between moderate and control groups **(P = 0.721)** and significant differences between sever Covid-19 and apparently health control group (P **< 0.001**).

The availability of angiotensin-converting enzyme 2 receptors may reportedly increase the susceptibility and/or disease progression of Covid -19. The enzyme dysfunction evidently contributes to the severity of Covid-19. Prior studies on SARS-COV illustrate significant correlations to ACE 2 expression in vitro **(Zoufaly, *et al.*, 2020)**. The mechanism for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections requires the binding of the virus to the angiotensin-converting enzyme-2 receptor, well-known for its role in counteracting ACE which is involved in modulating blood pressure and establishing blood pressure homeostasis **(Bosso, *et al.*,2020).** Recently, a critical debatable question has arisen whether using anti-hypertensive medications will have a favorable impact on people infected with SARS-CoV-2 or a deleterious one, mainly because angiotensin-converting enzyme inhibitor (ACEI) and angiotensin-receptor blocker (ARB) therapy can modulate the expression of ACE2 protein. Thev role of ACE2's in mortality rate among Covid-19 patients comorbid with hypertension and critically evaluate the current debate about the usage or discontinuation of ACEI/ARB anti-hypertensive drugs. Moreover, the two opposing roles that ACE2 genetic variants might be playing in Covid-19 by reducing ACE2 receptor effectiveness and mitigating SARS-CoV-2 infectivity **(Li, *et al.*, 2020).**

Furthermore, limited clinical studies depict elevated plasma angiotensin II (ANG II) and aldosterone levels correlating to Covid-19 severity **(Liu, *et al.*, 2020).** Significant plasma ANG II level elevations were seen in 90.2% of the observed Covid-19 cases, especially in 100% of the critical Covid-19 cases **(Henry, *et al.*, 2020)**; although Henry *et al.* observed no differences in ANG II regarding disease severity, while Liu *et al.* further delineated markedly increased ANG II levels linearly associated to viral loads and lung injury **(Villard, *et al.*, 2020)**, and multivariate analyses observed aldosterone levels positively associated with severity.

Angiotensin over level can be considered the main reason for the enhanced inflammatory response of patients with hypertension, diabetes, and obesity. Indeed, considering the down regulation of ACE-2 following the virus entrance, the increased rate of Angiotensin-2 leads to hyperactivity of the RAS inflammatory axis. Therefore, higher rates of mononuclear cells will be activated, which causes diffused endothelial and pulmonary inflammation **(Verdecchia, *et al.*, 2020).** However a recommendation reported by some investigator indicate that different ACE-2 DNA methylation patterns may cause different responses to the virus in patients with hypertension, diabetes, and obesity.

Different types of medications can also affect the expression of ACE-2. Angiotensin receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEIs) are widely used to treat cardiovascular disease. They up-regulate ACE-2 expression in the heart in addition to their pharmacologic effects , **(Simões e Silva, *et al* .,2013).** Study showed that the majority of the ACE gene polymorphism (47%) is related to insertion/deletion (I/D) polymorphism, which is associated to the absence or presence of 287-bp sequence in 16 intron of ACE gene on chromosome 17q23 **(Sayed-Tabatabaei, *et al.*, 2006)**.

3.4. Association between Hypertension, T2DM with Covid-19

Table (3-3) show the significant correlation between moderate and severe Covid-19 as compared with control groups according to the hypertension and T2DM ($P<0.001$) .

These results showed that diabetic patients with Covid-19 had higher severe infection and case-fatality rates as compared with non-diabetic patients, and T2DM was associated with an increased risk of severe infection and mortality in patients with Covid-19. T2DM may be related to the activation of the renin-angiotensin system, and patients with T2DM are often treated with angiotensin-converting enzyme inhibitors (ACEIs) and/or angiotensin receptor blockers

(ARBs), which may both lead to the increased expression of ACE-2 in tissues, promoting virus absorption and increasing the risk of severe infection in patients with T2DM **(Holt, *et al.*, 2020).**

Table (3-3): Hypertension and type-2 diabetes mellitus comparison between moderate and severe covid-19 as compared with control group.

Parameter		Moderate Covid-19 N=54 (%)	Severe Covid-19 N = 59 (%)	*P.value	Healthy Control N = 63 (%)	**P .value
Hypertension	With	30(55.6%)	26 (44.1%)	**<0.001**	0 (0.0)	**<0.001**
	Without	24(44.4%)	33 (55.9%)		63 (100)	
T2DM	With	20 (37.0%)	28 (47.5%)	**<0.001**	0 (0.0)	**<0.001**
	Without	34 (63.0%)	31 (52.5%)		63 (100)	

P value= Prober value ; (%): Percentage ; N : Number ; T2DM : Type-2 diabetic mellitus.

*** Moderate VS.Control , ** Severe VS. Control**

Secondly, T2DM may induce the hypercoagulable state in patients with Covid-19, resulting in worse outcomes of these patients. Study have reported that diabetic patients with Covid-19 had increased risk of hypercoagulability, and many severe and fatal patients with Covid-19 seemed to eventually die of small pulmonary embolism **(Connors, J. M., & Levy, J. H. ,2020**).

Singh et al. have reported a prevalence of hypertension, diabetes and CVD in 21%, 11%, and 7% patients, respectively **(Singh, *et al.*, 2020)**. Yang et al. reported a prevalence of 17%, 8%, and 5% for hypertension, diabetes and CVD respectively, in patients with Covid-19 **(Yang, *et al.*, 2020)**. In contrast, an Italian study by Onder *et al.* found diabetes in nearly 36%, while CVD was associated in nearly 43% of 355 patients admitted with Covid-19 **(Onder, *et al.*, 2020)**.

Similarly, in a small study of 24 patients from United States, reported diabetes to be associated with 58.0% patient with Covid-19 **(Singh, *et al.*, 2020).**

Moreover, this condition makes it difficult to control a SARS/CoV-2 infection. Older adults suffering from underlying systemic diseases are more prone to acute respiratory distress syndrome and cytokine storms **(Guo, *et al.*, 2020)**. The most common comorbidities in one report were hypertension (30%), diabetes (19%), and coronary heart disease (8%) **(Zhou, *et al.*, 2020)**. Another report showed that the most frequent comorbidities in patients with Covid-19 who developed the acute respiratory distress syndrome were hypertension (27%), diabetes (19%), and cardiovascular disease (6%) **(Wu, *et al.*, 2020).**

In the early phase of the Covid-19 pandemic, it was proposed that antihypertensive treatment using ACE-2 inhibitors or angiotensin receptor blockers (ARBs) may contribute to adverse outcomes in patients with hypertension and Covid-19. Most of these studies have suggested that these anti hypertensive drugs might increase tissue expression and/or level of ACE-2 though some have reported no alternation or decrease of ACE-2 in response to the drug. Nevertheless, increased membrane expression of ACE-2 induced by these drugs can theoretically increase the chance of virus entry into organs **(Takeda, *et al.*, 2007)**. The rates of T2DM in subjects affected by Covid-19 vary, depending on the median age, the severity of illness and the location of the study population. In general, people with diabetes are at higher risk of developing complications, because of viral infection. The differences in response are likely the result of the degree of viral loud, host immune response, age of the patient, and presence of comorbidities **(Iacobellis , *et al.*, 2020)**.

This is one of the study, associating between diabetes and hypertension with Covid-19 outcomes. It was able to analyze confirmed Covid-19 ,30 (55.6%) moderate infection and that having hypertension, 26 (44.1%) patients of severe infection and they have hypertention, 20 (37.0%) patients with moderate infection

of Covid -19 and that having type 2 diabetes, 28(47.5%) of severe infection with Covid -19 and they have diabetes. We identified a moderate positive association between diabetes mellitus and hypertension with Covid-19 severity and mortality, and a strong positive association with the report of previous cardiovascular disease with both outcomes.

The link between diabetes and Covid-19 outcomes was previously detected in meta-analyses with a smaller number of studies **(Li, *et al.*, 2020 ; Wang, *et al.*, 2020)**. In a previous study which confirmed Covid-19 patients, being 48 with diabetes. Diabetes is an important risk factor for Covid-19 severity. There was a high heterogeneity that was addressed by random-effect analysis and meta-regression, using the mean age as a covariate, with no impact on results, indicating that diabetes risk was independent of age. In the present study, we identified increase in the risk of severity and mortality associated with Covid-19 in patients with T2DM which was similar to data reported by Kumar *et al.*, who showed for the association of diabetes with severity and mortality **(Kumar, *et al* ., 2020).**

Other recent study including approximately 30 studies each (16.003 and 6.452 patients, respectively) were performed aiming to investigate the association between solely diabetes with severity and mortality of Covid-19, but the criteria to define disease severity were more variable than those defined in the present study. The authors found that diabetes was associated with mortality. Patients with diabetes also have a more unfavorable outcome in others common infections such as Severe Acute Respiratory Syndrome (SARS-CoV) and Middle East Respiratory Syndrome (MERS-Cov) as was described in previous out brakes **(Chan*, et al.*, 2003 ; Morra, *et al.*, 2018).** Multiple pathophysiological mechanisms can support the association between DM and Covid-19 severity; however, much of knowledge is derived from SARS-COV infection rather than Covid-19. Compromised innate immune system due to chronic hyperglycemia,

pro-inflammatory state characterized by inappropriate and exaggerated cytokine response and underlying pro-thrombotic hypercoagulable have been implicated in this association **(Chan, *et al.*, 2003 ; Morra*, et al.*, 2018).** However, the association between hypertension and worse outcomes of Covid-19 infection may be due to the higher frequency of comorbidities and a more advanced age of these individuals. An Italian cross-sectional study did not find hypertension as an independent factor affecting the outcome of Covid-19. It is possible that mechanism mediated by ACE-2 receptor links type 2 diabetes, hypertension and cardiovascular disease with a higher risk of severe manifestations of Covid-19 infection. It was also documented that some medications can increase ACE-2 expression, such as ACE inhibitors (ACEI) and angiotensin 2 receptor blockers (ARBs). In this study, however, the association between the uses ACE or ARB and severity or mortality of Covid-19 was not conclusive **(Sriram, *et al.,*2020).**

3.5. Molecular Analysis of Angiotensin-Converting Enzyme-2 and Covid-19

Isolation of nucleic acids is the first step in most molecular analytical studies and recombinant DNA techniques. A large number of procedures, based on a variety methods and principles, exist for the extraction and purification of nucleic acid. They all share the common requirements that the biological material needs to be lysed, cellular nucleases must be inactivated, and finally, the desired nucleic acid has to be purified from the cellular debris. Blood samples of apparently healthy and Covid-19 samples were subjected to genomic DNA extraction within 24-48 hours of aspiration**.** The DNA extraction method eliminates the need for organic solvent extractions and DNA precipitation, allowing for rapid purification of many samples simultaneously**.** The concentration of genomic DNA extracted was determined and the band integrity was found to be different according to the amount of genomic DNA and its purity which depend upon the amount of WBCs in neonatal sample used. In addition, using fresh blood samples were found to be better than that stored at -

20 °C for several days, therefore, the genomic DNA should be applied as early as possible.

3.5.1. Measurement of DNA Concentration and Purity.

DNA concentration and purity were estimated by the measurement of A260/A280 ratio. Results were clarified in table (3-4). DNA samples were seemed to be pure (**1.90 ± 0.20)** and its concentrations determine as **mean ± SD** was (**35.67 ± 20.26)** µg/ml.

Table (3-4): DNA concentration and purity.

	Mean ± SD
DNA Concentration, (µg/ ml)	**35.67 ± 20.26**
DNA Purity	**1.90 ± 0.20**

DNA: Deoxyribonucleic acid ; SD: Standard deviation

3.5.2. Association between Angiotensin-Converting Enzyme-2 Receptor Gene Polymorphism with the Severity of Covid-19.

The genomic DNA extracted were subjected to electrophoresis through agarose gels which was detected by staining and then visualized by illumination with UV light to confirm the presence and integrity of the extracted DNA. The visualization method of DNA extracted was performed by staining with the fluorescent dye ethidium bromide which is the most convenient and commonly used method to visualize DNA in agarose gels. Figure (3-1) genotype variation of the amplification fraction after polymerase chain reaction of ACE-2 receptor gene (rs4646994) of human samples obtained from Covid-19 sera by agarose gel electrophoresis (1.5% agarose) and then stained with ethidium bromide. Individuals with normal homozygous (DD) revealed band of (190 bp), while individuals with normal (II) revealed band of (490 bp), and the individuals with

heterozygous (ID) revealed two bands (190, 490 bp) respectively as shown in figures (3-1 ; 3-2).

The angiotensin-converting enzyme gene (ACE-2) located on chromosome 17q23.3, spansv21 kb, and comprises 26 exon and 25 intron. Exon 26 encodes for the functionally important membrane-anchoring domain of the ACE protein. Insertion (I allele) polymorphism had band in (490 bp) of an Alu repetitive element in an intron of the ACE gene that called homozygotes.

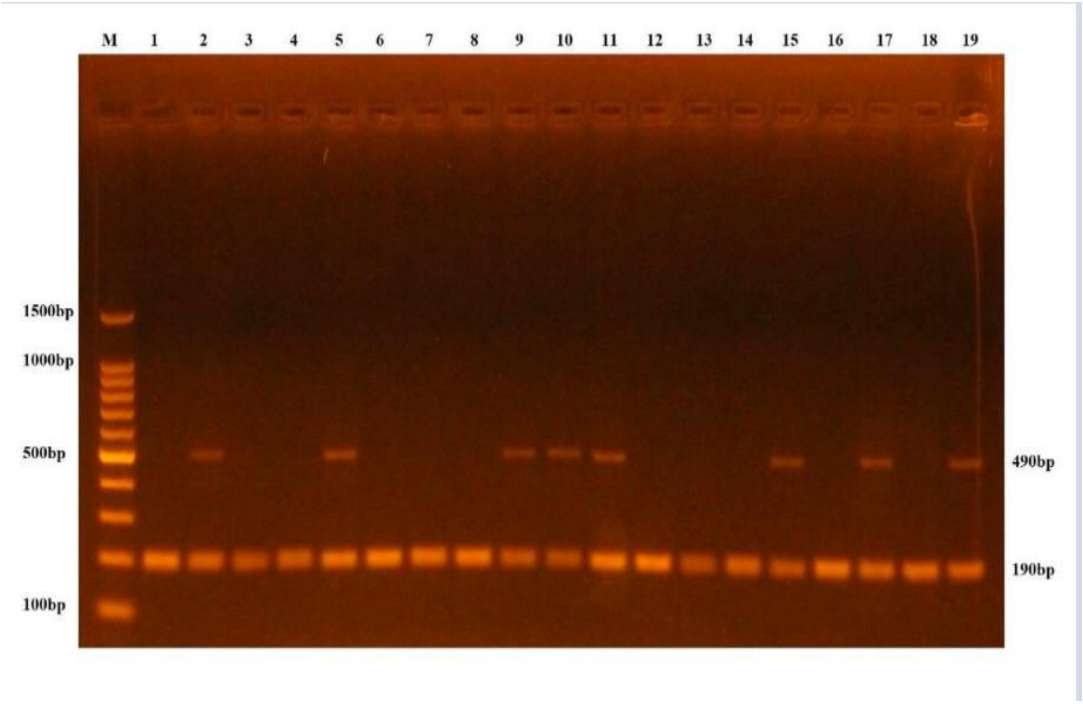

Fig.(3-1) : The amplification of rs4646994 region of human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. ; M: DNA Marker (Ladder 100bp). Lanes 1-19 resemble 190, 490bp PCR products.

Deletion genotype (D allele) had band in (190 bp) that lack the repetitive element also called homozygotes, while (ID) genotype had two band I and D in the same gene that mean in the same gene had insertion and deletion of an Alu repetitive element in intron of the ACE-2 receptor gene and it is called heterozygotes, so the two band in the same location differ in speed of migration as in figure (3-1).

The (DD) genotype showed band at 190 bp, (II) genotype showed band at 490 bp while (ID) genotype showed both bands at 190 and 490 bp. The size of each of (DD, II and ID) genotypes was indicated in table (3-8).

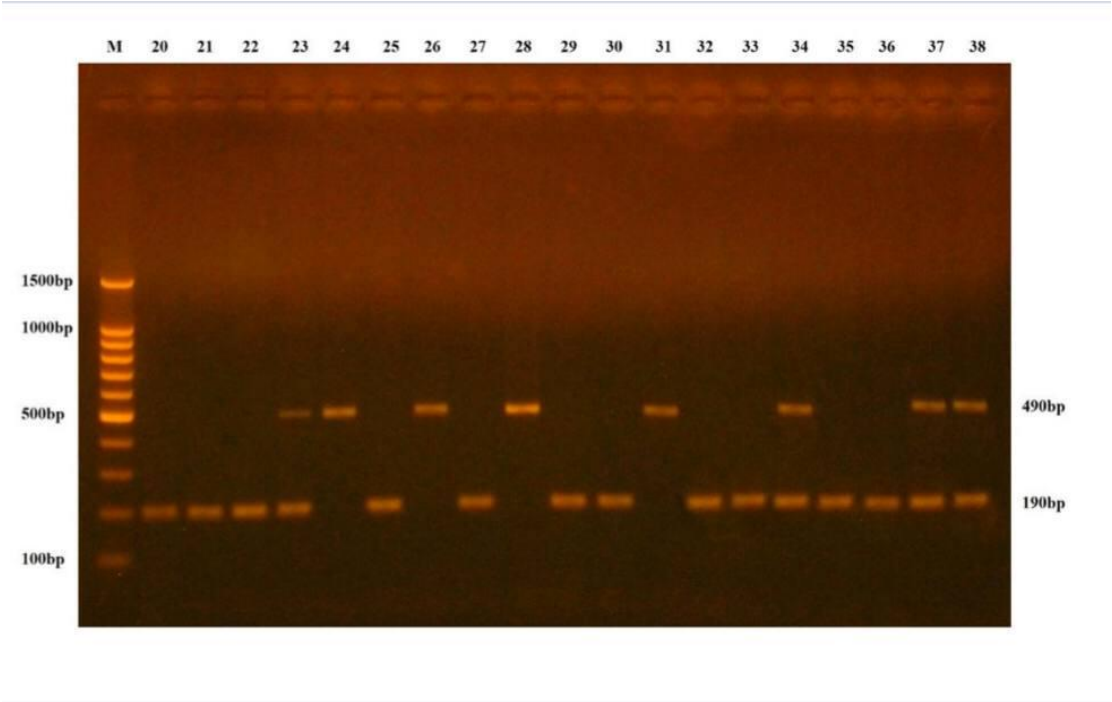

Fig.(3-2) : The amplification of rs4646994 region of human samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: DNA Marker (Ladder 100bp). Lanes 20-38 resemble 190, 490bp of PCR products.

Table (3-5): Size of bands of ACE-2 gene polymorphism (rs4646994) obtained from sera of Covid-19.

Genotype	No. of Bands		Size of band bp
Homozygous	DD	1	190
Homozygous	II	1	490
Heterozygous	ID	2	190 , 490

No: Number ; bp: Base pair ; DD: Deletion / Deletion ; ID: Insertion / Deletion ; II: Insertion / Insertion .

3.5.3. Angiotensin-Converting Enzyme Gene Polymorphism Alleles and their Relations with Age and BMI in Control and Covid-19 Patients

Table (3-6) show the comparison of age and body mass index in control group according to allele by unpaired t test. In this table show age and BMI are non-significant in control group according to the allele DD, ID. This means individuals with DD genotype showed approximately twice ACE levels as compared to II genotype individuals **(Rigat, B., *et al.,* 1990).**

Table (3-6): Comparison of age and body mass index of ACE-2 gene polymorphism in control group according to allele by unpaired t test

Parameters	Apparently Health Control Group N = 63		P. Value
	DD Allele N = 27 Mean ± SD	ID Allele N = 36 Mean ± SD	
Age, Year	41.96 ± 12.62	38.68 ± 11.42	0.291
BMI, (kg/m^2)	33.93 ± 6.41	32.75 ± 5.48	0.442

BMI: Body Mass Index ; N:Number ; SD: Standard Deviation ; DD: Deletion / Deletion ; ID: Insertion /Deletion

To date, few studies have been published that investigate the relationship between ACE-2 gene polymorphism and Covid-19 severity, but we are still lacking definite results **(Delanghe, *et al.*, 2020a ; Devic Pavlic, *et al.*, 2020 ; Gemmati, *et al.*, 2020 ; Hatami, *et al.*, 2020)**. In present study, observed that individual with 'DD' genotype showed significantly 3.69-fold higher risk of Covid -19 severity. Similarly, other studies found that ACE1 D/D-genotype showed association with Covid-19 related mortality **(Calabrese, *et al.*, 2020)**. Gomez *et al.* found D allele was significantly associated with hypoxemic as

compared to non-hypoxemic patients; however, 'DD' genotype individuals did not show any association with Covid-19 infection **(Gomez, *et al* ., 2020).**

Table (3-6) indicates the comparison of age and body mass index in moderate Covid-19 cases according to allele. In this table age and BMI are non-significant in moderate group according to the allele (ID and II). The (DD) allele not appeared in moderate Covid-19 infection group.

Table (3-7): Comparison of age and body mass index in moderate Covid-19 cases according to ACE-2 gene polymorphism allele obtained by polymerase chain reaction amplification.

Parameters	Moderate Covid-19 , N = 54		P .Value
	ID Allele N = 46 Mean ± SD	II Allele N = 8 Mean ± SD	
Age, Year	52.91 ± 14.62	47.13 ± 12.32	0.297
BMI, (kg/m^2)	33.07 ± 5.6	29.83 ± 4.38	0.126

N: Number ; BMI: Body Mass Index ; SD: Standard Deviation ; P value = Prober value ; ID: Insertion / Deletion ; II: Insertion / Insertion

The presence of this polymorphism could be involved in the hypertension process per se as previously mentioned, but also to be a synergistic risk factor for Covid-19 fatality. It is of great interest to investigate whether the increased risk of severe Covid-19 is due to the potentiation of the metabolic dysfunction by these alleles (acting as synergetic) or just because of such comorbidities. Thus, we performed an additional analysis adjusting for age, BMI and number of comorbidities. Our results show that D alleles remained associated with Covid-19 severity after adjustment for these factors, suggesting that both the metabolic comorbidities and the D allele act synergistically on Covid-19 outcome.

Table (3-8) show the comparison of age and body mass index of DD allele between severe covid-19 cases and control group. In this table age is significant between severe and control groups according to the deletion/deletion allele DD, but BMI in significant. The DD allele found in severe and control group, not found in moderate group.

Table (3-8): Comparison of age and body mass index in severe Covid-19 cases according to ACE-2 gene polymorphism allele obtained by polymerase chain reaction amplification as compared with control DD allele.

Parameters	Severe Covid-19 DD Allele N = 59 Mean ± SD	Control DD Allele N = 27 Mean ± SD	P Value
Age, Year	59.1 ± 12.64	41.96 ± 12.62	**<0.001**
BMI, (kg/m^2)	33.07 ± 5.72	33.93 ± 6.41	0.537

N: Number ; BMI: Body Mass Index ; SD: Standard Deviation ; P value = Prober value ; DD: Deletion / Deletion allele found in sever Covid-19 and control group

The study has reported an association of the DD genotype of ACE-2 polymorphism and higher circulating levels of ACE-2 **(Schüler, *et al.* 2017)**, which could explain the higher susceptibility to develop severe forms of the disease in patients with the DD genotype, in addition to hypertension and T2DM. SARS-CoV-2 sequesters ACE-2 to invade cells, decreasing the bioavailability of ACE-2 which entails a reduction in the degradation of ANG II and an exacerbation of the damaging effects of ANG II **(Chung, *et al.*, 2020)**. Thus, the lung injury and inflammation caused by the reduced ACE-2 levels due to the viral infection, and also by the hypertension and diabetes may be worsened by ACE-2 genotypes that further increase ACE-2 levels, and hence ANG II levels, such as the DD genotype of ACE-2 analyzed. In our study we confirmed that ACE-2 levels are associated with the DD genotype of both polymorphisms, which in turn

is associated with greater severity of the disease in hypertensive and T2DM. Although according to our data we could not affirm a direct association with Covid-19 severity.

However, our results clearly revealed that polymorphisms of the ACE gene are related to the risk of developing severe Covid-19 (ICU admission) in hyper tense and diabetic patients. Our study confirms previously reported findings of another ACE polymorphism on Covid-19 patients with hypertension **(Gómez, *et al.*, 2020)** however; it did not corroborate the association with Covid-19 severity found by Gomez *et al.* and other authors for the ACE I/D polymorphism, regardless of comorbidities. Unlike these studies, we also found an additional association of D Allele with enhanced severity in hypertension and diabetic patients, and interestingly, we further found a higher prevalence of the DD, ID genotypes (all containing the D allele) among deceased ICU-patients, not described so far, confirming the deleterious effect of the D allele in Covid-19 outcomes. Precisely most of these patients were hypertensive.

3.5.4. Angiotensin-Converting Enzyme-2 Gene Polymorphism and its Relation with Hypertension and T2DM in Covid-19 Patients

Table (3-9) show the comparison of gene polymorphism of ACE-2 alleles (ID and II) with hypertension and diabetes mellitus type 2 moderate Covid-19 cases according and compared with severe and control alleles (DD). In this table hypertension and T2DM non significant in moderate group according to the allele ID and II alleles. In moderate group appears ID, II alleles were found only.

In present study, 113 Covid-19 patients were selected with demographic and clinical characteristics. We observed that high frequency of hypertensive patients (54.3%) as compared to diabetic (34.8%) as shown in table (3- 9). In contrast, de Abajo *et al.*, found higher prevalence of diabetes in 1339 Covid-19 cases from Madrid, Spain as compared with 13,390 matched controls (27.2% vs 20.3%;

crude odds ratio, OR, 1.50) (**de Abajo, *et al.*, 2020**). Similarity, Singh *et al.,* pooled different studies (N = 2209) and found higher percentage of hypertension (21%) as compared to diabetes (11%) and CVD (7%) **(Singh, *et al.*, 2020)**. In contrast, a meta-analysis of Covid-19 Patients (N = 1576 patients) reported percentage of different comorbid conditions such as hypertension (17%) > diabetes (8%) (**Yang, *et al.*, 2020**).

Table (3-9): Comparison of gene polymorphism of ACE-2 alleles (ID and II) with hypertension and type 2 diabetes mellitus in moderate Covid-19 cases and compared with severe and control alleles (DD).

Parameters		Moderate Covid-19		P Value	Severe Covid-19	Control	P Value
		ID Allele N = 46 (%)	II Allele N = 8 (%)		DD Allele N = 59 (%)	DD Allele N = 27 (%)	
Hypertension	with	25 (54.3%)	5 (62.5%)	0.720	26 (44.1%)	0 (0.0)	**<0.001**
	without	21 (45.7%)	3 (37.5%)		33 (55.9%)	27 (100%)	
T2DM	with	16 (34.8%)	4 (50.0%)	0.450	28 (47.5%)	0 (0.0)	**<0.001**
	without	30 (65.2%)	4 (50.0%)		31 (52.5%)	27 (100%)	

N: Number ; T2DM:Type 2 Diabetes Mellitus.; ID: Insertion / Deletion; II: Insertion /Insertion ; P value= Prober value

In table (3-9), hypertension, T2DM are significant between severe and control groups according to the allele DD, this may be due to that D allele was found only in severe Covod-19. Other report also found high mortality rate in diabetic individuals (14.5%) as compared to non-diabetic (5.7) Covid -19 cases **(Li, *et al.*, 2020).** Similarly, our study found that diabetic patients have 47.5 % higher risk for having severity/mortality in Covid-19 ($P < 0.001$). The presented study also suggested that hypertensive individual might have 44.1% higher risk of

severity / mortality. Similarly, a study suggested that hypertension may be risk factor for severity in Covid-19 **(Yang, *et al.*, 2020).**

Table (3-10) show a comparison of the presence of hypertension and diabetes mellitus type 2 in control group according to allele by Fisher exact test. In this table hypertension and T2DM are non-significant in control group according to the allele DD and ID. In control group, higher frequency of the DD genotype and lower frequencies of the ID allele and very low frequency of the II genotype.

Table (3-10): Comparison of the presence of hypertension and diabetes mellitus type 2 in control group according to control allele by Fisher exact test.

Parameters		Apparently Health Control		P. Value
		DD Allele N = 27 (N%)	ID Allele N = 36 (N%)	
Hypertension	With	0 (0.0)	0 (0.0)	1.000
	Without	27 (100%)	34 (100%)	
T2DM	With	0 (0.0)	0 (0.0)	1.000
	Without	27 (100%)	34 (100%)	

DD: Deletion / Deletion ; ID: Insertion / Deletion ; N: Number ; T2DM :Type 2 Diabetic Mellitus

Covid-19 is a highly contagious disease characterized by high mortality, especially for patients with severe comorbidities such as diabetes, hypertension, CVD and CKD . The documented history of diabetes has been stated to be an independent indicator of morbidity and death in SARS patients **(Li, B., *et al.*, 2020).** Diabetes hyperglycemia is suspected to cause immune response dysfunction, which fails to regulate the spread of invasive pathogens. Therefore, diabetic individuals are considered to be more prone to infections and incidence

of infectious diseases and it will increase associated comorbidities **(Berbudi, *et al.*, 2020)**. A study was released by the Chinese Centre for Disease Control and Prevention, which showed elevated mortality rate in people with diabetes (2.3%, total and 7.3%, diabetes patients) study was performed in 72,314 cases of Covid-19 **(Wu, Z., & McGoogan, J. M. ,2020)**.

Blood pressure homeostasis maintained by renin–angiotensin system (RAS) **(Kuba, *et al.*, 2006)**. RAS system modulated by ACE-1 and ACE-2, angiotensin I is converted into angiotensin II (ATII) by ACE1 and degraded bioactive bradykinin **(Baudin, 2002)**. Insertion / deletion (I/D) polymorphism has been correlated with levels of circulating and tissue ACE1 and influences almost half of the variability of serum ACE levels in the general population. The 'D' allele of ACEI/D is associated with higher ACE activity **(Tiret, *et al.*, 1992)**. This means individuals with DD genotype showed approximately twice ACE levels as compared to II genotype individuals **(Rigat, *et al.,* 1990).** The 'D' allele of ACE1 gene is significantly associated with hypoxemic group as compared to non-hypoxemic group **(Itoyama, *et al.*, 2004)**. Recently, Delanghe *et al.* found that the prevalence of Covid-19 in 33 countries has been substantially associated with ACE1 I/D polymorphism **(Delanghe, *et al.*, 2020b)**. A previous study in north Indian suggests that ACE insertion /deletion gene polymorphism is associated with hypertension **(Singh, G., & Bhanwer, A. J. S. (2019).**

3.5.5. Association between Diagnostic Biochemical Markers with ACE-2 Gene Polymorphism Alleles and their Relations with Severity of Covid-19.

Table (3-11) show the comparison of biochemical in moderate covid-19 cases according to allele. In this table all parameters are non-significant in moderate group according to the allele ID and II. Genetic polymorphisms in ACE indicate that the ACE I/D polymorphism, have been shown to affect ACE activity levels and confer susceptibility to hypertension **(Yang, X., , *et al.*, 2020)** type 2 diabetes **(Schüler, *et al.* 2017)**, overweight **(McAllister, E. J., *et al.*, 2009)**

nephropathy and certain cardiovascular and autoimmune diseases **(Jiang, *et al.*, 2013) .** More specifically, the DD genotype of the ACE I/D polymorphism has been associated to higher levels of serum ACE and higher levels of circulating IL-6 in patients with myocardial infarction. In contrast, the II genotype has been associated to lower circulating ACE levels. Since some of these processes have been reported to be involved in the pathogenesis of Covid-19, the DD genotype could predispose to complications of Covid-19 due to higher baseline ACE levels and its consequences **(Gómez, *et al.*, 2020)**. Indeed, an association of the DD genotype of the ACE I/D polymorphism with severe Covid-19 has been reported in hypertensive males **(Gómez, *et al.*, 2020)**. However, analyzing the I/D polymorphism is laborious and time-consuming and some authors have described a preferential amplification of the D allele **(Abdollahi, *et al.*, 2008)**. The ACE-2 gene polymorphisms are in complete linkage disequilibrium with the ACE I/D polymorphism, therefore they could be better prognostic markers.

Table (3-11): Comparison of biochemical in moderate Covid-19 cases according to ACE-2 allele as compared with control

Parameters	Moderate Covid-19 Group		
	ACE-2 Allele	Mean ± SD	P .value
Ferritin, (ng/ml)	ID Allele	602.24 ± 509.41	0.542
	II Allele	597.08 ± 269.63	
LDH , (U/l)	ID Allele	395.78 ± 240.34	0.784
	II Allele	341.46 ± 158.35	
CRP, (mg/dl)	ID Allele	9.56 ± 13.26	0.574
	II Allele	6.75 ± 7.74	
ACE-2 , (ng/ml)	ID Allele	3.14 ± 0.83	0.124
	II Allele	2.56 ± 1.29	
ALT , (U/l)	ID Allele	60.81 ± 39.25	0.233
	II Allele	68.27 ± 26.44	
AST , (U/l)	ID Allele	41.28 ± 19.32	0.896
	II Allele	38.39 ± 15.56	
ALP , (U/l)	ID Allele	89.87 ± 30.73	0.747
	II Allele	91.46 ± 41.14	

ID: Insertion /Deletion ; II: Insertion / Insertion ; LDH: Lactate dehydrogenase ; CRP: C- Reactive protein ; ACE-2: Angiotensin Converting Enzyme-2 ; ALT: Alanine Aminotransferase ; ALP: Alkaline phosphatase ; AST: Aspartate Aminotransferase ; SD: Standard Deviation.

(Table 3-12): Comparison of biochemical in ACE-2 Alleles, ID allele in moderate Covid-19 group as compared to ID allele in control and also the DD allele in severe Covid-19 as compared with control DD allele

Parameters	ID Allele in Moderate Covid-19 Group as compared with Control			DD Allele in Severe Covid-19 Group as compared with Control		P .value
	Group	Mean ±SD	P value	Group	Mean ±SD	
Ferritin, (ng/ml)	Moderate	602.24 ± 509.41	**<0.001**	Severe	765.04 ± 359.37	**<0.001**
	Control	81.36 ± 47.31		Control	70.08 ± 26.96	
LDH , (U/l)	Moderate	395.78 ± 240.34	**<0.001**	Severe	432.9 ± 211.86	**<0.001**
	Control	132.93 ± 37.95		Control	140.98 ± 39.41	
CRP, (mg/dl)	Moderate	9.56 ± 3.26	**<0.001**	Severe	15.02 ± 23.53	**<0.001**
	Control	0.34 ± 0.15		Control	0.36 ± 0.13	
ACE-2 , (ng/ml)	Moderate	3.14 ± 0.83	**0.001**	Severe	3.74 ± 0.85	0.345
	Control	2.55 ± 0.62		Control	3.66 ± 0.64	
ALT , (U/l)	Moderate	60.81 ± 19.25	**<0.001**	Severe	101.11 ± 266.69	**<0.001**
	Control	29.56 ± 11.71		Control	26.54 ± 9.7	
AST , (U/l)	Moderate	41.28 ± 12.32	**<0.001**	Severe	51.6 ± 53.43	**<0.001**
	Control	22.77 ± 8.33		Control	23.54 ± 8.78	
ALP , (U/l)	Moderate	89.87 ± 30.73	0.063	Severe	109.48 ± 39.34	**<0.001**
	Control	77.12±23.52		Control	73.66 ± 24.41	

LDH: Lactate dehydrogenase ,CRP :C- Reactive protein .,ACE :Angiotensin Converting Enzyme ,ALT : Alanine Aminotransferase.,ALP :Alkaline phosphatase ,AST : Aspartate Aminotransferase ., S.D :Standered Deviation. DD : Deletion /Deletion , ID : Insertion /Deletion

Table (3-13) show the comparison of biochemical in control group according to allele. In this table all parameters in control group are insignificant according to the allele DD and ID expect ACE-2 is significant.

Table (3-13): Comparison of biochemical in control group according to allele

Parameters	Group	Mean± SD	P Value
Ferritin, (ng/ml)	DD Allele	70.08 ± 26.96	0.581
	ID Allele	81.36 ± 47.31	
LDH (U/l)	DD Allele	140.98 ± 39.41	0.441
	ID Allele	132.93 ± 37.95	
CRP, (mg/dl)	DD Allele	0.36 ± 0.13	0.278
	ID Allele	0.34 ± 0.15	
ACE-2 (ng/ml)	DD Allele	3.66 ± 0.64	**<0.001**
	ID Allele	2.55 ± 0.62	
ALT (U/l)	DD Allele	26.54 ± 9.7	0.305
	ID Allele	29.56 ± 11.71	
AST (U/l)	DD Allele	23.54 ± 8.78	0.994
	ID Allele	22.77 ± 8.33	
ALP (U/l)	DD Allele	73.66 ± 24.41	0.420
	ID Allele	77.12 ± 23.52	

LDH: Lactate dehydrogenase ; CRP: C- Reactive protein ; ACE-2:Angiotensin Converting Enzyme-2 ; ALT: Alanine aminotransferase ; ALP: Alkaline phosphatase ; AST: Aspartate aminotransferase .; SD: Standard Deviation ; DD: Deletion / Deletion ; ID: Insertion /Deletion

In this table show ACE-2 was significantly to DD and ID alleles because the D allele is associated with higher ACE-2 activity. Mean ACE levels in DD carriers were approximately twice that in II genotype individuals. Therefore, we propose a hypothesis that ACE-2 gene polymorphism may play an important role in patients with Covid-19 who are susceptible to develop severe lung injury or ARDS **(Rigat, *et al.* 1990).**

Based in according to our result we can conclude that the severity of Covid-19 patients may depend on age, diabetes, hypertension and ACE-2 gene polymorphism. Our result suggest that a prospective paradigm of DD genotype that has the potential to help explain the susceptibility of the host response to SARS-Cov-2 infection and involve in numerous pathological. Thus, ACE-2 I/D polymorphism may be act as a useful tool to predict the development of disease and may have an influence on the treatment outcomes against the Covid -19 to establish a population-based therapeutic development .

Conclusion And Recommendation

conclusion and Recommendations

4.1.conclusion

1. Increased rate for both of parameters include CRP, Ferritin ,LDH ,ACE ALT,AST and ALP in severe infection with Covid -19 and moderate infection compared to control group .
2. Every severe pataients carried D/D genotype ,every moderate patients carried either I/D or II genotype.
3. Controle group carrid either D/D, I/D and I/I.The data observed indicated that if those control groups will be infected with covid-19, they considered as severe cases due to that the D-allele is associated with higher ACE-2 activity; therefore, patients that carry D-allele, especially those with the D/D genotype, are at higher risk of pulmonary morbidity and/or mortality
4. Most of infected patients were old ages and with chronic disease (T2DM and Hypertention).

4.2.Recommendations

I would like to recommend these titles for future research to other researchers .

1. Further studies involving a larger cohort and control group should be carried out to better understand the association between Covid -19 Severity and different genotypes of ACE with treatment response .

2. Research about another receptors for Covid -19 in body .For example NRP1

3. Further genetic studies for parents of Covid -19 according to the DD ,ID and II genotype .

4. Study where deletion and insertion occure in the sequense?

5. study we investigated the association of rs4341 and rs4343 polymorphisms of the ACE gene with Covid-19 outcomes in patients with different degree of severity. Identifying genetic variants that influence the severity of Covid-19 could be useful to better understand the physiopathology of the disease or identify new therapeutic targets, through the identification of effector transcripts of the genetic variants that underlie the phenotype. Besides, it could facilitate the early identification of patients genetically susceptible for severe Covid-19 to better monitor them and give them a more appropriate clinical management that may improve their outcomes.

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((Experimental Data))

Sample No.:	BMI:	Age:	Gender:
Chronic diseases: Hypertension T2DM Others		Smoking: Slightly Moderately Severe	
Inclusion criteria:			
Exclusion criteria:			

Biomarkers

Biomarker	Levels	Unit
Ferritin		
Total LDH activity		
C-reactive protein		
Angiotensin-converting enzyme		
Liver enzymes ALT AST ALP		

Molecular Analysis:

Angiotensin-converting enzyme receptor-2 gene polymorphism and various molecular studies.

الخلاصة

يتجمع فيروس كورونا (كوفيد -19) تحت مجموعة الأسرة الفيروسية التي تسبب المرض في الثدييات والطيور. تم تسمية فيروس كورونا الجديد الوبائي باسم "مرض فيروس كورونا 2019" من قبل منظمة الصحة العالمية (WHO) في جنيف ، سويسرا. هذا هو فيروس الجيل الثالث القاتل في عائلة كورونا الذي سبقته متلازمة الشرق الأوسط التنفسية (MERS) في عام 2012 ومتلازمة الجهاز التنفسي الحادة الوخيمة (السارس) في عام 2003. يُلاحظ حدوث عدوى SARS-CoV -2 , من المرجح أيضًا أن يصيب SARS-CoV -2 الأشخاص الذين يعانون من أمراض مصاحبة مزمنة مثل القلب والأوعية الدموية وارتفاع ضغط الدم والسكري. تحدث أعلى نسبة من الحالات الشديدة عند البالغين الذين تقل أعمارهم عن 60 عامًا.

الإنزيم المحول للأنجيوتنسين (ACE-2) هو بروتين موجود على سطح العديد من أنواع الخلايا. إنه إنزيم يولد بروتينات صغيرة - عن طريق تقطيع البروتين الأكبر أنجيوتنسينوجين - الذي ينتقل بعد ذلك إلى تنظيم الوظائف في الخلية. باستخدام بروتين يشبه السنبلة على سطحه ، يرتبط فيروس-SARS-CoV 2 بـ ACE -2 مثل إدخال مفتاح في قفل - قبل دخول الخلايا وإصابتها. ومن ثم ، فإن2- ACE يعمل كمدخل خلوي - مستقبل - للفيروس المسبب لـ-covid 19. يعمل ACE-2 كمستقبل لفيروس SARS-CoV -2 ويسمح له بإصابة الخلية. تحدد كمية المستقبلات ما إذا كان شخص ما قد أصيب بمرض أكثر أو أقل ، لأن فيروس SARS-CoV-2 يتطلب ACE2 لإصابة الخلايا ولكن العلاقة الدقيقة بين مستويات ACE-2والعدوى الفيروسية وشدة العدوى.

الهدف من المشروع البحثي هو دراسة العلاقة بين المؤشرات الحيوية المختلفة بما في ذلك مستوى الإنزيم المحول للأنجيوتنسين وتعدد الأشكال الجيني لمستقبلاته مع شدة الإصابة بفيروس كوفيد -19 في الوباء العراقي في محافظة كربلاء.

أجريت هذه الدراسة على 176 شخصًا تم تشخيصهم من قبل الطبيب وتم تقسيمهم إلى ثلاث مجموعات: 59 منهم مصابين بفيروس كوفيد -19 شديد العدوى ، و 54 منهم مصابين بفيروس كوفيد -19 معتدل و 63 منهم على ما يبدو كانوا يتمتعون بصحة جيدة وحضروا مستشفى للفحص. تم جمع المرضى في الحالات الحرجة والمتوسطة من مستشفى الحسين التعليمي ، مدينة الحسين الطبية ، مديريات صحة كربلاء ، كربلاء - العراق خلال ايلول 2020 - آذار 2021 بأعما تراوحت بين (23-88) سنة. تم استخلاص الحمض النووي الجيني من الدم ثم دراسة التنميط الجيني لمستقبل ACE-2 باستخدام بادئات خاصة باستخدام تفاعل البلمرة المتسلسل.

تضمنت المؤشرات الحيوية والقياسات البشرية التي تم تحديدها: العمر ، مؤشر كتلة الجسم ، الفيريتين ، نشاط نازعة هيدروجين اللاكتات ، (LDH) البروتين التفاعلي ، (CRP) C نشاط إنزيم تحويل(ACE - 2 Aniotensin-2) ،نشاط Alanine aminotransferase (ALT) Aspartate ،نشاط aminotransferase (AST) ومستويات نشاط الفوسفاتيز القلوي .(ALP)يشير النمط الوراثي لـ- ACE 2 الذي تم الحصول عليه في جميع عينات الدراسة المقدمة البالغ عددها 176 عينة (شديدة ، ومتوسطة ، واصحاء) تمت ملاحظة ثلاثة أنواع من الأليل:

1 - ACE-2 . حذف / حذف(D / D) تعدد الأشكال

2- ACE-2 إدراج / حذف(I / D) تعدد الأشكال

3 - ACE-2. ادراج / ادراج((I / I) تعدد الأشكال

كل مريض يحمل (D / D) كانوا مصابين بالعدوى الشديدة ويزداد إنزيم AC E-2 ،لكن المرضى الذين يحملون (I / I) ، (I / D) كانوا مصابين بفيروس 19- covid. ارتبط تعدد الأشكال الجيني 2- ACE ومستويات نشاط2- ACE مع شدة19- كوفيد . تم العثور على المجموعات الثلاث المختارة مختلفة فيما يتعلق بالفيريتين ، ALT ، ACE ، CRP ، LDH ، ASTو .ALK القيم المتوسطة للمتغيرات Ferritin و LDH و CRP و ACE و ALT و ASTو ALK أعلى بشكل ملحوظ في شديد ومتوسط مقارنة بالناس الاصحاء .(HC)

ومن الممكن استنتاج الى أنه في حالة إصابة مجموعات الاصحاء هذه بفيروس covid-19 في المستقبل سوف تكون اصابتهم شديدة بسبب ارتباط D-alleleبنشاط2-ACE العالي ؛ لذلك ، فإن المرضى الذين يحملون ، D-allele وخاصة أولئك الذين لديهم النمط الجيني ، D / D هم أكثر عرضة للإصابة بالأمراض الرئوية و / أو الوفيات.

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تعدد الأشكال الجينية لمستوى الإنزيم المحول للأنجيوتنسين -2 ومستقبلاته وعلاقتها بخطورة جائحة كوفيد -19 من العراقيين

رسالة ماجستير

مقدمة الى مجلس كلية الطب - جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

سوزان حليم كامل

بكالوريوس تحليلات مرضية – الجامعة التقنية الوسطى – 2003

اشراف

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