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Immunological and Allele Specific Genotyping Study Among Patients with SARS-CoV-2

A thesis

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(فَوَجَدَا عَبْدًا مِّنْ عِبَادِنَا آتَيْنَاهُ رَحْمَةً مِّنْ عِندِنَا وَعَلَّمْنَاهُ مِن لَّدُنَّا عِلْمًا)

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Falah

2023

Dedication

The person who had sacrificed her health in my upbringing and education…… My true love mother

To the most precious person in my life, who encouraged me to reach the beach of Success……..... My big brother Mudher

To my pride and honor of the absent … the present, my God has mercy upon his soul…..….....My father

To my dear hero brother …… … Eng. Abed al-Warith

To the kindest hearts …….. My sisters

To the sweet heart ……... My wife

To the twilight stars ……. Precious children (Temogen, Muetaz and Yanogen)

The absent person who, but always present ……..

Falah

2023

Supervision Certification

I certify that, this thesis was prepared under my supervision at the College of Medicine, University of Babylon, as a partial fulfillment of the requirements for the degree of Doctorate in science of Medical Microbiology.

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We the members of the examining committee, certify that we´ve read the *Ph.D.* thesis entitled (**Immunological and Allele Specific Genotyping Study Among Patients with SARS-CoV-2).** We have examined the student (**Falah Hasan Obayes AL-Khikani),** in its contents on 19/6/2023. In our opinion it is meets the standards of thesis for the Degree of Doctorate of philosophy in Medical Microbiology.

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Summery

The disease caused by SARS-CoV-2 known as COVID-19, was considered a pandemic by the World Health Organization (WHO) and had the characteristic of being highly contagious and of rapid spread, which led to changes in human habits with an impacted the global health

The current study included 125 patients and 60 enrolled as a control group for immunological and genotyping investigations. All patients were admitted to Marjan Medical City, Al- Shomali general hospital, and Al-Sadeq hospital's COVID‑19 ward in the period between January 2022 to July 2022. The diagnosis COVID‑19 in each patient was confirmed by SARS-CoV-2-positive RT-PCR. Single nucleotide polymorphism (SNPs) for IL-12A-rs568408 and TYK2-rs2304256 were detected by Allele specific-PCR method. ELISA test used to determine concentration of interleukin- 35 (IL-35) and presepsin (PSN) in serum.

Patients' group included 56 (44.8%) males and 69 (55.2%) females, whereas, control group included 28 (46.6%) males and 32 (53.4%) females and there was no significant difference in the frequency distribution of patients and control subjects according to sex (P = 0.87). As well as there was no significant difference regarding age between patients and control subjects (P = 0.65).

IL-35 showed statistical significant differences between patients 6.86± 2.31ng/ml and control group 3.86± 2.07 (ng/ml) (P< 0.0001). PSN showed statistical significant differences between patients 318.18± 226.62 (pg/ml) and control group 199.68± 39 (pg/ml) (P< 0.0001).

The distribution of both genotyping and allele frequencies of IL-12A rs568408 revealed significant differences between patients and control groups (P= 0.006 and p=0.001 respectively). The IL12A rs568408 AA and AG variant genotypes were associated with a significantly increased risk of COVID-19 [odds ratio (OR) = 5.19, 95% Confidence interval (CI): 1.13-23.82; P= 0.034] and [OR = 2.39, 95% CI = 1.16- 4.94, P= 0.018] respectively compared with the wild-type GG homozygote.

Logistic regression analysis revealed that the frequencies of the homozygous variant AA and heterozygous variant AC of TYK2-rs2304256 were 14.4 % and 22.4 % in COVID-19 cases and 3.3 % and 16.7 % in healthy controls, respectively. For AA genotype (OR= 5.46, 95% CI: 1.21-24.61; P= 0.027). While AC genotype (OR = 1.7, 95% CI = 0.76- 3.81, P = 0.196), at TYK2-rs2304256 demonstrated that AA genotype showed a statistically significant risk for COVID-19 in Iraq compared with the wild-type rs2304256 CC. The variant rs568408 AC/AA genotypes were associated with a significantly increased risk of COVID-19 (OR= 2.81, 95% CI: 1.37-5.78; P= 0.004), compared with the wild-type rs2304256 CC.

In conclusion, IL-35 and Presepsin showed statistical significant differences between patients and controls (P< 0.0001) for each one. IL-35 can be used in diagnosis of COVID-19. The IL12A rs568408 AA and AG variant genotypes were associated with a significantly increased risk of COVID-19. In TYK2-rs2304256, AA genotype showed a statistically significant risk for COVID-19 compared with the wild-type rs2304256 CC.

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List of Abbreviations

Abbreviations	Mean
ACE2	Angiotensin converting enzyme 2
ADE	Antibody-dependent enhancement
AEC	Absolute eosinophil counts
ARDS	Acute respiratory distress syndrome
AUC	Area under the curve
B regs	Regulatory B cells
bp	Base pair
CBC	Complete blood counts
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
Cor.	Correlation
COVID-19	Coronavirus disease 2019
COX-2	Cyclooxygenase-2
CRP	C- reactive protein
CT	Computed tomography
CXCL-10	Motif chemokine ligand 10
CXR	Chest X-ray
DAMP	Damage-associated molecular pattern

dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMVs	Double-membrane vesicles
dNTPs	Deoxynucleoside triphosphates
EBI3	Epstein–Barr virus-induced gene 3
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte colony stimulating factor
GRA	Granulocyte
GSB	Gel Solubilization Buffer
GST	Glutathione S-Transferase
HBe Ag	Hepatitis Be antigen
HE	Hemagglutinin
HIV	Human immunodeficiency virus
HRCT	High-resolution CT
HRP	Horse radish peroxidase
IAV	Influenza A virus
ICU	Intensive care unit

IEM	Immunolabeling electron microscopy
IFN	Interferon
IgG	Immunoglobulin G
IL-35	Interleukin- 35
INR	International normalised ratio
IQR	The interquartile range
JAK	Janus kinases
KCl	Potassium chloride
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MCMV	Murine CMV
MCP-1	Monocyte chemoattractant protein-1
MERS-CoV	Middle East Respiratory Syndrome-CoV
$MgCl_2$	Magnesium chloride
MIP-1A	Macrophage inflammatory protein 1
MoDCs	Monocyte-derived dendritic cells
NAAT	Nucleic acid amplification test
ng/mL	Nanogram per millilitre
NK cells	Natural killer cells
NOS2A	Nitric oxide synthase 2A

OD	Optical density
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns
PCT	procalcitonin
pg/mL	Picograms per millilitre
PLpro	Papain-like protease
PRRs	Pattern recognition receptors
PSN	Presepsin
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
RBCs	Red blood cells
RBD	Receptor binding domain
RCU	Respiratory care unit
RdRp	RNA-dependent RNA polymerase
rpm	Rotation per minute or revolution per minute
RT-LAMP	Loop-mediated isothermal amplification-based assay
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcriptase real-time PCR
SAA	Serum amyloid A protein
SARS-CoV	Severe Acute Respiratory Syndrome-CoV

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard Deviation
Sig.	Significance
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphism
SPIEM	Solid-phase immune electron microscopy
STAT	Signal transducer and activator of transcription
T reg	Regulatory T cell
TBE buffer	Tris-Borate-Acetate
TLC	Total leukocyte count
TNF	Tumour necrosis factor
Tris. HCl	TRIS Hydrochloride
TYK2	Tyrosine kinase 2
UV	Ultraviolet
VWF	Von Willebrand factor
WBC	White Blood Cell
WHO	World Health Organization

Chapter One

Introduction and Literature Review

Introduction:

The number of confirmed cases of the 2019 novel coronavirus (COVID-19) continues to rise worldwide; the outbreak of the novel COVID-19 is regarding a serious threat to global health and had the characteristic of being highly contagious and of rapid spread, which led to changes in human habits with an impacted the global economy and health (Perez-Ramos *et al*., 2023).

In general, the pathophysiology regarding to COVID-19 is still unclear at this time. The immune-inflammatory response appears to play a key role in severe SARS-CoV-2 infections, depending on the recent clinical and experimental information obtained from COVID-19 research (Li *et al*., 2021).

The virus is typically rapidly spread from one person to another via respiratory droplets produced during coughing and sneezing. It is considered most contagious when people are symptomatic, although transmission may be possible before symptoms show in patients. Time from exposure and symptom onset is generally between two and 14 days, with an average of five days. Common symptoms include fever, cough, sneezing and shortness of breath. Complications may include pneumonia, throat pain and acute respiratory distress syndrome **(**Hafeez *et al*., 2020).

The standard tool of diagnosis is by reverse transcription polymerase chain reaction (rRT-PCR) from a throat swab or nasopharyngeal swab. The infection can also be diagnosed from a combination of symptoms, risk factors and a chest CT scan showing features of pneumonia **(**Aslani and Jacob, 2023).

Viruses have devised a variety of strategies for evading the immune system, including altering or redirecting immunological responses. While it's

still possible that SARS-CoV-2 has other immune evasion or disruption mechanisms that have not to be fully discovered (Sette and Crotty, 2021).

The tyrosine kinase 2 (TYK2) proteins, encoded by the TYK2 gene, belongs to the Janus kinases (JAK) family and plays an important role in signal transduction in response to cytokines such as type I interferon (IFN) and pro-inflammatory and anti-inflammatory cytokines (Murray, 2007). TYK2 is involved in a variety of immunological processes, including the activation of natural killer cells, the maturation of B and Treg cells, and the differentiation of Th1 and Th17 cells, in addition to its participation in the IFN-I and other type I and II cytokine receptor pathways (Deng *et al*., 2019).

IL-35 is a newly discovered heterodimeric cytokine that belongs to the IL-12 family, which comprises IL-12, IL-23, IL-27, and IL-35 (Niedbala *et al*., 2007). Interleukin-35 is thought to have a function in the regulation of autoimmune illnesses, inflammatory diseases, bacterial and viral infectious diseases, and malignancies. Along with new methodologies for examining receptors and signal transduction pathways, enables IL-35 to be considered as a potential immunotherapy target (Zhang *et al*., 2019).

Presepsin is a plasma soluble form of CD14 (sCD14), which is cleaved from the main membrane-bound form of CD14. It is a member of pattern recognition receptors (PRRs), which is upregulated on the surface of the phagocytes as a receptor for various groups of ligands particularly bacterial LPS endotoxin. Presepsin was discovered as a novel indicator whose value was used in the diagnosis and management of sepsis (Yaegashi et al., 2005).

Aim of the study

In view of the fore introduction, we designed this study to evaluate the association between Tyrosine kinase 2 and IL-12A genes polymorphisms in patients with COVID-19 through the following objectives:

1- Possible correlation between SARS-CoV-2 and polymorphism in TYK2-rs2304256 gene polymorphism. As well as polymorphism in IL12A rs568408 gene that associated with SARS-CoV-2 as a risk factor by Tri- allele specific- PCR technique.
2- Relation between IL-35 and SARS-CoV-2. As well as association between presepsin in severe patients with COVID-19 and healthy group by ELISA technique.

1. 1. General properties of coronavirus:

Coronaviruses are members of the Nidovirales order and the coronaviridae family. The family is split into two subfamilies: Coronavirinae and Torovirinae. It can be classified into the four main genera: Alpha-, Beta-, Gamma-, as well as Delta-coronaviruses SARS-CoV-2 is considered firmly belongs to the beta-coronaviruses, according to phylogenetic study (Phan *et al*., 2018).

Coronavirus disease 2019 (COVID-19), due to contagion with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that stated as a pandemic lately, majority of cases are self-limiting; nonetheless, it can proceed to a severe form with a high death rate at any time (Huang *et al*., 2020. The Severe Acute Respiratory Syndrome-Coronavirus flare-ups in both 2002 and 2003, as well as the Middle East Respiratory Syndrome-Coronavirus (MERS) outbreak in 2012, showed the scope for newly developing coronaviruses to be transmitted from animal to the human and individual to another (De Groot *et al*., 2013). HCoV229E, HCoV-OC43, MERS-CoV, SARS-CoV, HKU1, and SARS-CoV-2 are among the seven human coronaviruses (HCoVs) presently known (Asghari *et al*., 2020; Zhu *et al*., 2020).

Although viruses of the Beta-coronavirus lineage B (MERS-CoV, SARS-CoV-2, and SARS-CoV) infect humans, there are other human coronaviruses that have the ability to cause just mild disorders, except in children, elderly, and immunocompromised persons (Graham *et al*., 2013). Lineage A of the Beta-coronavirus (HCoVOC43 and HCovKU1) appears to have originated in

rats (Lau *et al*., 2015). HCoV-NL63 and HCoV-229E (Alpha-coronavirus), on the other hand, are thought to have originated in bats (Woo *et al*., 2012).

In the previous twenty years, MERS-CoV and SARS-CoV have recognized as epidemics with very high fatality percent reached to 34.4 % and 9.5%, respectively. COVID-19 was the third highly epidemic illness discovered, having a lower fatality rate than SARS and MERS, albeit mortality rates varied by country (Petrosillo *et al*., 2020).

Members of the Coronaviridae family are positive single stranded RNA genome with spherical virions, measuring 120–160 nm in diameter, with a large RNA (about 27 kb long RNA that encodes non-structural proteins such as papain-like protease (PLpro), RNA-dependent RNA polymerase (RdRp), main protease or chymotrypsin-like (Mpro or 3CLPRO), RNA helicase encoded by the replicase, and other accessory and regulatory proteins (King *et al*., 2012).

By using the genomic structure, the pathogenicity and related virulence traits of SARS-CoV-2 have been discovered. The genome of SARS-whole CoV-2 has been sequenced, and it has 29,903 nucleotides (Wu *et al*., 2020). SARS-CoV-2 has genetic traits with SARS-CoV and coronaviruses that are similar to SARS in bats. According to, the genome of SARS-CoV-2 shares 82% of its nucleotides with those of human SARS-CoV (Chan *et al*., 2020). Further genetic research revealed that SARS-CoV-2 shares 79% of its characteristics with SARS-CoV and is 50% identical to MERS-CoV. (Lu *et al*., 2020). SARS-CoV-2 shares 96.2% of its DNA with RaTG13, a bat coronavirus (Yan *et al*., 2020).

Virus-neutralizing antibodies are primarily induced by the S protein. Furthermore, the antigenic drift and shift in the S and hemagglutinin (HE) proteins are very varied, implying substantial antigenic drift and changes. As a result of their ability to adapt to different settings by different mutation and genetic recombination, coronaviruses can effectively change their host range and tissue tropism (Graham *et al*., 2010).

Previous research suggested that some bat species mostly capable of spreading the coronavirus to animals, including raccoon, dog, and ferret-badger (Lau *et al*., 2005). Other studies (Zhou *et al*., 2018) have shown the relevance of bats can act as a reservoir to the virus and other mammal animals may be intermediate hosts that help genetic alterations of the virus, which can lead to host species diversification and higher disease severity. Bats were most likely the reservoir of COVID-19, a novel human pandemic virus (Wu *et al*., 2020).

1. 2. Pathogenesis associated with SARS-CoV-2:

In general, the pathophysiology regarding to COVID-19 is still unclear at this time. The immune-inflammatory response appears to play a key role in severe SARS-CoV-2 infections, depending on the recent clinical and experimental information obtained from COVID-19 research (Li *et al*., 2021).

A series of processes are required for SARS-CoV-2 to be inside the target cells and release its genomes into host cells. The spike proteins are used by the virus to detect transmissibility and viral tropism. It also targets human pulmonary epithelial cells that carry angiotensin converting enzyme 2 (ACE2) receptors on their surfaces, suggesting that RBD has a similar structure to SARS-CoV. After the S1-RBD attaches to the ACE2 receptor, host cell-

surface proteases like transmembrane serine protease 2 act on a critical cleavage spot on S2. As a result, membrane of the host is fused and viral infection is ensued. When the virus enters the cell, the uncoated genomic RNA is translated into certain polyproteins (pp1a and pp1ab), which are then structured into replication/transcription combinations with virus-induced double-membrane vesicles (DMVs). The complex then replicates and forms a nested array of subgenomic RNA, expressing protein molecules and auxiliary proteins, via genome transcription (Voto *et al*., 2020).

The complexes of both endoplasmic reticulums besides the Golgi organelles are mediate the assembly of newly generated viral particles. Lastly, particles of the virus are released and discharged into compartment of the extracellular milieu. As a result, the replication cycle of the virus and the development of the virus begin (Ashour *et al*., 2020). Because coronaviruses can proofread during replication, their mutation rates are lower than those of other RNA viruses (Cevik *et al*., 2020).

According to earlier study, SARS may have three phases: viral replication, immune hyperactivity, and pulmonary damage (Navas-Martn *et al*., 2004). The viremia phase, acute phase, and recovery phase have been postulated as the clinical stages of COVID-19 (Lin *et al*., 2020). According to the following phases, the course of infection may generally be gone through (Navas-Martn *et al*., 2004; Millet and Whittaker, 2015): Viral multiplication inside infected host cells, dysregulated immune system response, damage to several organs, and eventually recovery stage (Li *et al*., 2021).

Respiratory particles and aerosols transfer the infection from human to human. Once within the body, the virus binds to host receptors and enters host

cells either via endocytosis or by membrane fusion. Coronaviruses are made up of four viral structural proteins: surface spike (S), membrane (M), envelop (E), as well as nucleocapsid (N) proteins (Jackson *et al*., 2022).

The surface S protein that protrudes on the viral surface and it is considered the most important for host cell binding and penetration. It is made up of two essential subunits (S1 and S2), S1 in charge of attachment to the host cell receptor while S2 is in charge of fusion of the viral and host cellular membrane (Bosch *et al*., 2003) (Figure 1-1).

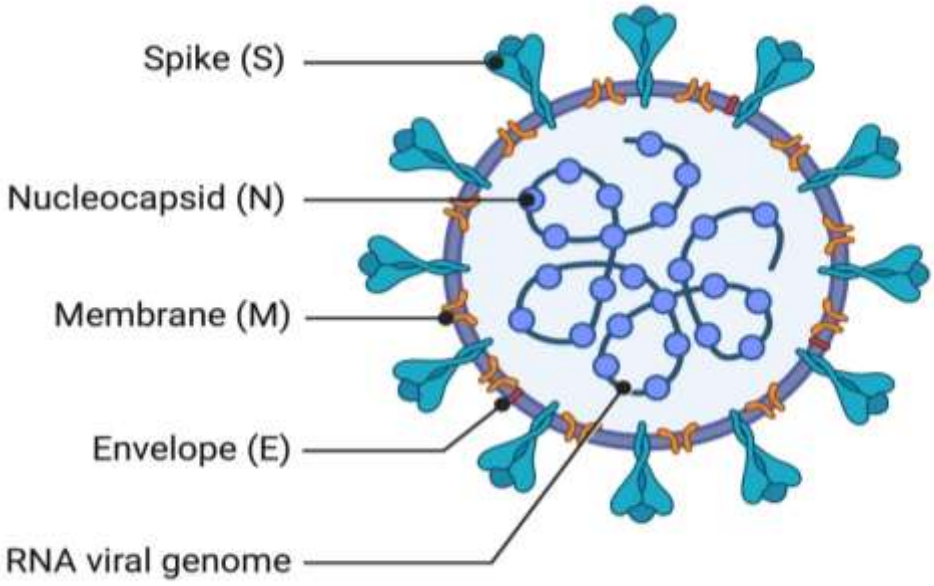

Figure (1-1): Structure of SARS-CoV-2 (Viral zone, 2022)

The receptor binding region of the S protein interacts to the peptidase domain of angiotensin-converting enzyme 2 (ACE 2) in the S1 subunit. The S2 subunit is substantially conserved in SARS-CoV-2 and is thought to be a potential antiviral target (Cevik *et al*., 2020).

SARS-CoV-2 binds to nasal epithelial cells in the upper respiratory tract during the asymptomatic phase. The ACE-2 receptor is the primary host receptor for viral entrance into cells, and it is shown to be abundantly expressed in adult nasal epithelial cells (Hoffmann *et al*., 2020; Wan *et al*., 2020). Local replication and proliferation of the virus, as well as infection of ciliated cells in the conducting airways, occur (Sims *et al*., 2005). This stage lasts a few days, and the immunological response produced at this time is minimal. The patients are very infectious, although having a low viral load at this time, and the virus may be diagnosed using nasal swab testing (Parasher, 2021).

The locations of infection and patient symptoms may be explained by the distribution of ACE 2 receptors in different organs. The ACE 2 receptor, for example, is located on the epithelial cells of various organs including the gut and endothelial cells in the kidney and blood arteries, which might explain gastrointestinal symptoms and cardiovascular issues (Monteil *et al*., 2020).

Much is yet unknown. Is endothelial dysfunction or pathological alteration inside the pulmonary tract due to direct viral infection, cytokine dysregulation, or coagulopathy, or are they multifactorial? Is it possible that direct effect of the viral invasion or may be coagulopathy contributes straightly to some of the ischemic complications like infarcts? (Cevik *et al*., 2020).

Septic shock and multi-organ dysfunction are also common in some cases. Early in COVID-19 disease, for example, the cardiovascular system is frequently engaged, as evidenced by the generation of extremely sensitive troponin and natriuretic peptides. Focal intra-alveolar bleeding and platelet-fibrin thrombi in tiny artery arteries are also detected, which is consistent with

the clinical setting of coagulopathy (Carsana *et al*., 2020). In certain cases, however, additional amplification of the immune response and accumulation of cytokines in other organs can result in significant tissue injury, or a cytokine release phenomenon (cytokine storm), which can lead to very dangerous outcomes such as capillary leak, thrombus development, and organ failure (Takahashi *et al*., 2020).

SARS-CoV-2 inhaled virus attaches to epithelial cells in the nasal cavity and begins reproducing. Both SARS-CoV2 and SARS-CoV1 use the ACE2 receptor (Hoffmann *et al*., 2020; Wan *et al*., 2020). According to in vitro investigations with SARS-CoV, the ciliated cells are the first cells attacked in the conducting airways (Sims *et al*., 2005). However, single-cell RNA reveals that conducting airway cells express just a little amount of ACE2 and that there is no apparent cell type preference, suggesting that this theory may need to be reconsidered (Reyfman *et al*., 2020).

1.3. Symptoms of COVID-19:

COVID-19 infection symptoms appear 2-5 days after the start of incubation (Li *et al*., 2020). The interval between the onset of COVID-19 symptoms and death, with a median of 14 days, was 6 to 41 days. The amount of time depends on the patient's age and immune system condition. Those older than 70 years compared to less than 70 years had shorter recovery times (Wang *et al*., 2020).

Around 80% of those infected will get a mild sickness that mostly affects the upper and conducting airways. These individuals can be monitored at home and treated symptomatically (Wu and McGoogan, 2020).

The most common symptoms of COVID-19 infection are fever, cough, and tiredness, while other signs and symptoms include sputum production, headache, haemoptysis, diarrhoea, dyspnea, and lymphopenia (Ren *et al*., 2020; Wang *et al*., 2020).

Although pneumonia was detected by a chest Computed tomography (CT) scan, other abnormalities including acute respiratory distress syndrome, acute heart injury, and a higher incidence of grand-glass opacities were also seen, all of which elevated the risk of death (Huang *et al*., 2020). Other people had multiple peripheral ground-glass opacities in the subpleural regions of both lungs, which most likely sparked a localized immune response and increased inflammation. Sadly, interferon inhalation treatment in some patients appeared to increase pulmonary opacities and had no clinical effect (Lei *et al*., 2020).

It's important to note that COVID-19's symptoms, such as fever, dry cough, dyspnea, and bilateral ground-glass opacities on chest CT scans, are comparable to those of prior betacoronaviruses (Huang *et al*., 2020). Nevertheless, COVID-19 had certain unique clinical traits, including the targeting of the lower airway as evidenced by upper respiratory tract symptoms as rhinorrhoea, sneezing, and sore throat (Lee *et al*., 2003; Assiri *et al*., 2013). The results of chest radiographs performed upon admission also show that some of the patients have an infiltration in the upper lobe of the lung, which is associated with greater dyspnea and hypoxemia (Phan *et al*., 2020). Diarrhoea and other gastrointestinal symptoms were present in COVID-19 patients, although they were not as common in MERS-CoV or SARS-CoV patients. In order to rule out a potential secondary transmission pathway, such as through patients or healthcare workers, it is crucial to analyse samples of feces and urine (Lee *et al*., 2003; Assiri *et al*., 2013). In

order to develop methods to stop and/or reduce transmission as well as therapies to manage the condition, it is essential to develop techniques to detect the various pathways of transmission, such as fecal and urine samples (Rothan and Byrareddy, 2020).

The virus migrates from the nasal epithelium to the upper respiratory tract through the conducting airways during invasion and infection of the upper respiratory tract. The disease appears as fever, lethargy, and a dry cough due to the involvement of the upper airways. There is a greater immune response during this phase involving the release of C-X-C motif chemokine ligand 10 (CXCL-10) and interferons (IFN-β and IFN-λ) from the virus-infected cells. The majority of patients do not progress beyond this phase as the mounted immune response is sufficient to contain the spread of infection (Parasher, 2021).

1. 4. Transmission of COVID-19:

The primary mode of transmission of SARS-CoV-2, like other coronaviruses, is through infected respiratory droplets, with viral infection occurring through direct or indirect contact with nasal, conjunctival, or oral mucosa when respiratory particles are inhaled or deposited on these mucous membranes.

The epithelium of the human respiratory system, comprising the oropharynx and upper airway, contains the majority of target host receptors. Infection can also spread through the conjunctiva and gastrointestinal tract, which can act as transmission gateways (Hui *et al*., 2020). As previously stated, transmission risk is determined by elements such as contact pattern,

environment, infectiousness of the host, and socioeconomic factors (Cevik *et al*., 2021).

Close range contact (such as 15 minutes face to face and within 2 m) accounts for the majority of transmission (Wölfel *et al*., 2020), and dissemination is most efficient inside homes and through gatherings of family and friends. Household secondary attack rates are ranging from 4% to 35% (the proportion of susceptible persons that become infected within a group of susceptible contacts with a main case). Infection risk is increased by sleeping in the same room as, or being married to, an infected person, while infection risk is reduced by isolating the sick person from the rest of the family (Cevik *et al*., 2021).

Other high-risk behaviors include eating in close proximity to an infected person, sharing meals, and participating in group activities. In comparison to outside settings, the risk of infection is significantly higher in enclosed surroundings (Cevik *et al*., 2021). A thorough study of transmission clusters, for example, discovered that the majority of superspreading episodes happened inside (Buitrago-Garcia *et al*., 2020). Aerosol transmission can still be an issue during a protracted stay in a busy, poorly ventilated indoor environment (meaning transmission might happen at a distance of more than 2 meters) (Cevik *et al*., 2021; Klompas *et al*., 2020).

The importance of faeces shedding in SARS-CoV-2 transmission, as well as the degree of fomite (through inanimate surfaces) transmission, are yet unknown. On smooth surfaces (stainless steel, plastic, glass) and at lower temperatures and humidity (eg, air conditioned surroundings), both SARS-CoV-2 and SARS-CoV-1 remain viable for several days (Chin *et al*., 2020;

Van Doremalen *et al*., 2020). As a result, illness can be transmitted from contaminated surfaces to the mucosa of the eyes, nose, and mouth via unwashed hands. This mode of transmission may have a role, particularly in institutions with common spaces, where the risk of environmental contamination is higher.

Both SARS-CoV-1 and SARS-CoV-2 are easily inactivated by conventional disinfectants, emphasizing the need of surface cleanliness and hand washing. SARS-CoV-2 RNA has been identified in stool samples, and RNA shedding lasts much longer than it does in respiratory samples; however, viral isolation from stool samples has proved difficult (Cevik *et al*., 2021). There are no documented studies on faecal-oral transfer. In the case of SARS-CoV-1, faecal-oral transmission was not thought to occur in most cases; nonetheless, one deadly epidemic was blamed on the virus being aerosolized and transmitted throughout an apartment building due to a malfunctioning sewage system (Kang *et al*., 2020). It's unclear whether SARS-CoV-2 will transmit in the same way.

It is spread mostly by respiratory droplet transmission, which happens when a person comes into close contact with someone who is coughing or sneezing. This happens when the host's mucosal surfaces, such as the eyes, nose, and mouth, are exposed to infective respiratory droplets (Huang *et al*., 2020). The virus can also be transmitted by fomites such as bedsheets, blankets, kitchen utensils, thermometers, and stethoscopes that are used by or on the infected person. COVID-19 has not been reported to spread through the air, except in the case of procedures that produce aerosols, such as endotracheal intubation, bronchoscopy, open suctioning, nebulization with oxygen, bronchodilators or steroids, bag and mask ventilation before

intubation, tracheostomy, and cardiopulmonary resuscitation (Ong *et al*., 2020).

COVID-19 has a 5–6 day incubation period, although it can take up to 14 days to show symptoms after being exposed to the virus. Infected individuals can be infectious and transfer the virus to healthy persons in the community during this period, often known as the 'presymptomatic' phase (Young *et al*., 2020).

1. 5. Immunity to SARS-CoV-2:

1.5.1. Innate immunity: The innate immune system is the initial line of defense, detecting viruses via pattern recognition receptors and triggering inflammatory pathways that aid in viral clearance. Innate immune responses help detect and eliminate infected cells, coordinate and speed the development of adaptive immunity, and restrict viral entrance, translation, replication, and assembly. Pattern recognition receptors (PRRs) on cell surfaces, endosomes, and cytosols respond to pathogen-associated molecular patterns (PAMPs) by triggering inflammatory responses and programmed cell death, which restrict viral infection and facilitate clearance (Kanneganti, 2020).

Viruses have devised a variety of strategies for evading the immune system, including altering or redirecting immunological responses. While it's still possible that SARS-CoV-2 has other immune evasion or disruption mechanisms that have yet to be discovered (Sette and Crotty, 2021).

Lympopenia is a prevalent symptom in SARS, MERS, and COVID-19 patients, and it can predict the development of pneumonia and the progression to respiratory failure (Chen *et al*., 2020; Huang *et al*., 2020). More

importantly, while the infection and immune patterns of SARS-CoV-2 are still being studied, elevated plasma levels of TNF- α, IL-2, IL-7, IL-10, granulocyte colony stimulating factor (G-CSF), interferon- γ induced protein 10 (IP10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 (MIP-1A), and C-reactive protein (CRP) may be markers of severe infection in the early stages of infection (Chen *et al*., 2020; Huang *et al*., 2020), suggesting that hypercytokinemia-related immunopathology may serve a fundamental role in severe COVID-19. Because of their weakened immune responses and lower ability to heal injured epithelia, the elderly are particularly vulnerable. The elderly also have lower mucociliary clearance, which may make it easier for the virus to move to the lung's gas exchange units (Ho *et al*., 2001).

1.5.2. Adaptive immunity: A Th2-bias hypothesis led to the early focus on the T cell immunopathological model (Peeples, 2020). This theory has not been proven. Another possibility is T cell immunopathology in the lungs by cells that cannot be found in the blood. Such idea is also not supported by the available facts. While some data suggesting a connection between T cell responses and COVID-19 immunopathology, the overwhelming body of research points to innate immune cells in the lungs as the main mediators of immunopathology. Some people with severe COVID-19 have high frequencies of circulating CD8+ T cells with unknown specificities, in contrast to those who have the condition and have limited virus-specific T cell responses (Mathew *et al*., 2020), raising the possibility that these cells play a role in immunopathogenesis (Mathew *et al*., 2020; Moderbacher *et al*., 2020). One of the challenges is that intricate, intertwined unfavorable outcomes might appear after many other negative things have already happened in ICU

patients. There is an urgent need for larger studies of virus-specific T cell responses in acute illness, and studies that start earlier in the disease are more informative (Sette and Crotty, 2021).

The results that are now available are positive for vaccine development since SARS-CoV-2 adaptive immune responses are linked to infection control, and there isn't any evidence in the literature linking adaptive immunity to the severity of COVID-19 illness (Sette and Crotty, 2021).

Specific IgA, IgM, and IgG responses are part of the host humoral response to SARS-CoV-2. Approximately 10 days after the beginning of symptoms, the majority of COVID-19 patients have a particular Ab response (Swerdlow and Finelli, 2020). In a study of 82 confirmed and 58 probable COVID-19 cases, the specific IgM and IgA Abs were detected earlier while IgG was detected on day 14 (10-18 days) after symptom onset (Guo *et al*., 2020).

COVID-19 instances have been linked to autoantibodies. Transient autoreactivity in serum is prevalent in response to an acute viral infection. Autoantibodies induced by infections, on the other hand, can be harmful (e.g., *S. pyogenes* and rheumatic heart disease), and it's uncertain if autoantibodies triggered by SARS-CoV-2 infection are exceptionally common or harmful (Zou *et al*., 2020).

Various viral proteins in SARS-CoV-2 can induce humeral immune response, but the spike (S) protein and nucleocapsid are the ones most commonly utilized in serological diagnosis. Few antibodies are detected in the first four days of sickness, but they grow with time, with the majority of patients having a detectable response by four weeks. There have been reports

of a wide range of virus-neutralizing antibodies, and new research shows that they may correlate with severity but fade with time (Perera *et al*., 2020).

Longer follow-up investigations are needed to determine the duration and protectiveness of antibody and T cell responses. Cross-reactivity between CD-4 T cell responses to endemic human coronaviruses and SARS-CoV-2 appears, but their relevance in protection is unknown (Grifoni *et al*., 2020).

1.5.3. Immunity and lung damage: The immunological response to a viral infection can sometimes be more harmful than the virus itself. As a result, immunopathogenesis must be considered in addition to adaptive immunity's role in controlling COVID-19. While the findings suggest that COVID-19 lung disease is caused by hyperactive innate immunity, other theories to explore include T cells or antibodies play the central cause of immunopathogenesis.

Despite the fact that the term "antibody-dependent enhancement" (ADE) of infection is frequently used, ADE was not observed in SARS or MERS. Furthermore, antibody-associated disease pathology with specific SARS vaccines was not FcR-dependent and appears to be more likely caused by immune complex-driven inflammation (Sariol and Perlman, 2020). Rarely are viral infections documented for ADE in vivo. It's unclear if immune complex-driven inflammation occurs in COVID-19 patients with severe COVID-19. Spike, neutralizing, and nucleocapsid antibody quality are comparable in hospitalized and non-hospitalized cases (Piccoli *et al*., 2020). Spike antibodies may be involved in lowering viral loads, according to two studies that showed a preference for nucleocapsid antibodies over Spike antibodies in fatal cases. (Zohar *et al*., 2020; Atyeo *et al*., 2020)

It's unclear if SARS-CoV-2 might directly infect and impair immune cells given that ACE2 is expressed in immune cells including monocytes/macrophages and lymphocytes (Weiss and Leibowitz, 2011). Moreover, immune cells can travel about the body. Immune cells that have been exposed to SARS-CoV-2 may thereby let the virus to propagate throughout the body. The typical kind of damage caused by SARS-CoV-2 infection also affects the immune system, according to pathological studies using COVID-19 models. Spleen and lymphoid atrophy are connected to considerable cytokine activity, suggesting that SARS-CoV-2 may directly impair immune cells (Bermejo-Martin *et al*., 2020 ; Chan *et al*., 2020).

Endogenous damage-associated molecular pattern DAMPs, including DNA, RNA, ATP, heat shock proteins, high mobility group protein B1, and the extracellular matrix, can be released in response to cellular injury and necrosis. These substances can be recognized and activated by corresponding PRRs, which then encourages the release of cytokines and chemokines, which can exacerbate the inflammatory response and tissue damage, creating a vicious cycle (Eppensteiner *et al*., 2019). Both DAMPs and pathogen-associated molecular patterns (PAMPs) are expected to contribute to the systemic dysregulation of the innate immune response, which is thought to play a part in the emergence of MODS in COVID-19. SARS-CoV-2 may activate PRRs, resulting in cell death and organ failure as well as an innate immune response to the virus (Kanneganti, 2020).

Lower respiratory tract involvement and progression to acute respiratory distress syndrome (ARDS) Approximately one-fifth of all infected people reach this stage of the illness and experience severe symptoms. Via the host receptor ACE-2, the virus invades and infects type 2 alveolar epithelial cells, where it

begins replication to create additional viral nucleocapsids. Interleukins (IL-1, IL-6, IL-8, and IL-12), tumour necrosis factor (TNF- α), IFN- λ, and IFN- β, CXCL10, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 (MIP-1) are among the cytokines and inflammatory markers released by virus-infected pneumocytes. This 'cytokine storm' attracts neutrophils, CD4 helper T cells, and CD8 cytotoxic T cells, which ultimately become trapped in lung tissue. These cells are in charge of battling the virus, but they are also in charge of the resulting inflammation and lung harm. The host cell dies, releasing more virus particles, which infect nearby type 2 alveolar epithelial cells in the same way. There is extensive alveolar damage as a result of the chronic injury induced by sequestered inflammatory cells and viral replication, which leads to the loss of both type 1 and type 2 pneumocytes, resulting in acute respiratory distress syndrome (Xu *et al*., 2022; Cascella *et al*., 2022).

1. 6. Tyrosine kinase 2 gene:

This gene codes for a protein that belongs to the tyrosine kinase family, specifically the Janus kinases (JAKs). This protein binds to the cytoplasmic domain of type I and type II cytokine receptors and phosphorylates receptor subunits to propagate cytokine signals. It's also a part of the interferon signaling pathways for both type I and type III. As a result, it might be involved in antiviral immunity. Immunodeficiency has been linked to a mutation in this gene. This gene has been investigated for its role in coronavirus biology, and it plays a role in immune response or antiviral activity (Murray, 2007).

1.6.1. General information of Tyrosine kinase 2:

The tyrosine kinase 2 (Tyk2) proteins, encoded by the TYK2 gene, it plays an important role in signal transduction in response to cytokines such as type I interferon (IFN) and proinflammatory and antiinflammatory cytokines (Murray, 2007). Tyk2 is a component of the signal transducers and activators of transcription (STAT) signaling pathway that binds to the type I interferon-a receptor (IFNAR) on the cell surface of IFN-producing cells, contributing to the development of autoimmune and inflammatory illnesses (Kyogoku and Tsuchiya, 2007).

The four JAK proteins (JAK1, JAK2, JAK3, and TYK2) connect with different cytokine receptors in a specific way (Strobl *et al*., 2011). JAK1 and JAK2 have a wide range of activities, although JAK3 and TYK2 are predominantly involved in immune responses. TYK2 binds to receptor chains used by a variety of cytokines, including IL6-R, which is targeted by tocilizumab, an anti-IL6R monoclonal antibody used to treat RA (Strobl *et al*., 2011; McInnes and Schett, 2011).

The TYK2 gene is found on chromosome 19p13.2 and encodes Tyk2 Polymorphisms in the TYK2 gene have been linked to a variety of autoimmune illnesses, as well as endometriosis (Peluso *et al*., 2013).

TYK2 is a non-receptor protein that binds to the inactive version of the IFN-I receptor (IFNAR1) on the cell surface. After IFN- binds to IFNAR1, the TYK2 and JAK1 proteins are activated, causing the signal transducers and activators of transcription (STAT) 1 and 2 to be recruited and phosphorylated. STAT1/2 heterodimers then translocate to the nucleus, where they act as

important regulators of IFN-stimulated gene expression (Yamaoka *et al*., 2004).

TYK2 is also linked to the cytokine receptors IL-6, IL-10, IL-12, and IL-23, and is involved in the activation of these pathways (O'Shea and Plenge, 2012). IFN-I and other cytokines, as well as JAK kinase members, are known to have a role in the etiology of autoimmune disorders (O'Shea and Plenge, 2012; Deng *et al*., 2019). TYK2 is involved in a variety of immunological processes, including the activation of natural killer cells, the maturation of B and regulatory T (T reg.) cells, and the differentiation of Th1 and Th17 cells, in addition to its participation in the IFN-I and other type I and II cytokine receptor pathways. As a result, autoimmune disorders, particularly systemic lupus erythematosus (SLE), has been linked to dysregulated TYK2 expression (Deng *et al*., 2019).

1.6.2. Tyrosine kinase 2 functions:

The tyrosine kinase protein binds to the cytoplasmic domain of type I and type II cytokine receptors and phosphorylates receptor subunits to propagate cytokine signals. It's also a part of the interferon signaling pathways for both type I and type III. As a result, it might play a part in antiviral immunity (Shimoda *et al*., 2000).

Cytokines regulate the survival, proliferation, differentiation, and function of immune cells as well as cells from other organ systems, and hence play an important role in immunity and inflammation. As a result, treating these illnesses by targeting cytokines and their receptors is a viable option. To alter intracellular signaling, type I and II cytokine receptors collaborate with Janus family kinases (JAKs). Interleukins, interferons, and hemopoietins are

cytokines that activate Janus kinases, which then interact with their respective receptors (Kubo *et al*., 2003).

JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) are the four members of the mammalian JAK family When a scan for genes involved in interferon type I (IFN-1) signaling found Tyk2 as a crucial element, which is activated by a variety of cytokine receptors, the link between Jaks and cytokine signaling was first discovered (Chen *et al*., 2022).

Tyk2 has a larger and more significant role in humans than previously thought based on murine models, which suggest Tyk2 is predominantly involved in IL-12 and type I-IFN signaling. In human cells, Tyk2 loss has a more severe effect than in mice cells. Tyk2 has important influence on the transduction of IL-23, IL-10, and IL-6 signals, in addition to IFN and IL-12 signaling. Because IL-6 signals through the gp-130 receptor-chain, which is shared by a large family of cytokines including IL-6, IL-11, IL-27, IL-31, oncostatin M (OSM), ciliary neurotrophic factor, cardiotrophin 1, cardiotrophin-like cytokine, and LIF, Tyk2 could affect signaling through these cytokines as well. It was shown that IL-12 and IL-23 have similar ligand and receptor subunits that activate Tyk2.IL-10 is a critical anti-inflammatory cytokine, and IL-10 in mice suffer from fatal, systemic autoimmune disease (Shaw *et al*., 2006).

Tyk2 is triggered by IL-10, and its absence impairs the capacity to produce and react to this cytokine (Shaw *et al*., 2006). Immune cells are controlled by a variety of cytokines under physiological settings, and it has become obvious that cross-talk between distinct cytokine-signaling pathways

is important in the regulation of the JAK–STAT system (Shimoda *et al*., 2000).

1.6.3. Tyrosine kinase 2 (TYK2) deficiency:

Tyrosine kinase 2 (TYK2), which is connected with the receptors of type I IFN, interleukin (IL)-6, IL-10, IL-12, and IL-23, is one of the JAKs that plays a key role in the signaling of these cytokines (Strobl *et al*., 2011). A 22-year-old Japanese male patient with hyper-IgE syndrome and sensitivity to several infections, including Staphylococcus, mycobacteria, and herpes simplex virus, was the first to be diagnosed with TYK2 deficiency (Minegishi *et al*., 2006).

The patient's genomic DNA sequencing showed a homozygous frame-shift mutation in the TYK2 gene, resulting in a frameshift at codon 90 and premature translation termination. As a result, immunoblot analysis revealed that the patient's cells did not express any functional TYK2 protein. Type I IFN, IL-12, IL-23, IL-6, and IL-10 responses were practically absent in cells obtained from the TYK2-deficient patient. More subsequently, seven further TYK2-deficient individuals were subjected to a thorough immunological examination (Kreins *et al*., 2015).

Unlike the first TYK2-deficient patient, these TYK2-deficient patients' cells responded to type I IFN, IL-12, IL-23, and IL-10 with a reduced but not absent response. The study concluded that poor responses to IL-12 and type I IFN were to blame for all of the TYK2-deficient individuals' vulnerability to intracellular bacterial and/or viral infections (Kreins *et al*., 2015). The functional effects of a full TYK2-deficiency on clinical outcomes have been explained in all of these publications. The functional significance of additional

TYK2 variations (e.g., insertion, deletion, and substitution) is, nevertheless, little understood (Nemoto *et al*., 2018).

Two cases of immunodeficiency were linked to novel heterozygous mutations in the four-point-one, ezrin, radixin, moesin (FERM) domain region of TYK2 in one study. The patients had significant T-cell lymphopenia, with low naive CD4+ T-cell numbers, unlike prior studies on TYK2 deficiency. Furthermore, both individuals developed B-cell lymphoma linked to the Epstein-Barr virus (EBV). In the patient's cells, The cellular activities are similar to but not identical to those described earlier in the TYK2-deficient patient (Nemoto *et al*., 2018).

Tyrosine kinase 2 (Tyk2)-deficient mice show a specific deficiency in in vivo protection against particular viruses. Tyk2 is required for the defense against murine CMV (MCMV). In the absence of Tyk2, in vivo MCMV challenges demonstrated poor viral clearance from organs and lower mouse survival. In comparison to wild-type macrophages, MCMV replicates to considerably greater titers in Tyk2-deficient macrophages, according to in vitro experiments. An essential role of type I IFN (IFN-αβ) was appeared in the control of MCMV replication, with a prominent role of IFN-β. MCMV infection leads to the activation of STAT1 and STAT2 in an IFN-αβ receptor 1-dependent manner. Consistent with the role of Tyk2 in IFN-αβ signaling, activation of STAT1 and STAT2 is reduced in Tyk2-deficient cells. However, lack of Tyk2 results in impaired MCMV-mediated gene induction of only a subset of MCMV-induced IFN-αβ-responsive genes. Taken together, the data demonstrate a requirement for Tyk2 in the in vitro and in vivo antiviral defense against MCMV infection. In addition to the established role of Tyk2 as an amplifier of Jak/Stat signaling upon IFN-αβ stimulation, the provided

evidence for a novel role of Tyk2 as a modifier of host responses (Strobl *et al*., 2005).

1. 7. IL-12A gene:

This gene codes for a cytokine component that operates on T and natural killer cells and has a wide range of biological functions. The cytokine is a disulfide-linked heterodimer consisting of a 35-kD component expressed by this gene and a 40-kD cytokine receptor family subunit. The activator of transcription protein STAT4 regulates lymphocyte responses to this cytokine. The cytokine's signaling pathway in innate immunity is shown to be dependent on nitric oxide synthase 2A (NOS2A/NOS2) (Yuzhalin and Kutikhin, 2012).

1.8. Interleukin-35 (IL-35)

1. 8.1. General features and functions of Interleukin-35:

IL-35 discovered heterodimeric cytokine that belongs to the IL-12 family, which comprises IL-12, IL-23, IL-27, and IL-35 (Niedbala *et al*., 2007). Chain (p19, p28, or p35) and chain (p40 or Epstein–Barr virus-induced gene 3 (EBI3)) are the two subunits that make up each of the four members. P35 and EBI3 combine to generate IL-35, which shares p35 with IL-12 and EBI3 with IL-27. Despite their structural similarities, the biological functions of the cytokines of the IL-12 family differ to some extent (Vignali andKuchroo, 2012). IL-35 has previously been studied primarily in relation to infection, inflammation, and auto-immune disorders (Wirtz *et al*., 2011).

All members of the IL-12 family are heterodimers, and they are only active when both subunits are present, as previously stated. Because IL-12 and

IL-23, as well as IL-27, IL-35, and IL-39, share one subunit, these cytokines compete for subunits and receptors. As a result, their output levels may differ (Guo *et al*., 2019).

Unlike other members of the IL-12 family, which are known to be produced largely by activated antigen-presenting cells. It has been discovered that it is secreted by a variety of cell types and organs, including regulatory B cells (Wang *et al*., 1014), dendritic cells (Dixon *et al*., 2015), endothelial cells, smooth muscle cells, and monocytes (Sha *et al*., 2015).

Unlike other members of the IL-12 family, IL-35 has four receptors: GP130–GP130, IL-12R2–IL-12R2, IL-12R2–GP130, and IL-12R2–IL-27R2. Following IL-35's interaction to its receptors, downstream signaling occurs via a unique heterodimer produced by STAT1 and STAT4, resulting in the production of target genes such as EBI3 and P35. These target genes trigger a feedback loop that boosts IL-35 expression (Collison *et al*., 2012). STAT1, STAT3, STAT4, JAK1, and JAK2 components make up the IL-35 signaling pathway (Floss *et al*., 2017)

Other members of the IL-12 cytokine family have a lot in common with IL-35 in terms of structure and makeup. Its function, on the other hand, differs significantly from that of the other members, to the point where it has consequences that are diametrically opposed to those of the other members. Treg cells that produce IL-35 have a lot of therapeutic promise. Furthermore, IL-35 works as an inhibitory cytokine in the immune system, modulating malfunctioning T cells, activating bone-marrow-derived immunosuppressive cells, and regulating various immune-related inflammatory factors. As a result, the control of IL-35 is critical in immunological diseases. B cells can

also release IL-35 and have an immunosuppressive effect in autoimmune disorders and bacterial infectious diseases. These findings imply that a variety of stimulants and immunological microenvironments can influence IL-35 production (Zhang *et al*., 2019).

Interleukin-35 is also thought to have a function in the regulation of autoimmune illnesses, bacterial and viral infectious diseases, and malignancies. IL-35 may be considered as a potential immunotherapy target (Zhang *et al*., 2019).

The ability of IL-35 to bind to distinct receptor subunits is, however, dependent on the cell type. IL-35 signaling activates STAT1 and STAT4 in T cells via three receptor subunits: GP130–GP130, IL-12R2–IL-12R2, and IL-12R2–GP130 (Garbers *et al*., 2015). GP130, IL-27R, IL-12R1, and IL-12R2 are the four receptor subunits found in regulatory B cells. Interestingly, rather than GP130 and IL-12R1, IL-35 signaling in B cells happens through IL-27R and IL-12R2, which activate STAT1 and STAT3, according to a research that used short interfering RNA to silence each component separately (Olson *et al*., 2013).

Furthermore, investigations have demonstrated that IL-35 expression is reduced in chronic obstructive pulmonary disease (COPD), a type of chronic bronchitis and emphysema marked by airflow restriction (Himani *et al*., 2018). Furthermore, lower IL-35 levels were inversely linked with smoking status, suggesting that IL-35 might be used as a biomarker to predict chronic obstructive pulmonary disease development. According to the findings, IL-35 is an excellent indication of allergic inflammation and can be utilized as a biomarker (Zhang *et al*., 2019).

Advances in IL-35 research, along with new tools for examining receptors and signaling cascades, allow IL-35 to be considered as a novel therapeutic target for immune modulation. The prospect of combining IL-35 into innovative treatment techniques to treat severe immunological disorders suggests that IL-35 will have more clinical importance in the near future. IL-35 can be used as a therapy method for immune-related disorders because of its many immunomodulatory effects in various diseases. If the expression of IL-35 is low in an illness, for example, IL-35 recombinant protein can be used to treat it. If the expression of IL-35 is considerably increased in an illness, IL-35 can be inhibited (Zhang *et al*., 2019).

1.8.2. Mechanisms action of IL-35:

IL-35 reduces inflammatory responses by modulating different cytokines and thereby controlling STAT signaling, unlike IL-12 and IL-23 (Liu *et al*., 2019). As a result, IL-35 can activate STAT1 and STAT4 in T cells and trigger STAT1 and STAT3 in B cells when it binds to IL-35R. IL-35 suppresses the development of monocyte-derived dendritic cells (MoDCs) by activating the STAT 1/3 pathways while concurrently suppressing the p38 MAPK and NF-κb signaling pathways, reducing pro-inflammatory activities (Chen *et al*., 2018).

IL-35 reduces T cell proliferation by counteracting the action of IL-12, which is critical in generating the positive feedback that connects T cells and IFN-γ. IL-35 causes significant damage to CD8+T cells, resulting in immunity breakdown or immunological malfunction (Liu *et al*., 2019). Furthermore, research shows that a lack of IL-10 promotes the production of IL-35, which may decrease Th2 cell development and cytokine synthesis. Another

fascinating fact is that CD4 T cells cause the development of IL-35, which lowers IFN-γ, but CD4 T cell-induced IL-12 substantially raises IFN-γ (Tao *et al*., 2018).

Eosinophilia, allergic asthma, and eosinophil-associated gastrointestinal illnesses are among the conditions in which increased eosinophils may be harmful. A study suggests that IL-35 reduces airway eosinophilia by inhibiting the synthesis of the eosinophil-attracting chemokines implying that IL-35 might be a potential treatment method for lowering eosinophil tissue recruitment in disorders like asthma (Kanai *et al*., 2017).

Increasing in IL-35 are due to the presence of regulatory T cells (Tregs) and regulatory B cells (Bregs) (Yang *et al*., 2019). Treg cells can promote the production of new Treg cells by inducing the transformation of Treg (iTreg) from CD4+Foxp3-T cells. IL-35-induced Treg cells (iTr35) produce more IL-35, and type 1 Treg cells (Tr1) use IL-35 to suppress the immune response (Xiang and Xie, 2015).

1. 8.3. Effect action of IL-35:

There are still only a few studies on IL-35, so the functions of IL-35 in combatting viral infection are not yet well understood (Guo *et al*., 2019). IL-35 is not favorable to Th17 and Th1 cell proliferation due to IFN-γ induced negative regulation, and it counteracts the activities of IFN-γ and TNF-α via STAT1 during HBV infection (Shao *et al*., 2017) Furthermore, via targeting HNF4, IL-35 increases HBV replication and transcription, resulting in higher amounts of HBV 3.5 kb mRNA, HBV core protein, and hepatitis Be antigen (HBeAg) secretion (Tao *et al*., 2018). IL-35 enhances immunological

tolerance by inhibiting pro-inflammatory cytokine expression in chronic HBV infection (Shao *et al*., 2017).

IL-35 may decrease IAV protein production by activating type I and type III IFNs (Wang *et al*., 2016). Furthermore, for IL-35 to operate properly, the STAT1 and STAT4 signaling pathways must be active (Guo *et al*., 2019).

The function of IL-35 during viral infection is poorly understood. Patients with seasonal influenza A virus (IAV) had higher amounts of IL-35 in their peripheral blood mononuclear cells and throat swabs than healthy people. IAV infection elevated IL-35 mRNA and protein levels in human lung epithelial and primary cells. IAV-induced IL-35 transcription is controlled by NF-B, according to another research. Select inhibitors of cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase dramatically reduced IL-35 expression, indicating that they are involved in IL-35 expression (Chen *et al*., 2016).

IL-35 may have inhibited IAV RNA replication and viral protein synthesis by inducing type I and III interferons (IFN), which then activated downstream IFN effectors such double-stranded RNA-dependent protein kinase, 2,5-oligoadenylate synthetase, and myxovirus resistance protein. Enterovirus 71, and vesicular stomatitis virus all showed significant antiviral action in the presence of IL-35. IL-35 is a new IAV-inducible cytokine that has antiviral action when produced (Wang *et al*., 2016).

To research post-influenza pneumococcal pneumonia and the function of IL-35 in host defense against post-influenza pneumococcal pneumonia, a mouse model was established. During mouse influenza infection, pulmonary IL-35 was quickly upregulated, which was largely mediated through the type I IFN-α/β receptor signaling pathway. In influenza-infected mice, secondary

pneumococcal infection resulted in a synergistic IL-35 response. Clinical investigation revealed that IL-35 levels in patients with influenza infection were much higher than in healthy people, indicating that influenza infection might cause IL-35 synthesis in human peripheral blood mononuclear cells. These findings imply that IL-35, at least in part, contributes to secondary pneumococcal pneumonia susceptibility by suppressing the early immune response (Chen *et al*., 2016).

Interleukin 35 (IL-35) is a cytokine made up of an Epstein-Barr virus–induced gene 3 chain and an IL-12 p35 chain that is mostly generated by regulatory T cells (Treg cells). IL-35 promotes cancer growth by causing tumorigenicity, protecting cancer cells from apoptosis, and facilitating cancer progression (Yazdani *et al*., 2020).

1. 9. Diagnosis of COVID-19:

Early diagnosis and isolation of suspected patients play a vital role in controlling this outbreak (Team, 2020). The specificity and sensitivity of various diagnostic procedures varies depending on the population and type of equipment used (Leeflang *et al*., 2013).

Epidemiological data, clinical symptoms, and some adjuvant technologies, such as nucleic acid detection and immunological tests, are utilized to make a COVID-19 diagnosis. In addition, high-throughput equipment (biosafety level-3) is required for the separation of SARS-CoV-2 to ensure worker safety. There are three main concerns: (i) reducing the number of false negatives by detecting small amounts of viral RNA; (ii) avoiding the number of false positives by correctly distinguishing positive signals from different

pathogens; and (iii) a high capacity for testing a large number of samples quickly and accurately (Caruana *et al*., 2020).

Despite significant attempts to limit the illness, the virus has remained widespread. Proper diagnosis utilizing quick technology, on the other hand, is critical. Individuals with new SARS-CoV-2 (or COVID19) have a wide range of symptoms, ranging from asymptomatic to acute respiratory distress syndrome and multi-organ failure. As a result, diagnosing COVID-19 accurately is difficult. COVID-19 is routinely diagnosed based on epidemiological history, clinical signs, and laboratory detection methods such as computed tomography (CT) scans, nucleic acid amplification test (NAAT), and serological approaches (Corman *et al*., 2020)

Specimens such as nasopharyngeal and/or oropharyngeal swabs, bronchoalveolar lavage fluid, sputum, bronchial aspirate, or blood are typically advised for early diagnosis of SARS-CoV-2 infection (Zou *et al*. 2020). For an accurate and timely identification of the causal agent, laboratory testing, in addition to clinical and epidemiological studies, is critical. This is also known to help with quarantine effectiveness (Rai *et al*., 2021).

The virus is spreading locally, but there is just a modest innate immune response. Nasal swabs can identify the virus at this stage. These people are infectious, even if their viral burden is low. The viral RNA RT-PCR result may be beneficial in predicting viral load, future infectivity, and clinical course. These investigations may be able to detect super spreaders. The sample collecting process would have to be standardised for the RT-PCR cycle number to be helpful. Swabs from the nose may be more sensitive than swabs from the throat (Mason, 2020).

Overall, the scientific community has developed various approaches beneficial for appropriately detecting a suspected case of COVID-19 infection in a short period of time. However, not only the test to be utilized, but also the patient's medical history, the timing of the suspected SARS-CoV-2 exposure, the type of sample to be obtained and analyzed, and how to interpret the result are all factors to consider when diagnosing COVID-19. Only by combining all of these factors will it be feasible to make an accurate COVID-19 infection diagnosis and successfully control the COVID-19 pandemic (Falzone *et al*., 2021).

1.9.1. Clinical presentation:

The majority of people infected with the virus will get a typical cold or flu, with only a few remaining asymptomatic. The condition will manifest itself in minor symptoms in 80% of patients (Hafeez *et al*., 2020). Adults have the highest immunity to resist the virus, but they are also more prone to transfer it. A recent study of nearly 140 patients at Wuhan University's Zhongnan Hospital identified a variety of symptoms that led to the development of COVID-19. Nearly all of the patients developed a fever with an extremely high temperature, while more than half of the patients also experienced fatigue and a dry cough. A dry cough and trouble breathing affected one-third of the patients (Lee *et al*., 2022).

According to the Chinese CDC, around 80% of coronavirus infections are light, 15% of patients have developed severe cases, and 5% of patients have been dangerously sick. A day-by-day analysis of coronavirus symptoms demonstrates how symptoms increase in average people and how COVID-19, the disease, progresses from poor to worse (Lee *et al*., 2022).

Day 1: The patient has fever, weariness, muscular discomfort, and a dry cough on the first day of the symptom. A few of them may have nausea and diarrhoea a few days before symptoms emerge. Day 5: Patients may experience breathing difficulties, particularly if they are old or have a medical condition. Day 7: These are the symptoms that led to the patient being admitted to the hospital, according to a Wuhan University research. Day 8: According to the Chinese CDC, 15% of patients get acute respiratory distress syndrome (ARDS), a disease in which fluid builds up in the lungs and is usually deadly. This is more common in severe situations. Day 10: As the illness progresses, the symptoms increase, and the patient is transferred to the intensive care unit (ICU). Patients with milder symptoms are more likely to experience stomach discomfort and hunger loss. Only a small %age of people die. The current mortality rate is around 2%. Day 17: Patients who recover are usually discharged from the hospital after two and a half weeks (Hafeez *et al*., 2020).

However, identifying symptoms in the early stages of an infection can be challenging (Lee *et al*., 2022). For verified coronavirus disease 2019 cases, symptoms have varied from mild to severe sickness and death. COVID-19 emergency warning indications include constant discomfort or pressure in the chest, difficulty breathing, disorientation, and pale lips or face, all of which require rapid medical intervention. Pneumonia develops when the disease worsens (Amanatidou *et al*., 2022). Because the virus was just recently discovered, the incubation time has yet to be defined. Symptoms might develop as soon as three days after exposure or as late as 13 days later, according to the new research. According to new study, the incubation time is roughly five days on average (Lee *et al*., 2022).

1.9.2. Serological diagnosis:

One of the most significant diagnostic tools in disease monitoring is detecting antibodies against a virus in infected patients. Though RT-qPCR is the most widely used method for diagnosing SARS-CoV-2 active patients, viral RNA becomes nearly undetectable 14 days after infection; moreover, false-negative findings might occur owing to inappropriate viral sample handling. These difficulties necessitate the development of simple test kits that identify human antibodies produced in response to viral infection. The detection of antibodies generated in response to viral infection (IgG and IgM) and/or viral antigen using enzyme-linked immunosorbent assays is the basic premise underpinning antibody-based immunodiagnostic (ELISA).

Antigen-specific antibodies can be discovered in a patient after 3 to 6 days, while IgG can be detected later in an infection, according to studies. These assays may be used to offer information on both present and previous illnesses, and they can be scaled up to evaluate thousands of samples in labs with limited resources. It may also be used in disease monitoring programs to have a better knowledge of the infection rate in the community. Although serological assays may detect both ongoing and previous infections, their effectiveness in verifying SARS-CoV-2-specific antibody responses to detect past infections is well established (Wang *et al*., 2003; Lee *et al*., 2010).

A study in China found that the titer of virus-specific antibodies in asymptomatic COVID-19 patients is much lower than in symptomatic COVID-19 patients (Long *et al*., 2020). The presence of IgM antibodies implies current viral infection, whereas the presence of IgG antibodies indicates past SARS-CoV-2 infection. As a result, immunodiagnostic tests are

also crucial for the development of COVID-19 vaccines. This also aids in determining the level of infection in those who are not currently infected (Rai *et al*., 2021).

There have been reports of a wide variety of virus-neutralizing antibodies, and new research shows that they may correspond with the severity of disease but fade with time (Cevik *et al*., 2020).

Quick antigenic and rapid antibody tests are faster than RT-PCR-based procedures, with execution durations of 15-30 minutes, a cheaper cost, and a simpler approach that does not require the presence of highly skilled individuals (Pilarowski *et al*., 2021).

The low viral load and low antibody response observed in some patients are primarily responsible for the low sensitivity and high false-negative results; however, as previously stated, the likelihood of obtaining a positive test is also dependent on the time of the presumed infection and the test execution time. Indeed, while viral antigens can be identified in samples within a short period of time following infection, their longevity and stability in biological samples are restricted, making it difficult to appropriately identify these proteins (Jacobs *et al*., 2020).

Rapid antibody tests are primarily intended to detect IgM and IgG antibodies, which are not created by the body right away but begin to appear in the bloodstream. As a result, depending on the period of the suspected infection, it's critical to employ the most appropriate test. It has been recommended to employ quick testing for the identification of IgA to speed up the diagnosis of COVID-19 infection (La Rosa *et al*., 2020).

1.9.3. Radiological findings:

Radiological investigations help clinicians to correctly diagnose COVID-19 infection in the case of a suspicious case of pneumonia. Chest X-ray (CXR) and computed tomography (CT) are the most powerful radiological imaging tools for diagnosing COVID-19 pneumonia (Chang *et al*., 2020).

1.9.3.1. Chest X-ray:

In the early stages of the disease, a chest X-ray is frequently inconclusive and may not reveal any major alterations. Bilateral multifocal alveolar opacities develop as the infection advances, which may be coupled with pleural effusion (Rajnik *et al*., 2021). CXR is commonly used to diagnose pulmonary abnormalities after lung damage caused by infectious or cancerous disorders (Kang *et al*., 1996). CXR was frequently employed to detect multifocal opacities affecting mostly the lung interstitial space and alveoli in patients with COVID-19-related symptomatology during the initial phase of the COVID-19 epidemic (Larici *et al*., 2020).

CXR is mostly used for patients with moderate to severe symptomatology who are suspected of COVID-19 infection and have interstitial opacities (71.7%) or alveolar opacities (60.5%), which typically involve both lungs (64.5%) (Ippolito *et al*., 2020). These radiological abnormalities worsen with time as symptoms worsen, and they are most commonly seen in older individuals with preexisting pulmonary parenchyma changes (such as patients with chronic obstructive pulmonary disease) who have both bilateral interstitial and alveolar opacities (Li *et al*., 2020).

1.9.3.2. Computed tomography:

CT Even in the early stages of the disease, high-resolution CT (HRCT) is the tool of choice for detecting COVID-19 pneumonia. Multifocal bilateral 'ground-glass' regions associated with consolidation and a patchy peripheral distribution, with increased involvement of the lower lobes, are the most typical signs. A reversed halo sign,' defined as a central region of patchy opacities surrounded by a peripheral ring with consolidation, is also present in certain cases. Pleural effusion, cavitation, calcification, and lymphadenopathy are among the other findings (Rajnik *et al*., 2021).

One of the earliest live imaging tools for detecting pneumonia-related infections is chest computed tomography (CT). It has previously been frequently utilized to detect lung anomalies in SARS and MERS, and has been proven to be more sensitive than X-rays (Memish *et al*., 2014). The approach has been used in hospitals for the diagnosis of COVID-19. The approach, however, has its own set of constraints. For example, chest radiography had a sensitivity of 69 % in a retrospective study of 64 patients in Hong Kong, compared to 91 % in RT-PCR. On a chest radiograph, 20% of the RT-PCR positive subjects did not reveal any lung abnormalities (Wong *et al*., 2020).

In another research, 75 % of RT-PCR negative patients had chest CT abnormalities, with 48 % of them expected to be COVID19 positive (Ai *et al*., 2020). Furthermore, because it can overlap with other illnesses including influenza, SARS, and MERS, chest computed tomography alone could result in false positive results. In light of these considerations, the majority of health commissioners have lately dropped chest CT scanning as diagnostic criteria for suspected COVID-19 cases. However, employing a combination of chest

CT scans and RT-PCR methods, these diagnostic uncertainties can be efficiently resolved. Furthermore, chest CT imaging might be beneficial in clinical settings for monitoring COVID-19 development and therapy impact (Rai *et al*., 2021).

Despite the inexpensive cost and quick radiological results achieved by CRX, some lung abnormalities are not clearly apparent by this method. As a result, in addition to CRX, CT scan is commonly used to better visualize lung abnormalities, which are mostly characterized by bilateral interstitial ground-glass opacities (Cui *et al*., 2020). The CT scan, in particular, has a high resolution power and a sensitivity of 95-100 %, but the specificity is limited since this technology does not allow for the differentiation of pulmonary abnormalities associated with various etiological agents other than SARS-CoV-2 (Kovács *et al*., 2021).

1.9.4. Molecular diagnosis:

Since viraemia is typically detected early in the course of an illness, nucleic acid amplification tests (NAAT) are the most sensitive assays and frequently used test to identify early viral infections. Many NAAT techniques, including reverse transcriptase real-time PCR (RT-qPCR), loop-mediated isothermal amplification-based assay (RT-LAMP), microarray, and high-throughput sequencing, have been developed for the quick and accurate diagnosis of COVID-19. On the other hand, SARS-CoV-2 RNA of the highest caliber is needed for NAAT. As advised by the WHO and CDC, probe-based RT-qPCR has long been considered the gold standard method for identifying SARS-CoV-2 and is currently one of the most widely used assays for population screening in many countries (Loeffelholz and Tang 2020).

Several RT-qPCR techniques were employed after the initial epidemic to locate SARS-CoV-2 in clinical samples. RT-qPCR experiments were used to target the RNA dependent RNA polymerase (RdRp), nucleocapsid (N), envelope (E), spike (S), and ORF1b or ORF8 regions of the SARS-CoV-2 genome (Reusken *et al*., 2020). The WHO recommends employing an RT-qPCR-based assay targeting the E gene for SARS-CoV-2 screening, followed by a confirmatory test targeting the RdRp gene. The CDC advised utilizing an RT-qPCR test that used the N1 and N2 nucleocapsid protein genes (Holshue *et al*., 2020).

The upper respiratory tract is sampled using nasopharyngeal and oropharyngeal swabs, while the lower respiratory tract is sampled using expectorated sputum and bronchoalveolar lavage (only for mechanically ventilated patients). The samples are delivered to the laboratory after being kept at 4°C for amplification of the viral genetic material via a reverse-transcription procedure. This entails either reversetranscription PCR (RT-PCR) or real-time RT-PCR to create a double-stranded DNA molecule from the existing viral RNA (Bhadra *et al*., 2015).

The amplified genetic material and the conserved parts of the SARS-CoV-2 genetic code are recognized. In situations of a positive test, the test should be repeated for confirmation, as well as to confirm viral clearance in COVID19 positive patients. The sensitivity of these tests is low; for example, 53.3 % of COVID-19-confirmed patients had positive oropharyngeal swabs, and 71 % of COVID-19-confirmed patients had positive RT-PCR results with sputum samples (Zhang *et al*., 2020). After 2–8 days, the RT-PCR findings are frequently positive (Huang *et al*., 2020).

For a mean of 17 days, reverse transcription polymerase chain reaction (RT-PCR) tests can detect viral SARS-CoV-2 RNA in the upper respiratory tract; however, detection of viral RNA does not always imply infectiousness, and viral culture from PCR positive upper respiratory tract samples has only been positive once beyond nine days of illness (Cevik *et al*., 2020).

The gold standard approaches for making a confirmed diagnosis of COVID-19 infection are RT-PCR-based molecular assays. Since the complete sequencing of the SARS-CoV-2 genome (Wu *et al*., 2020), researchers from various countries have started developing molecular primers and probes specific to SARS-CoV-2 RNA sequences in order to distinguish COVID-19 infections from other pathologies with similar symptoms, such as seasonal flu or bacterial infections (Silva *et al*., 2020).

Because of the low sensitivity of the primers and probes used, or the inaccuracy of the entire RT-PCR procedure, a significant fraction of COVID-19-positive patients were identified as false-negative during the early stages of the pandemic, when diagnostic techniques had not yet been optimized and standardized (false-negative rates ranging from 38 % at the day of symptom onset to 67 % before one day from the onset of symptoms or to 66 % after one day from the (Kucirka *et al*., 2020).

1.9.5. Viral culture and electron microscopy

Viral culture has represented the fundamental method that allows the identification of SARS-CoV-2 as a novel causative agent of human pneumonia (Zhu *et al*., 2020). Despite the difficulty of obtaining a viral culture in vitro and the length of time required, viral isolates constitute a watershed moment in the identification of new viral infections (Leland and

Ginocchio, 2007). In the case of SARS-CoV-2 infection, viral culture was critical in the early stages of the outbreak before alternative diagnostic tests were developed.

Zhu and his colleagues, 2020 were the first to isolate SARS-CoV-2 virus isolates from clinical material and use transmission electron microscopy to investigate cytopathic effects. After this study, additional research groups isolated SARS-CoV-2 with the goal of studying its structural properties and molecular interaction with infected cells (Leung *et al*., 2020). Other cell lines, such as the Vero and LLC-MK2 cell lines, have been used for these purposes; using electron microscopy and cells infected with clinical specimens obtained from COVID-19 patients, it was possible to identify the virus's ultrastructural details, the virus's interaction with cells, and the resulting cytopathic effects (Zhao *et al*., 2020).

It's worth noting that electron microscopy was one of the first approaches for discovering new diseases, allowing structural traits to be identified. Solid-phase immune electron microscopy (SPIEM) and immunolabeling electron microscopy (IEM), which are based on the observation of cells blocked in the surface of a grid and the observation of antibody-antigen complex occurring in infected cells, respectively, are the two main applications of electron microscopy in viral infections (Akilesh *et al*., 2021).

In general, viral culture and electron microscopy are crucial approaches for observing the virus's main properties. These two approaches were used to identify the usual structure of coronaviruses, which is characterized by a nucleocapsid encased inside a crown-like envelope made up of spike proteins in the case of SARS-CoV-2. In terms of cytopathic consequences, both

approaches showed a wide spectrum of cellular changes, the most prominent of which was the creation of plaques with a net-like structure or joined cells. Deformed cilia with a granular structure and disorganized polarity are also observed in these plaques, which are made up of multinucleated syncytial cells. Double-membrane vesicles and damaged mitochondria were also seen in SARS-CoV-2-infected cells. Finally, viral infections caused the endoplasmic reticulum to expand and the number of secretory vesicles to grow (Zhao *et al*., 2020).

Despite their relevance, both viral culture and electron microscopy have drawbacks that restrict their application in therapeutic settings. Viral culture is time-consuming and needs specialized equipment as well as a high level of biosecurity. As a result, the CDC recommends using SARS-CoV-2 virus culture only in laboratories with level 3 biosafety cabinets for research purposes (Bain *et al*., 2020).

Electron microscopy, on the other hand, is not frequently utilized since it needs expensive gear and highly educated workers with particular abilities in sample preparation and image interpretation. Furthermore, this method has a limited diagnostic sensitivity and specificity, and the best findings can only be achieved if adequate viral cultures are available (Wu *et al*., 2020).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipments and Instruments

Table 2-1: Equipments and Instruments used in this study

No.	Equipment &	Company/ country
1.	Digital camera	Samsung/ china
2.	ELISA	BioTek/ USA
3.	ELISA reader	BioTek/ USA
4.	Exispin vortex	Bioneer/ Korea
5.	Gel	Bioneer/ Korea
6.	Hematology	Minami-Ku Kyoto/
7.	High Speed Cold	Eppendorf
8.	Incubator	Memmert/Germany
9.	Microcentrifuge	Biobasic/ Canada
10.	Micropipettes 5-	CYAN/ Belgium
11.	Microwave	Argose/Germany
12.	Refrigerator	Concord /Lebanon
13.	Sensitive	Sartorius/Germany
14.	Thermocycler	BioRad/ USA
15.	UV	ATTA/ Korea
16.	Vortex	CYAN/ Belgium
17.	Water Bath	Memmert/Germany

2.1.2. Kits

Table 2-2: The genetic kits used in this study with their companies and countries of origin

Kit	Company	Country
gSYAN DNA Extraction	**Geneaid**	**Taiwan**
GST buffer		
GSB buffer W1 buffer		
Wash buffer Elution buffer		
GD column Collection tube 2ml		
Proteinase K 10mg/ml		
GoTaq® G2 Green Master	**Promega**	**Korea**
Taq DNA polymerase dNTPs (dATP, dCTP, Tris.HCl pH 9.0		
KCl $MgCl_2$		
Loading dye		

Table 2-3: The ELISA kits used in this study with their companies and countries of origin

	Company	Country
Interleukin- 35 (IL-	**Bioassay**	**China**
presepsin	**Bioassay**	**China**

2.1.3. Primers

Allele specific-PCR primers for ***TYK2-rs2304256 and IL-K12A rs568408*** **gene polymorphism** were designed in this study using NCBI-SNP data base and Primer1 Allele Specific-PCR primers design online. These primers were provided from (Scientific Researcher. Co. Ltd. Iraq) as following tables:

Table 2-4: The TYK2-rs2304256 Allele specific-PCR primers with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
Wild type Reverse Primer	GCCAAGGCTCACAAGGCAT	120 bp
Mutant Reverse Primer	GCCAAGGCTCACAAGGCAG	
Common Forward Primer	GATGCTGACACAGTGCTCTT	

Table 2-5: The IL-12A rs568408 Allele specific-PCR primers with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
Wild type Reverse Primer	GATGGGACTATTACATCCACAGA	205 bp
Mutant Reverse Primer	GATGGGACTATTACATCCACAGG	
Common Forward Primer	TGCTTACATGTTTGTTTCCA	

2.1.4. Chemicals

Table 2-6: All the chemicals materials that used in this study with their company and country of origin

Chemical	Company and Origin
Absolute Ethanol	Scharlau (Spain)
Agarose	BioBasic (Canada)
TBE buffer 10X	BioBasic (Canada)
Ehidium Bromide	BioBasic (Canada)
DNA Marker Ladder	INtRON (Korea)
Free nuclease water	Bioneer (Korea)

2.2. Methods

2.2.1. Patients

This is a case control study enrolled a total of 125 severe COVID-19 patients. All patients were admitted to Marjan medical city, Al-shomali general hospital, and Al-Sadeq hospital's COVID‑19 ward in the period between January 2022 to July 2022. Their ages ranged from 16 to 90 years. The diagnosis COVID‑19 in each patient in this study was confirmed by SARS-CoV-2-positive RT-PCR provided by general health laboratory in Babylon city. The total numbers of control group were 60 people without infections or other chronic diseases.

Blood specimens were collected by venipuncture; five ml of venous were drawn by disposable syringe under sterilization technique and putting in gel

and EDTA tubes then gel tube allowed to clot after that serum was separated by centrifugation 2500 rpm for 10 minute. The serum has been collected in Eppendorf tube then stored at -70 ^{o}C to be used for ELISA test to determine concentration of IL-35 and presepsin in serum. EDTA tube stores at -70 ^{o}C to be used for genetic purposes (Figure 2-1).

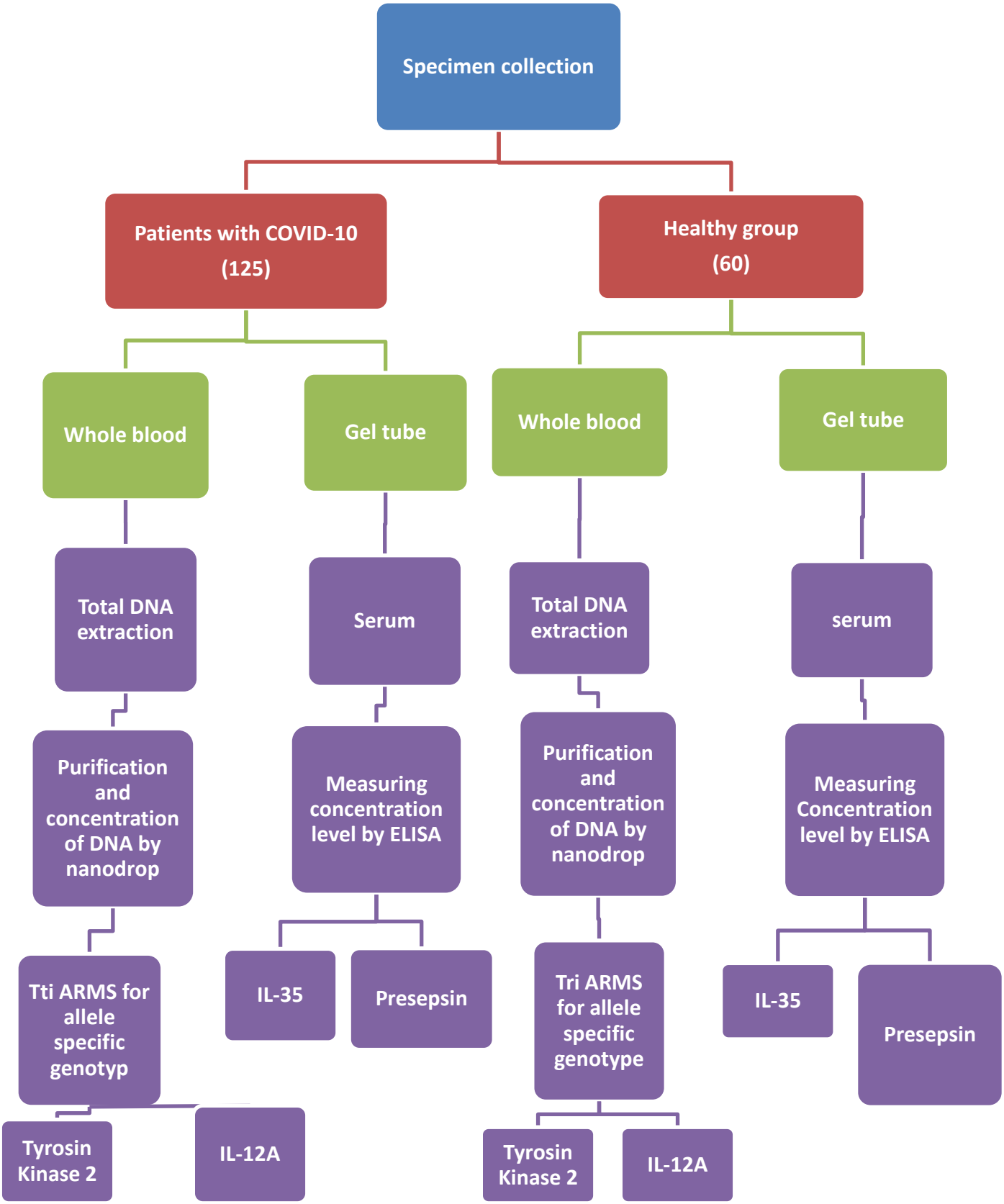

Figure 2-1: Study design

2.2.2. Serological tests:

2.2.2.1: ELISA assay

This assay employed the quantitative sandwich enzyme immunoassay techniques. The micro ELISA plate had been pre-coated with an antibody specific to IL-35 and presepsin. Then antigen was bound to immobilized capture antibody, standard and samples were pipetted into the well and any IL-35 and presepsin present were bound by the immobilized antibody.

After removing any unbound substance by simple washing procedure. Biotin -conjugated antibody specific for the markers were added to the wells. After washing, Avidin conjugated Horse radish peroxidase (HRP) was add to each microplate well and then incubated and then wash to remove any unbound Avidin -enzyme reagent, substrate solution was added specific to the enzyme in the well. The color intensity produced was directly proportional to the amount of biomarkers bound in the initial step. The enzyme-substrate reaction was terminated by the addition of a stop solution and the color turns yellow.

The optical density (OD) was measured spectrophotometer at a wave length of 450nm.The OD value was proportional to the concentration of IL-35 and presepsin, then calculate the concentration of substances in the sample by comparing the OD of the samples to the standard curve.

2.2.2.2. Procedure Assay

1. Prepare all reagents, standard solutions, and samples. Before use, bring all reagents to room temperature. The experiment is carried out at room temperature.

2. Establishing the quantity of strips needed for the test by placing the strips in the frames. The strips should be kept between 2 and 8 °C.

3. Filling a standard well with 50 µl of standard solution.

4. Adding 40 µl of material to the sample wells, 10 µl of anti-IL-35 antibody, 50 ml of streptavidin-HRP to the sample wells, and standard wells (Not blank control well) at 37 °C, incubate for 60 minutes.

5. Removing the sealant and using a wash buffer to wash the plate five times. For every wash, soaking wells in a minimum of 0.35 ml of wash buffer for 30 to 1 minutes.

6. Pouring 50 µl of substrate solution A into each well, followed by 50 µl of substrate solution B. Plate should be incubated for 10 minutes at 37 °C in the dark.

7. Filling each well with 50 µl l of Stop Solution, and the blue hue will instantly become yellow.

8. After 10 minutes of applying the stop solution, calculating the optical density (OD value) of each well using a microplate reader set to 450 nm.

2.2.2.3. Calculation of results

The ELISA results were calculated based on the readings for each standard and samples optical density. Then create a standard curve by plotting OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph in excel office program.

1-First, calculated the O.D value for each standard and sample, then construct the standard curve (Figure 2-2 and 2-3).

2- Determined the amount of IL-35 and presepsin in each sample, by locating the O.D according to their site in curve.

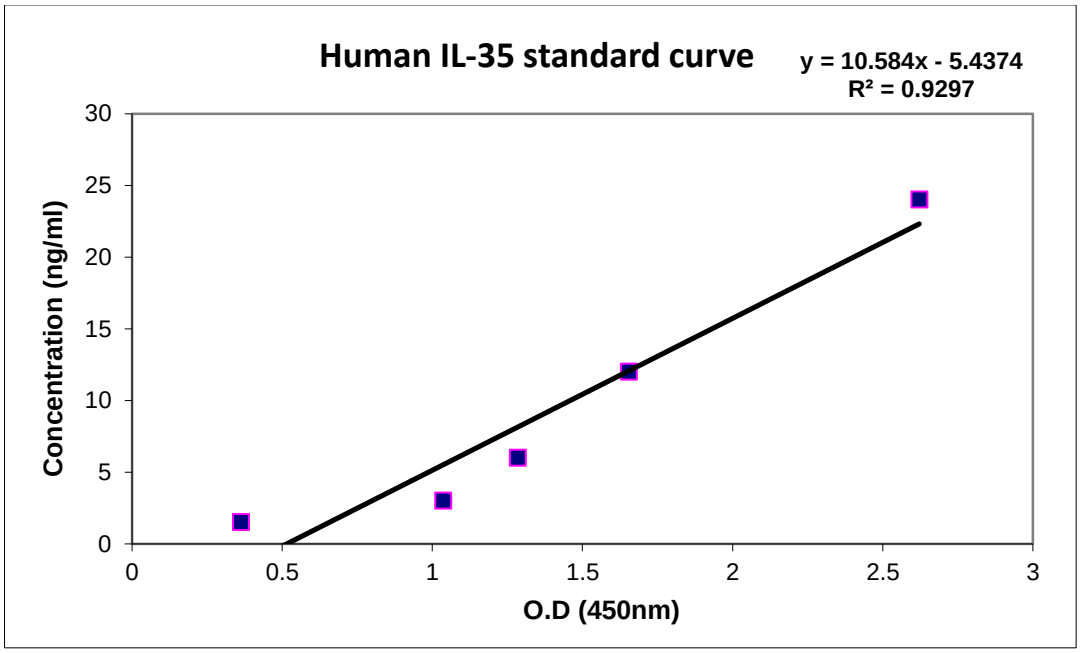

Figure 2-2: Standard curve for human IL-35 in ELISA

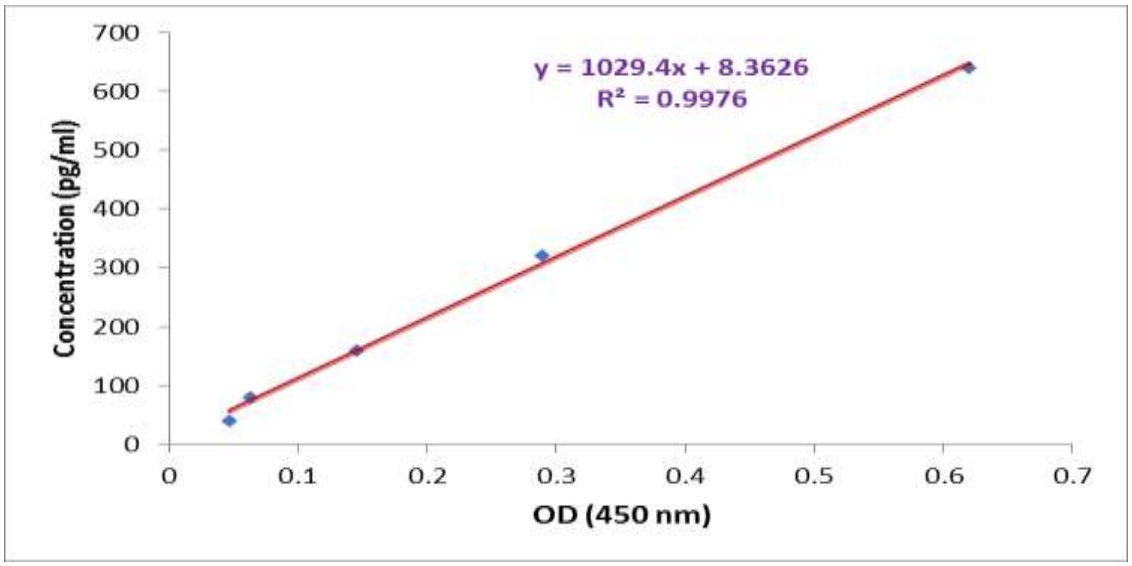

Figure 2-3: Standard curve for human Presepsin in ELISA

2.2.3. Genotyping study:

2.2.3.1. Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA kit extraction kit (Frozen Blood) Geneaid, Taiwan that done according to company instructions as following steps:

1. A 200µl of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 30µl of proteinase K and mixed by vortex. And incubated at 60℃ for 5 minutes.
2. After that, 200µl of lysis buffer GSB was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃ for 10 minutes, and inverted every 3 minutes through incubation periods.
3. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.
4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000 rpm for 5 minutes. And the 2 ml collection tube containing the flow. Through were discarded and placed the column in a new 2 ml collection tube.
5. 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000 rpm for 30 seconds. The flow was discarded and placed the column back in the 2 ml collection tube.
6. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000 rpm for 30 seconds. The flow was discarded and placed the column back in the 2 ml collection tube.

7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated elution buffer were added to the center of the column matrix.

9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.2.3.2. Genomic DNA estimation

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.

3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

2.2.3.3. Allele specific-PCR Method:

Allele specific-PCR assay was performed for detection and genotyping of IL-12A and TYK2 genes polymorphism in 125 patients and 60 healthy control blood samples. This method was carried out as following steps:

A- Allele Specific-PCR master mix preparation

Allele Specific-PCR master mix was prepared by using (**GoTaq® G2 Green Master Mix kit**) and this master mix done two reactions for each samples according to company instructions as following tables:

Table 2-7: Wild type allele AS-PCR reaction Mix:

Allele specific-PCR Master mix	Volume
DNA template	5µl
Wild type primers (10pmol)	2µl
Common Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

Table 2-8: Mutant type allele AS- PCR reaction Mix:

Allele Specific-PCR Master mix	Volume
DNA template	5µl
Mutant type primers (10pmol)	2µl
Common Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

After that, these PCR master mix component that mentioned in table above were transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (BioRad. USA).

B- Allele Specific-PCR Thermocycler Conditions

PCR thermocycler conditions were done as following tables:

Table 2-9: PCR thermocycler conditions

PCR step	Temp.	Time	repeat
Initial	95°C	5min.	1
Denaturation	95°C	30 sec.	35cycle
Annealing	58°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

C- Allele Specific-PCR product analysis

The Allele Specific-PCR products were analyzed by agarose gel electrophoresis following steps:

1- 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

1- Then 3µL of ethidium bromide stain were added into agarose gel solution.

2- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.

3- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.

4- 10µl PCR product were added in to each comb well and 3µl of (1000 bp Ladder) in First well.

5- The electric current was performed at 100 volt for 1hour

6- The Allele Specific-PCR products were visualized by using UV trans-illuminator.

2.2.4. Ethical approval

The college of medicine, university of Babylon granted ethical approval. Before taking the sample, the patients and his relative were asked for their permission. Sampling, health and safety precautions were implemented.

2.2.5. Statistical analysis:

The collected data were recorded using Microsoft Excel (Microsoft, Redmond, WA, USA). All records of the included patients were anonymized prior to analysis. Statistical analyses were performed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA).

The Shapiro–Wilk test was used to assess the normality of the data distribution. Categorical variables were expressed as numbers and percentages, the chi-square test was used for comparisons. Normally distributed continuous variables presented by mean and standard deviation. The independent samples t-test was used to compare two sample means with normally distributed. Multiple logistic regression analysis used for genotyping

analysis. All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

Chapter Three

Results and Discussions

3. Results and Discussions:

3.1. Demographic characteristics of patients and control subjects

The present study enrolled 125 patients with COVID-19 and 60 apparently healthy subjects. The demographic characteristics of patients and control subjects are shown in (Table 3-1). The median age of patients was 75 (62-85) years, the smallest age was 15 years old and the biggest age was 90 years old. The median age of control subjects was 74 (64-82) years and there was no significant difference between patients and control subjects (P = 0.65). Ages were divided in to five categories for each patients and control groups. No significant differences between them was observed (P= 0.73) (Figure 3-1).

Patients' group included 56 (44.8%) males and 69 (55.2%) females, whereas, control group included 28 (46.6%) males and 32 (53.4%) females and there was no significant difference in the frequency distribution of patients and control subjects according to sex (P = 0.87) (Figure 3-2).

Table 3-1: Demographic characteristics of patients and control subjects

Characteristic	Patients (N = 125)	Control (N =60)	P value
Age (years)			
Median (IQR)	75 (62-85)	74 (64-82)	0.65
Range	15-90	22-89	
15-30, *n* (%)	10 (8 %)	4 (6.7 %)	0.73
30-45, *n* (%)	6 (4.8 %)	3 (5 %)	
45-60, *n* (%)	15 (12 %)	5 (8.3 %)	
60-75, *n* (%)	34 (27.2 %)	22 (36.7 %)	
75-90, *n* (%)	60 (48 %)	26 (43.3 %)	
Sex			
Male, *n* (%)	56 (44.8%)	28 (46.6%)	0.87
Female, *n* (%)	69 (55.2 %)	32 (53.4%)	

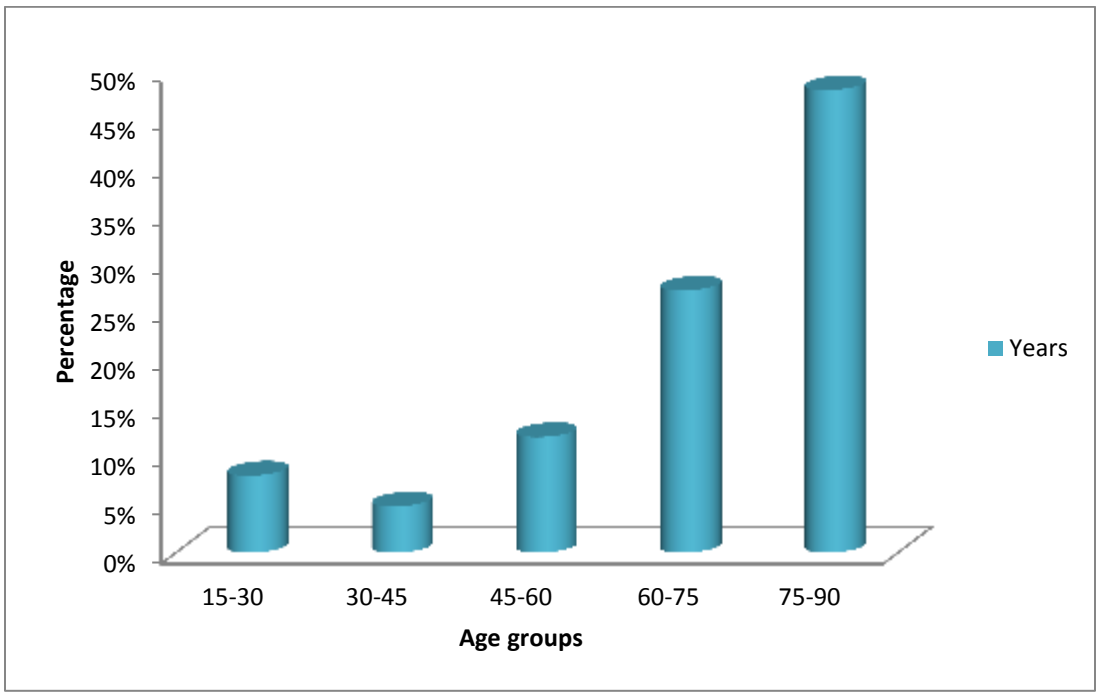

Figure 3-1: Distribution of the COVID-19 patients according to Age

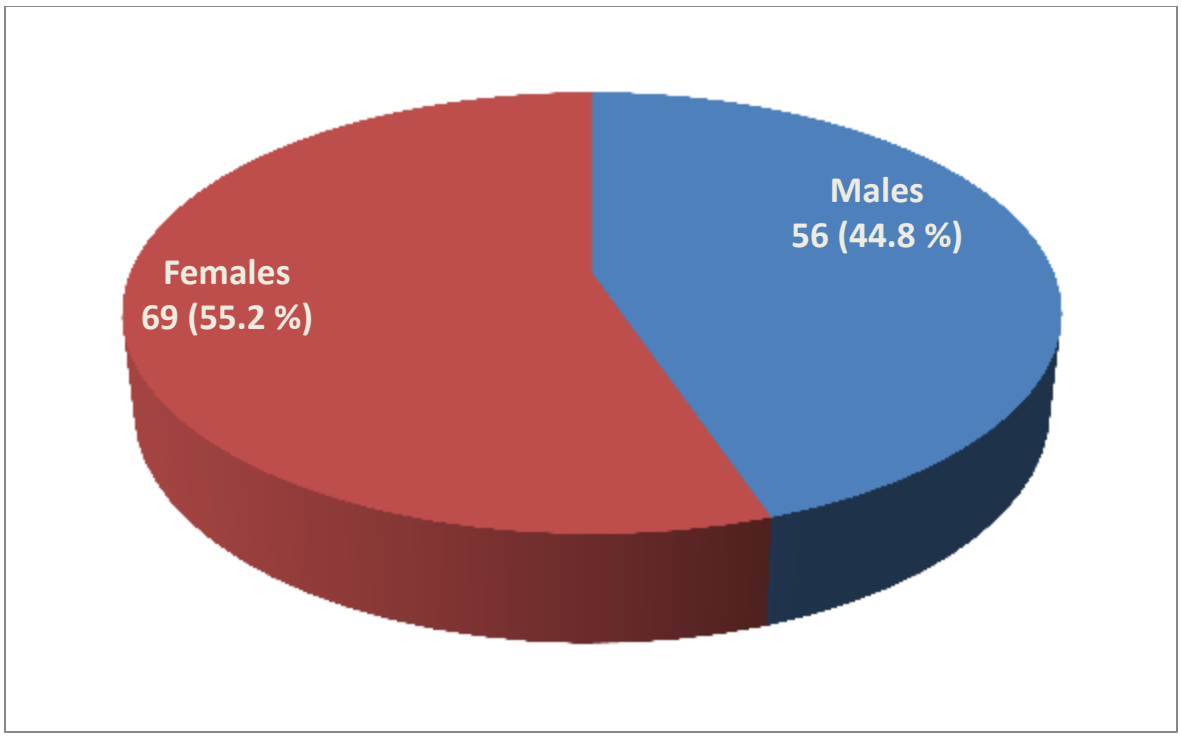

Figure 3-2: Distribution of the COVID-19 patients according to sex

An understanding of the sex and sex sensitivities of COVID-19 infection is a necessary component towards the creation of effective treatment options and therapies for the virus (Oertelt-Prigione, 2021).

The present study revealed that females were more infected than males; this finding was agreed and disagreed with other studies around the world. A study in the United States mentioned that 52.1% of COVID-19 patients who participated in the research were female, while 47.9% were male (Krueger *et al*. 2020). This finding was agreed with the current study. Another study also mentioned that among the total cases, 48.5% were male (Tian *et al*. 2020).

The public data set from the Chinese population that included the clinical data about COVID-19 found no significant sex differences in susceptibility to infection. Analysis revealed that patients' susceptibilities to COVID-19 infection were same in male and female patients (Jin *et al*. 2020).

Another study found that 51.4% of patients with confirmed COVID-19 cases were male, according to a different research that looked at 44,672 confirmed cases until February 11, 2020 (Team, 2020).

In this study, the median age of patients was 75 (62-85) years, the smallest age was 15 years old and the biggest age was 90 years old. Age group (75-90 years) represents the high percent (48%) of patients.

A study of 262 COVID-19 patients found that the age range of patients was from 6 months to 94 years old, and the median was 47.5 years old. Additionally, 77.4% of the patients were aged between 13 and 64 years old (Tian *et al*. 2020).

A retrospective study was conducted regarding 191 patients diagnosed with COVID-19 in Wuhan, China mentioned that the age range for all of the patients was 18 to 87 years old, and the median age was 56 years old (Zhou *et al*. 2020). In the study of 44,672 COVID-19 cases from China, taken through February 11, 2020, a total of 1,023 deaths occurred. Among these patients, 77.8% were between the ages of 30 and 69 years old (Team 2020).

SARS-CoV-2 enters the body through the angiotensin-converting enzyme-2 (ACE2) (Hoffmann *et al*. 2020). The sex differences in COVID-19 infection, severity, and mortality may be explained by variations in ACE2 expression brought on by sex hormones (Zaman *et al*. 2002). Moreover, these differences might be explained by sex-based variations in immune responses.

3.2. Inflammatory parameters:

IL-35 showed statistical significant differences between patients 6.86± 2.31ng/ml and control group 3.86± 2.07 ng/ml (P< 0.0001). The standard curve of ELISA and mean are showed in (Figure 3-3).

Sometimes the immune system's reaction to a viral infection is more destructive than the virus itself (Al-Khikani *et al*., 2022). In order to manage COVID-19, immunopathogenesis must also be taken into account in addition to adaptive immunity. As there have only been a few researches on IL-35, its roles in preventing viral infection are still not well known (Guo *et al*., 2019).

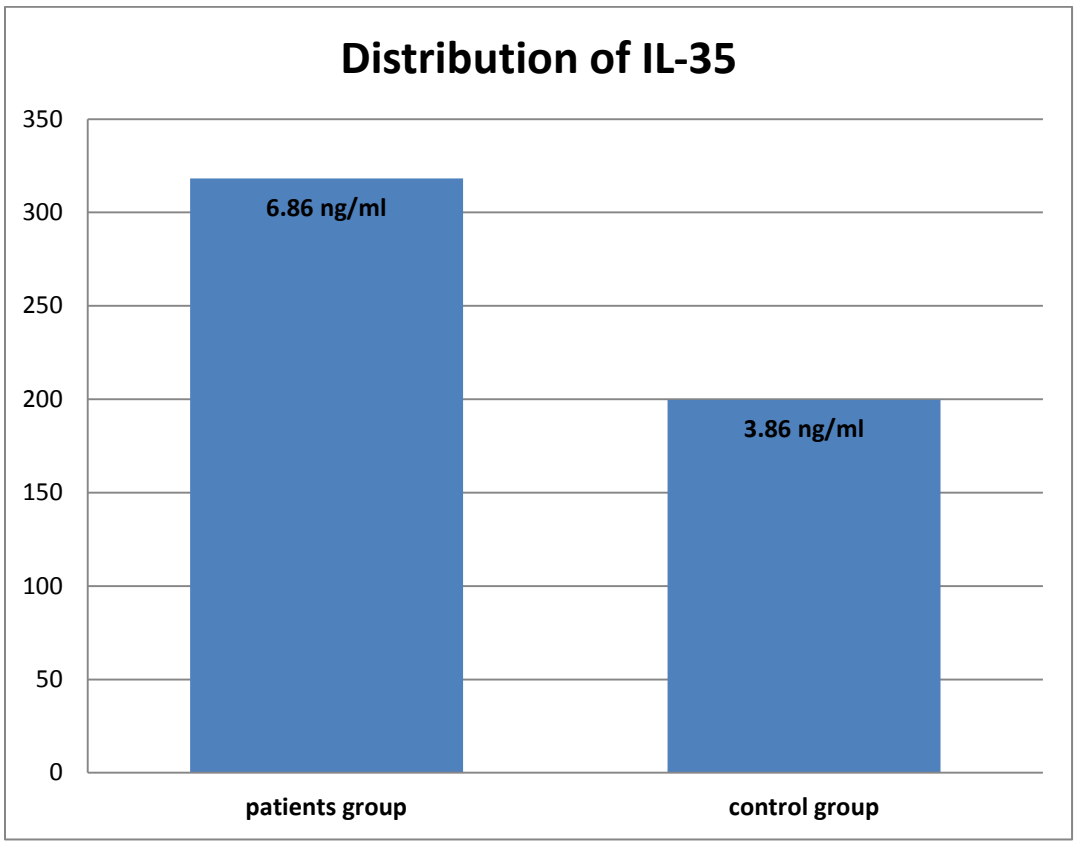

Figure 3-3: Distribution of human IL-35 among patients and control.

The function of IL-35 during viral infection is poorly understood. Patients with seasonal influenza A virus (IAV) had higher amounts of IL-35 in their peripheral blood mononuclear cells and throat swabs than healthy people. IAV infection elevated IL-35 mRNA and protein levels in human lung epithelial and primary cells. IAV-induced IL-35 transcription is controlled by NF-B, according to another research. Select inhibitors of cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase dramatically reduced IL-35 expression, indicating that they are involved in IL-35 expression (Chen *et al*., 2016).

To research post-influenza pneumococcal pneumonia and the function of IL-35 in host defense against post-influenza pneumococcal pneumonia, a clinical investigation revealed that IL-35 levels in patients with influenza

infection were much higher than in healthy people, indicating that influenza infection might cause IL-35 synthesis in human peripheral blood mononuclear cells. These findings imply that IL-35, at least in part, contributes to secondary pneumococcal pneumonia susceptibility by suppressing the early immune response (Chen *et al*., 2016).

Presepsin showed statistical significant differences between patients 318.18± 226.62 (pg/ml) and control group 199.68± 39 (pg/ml) (P< 0.0001). The standard curve of ELISA and mean are showed in (Figure 3-4).

This study showed no significant differences between males 273.55 pg/ml and females 284.90 pg/ml regarding the mean of presepsin (P= 0.67).

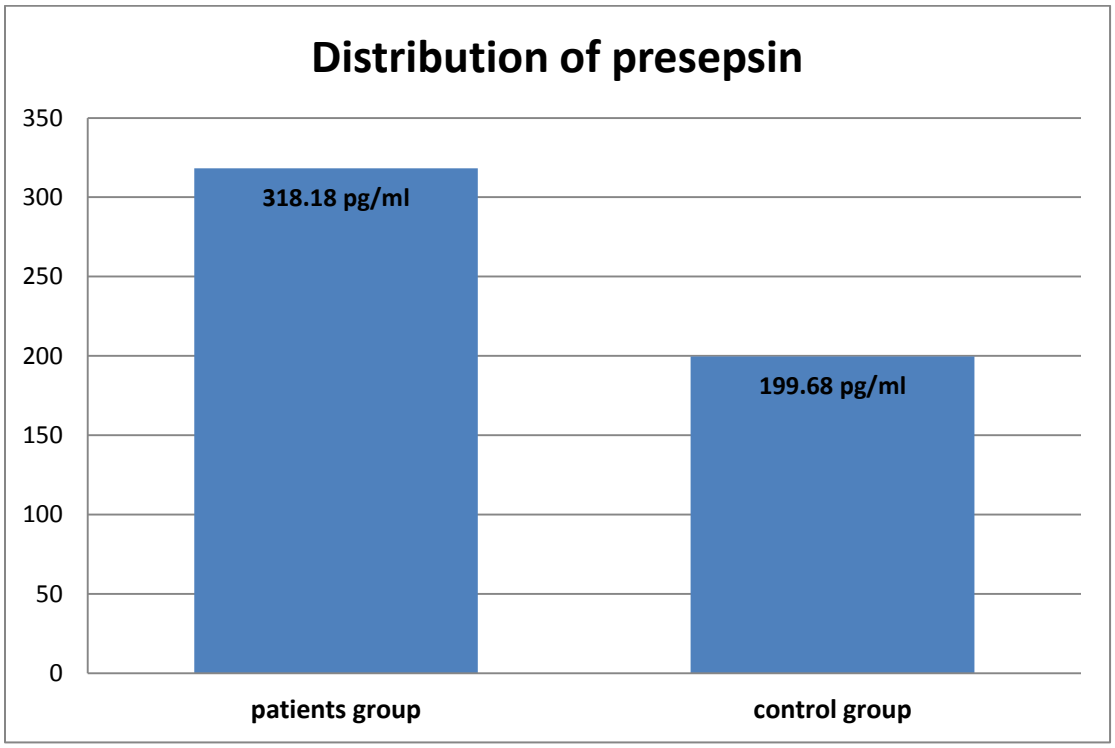

Figure 3-4: Distribution of presepsin among patients and control

The soluble CD14 subtype PSN has been suggested as a new biomarker in sepsis patients. Its importance in risk stratifying patients with sepsis and its capability to distinguish between patients with sepsis and those advancing to septic shock have been demonstrated in several investigations (Mabrey *et al*., 2021). It takes part in the first few steps of the septic process. The soluble CD14 subtype (PSN) is released into the plasma when an infectious pathogen activates monocytes. In the early stages of sepsis, PSN levels therefore keep rising. It has been postulated that the increase of PSN is caused by a dose-response mechanism of the host-pathogen interaction, which happens in the early stages of pathogen identification and remains raised for a number of days depending on the severity of the disease. There has not yet been enough study done to fully understand and examine the role of PSN in patients with COVID-19 (Assal *et al*., 2022).

In the current study, Presepsin showed statistical significant differences between patients ($P< 0.0001$). This finding is agree with another study applied on 85 patients who had COVID-19 infection found that the mean PSN levels in the patient group were considerably higher than in the control group. PSN levels showed a high association with creatinine levels and a slight positive link with CRP levels. The presepsin levels in the patient group that recovered and dying patients group did not differ significantly (Pişkinpaşa, 2022).

COVID-19 patients with severe/critical illnesses had PSN levels that are much greater (almost 3-fold) than COVID-19 patients without such illnesses. In a meta-analysis, Kondo *et al*. discovered that PSN had even higher diagnostic accuracies than procalcitonin for identifying mixed-pathogen sepsis in critically sick adult patients (Kondo *et al*., 2019). Regular evaluation of PSN in COVID-19 may offer useful clinical data for anticipating negative

consequences and assisting in clinical and therapeutic decision-making (Favaloro and Lippi, 2020).

The PSN prognostic value for predicting 30-day death in COVID-19 patients is also investigated in this study. In COVID-19 patients that were hospitalized, procalcitonin and PSN both performed comparably in terms of predicting 30-day death (Park *et al*., 2022). PSN has demonstrated adequate performance in predicting the worsening of severity in COVID-19, which can help doctors identify high-risk patients and choose treatment plans early on for the best use of available resources (Ahmed *et al*., 2021).

3.3. Genetic results:

3.3.1. IL-12A- rs568408

For genotyping and allele frequency, the present study enrolled 125 patients with COVID-19 and 60 apparently healthy subjects were frequency-matched by sex and age. The genotype and allele distributions of IL12A rs568408 in the cases and controls are shown in (Table 3-2). Overall, there was a significant difference in the distribution of IL-12A rs568408 genotypes between cases and control groups (P = 0.006).

Logistic regression analysis revealed that the frequencies of the homozygous variant AA and heterozygous variant AG of IL-12A rs568408 were 12 % and 36 % in COVID-19 cases and 3.3 % and 21.7 % in healthy controls, respectively. For AA genotype (OR= 5.19, 95% CI: 1.13-23.82; P= 0.034). While GA genotype (OR = 2.39, 95% CI = 1.16- 4.94, P = 0.018), at IL-12A rs568408 demonstrated a statistically significant risk for COVID-19 in Iraq. The variant rs568408 GA/AA genotypes were associated with a

significantly increased risk of COVID-19 (OR= 2.76, 95% CI: 1.40-5.47; P= 0.003), compared with the wild-type rs568408 GG.

The combined variant genotypes GA + AA did not further elevate COVID-19 risk that the OR of AA alone (2.76 versus 5.19), compared to the wild-type GG genotype, indicating that A-allele of IL-12A rs568408 behaves as a recessive determinant to COVID-19 risk. (Table 3-2 and Figure 3-5).

Table 3-2: Genotyping and allele frequency of IL-12A- rs568408 in COVID-19 patients and control

Variables	Patients N= 125	Control N= 60	OR (95% CI)	P value
IL-12A rs568408				
GG	65 (52 %)	45 (75 %)	1	1
AA	15 (12 %)	2 (3.3 %)	5.19 (1.13-23.82)	**0.034**
GA	45 (36 %)	13 (21.7 %)	2.39 (1.16- 4.94)	**0.018**
AA+ GA	60 (48 %)	15 (25 %)	2.76 (1.40-5.47)	**0.003**
P trend				**0.006**
Allele frequency				
G	175 (70 %)	103 (85.83%)		
A	75 (30 %)	17 (14.17 %)	2.59 (1.45- 4.63)	**0.001**
Recessive model				
GG+GA	110 (88 %)	58 (96.7 %)		
AA	15 (12 %)	2 (3.3 %)	3.95 (0.87- 17.88)	0.056
Additive model				
GG	65 (52 %)	45 (75 %)		
AA	15 (12 %)	2 (3.3 %)	5.19 (1.13- 23.82)	**0.021**

Dominant model				
GG	65 (52 %)	45 (75 %)		
AA+ GA	60 (48 %)	15 (25 %)	2.67 (1.40- 5.47)	**0.003**

The distribution of allelic frequencies of rs568408 in IL-12A showed that the A allele was associated with increased risk of COVID-19, compared to the G allele (OR = 2.59, 95% CI = 1.45- 4.63, P = 0.001). The frequencies of the A and G alleles were 30 % and 70% in COVID-19 patients and 14.17 % and 85.83% in control respectively.

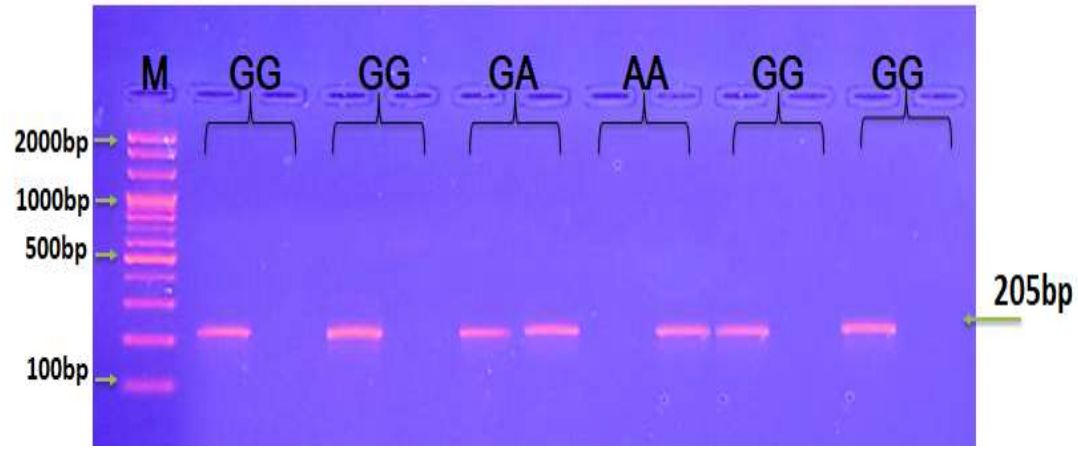

Figure 3-5: Agarose gel electrophoresis for IL-12A rs568408

In this image above showed the ARMS-PCR product analysis of IL-12A rs568408 gene polymorphism. Where M: marker (2000-100bp). The (GG) wild type homozygote were showed in G allele only, the (AA) mutant type homozygote were showed in A allele only, whereas the (GA) heterozygote were showed in both G and A allele. The presence of G or A allele were observed at 205bp product size.

In the current hospital-based case-control study, we looked at the relationship between the Iraqi COVID-19 risk and the IL-12A rs568408 polymorphism. 125 COVID-19 patients and 60 healthy people of matched age and sex made up the sample size. We found that COVID-19 risk was determined genomically by SNPs at IL-12A rs568408. Our hypothesis that functional polymorphisms in IL-12 may contribute to the susceptibility of SARS-CoV-2 is supported by our finding.

We detected a genetic risk biomarker for predicting COVID-19 susceptibility, the AA genotype at IL-12A rs568408. This genotype was linked to an increased risk of osteosarcoma and esophageal cancer (Tao *et al*., 2012). Chen and colleagues demonstrated in 2009 that the genotypes of the IL-12A rs568408 GA/AA variant were significantly associated with an increased risk of cervical cancer. They proposed that rs568408 may interfere with the binding of miRNAs and exonic splicing enhancers, and three SNPs that are in mice-conserved regions may interfere with exonic splicing silencers (Chen *et al*., 2009).

A crucial cytokine in the fight against intracellular infections, interleukin-12, encourages the growth of Th1 cells, cell-mediated cytotoxicity, and interferon-gamma production (Al-Khikani, 2022). Various studies have shown that the two genes that code for IL-12, IL12A and IL12B, have numerous functional polymorphism sites that may have an impact on the development and spread of lung cancer (Sepesi *et al*., 2018) and tuberculosis (Taheri *et al*., 2017). Tan *et al*. find that the IL12A rs568408 variant may be a marker SNP associated with a greater risk of insufficient HBV clearance (Tan *et al*., 2016). However, Ben-Selma *et al*. suggested that IL-12A rs568408 and interactions with other genes may affect the course of chronic HBV

infection, underlining their potential use as predictive and diagnostic biomarkers (Selma *et al*., 2020). The IL-12 signaling pathway is crucial for HBV infection and may play a role in pathogenesis, according to other evidences (Wang *et al*., 2015; Yin *et al*., 2016).

In response to microbial stimuli, such as viral infection, dendritic cells and macrophages release IL-12, which interacts with the IL-12 receptor that is primarily expressed by activated T and NK cells. IFN-γ is secreted when IL-15, IL-12, type I IFN, and IL-18 are combined to increase the cytotoxic activity of NK cells. NK cells release IFN-γ, which stimulates macrophages to kill phagocytosed bacteria. Another well-known function of IL-12 is to promote T-helper 1 cell development (Presky *et al*., 1996). A considerably greater amount of IL-12 was produced in patients with mild COVID-19 during the beginning of the acute phase due to SARS-CoV-2 infection than in patients with moderate or severe symptoms and healthy controls. Interestingly, multiple investigations found that patients with severe COVID-19 had much fewer peripheral NK cells than healthy people, or people who have COVID-19 minor cases (Zheng *et al*., 2020). IL-12 induction is necessary to sustain NK cell numbers in the early stages of SARS-CoV-2 infection and this may contribute to the evasion of viral propagation seen in asymptomatic and mildly symptomatic patients (Tjan *et al*., 2021).

In another investigation, the distributions of IL-12A rs568408 genotypic and allelic frequencies showed significant differences between patients and controls with lung cancer (p=0.0036 and p=0.0005, respectively). When compared to the GG genotype, the IL-12A rs568408 AA genotype was specifically linked to a substantially increased risk of lung cancer (odds ratio = 2.41, 95% CI=1.36-4.29, p=0.0021) (Wu *et al*., 2018).

Another study mentioned that IL-12A rs568408 GA (p = 0.035), and rs568408 GG/AA (p = 0.034) were associated with an increased chance for the development of anti-hepatitis B virus surface antigen in hemodialysis patients. Patients bearing rs568408 AA had a 10.9-fold or 8.9-fold chance to develop antibodies compared with those carrying any other genotype (p = 0.005) or those who had both wild-type rs568408 GG (Grzegorzewska *et al*., 2012).

In a sample of the southeast Iranian population, research revealed that IL12A rs568408, polymorphism was not a significant genetic determinant for resistance or susceptibility to pulmonary tuberculosis (PTB). In patients and controls, the rates of the GA and AA genotypes of the IL12A rs568408 variation were 38.5%, 1.7%, and 37.3%, 1.1%, respectively (Taheri *et al*., 2017).

The genotype and allele frequencies of IL-12A rs568408 in asthma were significantly different between patients and controls (p= 0.001). The AC genotype of rs3212227 was associated with a significantly decreased risk of having asthma when compared to the AA genotype (p= 0.036). Individuals with the coupled genotypes (rs568408 AG and rs3212227 AC/CC) had a 2.05-fold increased risk of having asthma compared to those with all other genotypes (p= 0.001). When rs568408 GG and rs3212227 AC/CC were combined, the probability of developing asthma was significantly lower than when rs568408 GG and rs3212227AA were combined (p= 0.009) (Chen *et al*., 2011).

3.3.2. TYK2-rs2304256

The genotype and allele distribution distributions of TYK2-rs2304256 in the cases and controls are shown in (Table 3-3 and Figure 3-7). Overall, there was a significant difference in the distribution of TYK2-rs2304256 genotypes between cases and control groups (P= 0.019).

Logistic regression analysis revealed that the frequencies of the homozygous variant AA and heterozygous variant CA of TYK2-rs2304256 were 14.4 % and 22.4 % in COVID-19 cases and 3.3 % and 16.7 % in healthy controls, respectively. For AA genotype (OR= 5.46, 95% CI: 1.21-24.61; P= 0.027). While CA genotype (OR = 1.7, 95% CI = 0.76- 3.81, P = 0.196), at TYK2-rs2304256 demonstrated that AA genotype showed a statistically significant risk for COVID-19 in Iraq compared with the wild-type rs2304256 CC. The variant rs568408 CA/AA genotypes were associated with a significantly increased risk of COVID-19 (OR= 2.81, 95% CI: 1.37-5.78; P= 0.004), compared with the wild-type rs2304256 CC.

The combined variant genotypes CA + AA did not further elevate COVID-19 risk that the OR of AA alone (2.81 versus 5.46), compared to the wild-type CC genotype, indicating that A-allele of TYK2-rs2304256 behaves as a recessive determinant to COVID-19 risk (Table 3-3 and figure 3-6)

The distribution of allelic frequencies of rs2304256 in TYK2 showed that the A allele was associated with increased risk of COVID-19, compared to the C allele (OR = 2.60, 95% CI = 1.39- 4.87, P = 0.002). The frequencies of the A and C alleles were 25.6 % and 74.4% in COVID-19 patients and 11.76 % and 88.33% in control respectively.

Tyrosine kinase 2 (Tyk2), a member of the Janus kinase (JAK) family, is associated with the cytoplasmic domain of type I and II cytokine receptors. The Tyk2 gene is located on chromosome 19p13.2. It has been reported that polymorphisms at rs2109069 near the Tyk2 gene might be associated with COVID-19 outcome (Pairo-Castineira *et al*., 2021).

Table 3-3: Genotyping and allele frequency of TYK2-rs2304256 in COVID-19 patients and control

Variables	Patients N= 125	Control N= 60	OR (95% CI)	P value
TYK2-rs2304256				
CC	79 (63.2 %)	48 (80 %)	1	1
AA	18 (14.4 %)	2 (3.3 %)	5.46 (1.21-24.61)	**0.027**
CA	28 (22.4 %)	10 (16.7 %)	1.7 (0.76- 3.81)	0.196
AA+ CA	46 (36.8 %)	12 (20 %)	2.81 (1.37-5.78)	**0.004**
P trend				**0.019**
Allele frequency				
C	186 (74.4%)	106 (88.33%)		
A	64 (25.6%)	14 (11.67%)	2.60 (1.39- 4.87)	**0.002**
Recessive model				
CC+CA	107 (85.6 %)	58 (96.7 %)		
AA	18 (14.4 %)	2 (3.3 %)	4.87 (0.09- 21.76)	**0.032**
Additive model				
CC	79 (63.2 %)	48 (80 %)		
AA	18 (14.4 %)	2 (3.3 %)	5.46 (1.21- 24.61)	**0.015**
Dominant model				
CC	79 (63.2 %)	48 (80 %)		
AA+ CA	46 (36.8 %)	12 (20 %)	2.32 (1.12- 4.83)	**0.021**

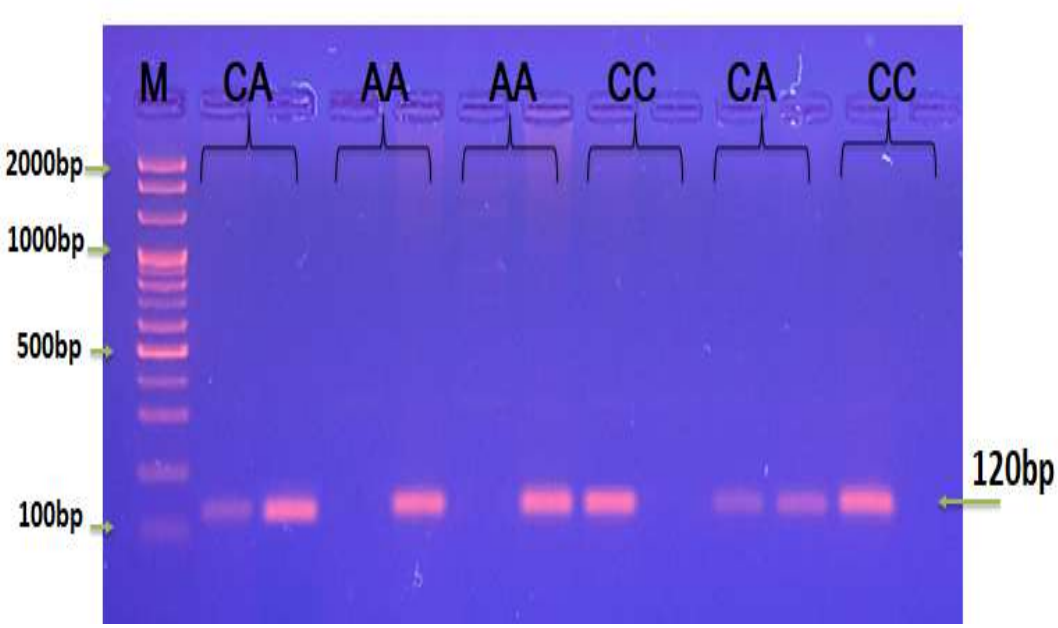

Figure 3-6: Agarose gel electrophoresis for TYK2-rs2304256

In this image above showed the ARMS-PCR product analysis of TYK2-rs2304256 gene polymorphism. Where M: marker (2000-100bp). The (CC) wild type homozygote were showed in C allele only, the (AA) mutant type homozygote were showed in A allele only, whereas the (CA) heterozygote were showed in both C and A allele. The presence of C or A allele were observed at 120bp product size.

The current study found that frequencies of the homozygous variant AA and heterozygous variant CA of TYK2-rs2304256 were 14.4 % and 22.4 % in COVID-19 cases and 3.3 % and 16.7 % in healthy controls, respectively.

TYK2 plays an important role in immunity because it encodes a nonreceptor tyrosine kinase that is constitutively expressed across different immune cells and activates dendritic cells to present self-antigens to autoreactive T-cells (Dendrou *et al*., 2016). Moreover, TYK2 has a key role in regulating the intracellular signaling of several cytokines and IFN-I (Marroqui

et al., 2015). TYK2 has been associated with other autoimmune diseases in different populations (Dendrou *et al*., 2016; Westra *et al*., 2018).

Another study mentioned that TYK2 rs74956615 was associated as a risk factor in patients with COVID-19, OR was 1.6 (P< 0.0001). TYK2 is one of four gene targets for JAK inhibitors such as baricitinib44, one of the nine candidate drugs that we used in the creation of our a priori target list. As opportunities for therapeutic intervention, particularly experimental therapy, are more abundant in later, more-severe cases of disease, it is important that the results also reveal genes that may act to drive inflammatory organ injury. High expression of TYK2, are associated with life-threatening disease; and transcriptome-wide association in lung tissue revealed that high expression of the monocyte–macrophage chemotactic receptor CCR2 is associated with severe COVID-19 (Pairo-Castineira *et al*., 2021).

3.4. Impact of IL-35 and presepsin on other inflammatory parameters:

There is no impact of IL-35 and presepsin on each other (r= -0.05; P= 0.57). As well as no impact on other inflammatory parameters including lactate dehydrogenase (LDH), D dimer, ferritin (P< 0.05) except there is negative correlation between presepsin and C- reactive protein (CRP) (r= 0.21; P= 0.018) that's mean presepsin may has negatively direct effect with CRP. (Table 3-4).

Table 3-4: Impact of IL-35 and presepsin on other inflammatory parameters

Variables		**Presepsin**	**IL_35**
LDH	Cor.	-0.118	0.082
	Sig. (2 tailed)	0.190	0.366
D dimer	Cor.	-0.130	0.068
	Sig. (2-tailed)	0.147	0.453
Ferritin	Cor.	-0.170	-0.062
	Sig. (2-tailed)	0.059	0.494
CRP	Cor.	**-.211-***	0.035
	Sig. (2-tailed)	**0.018**	0.695
Presepsin	Cor.		-0.051
	Sig. (2-tailed)		0.572

There are still only a few studies on IL-35 and presepsin, so the functions of these cytokines in combatting viral infection are not yet well understood (Guo *et al*., 2019).

Eosinophilia, allergic asthma (Giovannini-Chami *et al*., 2016), are among the conditions in which increased eosinophils may be harmful. A research suggests that IL-35 reduces airway eosinophilia by inhibiting the synthesis of the eosinophil-attracting chemokines, implying that IL-35 might be a potential treatment method for lowering eosinophil tissue recruitment in disorders like asthma (Kanai *et al*., 2017).

Investigations have demonstrated that IL-35 expression is reduced in chronic obstructive pulmonary disease (COPD), a type of chronic bronchitis and emphysema marked by airflow restriction (Himani *et al*., 2018). Furthermore, lower IL-35 levels were inversely linked with smoking status, suggesting that IL-35 might be used as a biomarker to predict chronic obstructive pulmonary disease development (Jiang *et al*., 2018). As well as IL-35 is an excellent indication of allergic inflammation and can be utilized as a biomarker (Zhang *et al*., 2019).

The function of IL-35 during viral infection is poorly understood. Patients with seasonal influenza A virus (IAV) had higher amounts of IL-35 in their peripheral blood mononuclear cells and throat swabs than healthy people, according to this study. IAV infection elevated IL-35 mRNA and protein levels in human lung epithelial and primary cells. IAV-induced IL-35 transcription is controlled by NF-B, according to another research. Select inhibitors of cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase dramatically reduced IL-35 expression, indicating that they are involved in IL-35 expression (Chen *et al*., 2016).

According to reports, PSN is a new biomarker for sepsis. Numerous studies have demonstrated that PSN is helpful for diagnosing sepsis and may also be able to predict the severity and mortality of the condition. Additionally, higher PSN may serve as a biomarker in the prognostic evaluation of COVID-19 patient (Zeng *et al*, 2020).

The PSN prognostic value for predicting 30-day death in COVID-19 patients is also investigated in this study. In COVID-19 patients that were hospitalized, PSN was performed comparably in terms of predicting 30-day

death (Park *et al*, 2022). PSN has demonstrated adequate performance in predicting the worsening severity of COVID-19, which can help doctors identify high-risk patients and choose treatment plans early on for the best use of available resources (Ahmed *et al*, 2021).

3.5. Vaccination in COVID-19 patients:

The most common patients in the current study were non- vaccinated 118 (94.4%), whereas vaccinated patients were 7 (5.6%) (Figure 3-7).

Patients that have tow dose of COVID-19 vaccine were 5 patients; all these patients were survivor. Patients with one dose vaccine were 2; both patients were non-survivor

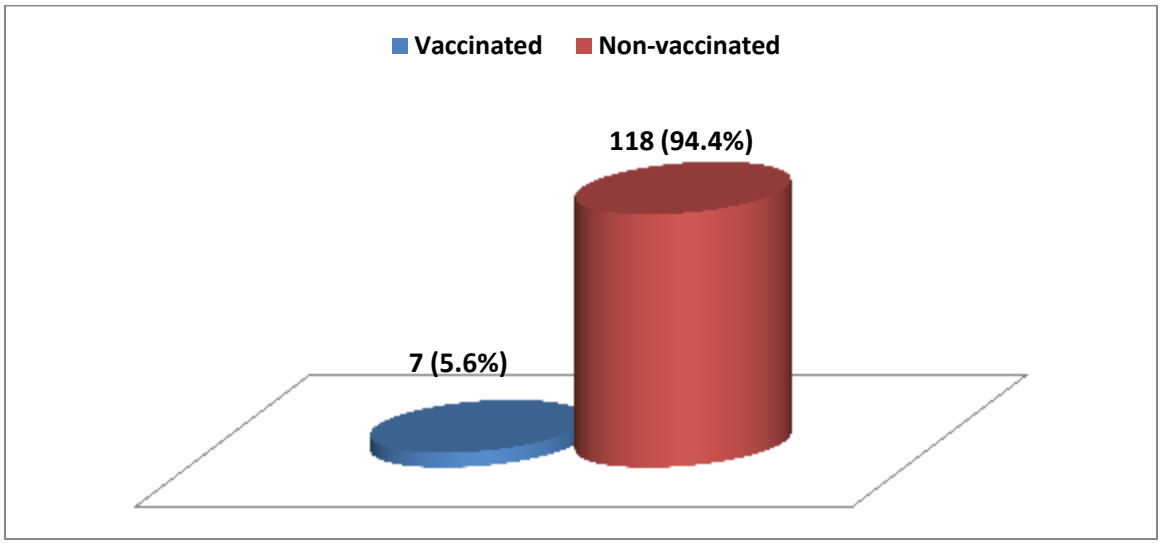

Figure 3-7: Vaccination among patients with COVID-19

The majority of the population remains susceptible to SARSCoV-2 infections (WHO, 2020). Thus, vaccine development has been a high priority. Vaccination remains a key preventive measure to reduce disease burden and mitigate future outbreaks.

The most common patients in the current study were non- vaccinated 118 (94.4%), whereas vaccinated patients were 7 (5.6%).

In a study suggests that a vaccine could have a substantial impact on reducing incidence, hospitalizations, and deaths, especially among vulnerable individuals with comorbidities and risk factors associated with severe COVID-19. Vaccination with reduced efficacy in elderly and comorbid individuals still markedly reduced hospitalizations and deaths. Non-ICU hospitalizations, ICU hospitalizations, and deaths would be reduced by 63.5%, 65.6%, and 69.3%, respectively, over 300 days from the start of vaccination. (Moghadas *et al*., 2021)

Results from a study mentioned that among 1983patients with COVID-19, the median age, 59 years [IQR, 45-69]. Unvaccinated patients accounted for 84.2% (1669/1983) of COVID-19 hospitalizations. Hospitalization for COVID-19 was significantly associated with decreased likelihood of vaccination (Tenforde *et al*., 2021).

Relevance vaccination with an mRNA COVID-19 vaccine was significantly less likely among patients with COVID-19 hospitalization and disease progression to death or mechanical ventilation. These findings are consistent with risk reduction among vaccine breakthrough infections compared with absence of vaccination (Bajema *et al*., 2021).

Among 1983 COVID-19 case patients, vaccine breakthrough patients compared with unvaccinated patients tended to be older (median age 67 vs 53 years) (Tenforde *et al*., 2021).

Unvaccinated patients accounted for 93.9% (261/278) of cases with disease progression to death or invasive mechanical ventilation. Among

patients hospitalized with COVID-19, death or invasive mechanical ventilation was associated with a lower likelihood of vaccination (OR= 0.33). Restricting to cases admitted with hypoxemia death or mechanical ventilation was also associated with a lower likelihood of vaccination (OR= 0.30) (Tenforde *et al*., 2021).

Conclusions and Recommendations

Conclusions:

1. IL-35 and Presepsin showed statistical significant differences between patients and controls (P< 0.0001) for each one.
2. The IL12A rs568408 AA and AG variant genotypes were associated with a significantly increased risk of COVID-19. In TYK2-rs2304256, AA genotype showed a statistically significant risk for COVID-19 compared with the wild-type rs2304256 CC.

Recommendations:

1. More single nucleotide polymorphisms may be studied in the IL-12A gene and TYK-2 gene to get more information about possible association to COVID-19.
2. Study of IL-35 and presepsin effect in the severity of COVID-19 patients.
3. Study the immunological therapy of IL-35 in viral infection generally and in COVID-19 especially.

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الخلاصه:

اعتبر المرض الناجم عن SARS-CoV-2 المعروف باسم COVID-19 وباء من قبل منظمة الصحة العالمية (WHO) وكان له خاصية العدوى الشديدة وسرعة الانتشار ، مما أدى إلى تغييرات في عادات الإنسان مع تأثره على الصحة العالمية.

شملت الدراسة الحالية ١٢٥ مريضا و ٦٠ مسجلين كمجموعة ضابطة في فحوصات المناعية والتنميط الجيني. تم إدخال جميع المرضى إلى مدينة المرجان الطبية ومستشفى الشوملي العام وجناح كورونا فايروس المستجد بمستشفى الصادق في الفترة من يناير ٢٠٢٢ إلى يوليو ٢٠٢٢. تم تأكيد تشخيص كورونا فايروس المستجد لكل مريض بواسطة- SARS-CoV-2 إيجابي .RT-PCR تم الكشف عن تعدد أشكال النوكليوتيدات الفردية (SNPs) لـ IL-12A- rs568408 و-TYK2 rs2304256 بواسطة طريقة Allele-PCR المحددة. استخدم اختبار ELISA لتحديد تركيز إنترلوكين ٣٥ (IL-35) وبريسيبسين (PSN) في مصل الدم.

اشتملت مجموعة المرضى على ٥٦ (٤٤,٨٪) من الذكور و ٦٩ (٥٥,٢٪) من الإناث ، بينما ضمت المجموعة الضابطة ٢٨ (٤٦,٦٪) من الذكور و ٣٢ (٥٣,٤٪) من الإناث ولم يكن هناك فرق معنوي في التوزيع التكراري للمرضى والسيطرة. الموضوعات حسب الجنس.(P = 0.87)

كان متوسط عمر المرضى ٧٥ (٦٢-٨٥) سنة ، أصغر عمر كان ١٥ سنة وأكبر عمر كان ٩٠ سنة. كان متوسط عمر الأشخاص الضابطة ٧٤ (٦٤-٨٢) سنة ولم يكن هناك فرق كبير بين المرضى والأشخاص الضابطة.(P = 0.65)

أظهر IL-35 فروق ذات دلالة إحصائية بين المرضى ٦,٨٦ ± ٢,٣١ ng / ml ومجموعة التحكم ٣,٨٦ ± ٢,٠٧ (نانوغرام / مل .(P <0.0001) (أظهر PSN فروق ذات دلالة إحصائية بين المرضى ٣١٨,١٨ ± ٢٢٦,٦٢ (pg / ml) ومجموعة التحكم ١٩٩,٦٨ ± ٣٩ P) (pg / ml) (<0.0001).

كشف توزيع كل من ترددات التنميط الجيني والأليل لـ IL-12A rs568408 عن فروق ذات دلالة إحصائية بين المرضى ومجموعات المراقبة (P = 0.006 و p = 0.001 على التوالي). ارتبطت الأنماط الجينية المتغيرة IL12A rs568408 AA و AG بزيادة كبيرة في خطر الإصابة بـ

مرض كورونا المستجد بنسبة الأرجحية 5.19 = (OR) ، فاصل الثقة ٩٥ ٪ 1.13-23.82 :(CI)؛ [OR = 2.39وP = 0.034] ، CI = 1.16- 4.94٪95 ، [P = 0.018على التوالي مقارنة مع الزيجوت المتماثل GG من النوع البري.

أظهر تحليل الانحدار اللوجستي أن ترددات المتغير متماثل اللواقح AA والمتغير المتغاير الزيجوت AC لـ TYK2-rs2304256 كانت ١٤,٤٪ و ٢٢,٤٪ في حالات COVID-19 و ٣,٣٪ و ١٦,٧٪ في الضوابط الصحية على التوالي. بالنسبة للنمط الجيني5.46 = (OR AA ، 95٪ :CI 1.21-24.61؛ .(P = 0.027 بينما أظهر التركيب الوراثي1.7 = (OR AC ، 95 ٪ -0.76 = CI 3.81 ، (P = 0.196، فيTYK2-rs2304256 ، أظهر النمط الوراثي AA خطرًا ذا دلالة إحصائية لـ COVID-19 في العراق مقارنة مع النوع البري .rs 2304256 CC ارتبطت الأنماط الجينية المتغيرة rs568408 AC / AA بزيادة كبيرة في خطر الإصابة بـ = (OR COVID-19 2.81 ، 95٪ CI: 1.37-5.78 ؛ (P = 0.004 ، مقارنةً بالنوع البري.rs 2304256 CC

في الختام ، أظهر IL-35 و Presepsin فروق ذات دلالة إحصائية بين المرضى والضوابط(P <0.0001) لكل واحد. يمكن استخدام IL-35 في تشخيص .COVID-19 ارتبطت الأنماط الجينية المتغيرة IL12A rs568408 AA و AG بزيادة خطر الإصابة بـ COVID-19 بشكل ملحوظ. في TYK2-rs2304256 ، أظهر النمط الجيني AA خطرًا ذا دلالة إحصائية لـ COVID-19 مقارنةً بالنوع البري.rs2304256 CC

جمهورية العراق
وزاره التعليم العالي والبحث العلمي
جامعه بابل/ كليه الطب
فرع الاحياء المجهرية

دراسة مناعية و التنميط الوراثي المتخصص للاليل في المرضى المصابين بفايروس كورونا المستجد

رسالة مقدمة الى
مجلس كلية الطب جامعة بابل
كجزء من متطلبات نيل شهادة الدكتوراه فلسفة في العلوم /الاحياء المجهرية الطبية

من قبل
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بكالوريوس الكليه التقنيه الطبيه بغداد (٢٠١٣)
ماجستير الاحياء المجهرية الطبيه (٢٠٢٠)

بأشراف
الاستاذه الدكتوره زيتون عبد الرضا الخفاجي

١٤٤٤ هـ ٢٠٢٣ م