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Effect of Toll-like Receptor 7 *TLR7* Gene Polymorphism and Some Biomarkers on the Severity in COVID-19 Patients

A Thesis
Submitted to the Council of the College of Medicine / University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Clinical Biochemistry

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يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ

وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ (11)

صدق الله العظيم

المجادلة (11)

Supervisor Certification

We certify that this thesis entitled (Effect of Toll-like receptor 7 (TLR7) gene polymorphism and blood groups on the severity in COVID-19 patients) has been prepared under our supervision at the College of Medicine, University of Babylon, as partial fulfillment of the requirement for the master degree of science (M.Sc.) in Clinical Biochemistry.

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

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In the Name of Allah Most gracious Most Merciful

Either after:

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Zainab

Dedication

To.....

My beloved Iraq

To everyone who fell martyr in defense of the homeland

For my Father and Mother, My Lovely Children, Brothers and Sisters,

Special thanks for Alaa Hussein Sabaa who supported me throughout this work and I will always appreciate what he has done to me.

To every colleague who died because of this damn virus.

To…All people who helped me in my research.

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Summary

The latest threat to global health is the continuing outbreak of a respiratory disease that was recently given the name COVID-19. COVID.19 was recognized in December 2019. It was rapidly shown to be caused by a novel coronaviruses that is structurally related to the virus that causes severe acute respiratory syndrome (SARS).

The COVID-19 outbreak has posed critical challenges for the public health, research, and medical. The difference insusceptibility for infection of coronaviruses and disease progression is associated with a genetic background. As may be toll like receptor 7 gene polymorphism. ABO blood group may associated with severity of COVID-19 patients.

The present study was designed to investigate the Effect of Toll-like receptor 7 gene polymorphism and ABO blood groups on the severity in COVID-19 patients.

Toll Like Receptor7 gene located on the Xp22.2 chromosome. It is Pattern recognition receptors located on intracellular organelles which produce antiviral immunity by recognizing single-stranded RNA (ss-RNA). TLR7's ability to inhibit virus replication and release of pro-inflammatory cytokines and chemokines.

This study was included ninety patients with COVID-19, which were divided into three groups according to World Health Organization (mild, moderate, sever). From Diyala Province population in Iraq during the period from November 2021 to February 2022. The participants were asked to provide information about the nature, severity, and duration of their related symptoms , fever, nausea, diarrhea, loss of sense of smell or taste, fatigue, dyspnea, headache, cough, runny

nose, sore throat, and myalgia). In a standardized questionnaire, they answered questions about their COVID-19 infection.

By AFIAS instrument were determination of ferritin, D.dimer, CRP, by Human spectrophotometric (Germany) was done serum LDH, device Mindary BC-3000 Plus was use for Complete Blood Count, PCR-RFLP protocol was used to detect Toll Like Receptor rs179008 gene polymorphism.

Estimation of ferritin, D.dimer, C - reactive protein and Lactate dehydrogenase proved an increasing the level of these parameters with significant difference between the patients with COVID-19 in the three groups were p-value > 0.05.

The levels of the white blood cell in three groups revealed no significant difference between mild and moderate group were p value (0.228), positive significant difference between moderate and severe groups were p value (0.015) and positive significant difference between mild and severe groups were p value (0.000), the results showed positive significant difference and an elevation in level of neutrophil and reducing in lymphocyte count.

While, the study showed a non-significant difference in the levels of hemoglobin, Hematocrit, Mean platelet volume were $p > 0.05$.

In other hand found non-significant difference with ABO blood group when compared between three groups.

Regarding the genetic analysis, There was no significant difference in genotype frequency between three patient groups all modes, codominance, dominance and recessive ($p < 0.05$). Except for genotype (AT) in overdominant between mild and moderate groups it is a less effect than other genotype were p value (0.024). In addition, there was no significant difference in alleles frequency

(A/T) between patient groups. Therefore, based on odds ratio (OR) calculations, the genotypes or alleles don't act risk factor for the disease.

The current study concludes that patients when infected with coronaviruses were found some parameter elevated in blood, ABO blood group not effect with severity disease in COVID-19 patients and found model overdominant (AT) in TLR7 gene less effected than other genotype (AA, TT) on severity, and positive correlation between these biomarkers contribute in the predict progression to severe infection. So the laboratory test results can be used to differentiate mild from severe COVID-19 cases.

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List of Abbreviations

ABBREVIATION	KEY
ACE2	Angiotensin-converting enzyme 2
aPL	Antiphospholipid antibodies
AFIAS	Automated Fluorescence Immunoassay System
ARDS	Acute respiratory distress syndrome
CBC	Complete blood count
CD147	Cluster differentiation 147
CoVs	COVID-19
CRP	C-reactive protein (CRP) C-reactive protein
CT	Computed tomography
CTLs	Cytotoxic T lymphocytes
DAMP	Danger-associated molecular patterns
DCs	Dendritic cells
dsRNA	Double-stranded RNA
EDTA	Ethylene di amine tetra acetic acid
FiO_2	Fraction of inspiration oxygen
Hb	Hemoglobin
HCT	Hematocrit
ICU	Intensive care unit
IFN	Interferon
LDH	Lactate dehydrogenase
LYM	Lymphocyte
MERS	Middle East Respiratory Syndrome
MPV	Mean platelet volume

MyD88	Myeloid differentiation factor-88(MyD88)
MyD88	Adaptor molecule
NLR	Neutrophil to lymphocyte ratio
NEU	Neutrophil
NK	Natural killer
PAMPs	Pathogen-associated molecular patterns
PaO_2	Partial arterial oxygen percentage
PRRs	Pattern recognition receptors
RBCs	Red blood cells
SARS-CoV-1	Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
ss-RNA	Single-stranded RNA
TLR	Tool-Like Receptors
TMPRSS2	Transmembrane protease
URI	Upper respiratory infections
WBC	White blood cell

Chapter One
Introduction
and
Literature Review

1. Introduction

1.1 COVID-19

1.1.1 History

Coronavirus disease 2019 (COVID-19) is the third plague of this century, and it has been designated as the sixth international health issue. The World Health Organization (WHO) has declared a global health emergency on January 30, 2020 [1,2]. The International Committee on Virus Taxonomy of Viruses named it (severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [3]. On December 2019, A cluster of individuals with pneumonia of unknown etiology was discovered in Wuhan, Hubei Province, China[4,5]. The Chinese Center for Disease Control and Prevention (China CDC) and Wuhan City health authorities detected a novel coronavirus from lower respiratory tract samples of pneumonia patients on January 7, 2020, and released the genomic sequence on 11 January, 2020. In less than three months after the unknown pathogen was discovered, it had spread to at least 114 nations and killed nearly 4,000 people[5]. It's the seventh coronavirus to infect people; four of the others (Human coronavirus 229E, Human coronavirus NL63, Human coronavirus OC43, and Human coronavirus HKU1) only cause minor cold symptoms. Severe acute respiratory syndrome coronavirus 1(SARS-CoV), Middle east respiratory syndrome (MERS-CoV), and Severe acute respiratory syndrome coronavirus 2(SARS-CoV-2), on the other hand, are capable of causing serious symptoms and even death[6]. This is a worldwide crisis that requires the joint efforts of all humanity s to prevent it [7]. The difference insusceptibility for infection of coronaviruses and disease progression is associated with a genetic background[8].

1.1.2 Classification of the Coronavirus

The most well-known viruses are divided into groups based on their phylogenies with viruses that have previously infected the same host, as well as their genotype[9]. Coronaviruses belong to the Coronavirinae subfamily of the Coronaviridae family and the Nidovirales order. Alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus are the four genera within this subfamily[10]. Human coronaviruses are typically respiratory pathogens of seven types: 229E, NL63, OC43, KHU1, MERS-Cove, Coronavirus (SARS-Cove), and others (SARS-CoV-2). MERS-Cove, SARS-Cove, and SARS-CoV-2 are all members of the genus Betacoronavirus [11]. All viral in the Nidovirales order are enclosed, nonsegmented positive-sense RNA viruses, It carries the biggest genome among the previously known RNA viruses[12].

1.1.3 Origin of SARS-CoV-2

Scientists have questioned the origins of the new coronavirus SARS-CoV2 discovery[13]. It's been suggested that SARS-CoV-2 is the result of laboratory experiments. However, genomic evidence contradicts this theory, indicating that SARS-CoV-2 did evolve from a previously identified virus backbone[14]. It is, therefore, believed that the SARS-CoV-2 also originated from bats and, after mutating, was able to infect other animals. The mutation increased the receptor binding domain (RBD) affinity to angiotensin-converting enzyme 2 (ACE-2) in humans, but also other animals such as ferrets and Malayan pangolins (Manis javanica; a long-snouted, ant-eating mammal sold illegally for use in traditional Chinese medicine), but also decreased the RBD affinity to ACE-2 found in

rodents and civets. The pangolin is believed to be the intermediate host of SARS-CoV-2[15].

1.1.4 Structure of the Coronavirus

Structurally, Spherical or pleomorphic particles ranging in length from 80 to 160 nm the presence of single-stranded (positive-sense) RNA coupled with a nucleoprotein was found in a capsid containing matrix protein[16]. SARS-CoV.2 has four major structural proteins: spike (S) glycoprotein, small envelope (E)

glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein, as well as a number of auxiliary proteins[17].

Spike or S glycoprotein is a polyprotein of 1273 amino acids[18]. It is a transmembrane protein located in the outer region of the virus with a molecular weight of 150 k Da. S protein generates homotrimers protruding in the viral surface and was aids envelope virus attachment to host cells by binding to (ACE2), which is abundant in lower respiratory tract cells. The ectodomain of S protein in all protein of CoVs has a domain organization, separated into 2-domains, S1 and S2. With the receptor binding domain, part S1 assists in the binding of host receptor, whilst part S2 is in charge of mediating virus fusion and transmitting to host cells.[19,11] S1 and S2 subunits constitute the spike glycoprotein[20].

The (N) proteins are the structural phosphoprotein of 43–46 K Da, N is the unique protein that responsible for binding to the CoV RNA genome, making up the nucleocapsid[21], The discovery of N in the endoplasmic reticulum (ER)-Golgi area because the protein is bound to RNA, the protein is involved in processes related to the viral genome, the viral replication cycle, and the cellular response of host cells to viral infections[22]. Shown figure 1.1

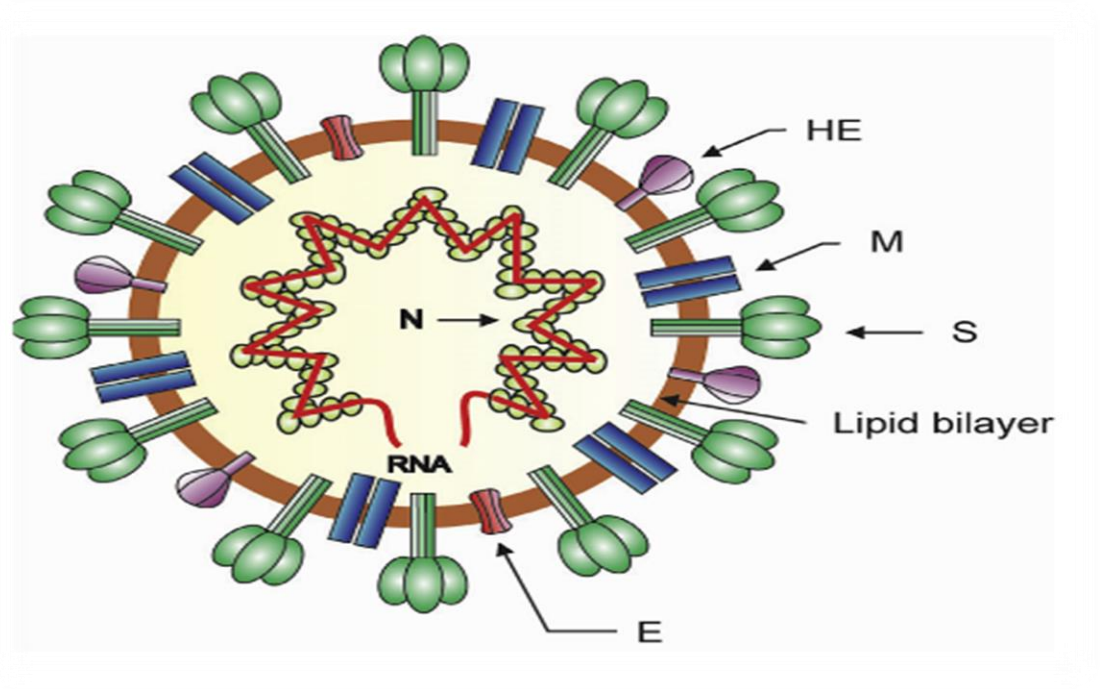


Figure 1.1 Structure of coronaviruses[23]

The membrane (M) glycoprotein is another important part of this virus which is the abundant envelope protein of CoV. Plays a role in determining the shape of the virus envelope. This protein has the ability to bind to all structural proteins. Binding with protein M helps to stabilized nucleocapsids or N proteins and promotes completion of viral assembly by stabilizing the N protein. RNA complex, inside the internal virion play a vital role in instituting the viral envelope's shape. While the E protein is a small protein in the CoV structure the functions in the production and maturation of this virus[22].

1.1.5 Epidemiology of Coronavirus

The first infection occurred in December 2019[24]. At the first injury was very low. But, In January 2020 it reached a breaking point. The migration of people before the Chinese lunar New Year resulted in a substantial increase of infected patients in places outside of Hubei Province during the second part of this month[25].

After an exponential increase till January 23, 2020, the virus spread across countries and drew widespread attention. Human-to-human infection has been observed in clusters of affected family members and medical staff[26].

In late January 2020, the World Health Organization recorded over 10-thousands cases of COVID-19 infections through China. [24].

1.1.6 Disease severity

COVID-19 disease severity was determined using the Chinese COVID-19 treatment guideline (version 6.0)[27]. COVID-19 patients were classified as mild, common (moderate), severe, or critical, depending on the severity of their disease. Modest type was described as symptoms that were mild and did not show up on imaging. The most common form was described as having respiratory tract symptoms and imaging with pneumonia. The term "severe type" was defined as meeting any of the following criteria:

1- Respiratory distress and a respiratory frequency of less than 30 per minute.
2- At rest, blood oxygen saturation is 93 %.
3- Partial arterial oxygen pressure (PaO_2)/fraction of inspiration oxygen (FiO_2) PaO_2/FiO_2 ratio $\leq$ 300 mmHg.
4- Within 24–48 hours, lung infiltrates exceed 50%.

Any of the following criteria were used to determine critical type:

1- Failure of the normal respiration, requiring the use of mechanical ventilation.

2- Shocking takes place.

3- When common with another organ failure, ICU monitoring and therapy is required[27].

1.1.7 Entry and Life Cycle SARS COV-2

This case similar to other viral infections, asymptomatic disease occurs in a significant number of patients but in the majority of the patients, a first week, self-limiting viral respiratory disease typically occurs which ends with the development of neutralizing antiviral T cell and antibody immunity [28].

The virus can enter the human body through its receptors ACE2 which are found in various organs such as the heart, lungs, kidneys, and gastrointestinal tract, thus facilitating viral entry into target cells. The process of COVID-19 entering into the host cell begins through the attachment of the S glycoprotein to the receptor, the ACE2 in the host cells (such as in type II pneumocystis in the lungs)[29]. This binding takes place in the S protein of SARS-CoV.2 receptors' binding domain, which has 331 to 524 residues and may bind strongly to human ACE2. The entry and binding processes are then followed by fusion of the viral membrane and host cell [11].

After fusion occurs, the type II trans membrane serine protease (TMPRSS2) that is present on the surface of the host cell will clear the ACE2 and activate the receptor attached spike like S proteins [30,[31]. This activation of the S proteins causes conformational changes in the viral and allowing it to enter cells. Both of these proteins (TMPRSS2 and ACE2) are the main determinants of the entry of this virus[32].As shown in figure 1.2

Based on the research of Sungnak *et al*. nasal epithelial cells, specifically goblet/secretory cells and ciliated cells, display the highest ACE2 expression throughout the respiratory tract[33].

Furthermore, entered SARS-CoV will subsequently release its genomic material in the cytoplasm and become translated in the nuclei. The genomic material released by this virus is mRNA that is ready to be translated into protein. In its genome range, this virus is complemented by about 14, each of which encodes a variety of proteins both structural and non-structural that play a role in its survival as well as virulence power [34].

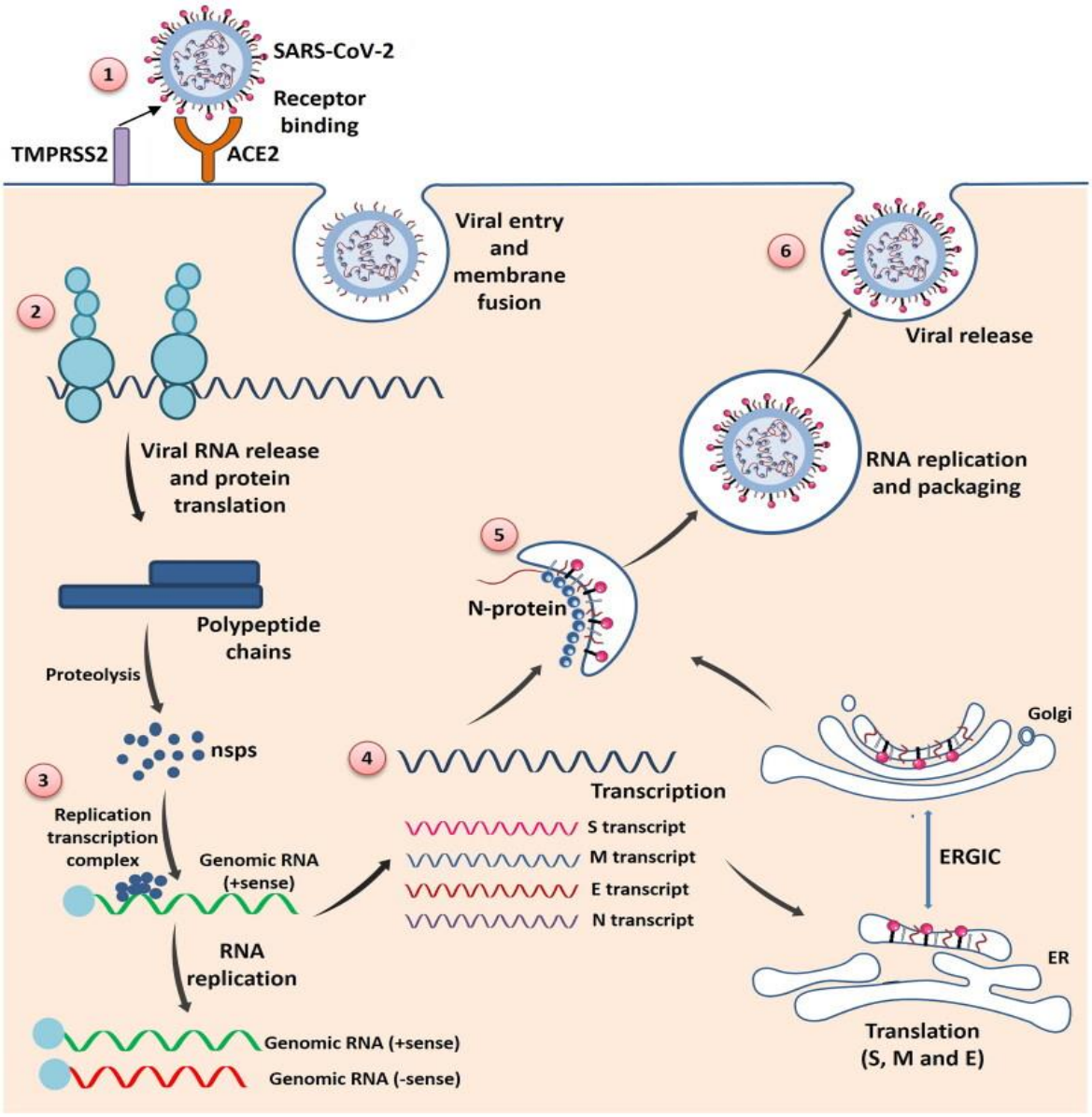


Figure 1.2 Replication of coronaviruses[35]

1.1.8 Risk factors of Coronavirus

Patients with CoVs infection can have symptoms ranging from mild to severe with a significant portion of the population is asymptomatic carriers, The most commonly reported symptoms are fever ,cough and shortness of breath[36]. Cough (with or without sputum), loss of sense of smell or taste, discomfort

arthralgia, weakness, fatigue, or myalgia, chest tightness, excessive mucus production with expectoration, hemoptysis, and dyspnoea are some of the symptoms that might occur[37,38], Gastrointestinal symptoms such as vomiting, diarrhea and abdominal pain are observed in some patients with COVID-19[38,39].

Patients with comorbidities including hypertension or diabetes mellitus are more susceptible to developing a more severe course and progression of COVID-19 illness. Furthermore, elderly patients, particularly those 65 and older who have comorbidities and infected, have a higher rate of intensive care unit (ICU) hospitalization and mortality from the COVID-19 disease[40] .

Although the lung is the primary target of coronavirus infection, which is ACE2 receptors are wide distribution in this organs[41], may cause harm to the heart, gastrointestinal tract, kidneys, liver, central nervous system and eyes, all of which must be continuously monitored[42]. COVID-19 has a median incubation period of 4 days, with an interquartile range of 2 to 7[43,44]. In six days, one COVID-19 infected person can infect three additional people, and those three newly infected people can infect nine more people[45] .

1.2 Parameters of COVID-19 infection and severe Progression

In patients with severe disease compared to moderate systemic disease, a patterns of inflammatory, biochemical, Coagulation and hematologic biomarker abnormalities has been found.

1.2.1. Ferritin

Ferritin is an intracellular protein that can store iron, and plays a critical role in inflammatory diseases, such as infection. Ferritin is primarily used in clinical medicine as a serum measure of total body iron storage. In cases of iron deficiency and overload, serum ferritin serves an important role in both diagnosis and management[46]. The cytokine storm is an unregulated and disordered immune in the immunopathogenic mechanism of coronavirus similar to that seen in severe influenza. During the course of the disease, inflammatory cytokines such as TNF-, IL-6, IL-12, and IL-8 are released in large amounts. possible outcomes acute respiratory distress syndrome (ARDS) and systemic organ failure. Evidence suggests that serum ferritin, d-dimer, LDH, and CRP levels rise as the condition worsens, indicating a higher chance of death[47].

Hyperferritinemia is a condition induced by an infection's excessive inflammation and represents an indicator to identified high-risk patients in order to direct the treatment intervention to control inflammation[48,49]. Blood ferritin which is a well- known complication of virus infection, is closely linked to CoV-19 patients' poor recovery[47,50,51]. Serum ferritin is especially interesting due to its Possible diagnostic and prognostic effect. In this study, determine the potential relationship of ferritin level with severe condition of COVID-19 patients.

1.2.2 C-Reactive protein

C-reactive protein (CRP) is a well-known indicator of systemic inflammation and infection. CRP binds to phosphocholine in pathogens and host cell membranes as an acute-phase reactant protein made in the liver in response to interleukin-6 and is a frequently used biomarker of inflammation and functions as an opsonin to aid in phagocytosis and clearing. CRP that is coupled to a ligand

activates the complement system's classical pathway as well as an important component of innate host defence[52]. Prior to the COVID-19 international pandemic, infectious aetiologies were responsible for up to 90% of all CRP rises, the majority of which were caused by bacterial pathogens[53[54]. CRP levels have also been found to be elevated in severe viral illnesses, such as pneumonia, and SARS-CoV-2 infections[55,56]. CRP concentrations have also been linked to respiratory failure necessitating mechanical ventilation in recent studies. A five-fold increased risk of acute respiratory distress syndrome, CRP has been linked to extrapulmonary disease in COVID-19, and many studies have found a link between CRP levels and myocardial injury. CRP concentrations in COVID-19 infection reflect severity of infection as well as the intensity of the acute inflammatory response[57,58].

1.2.3 D-dimer

D-dimer, a degrading product of cross-linked fibrin generated during of the coagulation system activation, As a result, it reflects still active of hemostatic and thrombolytic system[59].

Coagulopathy is a significant symptom of infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is linked to poor illness outcomes[60]. Infection with SARS-CoV-2 causes both venous and arterial thrombosis[61]. The most common kind of arterial thrombosis was ischemic stroke. additional, arterial events recorded include myocardial infarction, microvascular thrombosis in several organ which is lungs, bowel, limb and skin[62][63].

A systematic review reported that a normal range of a highly sensitive D-dimer level accurately ruled out deep venous thrombosis (DVT) in patients classified as having a low or moderate clinical probability of DVT. Thus, it is important to monitor the level of D- dimer the incidence and characteristics of DVT in acute stroke patients[64].

Previous research has looked at D-dimer as a predictor of outcomes and a measure of disease severity[65],66]. After receiving anticoagulant therapy, the level of D-dimer decreased gradually, which means that D-dimer can not only predict thrombosis but also monitor the effectiveness of anticoagulants[67].

1.2.4 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is an enzyme that is involved in the converting of lactate to pyruvate in most body tissues' cells and it is released in to [68,69]. it is made up of four peptide chains of two types: Muscle (M) and Heart(H), each of which is genetically controlled separately[70]. Its activity can be found in the cytoplasm of numerous body cells, and enzyme concentrations in different tissues range from 1,500 to 5,000 times higher than those seen in serum. As result, leaking of the enzyme from even a little quantity of injured tissue increases the observed serum activity of LDH to a significant extent. LDH is a good biomarker for acute inflammatory damage in upper respiratory infections (URI), these cytokines, unlike LDH, do not immediately represent cellular damage — acute-phase cytokines are mediators of inflammation, but LDH is the result of inflammatory injury[71]. LDH is released in to the bronchoalveolar space when damage of cytoplasmic cell membrane. LDH elevation can potentially be a sign of underlying lung damage and inflammation[72]. Elevated LDH and neutrophil count were also indicator of severity viral. LDH levels are elevated in about 75%

of the cases involving new coronaviruses (SARS-CoV-2). LDH increases are linked to a higher likelihood of severe COVID-19. It can be used to make predictions for severe disease during hospitalization in order to ensure that patients receive correct clinical treatment[73].

1.2.5 Hematologic Biomarkers

COVID19 can produce a variety of alterations in peripheral blood parameters such as lymphopenia and neutrophilia, which should be examined early on in COVID-19 and are assumed to be strongly linked to the severity of the disease[74,75,76].

Furthermore, complete blood count (CBC) is one of the most commonly tests performed in the clinic, and it takes very little time. 80% of critically ill adult COVID-19 patients had lymphopenia, according to many studies[39]. These findings imply that lymphopenia may be linked to the severity of an illness. The hyperinflammatory response and subsequent cytokine production of COVID-19 infection drive an exaggerated neutrophil, macrophage, and monocyte infiltration into the lung parenchyma[77]. Finding that severe cases had lower lymphocyte, higher leukocyte, and higher Neutrophil to lymphocyte ratio (NLR) counts, as well as lower percentages of monocytes, eosinophils, and basophils, when compared to mild forms[78]. It is known that SARS-CoV-2 invades human cells by binding to (ACE-2) receptor, which is primarily found in the lungs, heart, and gastrointestinal tract. These receptors are also expressed on the surface of lymphocytes. Consequently, SARS-CoV-2 may bind directly to these cells and cause lysis. Infection also results in the production and release of multiple inflammatory cytokines, as described above. This potent cytokine activation can promote

lymphocyte apoptosis and lead to atrophy of lymphoid organs, thus decreasing lymphocyte regeneration[74].

Furthermore, CD4+T cells play an essential role as immune modulators, including downregulation of the inflammatory response. Consequently, lymphopenia may contribute to the hyperinflammation cascade[77]. The number of CD8 T lymphocytes in the blood was found to be an independent predictor of COVID-19 severity and treatment success[79]. Eosinophil count was shown to be lower in COVID-19 participants in a study[80]. The number of eosinophils in the blood has been linked to the number of lymphocytes, it could be a sign of a poor prognosis [81]. NLR has been demonstrated to be an independent risk factor for severe illness in COVID-19 patients[82].

NLR elevation could be caused by inflammatory cytokine dysregulation or an abnormal increase in pathogenic low-density neutrophils. and the overexpression of genes implicated in the lymphocyte cell death pathway as a result of the SARS-CoV-2 infection mechanism[83]. Because platelet count is a biomarker that has been linked to illness severity and mortality risk in the intensive care unit (ICU) [84,85]. The amount of platelets in COVID-19 patients was found to be considerably lower[86,87]. Platelets in the lungs may be activated by damaged lung tissue and pulmonary endothelial cells, leading in aggregation and the production of microthrombi, boosting platelet consumption[88].

1.3 Relationship between ABO blood group system and COVID-19

In 1901, the ABO blood group system was discovered[89]. It is formed by three alleles: A, B, and O, which are all coded by the ABO gene. On red blood cells (RBCs), the combination of these three alleles results in six genotypes and four phenotypes. Antigens on (RBCs) are combined with antibodies in the plasma, as shown in Table 1.1

Table1.1 Antigen and antibodies for each blood group

ABO blood group	Antigen	Antibody
A	A	Anti-B
B	B	Anti-A
O	None	Anti- A and B
AB	A and B	None

The ABO blood group polymorphism has been linked to the susceptibility and outcomes of a variety of diseases and infections. Such as SARS-COV-2 [90][91]. Furthermore, mounting data suggests that the ABO blood group may have a role in SARS-CoV-2 infection susceptibility and severity[92]. Differences in blood group antigen expression can increase or decrease host susceptibility to many infections. Blood group antigens can play a direct role in infection by serving as receptors and/or coreceptors for microorganisms, parasites, and viruses. In addition, many blood group antigens facilitate intracellular uptake, signal transduction, or cell adhesion through the organization of membrane microdomains. Blood group antigens can modify the innate immune response to infection[93]. Several research looked into the link between ABO blood groups and COVID-19 severity. According to reports that the highest number of COVID-19 individuals with severe symptoms was blood group (A)[92,94]. The blood group O was found to have the least severe symptoms[95]. (found that is Anti-A, anti-B and anti-A,B antibodies, which are prevalent in people with blood groups O, and absent in the other blood groups appear to block the interaction between SARS-CoV-2 and the angiotensin converting enzyme 2 (ACE2) receptor, which is expressed by the target cells in the host)[96]. shown in Figure 1.3

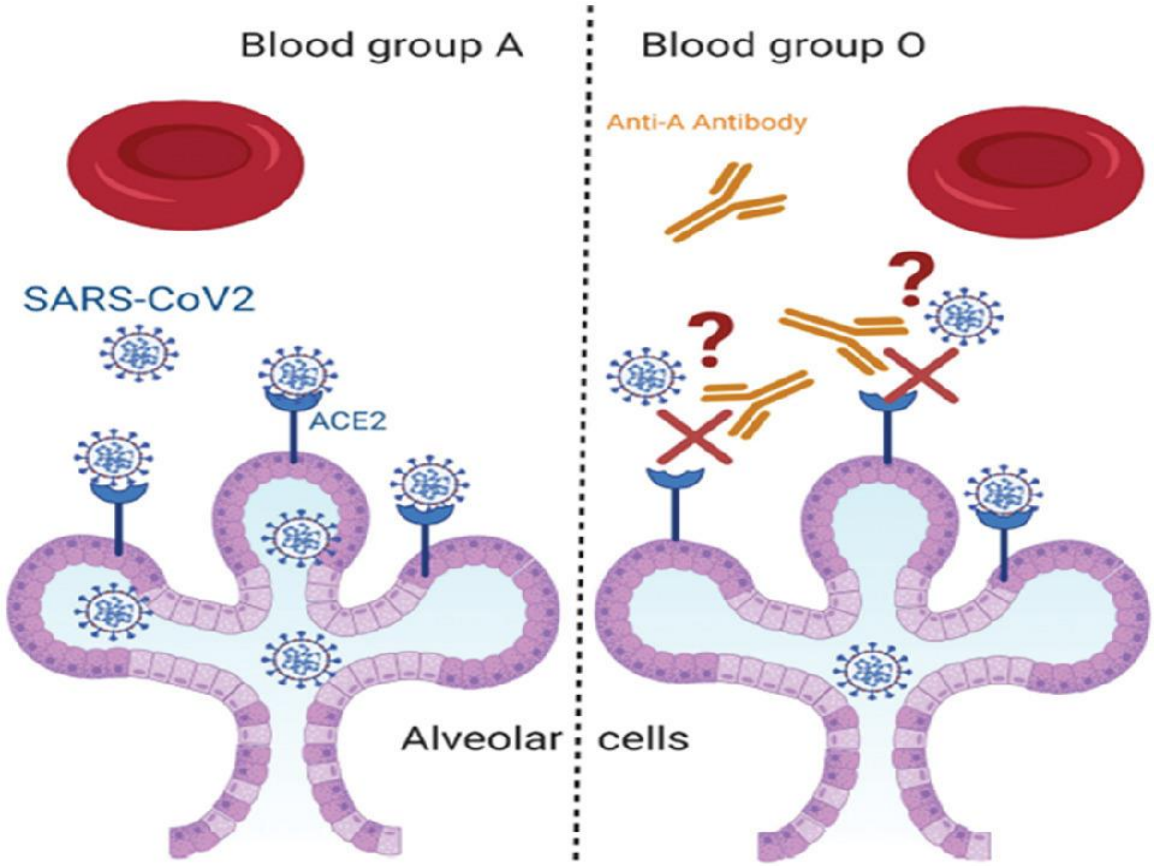


Figure 1.3 Interference of blood groups with SARS-CoV-2 adherence to host cells[97]

1.4 Tool-Like Receptors

Toll like receptors are protien that are involved in the immunity in vertebrates is divided into innate and adaptive immunity, innate immunity is an old kind of host defense against pathogens and exhibits important role in activation and creation of the adaptive immunity by inducing cytokines, chemokines and other co-stimulatory molecules. As a result, T and B cells are activated in host humans and a large number of different T and B cell receptors are product[98], the innate immune system recognizes a nonclonal pattern of molecules by so called pattern recognition receptors (PRRs). They bind conserved molecular structures as parts of pathogens (pathogen-associated molecular patterns, or (PAMPs).

The group of PRRs is large and diverse. They can be secreted or expressed on the cell surface or in intracellular compartments. One of the most important groups of pattern recognition receptors is the toll like receptor (TLR) family[99], it is comprises ten members (TLR1–TLR10). TLRs are located on the cell surface or in endosomes from immune and nonimmune cells (such as glia and neurons), such as TLR-1, -2, -4, -5, -6, -10 or in the endosome compartment, such as TLR-3, -7, -8, -9[100], whereas *TLR7* and *TLR8* are encoded on the X-chromosome. Recently TLR11 has been identified in mice as an important defense mechanism against bacterial infection but it was not yet found in humans[99]. while TLR3 recognizes viral double-stranded RNA (dsRNA). TLR7 recognizes viral single-stranded RNA and is therefore, likely to be implicated in clearance of SARS-CoV-2[101].

TLRs are essential proteins that recognize foreign and self-molecular signatures and offer host surveillance [102,103]. TLRs are type I transmembrane glycoproteins with three structural elements: (i) An N-terminal intracellular toll-interleukin 1 receptor domain, required for signal transduction, (ii) a central transmembrane domain, and (iii) an extracellular C-terminal rich in leucine repeats which provides variation between patient TLRs[104]. TLRs may recognize a set of

pathogen-associated molecular patterns (PAMPs) and respond by triggering a strong inflammatory response to neutralize and eradicate invading germs[102][103]. TLRs also respond to danger-associated molecular patterns (DAMP), which are released by injured, stressed, or necrotic cells, even when the cells are not infected [105]106] . The end product of inflammation, produced through the myeloid differentiation factor-88(MyD88)-dependent pathway (TLR1, 2, 4-10)[107] or the toll/IL-1-domain-containing adapter-inducing interferon-beta (TRIF)-dependent pathway (TLR3 and 4)[108]. Is found in all TLRs, regardless of where the activating ligand comes from. TLRs have been found to express themselves across the human respiratory system[109].

TLRs on the cell surface have been proposed as potential therapeutic targets in COVID-19, after molecular docking studies revealed direct interaction between S protein and TLR1, 4, and 6[110]. TLRs (TLR3, TLR7, and TLR8), which are important for recognizing pathogenic nucleic acids, are also found on the membranes of intracellular organelles (endosomes, lysosomes, and endolysozomes)[111]. As shown in figure 1.4

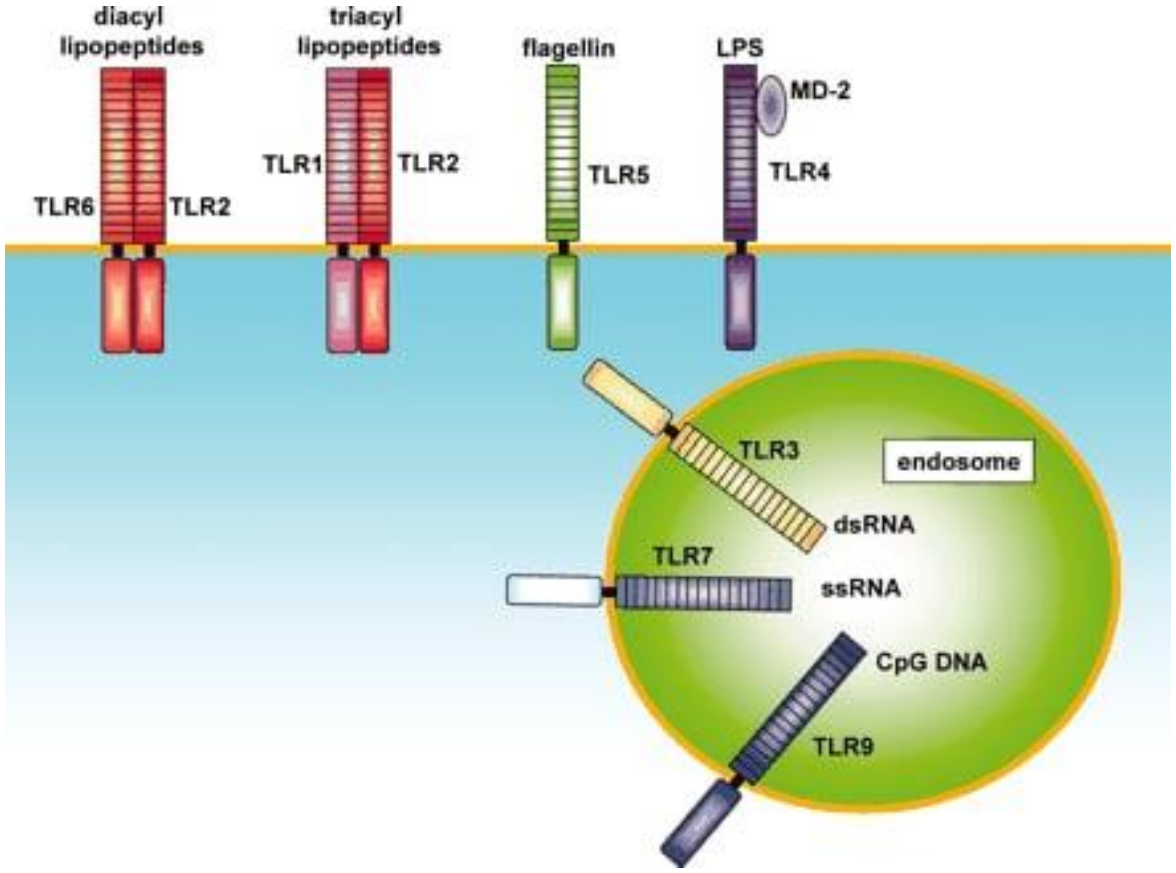


Figure 1.4 Ligand and TLRs[98]

The TLR family members recognize specific patterns of microbial components. TLR2 is essential in the recognition of microbial lipopeptides. TLR1and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides. TLR3 is implicated in the recognition of viral dsRNA, TLR4 is the receptor for LPS. TLR5 recognizes flagellin. TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR9 is essential in DNA recognition [98].

1.4.1 Relationship between Toll-Like Receptor 7 and COVID-19

COVID-19 recently has become a serious global pandemic. Age, gender, and comorbidities are known to be common risk factors for severity but are not enough to fully explain the magnitude of their effect on the risk of severity of the disease SNP (Single Nucleotide Polymorphism) is a type of mutation. Also, all mutations cannot be considered as SNP. The difference between SNP and Mutation As shown in the Table 1.2.

Table 1.2 Summarizing the difference between SNP and Mutation

SNP	**Mutation**
SNP is a change in the single-nucleotide of a genome. Also, it is a type of mutation.	Mutation is the variation in DNA base pairs caused due to insertion, deletion, duplication or substitution of base pairs
The variation is seen only in a single nucleotide.	The variation can be due to changes in many or even a single nucleotide
The SNP variation is available in a minimum of 1% of the population	The mutation frequency is available in less than 1% of the population.
Example – In the sequence ATAGC, the substitution of G by C will produce ATACC. This change in a single nucleotide is termed as SNP.	The mutations are of different types. The missense mutation, silent mutation and nonsense mutation are some of them.

(SNPs) in several genes have been reported as a genetic factor contributing to COVID-19 severity. The TLR7 gene span 16 kb apart, located on the Xp22.2 chromosome [9]. rs179008 SNP in TLR genes may contribute to disease progression [6].

TLR7 is PRRs located on intracellular organelles[112[113]. Which produce antiviral immunity by recognizing single-stranded RNA (ss-RNA) oligonucleotides containing guanosine- and uridine-rich sequences from RNA viruses Recognition occurs in the endosomes of plasmacytoid dendritic cells (DCs) and B cells and the consequent activation of pro-inflammatory pathways[114,115]. as show in figure 1.5, TLR7's ability to inhibit virus replication has been established in MERS-CoV[116]. As viral ssRNA binds to TLR7/8 upon entrance into the cell, increasing activation and antiviral immunity[117]. TLR7 activation causes the adaptor molecule MyD88 to be recruited, resulting in the release of pro-inflammatory cytokines and chemokines[118], IFNs of type I (IFNalpha and IFN-beta) and III (IFNalpha and IFN-beta) (IFN-lambda)[119]. It has been demonstrated to help in viral clearance and replication reduction. It's still unclear if TLR7/8 can interact directly with the SARS-CoV-2 S protein once it enters host cells. However, because of their antiviral immunity and ability to sense ssRNA, they have been suggested as potential SARS-CoV-2 therapeutic targets.

TLR7/8 in the endosome has been shown to identify viral genomic ssRNA from positive-sense RNA viruses[120[121]. TLR7's protective and antiviral involvement in SARS-CoV-2 infection is backed up by the catastrophic COVID-19 consequences.

Inheritance of a four-nucleotide deletion or a missense mutation resulted in lower TLR7 mRNA expression, adverse effect on TLR7 structure and

functionality, and defective type I and II IFN production[122]. Activation of TLR7/8 during SARS-CoV-2 may cause an enhanced inflammatory response, which could result in severe and potentially deadly immunopathological effects in COVID-19 patients, due to the simultaneous release of pro-inflammatory cytokines and chemokines[123]. Patients with COVID-19 have higher levels of pro-inflammatory cytokines and chemokines in their blood, which are produced by the TLR7/8 pathways[118]. This could be because antiphospholipid antibodies (aPL) (a TLR7/8 activating DAMP) are recognized by TLR7/8. aPLs are a type of autoantibody that binds to negatively charged phospholipids, causing self-tolerance to be disrupted and autoimmune reactions to be launched against host phospholipids[124][125]. When compared to healthy individuals, patients infected with SARS-CoV-2 exhibited higher concentrations of circulating aPLs, according to a study looking at the presence of aPLs in severe and critical COVID-19 patients[126].

Together, previous study findings suggest that activating TLR7 could be used as a therapeutic treatment to improve viral immunity and clearance. Imiquimod is a dual TLR7/8 agonist that has been proposed as a potential COVID-19 pharmacological therapy[127].

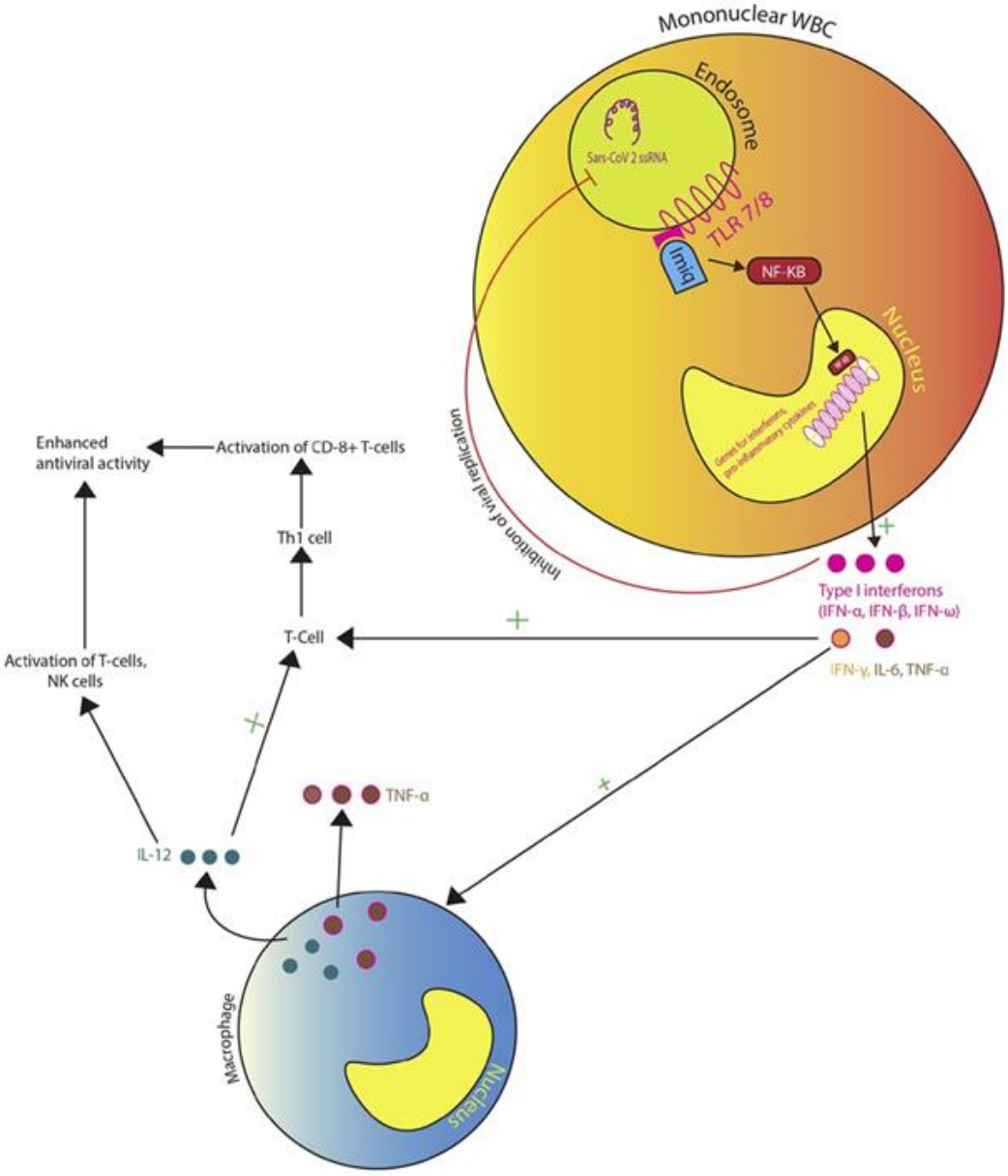


Figure 1.5 Intracellular TLR signaling and trafficking in pDCs[101]

Aim of the study

The principle objects of the present study can be summarized as show below:

1- To investigate the presence of effect the rs179008 (A>T) SNP polymorphism in *TLR7* gene on severity of COVID-19.

2- Determination the associated between LDH, C-reactive protein, D-dimer, Ferritin, Complete blood count with severity.

3-Determination effect ABO blood group on severity

Chapter Two
Materials and Methods

2. Materials and Methods

2.1 Ethical Issues

The ethical issues in the study were depended on the following:

1- Agreement of Babylon Medical College (University of Babylon, Iraq) and Biochemistry department at the same institution.

2- Approval of the Research and Development Department of the Diyala Health Directorate in Diyala Province.

3- The goals and methods of this research have been clarified to all members in the present research in order to obtain their oral approval.

2.2 Materials

2.2.1 Chemicals and kits

Table 2.1 Lists of the chemical substances and kits that used in this study.

NO	Chemicals	Company	Origin
1	100 bp step DNA ladder	Cyntol	Russian
2	Agarose	Himedia	India
3	Blood group kit		Canada
4	Complete blood count (CBC)	Human	Germany
5	C-Reactive Protein (CRP)	AFIAS	Korea
6	D-Dimer	AFIAS	Korea
7	EDTA	Promega	USA
8	Ethidium bromide	Himedia	India
9	Ferritin kit	AFIAS	Korea
10	Kit for PCR Master mix	Cyntol	Russia

11	Lactatdehydrogenase (LDH)	Human	Grmany
12	Molecular grad water	Promega	USA
13	Primer	Macrogen	Korea
14	Restriction enzyme	Sib	Russian
15	Tris /HCL	Himedia	India
16	Tris base	Himedia	India

2.2.2 Instruments and Tools

Table 2.2 List for commonly used instruments and tools are used in the study

NO	Instruments and Tools	Company	Origin
1	Centrifuge	Hitachi	Germany
2	Different size micropipettes	Dragon	German
3	Disposable syringe	Al Sheghaf	China
4	Spectrophotometer	Human	Germany
5	Gel tube & EDTA tube & Sodium strait tube	Q . l.las	China
6	Plastic disposable tips (different sizes)	JRZ	Lebanon
7	Slide	Ground edges	China
8	Shaker incubator	Bioneer	Korea
9	Water bath	Grant	England
10	Vortex Mixer	Digisystem	Taiwan
11	Spin Column, Eppendorf tube	Cleaver	England
12	Gel imaging device	ATTA	Japan

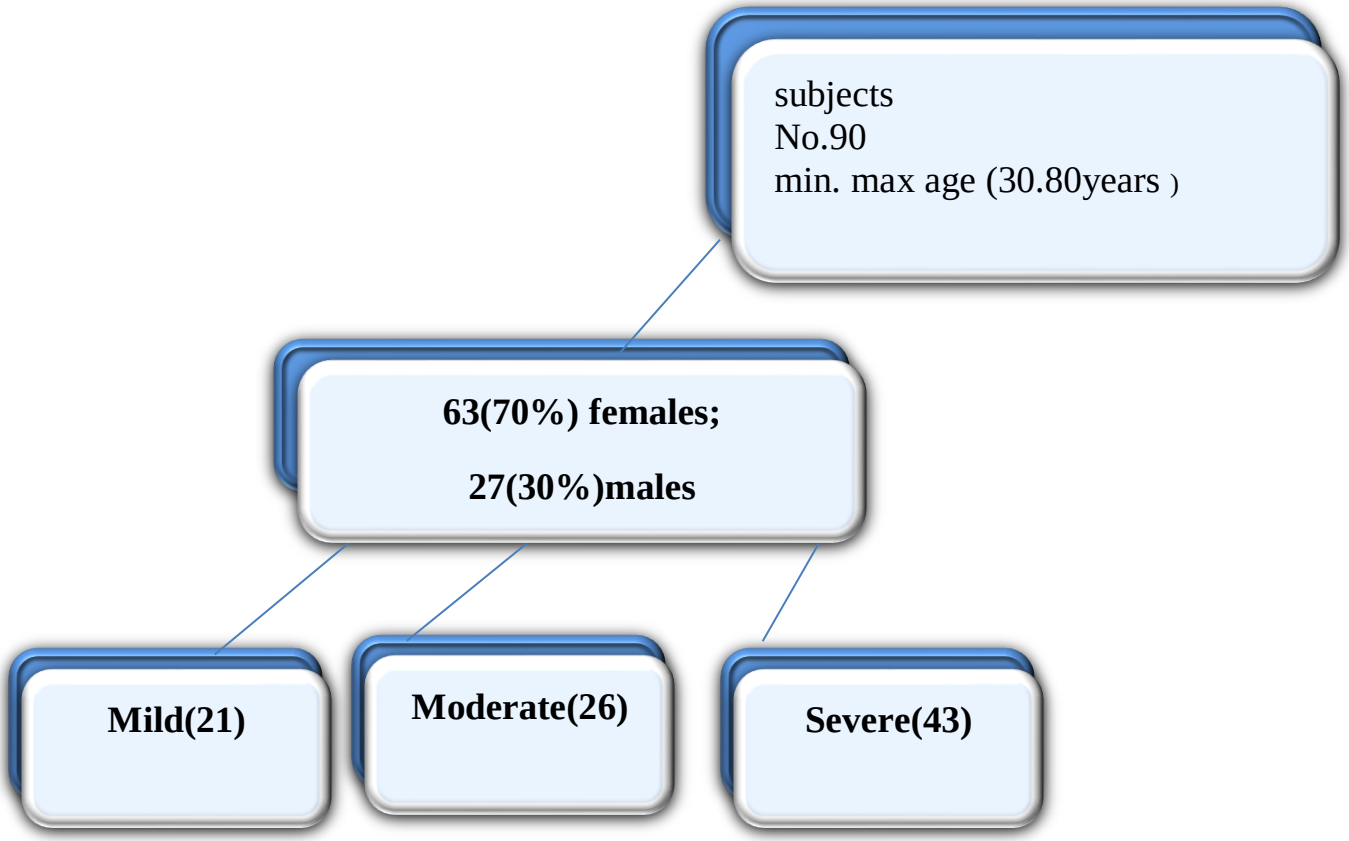
13	Horizontal gel electrophoresis ,Hot plate	ATTA	Japan
14	Incubator	Bindre	Germany
15	Micro-Centrifuge	Hittch	Germany
16	Scan drop	Analytik jena	Germany
17	Plan tube(5ml)	AFCOVAC	Jordan
18	PCR Thermocycler	Biometra	Germany
19	UV transiluminator	Quantum	france

2.3 Subjects

2.3.1 Study Design

This study was designed as cross-sectional study.

The period of sample collection extended from November 2021 to February 2022.



Patients

In this study (90) patients were participated whom infected with coronavirus, and their age range from (30-80 years), 63 females and 27 males. Then divided in to three groups 21 mild, 26 moderate, 43 sever. Collected from Al Mugdadyia General Hospital and Baquba General Hospital (Diyala, Iraq) diagnosed with real time reverse transcriptase polymerase RT.PCR and chest computed tomography,

Exclusion criteria: The study excluded patients with other chronic diseases, systematic immune disease, chemotherapy, pregnancy.

2.3.2 Sample collection

Drawn five milliliters blood from venous by medical syringes from patient and put into Sodium Citrate for D-dimer use and 2ml into EDTA tube for CBC, Blood group, genotype and other blood placed into gel tube and left for less than fifteen min in room temperature to coagulation than for 10 min Centerfuge at (3000 X g) for separation serum and divided into three Eppendorf tube and at (-20 ºC) stored until the time of estimation biochemical.

2.4 Methods

2.4.1 Determination AFIAS D-dimer

Determination of D-dimer was done using automated fluorescence immunoassay system (AFIAS) instrument, which based on fluorescence immunoassay (FIA) technique for the quantitative determination of D-dimer in human serum for *in vitro* diagnostic use only

2.4.1.1 Principle

The test employs a sandwich immunological detection method, in which the detector antibody in the buffer binds to antigen in the sample, generating antigen-antibody complexes, which then move onto the nitrocellulose matrix, where they are collected by the other immobilized-antibody on the test strip. The more antigens in the sample, the more antigen-antibody complexes form, resulting in a higher fluorescence signal on the detector antibody, which is processed by the instrument for AFIAS testing to determine the quantity of D-Dimer in the sample.

2.4.1.2 Procedure

1- A volume of 100µL of sample was dispensed into the sample well of the cartridge.

2- In the cartridge holder, the cartridge was placed.

3- A tip was placed in the cartridge's tip hole.

4- On the screen, the "START" icon was tapped.

5- After 12 minutes, the test result will appear on the screen.

2.4.1.3 Reference Range

Cut off 500 ng/mL

2.4.2 Determination of Serum Ferritin

2.4.2.1 Principle

The test employs a sandwich immunological detection approach, in which the detector antibody in the buffer binds to antigen in the sample, generating antigen-antibody complexes, which then move onto the nitrocellulose matrix, where they are collected by the other immobilized-antibody on the test strip. The more antigens in the sample, the more antigen-antibody complexes form, resulting in a higher fluorescence signal on the detector antibody, which is processed by the instrument for AFIAS testing to determine the quantity of ferritin in the sample.

2.4.2.2 Procedure

1- A volume of 100µL of sample was dispensed into the sample well of the cartridge.

2- In the cartridge holder, the cartridge was placed.

3- A tip was placed in the cartridge's tip hole.

4- On the screen, the "START" icon was tapped.

5- After 12 minutes, the test result will appear on the screen.

2.4.2.3 Reference Value

Women (20 – 250) ng/mL

Men (30 – 350) ng/mL

The working range of AFIAS is 10-1000 ng/ML

2.4.3 Determination of Serum C- Reactive Protein

2.4.3.1 Principle

A sandwich immune detection technique is used in the test. Antigens in the sample bind to the detection antibodies in the buffer. Antigen-antibody complexes develop and move onto the nitrocellulose matrix, where they are collected by immobilized antibodies on the test strip. With more antigens in the sample, more antigen-antibody complexes develop, resulting in a high fluorescence signal from the detector. This is processed by the device to indicate CRP concentration in AFIAS tests

2.4.3.2 Procedure

1- A volume of 100µL of sample was dispensed into the sample well of the cartridge.

2- In the cartridge holder, the cartridge was placed.

3- A tip was placed in the cartridge's tip hole.

4- On the screen, the "START" icon was tapped.

5- After 12 minutes, the test result will appear on the screen.

2.4.3.3 Reference Values

The cut off value of instrument is 10 mg/L and the working rang of the AFIAS for CRP is 0.5-200 mg/L.

2.4.4 Determination Serum Lactate Dehydrogenase

2.4.4.1 Principle

Lactate dehydrogenase catalyzes the reversible reaction of L-lactate and NAD+ to produce pyruvate and NADH or vice versa. The reversible reaction is monitored spectrophotometrically by measuring the increase in NADH at 340 nm.

Pyruvate + $NADH^{+}$ + H → Lactate+ NAD

2.4.4.2 Reagent

Reagent	Contents	Concentration
Buffer/Substrate	TRIS buffer(PH 7.35) Pyruvate Sodium azide	62.5mmol/L 1.5mmol/L 0.095mmol/L
Substrate	NADH Sodium azide	0.75mmol/L 0.095%

2.4.4.3 Preparation of Reagents

Preparation of working reagent was done by addition of 1ml from bottle SUB to one bottle of BUF and shake for 5min.

2.4.4.4 Procedure

Pipette into cuvettes at 37 ºC	
Sample	10µL
Working reagent	1000µL

Then the mixture was mixed and incubated. The absorbance was read after 1, 2, and 3 min.

2.4.4.5 Calculation of Results

The absorbance reading change was recorded per time (ΔA/min)

LDH activity (U/L) was calculated by multiplying (ΔA/min) by 16030 factor.

The conversion factor of the traditional unit (U/L) in SI unit is equal to (kat/L).

1 (U/L) =16.67 × 10^{-3} (kat/L).

1 (kat/L) = 60 (U/L).

2.4.4.6 Reference Values (225-450) U/l

2.3.5 Determination of Complete Blood Count by Mindary BC-3000 Plus

Mindary BC-3000 Plus instrument is fully automated analysis on whole blood collected on EDTA tubes. The ratio between EDTA and whole blood must be between1 to 2 mg per ml of blood. The samples should be used at room temperature no longer that 4 hours after collection.

2.4.5.1 Diluent

Principle

The diluent is designed for diluting the whole blood prior to counting and sizing of RBC/WBC/PLT. It maintains stability RBC/PLT during counting.

Components

Diluent contains the following compound: sodium sulphate (11 g/L), sodium chloride (5 g/L), N,N-dimethylurea (1.5 g/L), buffer (3 g/L), preservatives (1 g/L).

2.4.5.2 Cleaning Reagent

Principle

Cleaning reagent is designed to remove protein contaminants from the measurement system analyzer after each blood sample analysis.

Components

Cleaning reagent contains the following compound: sodium chloride (5 g/L), sodium sulphate (11 g/L), sodium hydroxide (0.1 g/L), preservatives (1 g/L), and nonionic surfactant (2 g/L)

Enzymatic Cleaner Forte

Enzymatic cleaner forte contains the following compound: Sodium chloride (5 g/L), sodium sulphate (11 g/L), sodium hydroxide (0.1 g/L), preservatives (1g/L), nonionic surfactant (2 g/L), proteolytic enzymes (6 g/L) and dye (0.02 g/L).

2.4.5.3 Lysing Reagent Free:

Principle

Lysing reagent uses to obtain the measurement of the haemoglobin, counting and differentiation of the white blood cells. Use in combination with the diluent, this reagent lyses the red blood cells and protects the state of the

leukocytes to permit the differentiation in three populations (lymphocytes, monocytes, granulocytes).

Components

Lysing reagent contains the following compound: quaternary ammonium salts (33 g/L), sodium chloride (1g/L), sodium sulphate (3 g/L), organic buffer (2 g/L), preservatives (2 g/L).

2.4.6 ABO Blood Group

Procedure

1- First, a glass side was prepared and marked with three circles after cleaning the slide.

2- Anti-A, Anti-B and Anti-D in the first, second and third circle respectively were added with the help of a dropper.

3- By use pipette, three drops of the antigen were added on anti A,B, and D in glass slid.

4- The blood sample was mixed gently with the help of a toothpick and waited for a minute to observe the result.

2.4.7 Genetic Study

2.7.1 Extraction of DNA and Genotype Analysis

DNA was extracted by novel DNA extraction protocol from frozen blood [128].

2.4.7.1.1 DNA Extraction

1- A volume of 500µl of the blood was transferred to 1.5ml of the Eppendorf tube, after the frozen sample is thawed.

2- A volume of 1 ml of the washing buffer was added to the blood, mixed, and incubated for 10 minutes at room temperature in a spinning mixer.

3- After centrifugation for 1 min at 10000×g, the supernatant was discarded.

4- A volume of 1 ml of washing buffer was added, the pellet was suspended and centrifuged for 1min at 10000×g, and then the supernatant was discarded.

5-The precipitate was suspended with 2000 µl of 2mmol EDTA solution.

6- A volume of 400 µl of the extraction solution are added and suspended well until complete dispersal, then placed at a temperature of 60 ºC for 15 minutes.

7- Then it cools down to room temperature, 100 µl of sodium acetate are added, and mixed by vortex mixer for a minute.

8- The mixture was centrifuged for 10 minutes at 10000×g and after that the sediment is neglected and the remaining solution is placed in the column.

9- Column was centrifuged at 10000×g for 30 seconds again.

10-Column was washed twice with 600µl of column wash solution, then centrifuged at 10000×g for 30 seconds and this step was repeated once again,

11-The column is dried after removal of the filtrate using centrifuge at 12000×g for three minutes to remove washing solution.

13-The collection tube is discarded and the column is moved to a 1.5 ml sterile Eppendorf tube.

14- A volume of 100 µl of solution (Elution buffer) is added to the center of the column and left for 5 minutes. After addition, it is ensured that all the solution is absorbed from the column and the solution is not left until complete absorption.

15-Then the column was centrifuged at 10000×g, and the filtrate was takes which represents pure DNA.

2.4.7.2 DNA Spectrophotometry

The DNA quality and quantity was measured by nano-drop, using the scanning ability of diode array from 200 to 320 nm wave length, the absorbance profile then processed and analyzed to estimate the DNA quality and quantity by calculating the 260/280 and 260/230 ratios. A sample would be re-extracted if the 260/280 ratio was less than 1.7 and/or the 260/230 ratio was less than 1.7. as show figure 2.1

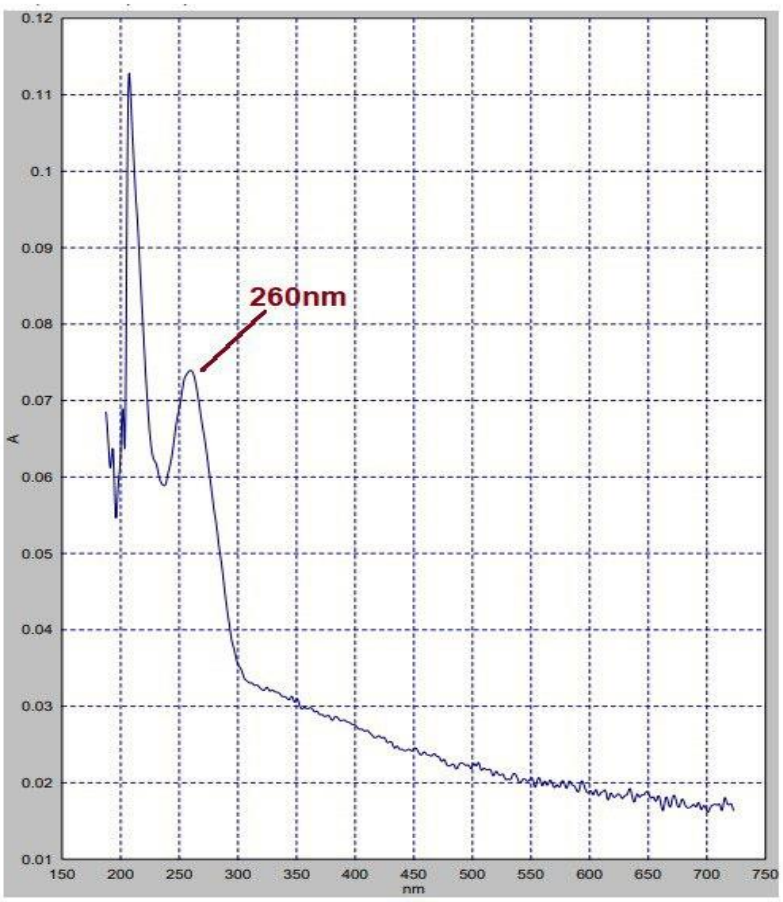


Figure 2.1 Measurement of DNA purity by using scan drop device.

2.4.7.3 Gel Electrophoresis for Analyze DNA Quality

The molecular weight and the integrity of extracted DNA were determined by agarose gel electrophoresis, the electrophoresis was carried out according to[129] . as describe below in brief:

1- The gel (1%) was prepared by dissolving 0.5 g of agarose in 50 ml of 1X TBE buffer and heated by microwave oven for 2 minutes.

2-The homogenized agarose then cooled to 55ºC by water bath.

3- A volume 50µl of ethidium bromide stock (1mg/ml) solution was added to the gel and mixed by swirling.

4- The gel then poured to the gel try and let to polymerize for 30 minutes.

5- The polymerized gel then transferred to the electrophoresis devise and submerged with 0.5x TBE running buffer.

6- Five microliters of extracted DNA was mixed with 1µl of lauding buffer and loaded carefully by mechanical pipet to the gel wells.

7- The electrophoresis was carried out by setting the device on 50 volts and 40mA for 60 minutes.

8- The gel then imaged and the image analyzed by CS analyzer® software to determine the extracted DNA molecular weight as shown in figure 2.2.

Figure 2.2 Agarose gel electrophoresis for extracted DNA

DNA molecules phosphate backbone is negatively charged and therefore DNA fragments migrate to positively charged anode when put in electric fields. DNA's weight/charge ratio is consistent, such that the distance traveling is

inversely proportional to the log of molecular mass, DNA molecules is separated by size inside an agarose gel.

Electrophoresis is the leading model for the movement of DNA through an agarose gel and is used for pushing the remaining molecule forward.

The migration rate of a DNA molecule through a gel is represented by:

* DNA molecule sizes.
* Agarose concentrations.
* Conformation of DNA.
* Applied voltage.
* Ethidium bromide presence.
* Agarose type
* Buffer for electrophoresis

2.4.7.4 Primers Design for PCR-RFLP

The primers were designed by the aid of NCBI-primer BLAST online software. (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi? LINK_LOC =BlastHome), at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome, then the primers pair was selected according to the demand criteria such as: product length, the similarity of melting temperature, primers length, specificity, etc. Then the mutation was interred according to the design demands, as shown in figure 2.3 and Table 2.3.

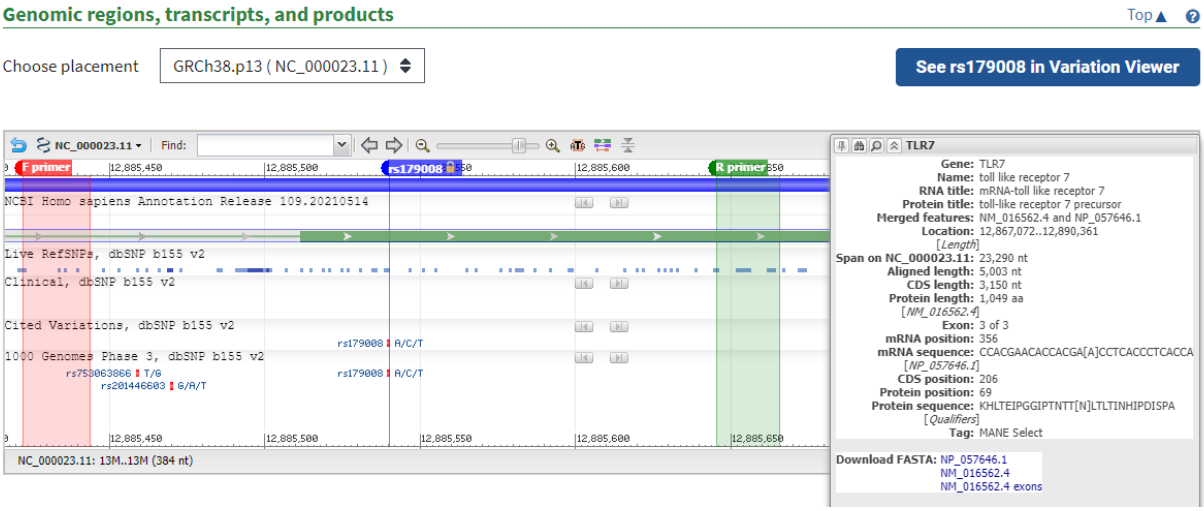


Figure 2.3 Represents the location of the rs179008 and the genetic variant

Table 2.3 Sequences of Primer for PCR-RFLP (Tm: Melting Temperature, R: Reverse Primer, F: Forward Primer)

	Sequence(5->3)	Start	Stop	Tm	GC %
Zh08f	GTTGCAAAAGAGAGGCAGCAA	1288 5423	1288 5443	59.93	47.62
Zh08r	CTGTGCAGTCCACGATCACA	1288 5665	1288 5646	60.32	55.0

2.4.7.4.1 Checking Primers Ability to Form Secondary Structures

The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html), the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin.

2.4.7.4.2 Checking the Dimer Formation Ability of the Primers Pair

Each primers pair was checked for dimer formation by the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.©, the sensitivity of the software was adjusted to the value 2, the primer pair would be rejected if it made any dimers in this degree of sensitivity.

2.4.7.5 Restriction Enzyme Selection

The selection of the suitable restriction enzyme was performed by the aid of WatCut online software (http://watcut.uwaterloo.ca/template). It was selected according to several criteria such as compatibility of the produced primers, cost and availability, as shown in figure 2.4.

GTTGCAAAAGAGAGGCAGCAAATGGG/AATTTTTAATTCTGATTCTTGGTATGTTTTAGAACAATGATTTGTTC
TTTCTTATACTTTCAGGTGTTTCCAATGTGGACACTGAAGAGACA/AATTCTTATCCTTTTTAACATAATCCT
AATTTCCAAACTCCTTGGGGCTAGATGGTTTCCTAAAACTCTGCCCTGTGATGTCACTCTGGATGTTCCAAAGA
ACCATGTGATCGTGGACTGCACAG

Figure 2.4 Represents the target amplicon sequence for genotyping (rs179008) TLR7 is located on the X chromosome, so men have one allele, either A or T, and women have two alleles, A, T, or AT. Sequences marked in gray represent primer, large red A represent the A allele of rs179008. The sequences marked in green are the sequences recognized by the restriction enzyme called Restriction site.ACSI (Restriction enzyme) is recognize restriction site R AATTY. During the digestion by ACSI restriction enzyme, The T allele will produce 26 and 217bp fragments, while A allele produce 26, 92 and 125 bp fragments

2.4.7.6 Primers Preparation

Specific primers were used for the PCR protocol. Primer pairs were provided by Macrogen company as a lyophilized product of various concentrations of pico moles. According to Macrogen company. At the beginning tube contains

lyophilized primers was centrifuged at 10000 ×g for 1 min due to the distribution of material on the walls of tube. Lyophilized primers were dissolved in deionized distilled water to prepare 100 pmol / µL as a final concentration as stock solution.

The stock solution was mixed by vortex mixer and then centrifuged for 5 sec at 4000 ×g. Then a volume of 10µL of each primer (stock solution) diluted with 90µL of nuclease free D.W. to prepare working solution with concentration of 10 pmol/µL and kept at -20 °C until used. It was recommended that the primers should be distributed into single-use aliquots to limit the freeze-thawing of primers and therefore will extend their life.

2.4.7.7 Polymerase Chine Reaction (PCR) Amplification TLR Gene for PCR-RFLP Analysis

Single nucleotide polymorphism of the TLR7 gene(rs179008) is located on X chromosome. It was amplified by a programmable thermal cycler gradient PCR system with specific primers for TLR 7gene.

Optimizations of PCR condition were conducted by employing different annealing temperature. The reaction ingredient and thermocycling condition are listed in Table 2.4.

Table 2.4 Optimization of PCR Thermo cycling condition

Name of cycle	Temp C	Time	No of cycle
Initial denaturation	94	5min	1 cycle
Denaturation	94	30sec	35 cycle
Annealing	55-66	30sec	
Extension	72	30sec	
Final extension	72	5min	35 cycle

The final PCR reaction for amplification of TLR7 The contains the components showed in Table 2.5.

Table 2.5 PCR reaction components for amplification of TLR7

No.	Component	Volume
1	DNA	2µl
2	Master mix	8 µl
3	Forward Primer	1 µl
4	Reverse Primer	1 µl
5	$MgCl_2$	0.5 µl
6	Nucleases free water	7.5 µl
7	Final reaction volume	20 µl

After PCR optimization the annealing temperature was fixed as 60 ºC for further PCR amplification because it was produce the best amplicon.

The PCR products were submitted to electrophoresis on agarose gel (2%) and stained using ethidium bromide, as shown in figure 2.5.

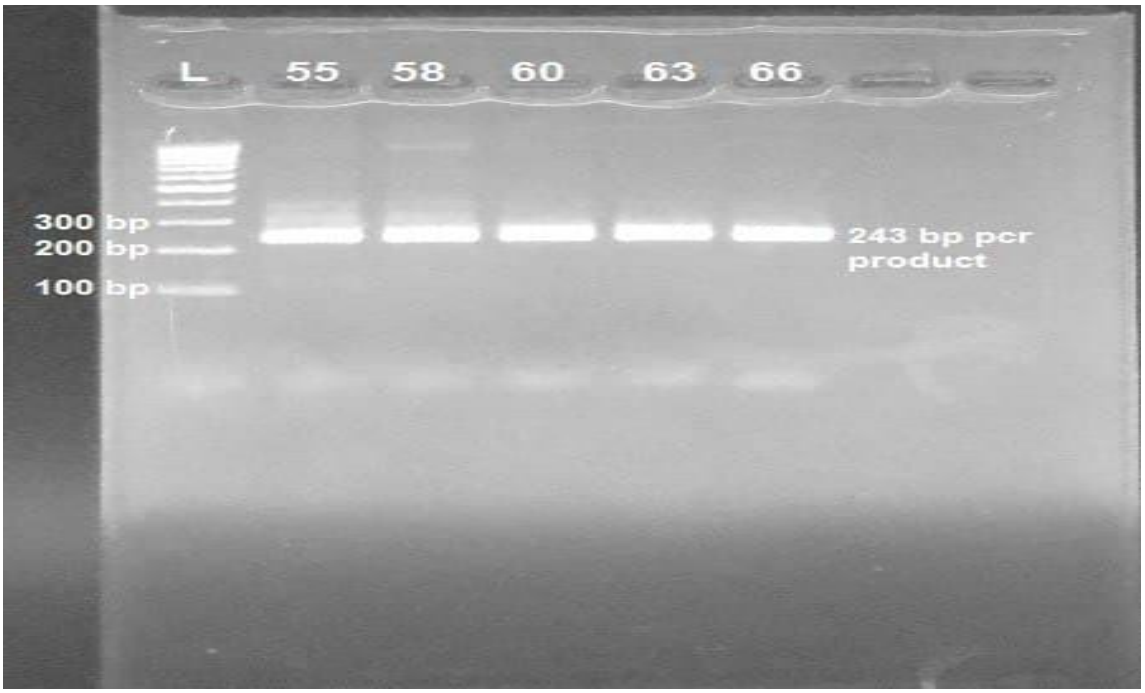


Figure 2.5 Agarose gel electrophoresis for PCR optimization.

2.4.7.8 PCR-RFLP Conditions for rs179008 Analysis.

2.4.7.8.1 Restriction Digestion for PCR-RFLP

PCR-restriction fragment length polymorphism (RFLP) was used to investigate the polymorphism in rs179008 of patients with COVID-19 involved in this study.

The restriction reaction for PCR-RFLP of TLR7 was carried out by the following protocol.

1- A volume of 10µl of PCR product was pipette into Eppendorf tube.

2- Two units of the selected restriction enzyme were added.

3- Restriction buffer 1.5µl (each restriction enzyme has its restriction buffer supplied by the manufacturer).

4- A volume of 0.15µl of bovine serum albumin was added.

5- The reaction mixture then completed to 15µl by molecular grad water.

6- The reaction mixture was incubated in 37 ºC in water bath for over night.

7- The RFLP products were loaded to electrophoresis on agarose gel (2%) and stained using ethidium bromide.

2.5 Statistical Analysis

The statistical program for social sciences (SPSS) version 23 and Microsoft Office Excel 2010 were used to collect, summarize, analyze, and present the data. Quantitative (numeric) data were initially tested for normality distribution using the Kolmogorov-Smirnov test, whilst qualitative (categorical) variables were reported as number and percentage, and then accordingly normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion), while those numeric variables that are not normally distributed were expressed as median (an index of central tendency) and inter-quartile range (an index of dispersion).

The following statistical tests were used:

1. Chi-square test was use to evaluate association between any two categorical variables provided that less than 20 % of cells have expected count of less than 5.
2. One way analysis of variance (ANOVA) was used to evaluate difference in mean of numeric variables among more than two groups provided that these numeric variables were normally distributed; One way ANOVA was followed by pos hoc LSD test to evaluate individual differences in mean values between any two groups
3. Risk estimation was done using odds ratio calculation with corresponding 95 confidence interval. The level of significance was considered at P-value of equal or less than 0.05. The level of high significance was considered at P-value of equal or less than 0.01

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Demographic characteristics of patients enrolled in this study

As a results patients participate in the present study divided in to three groups were mild 21(23.3%), moderate 26(28.9%), and sever 43(47.8%) respectively, as shown in figure 3.1. And divided according to Sex. As show in table 3.1

Figure 3.1 Patients distribution according to severity in to three categories

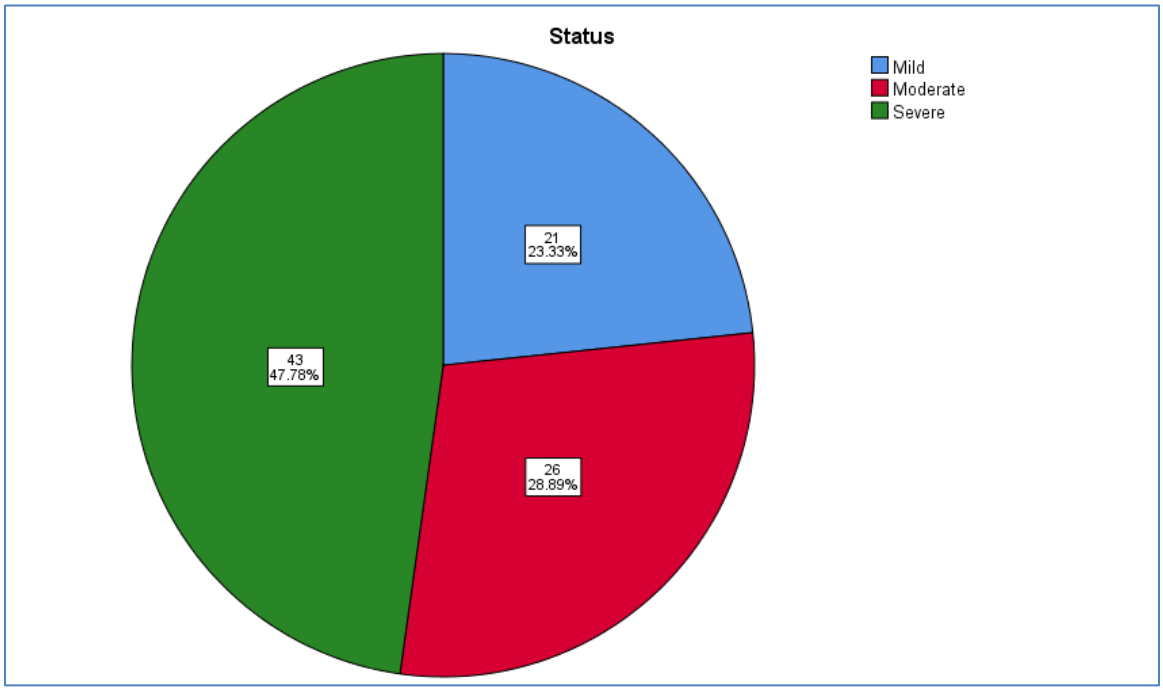


Table 3-1 Patients distribution according to gender

Parameters		**NO (Mild)**	**NO (Moderate)**	**NO (Severe)**
Participants	female	14	16	33
	male	7	10	10

3.1.1 Age of Patients

The mean age of patients in the present study are shown in Table 3.2 in which mild patients ages were 32.48 ±15.39, moderate 57.04±16.91, and severe 66.51±14.186 years respectively. There were a significant difference between three groups ($p < 0.000$).

Table 3.2 Comparison of mean age between categories

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
Age (year)	32.48±15.39	57.04±16.91	66.51±14.186	0.000*
				0.000**
				0.015***

*Mean comparison of mild with moderate
** Mean comparison of mild with severe
*** Mean comparison of moderate with sever

The results of the present study showed that there is no significant difference between sex of COVID-19 patients. When comparison between three groups were found p-value 0.716 between mild and moderate, p-value 0.391 of mild and severe, p-value 0.177 when comparison of moderate with sever.

In current study older patients (≥65 years old), were more likely to have a severe type of COVID-19.

The Chinese mainland's total number of confirmed cases had reached 76,936, with 2,442 individuals dying as a result of the disease, according to the

Chinese health authority. The majority of the 2,442 patients were old and two-thirds of them were men[130]. Patients who were 65 years old or older found to be more likely to develop a severe form of COVID-19. They find that men tended to acquire COVID-19 more than women. According to the clinical classification of

severity, in the public data collection of COVID-19, they also discovered that the percentage of older age (≥65 years) was majority in the severe patients. They also find that 60% of male patients were died in comparison to 40 % for females. While the total percentage of people infected with the virus was 57% females and 43% males[130,131].

This fact could be affected by both physiological aging, in particular, the higher prevalence of frailty and comorbidities in older adult patients, which lead to a decline in functional reserve, which decreases intrinsic capability and resilience and impairs the fight against infections[132].

In immunopathology, vulnerability to an infection in the elderly is usually explained by immunosenescence[133]. Immunosenescence is quite complicated. Briefly, in old age, the production of naïve T and B cells decreases, and the function of innate immune cells is impaired; hence, cells involved in the innate immunity do not get activated efficiently during an infection, and progression to an adaptive immune response does not occur in a coordinated manner[134].

Previous study also showed COVID-19 had been demonstrate increase in the numeral of cases and a high risk of severe illness with the progressing age[135].

These changes reduce the effectiveness of viral clearance and increase the likelihood of triggering a dysregulated immune response in which cytokines are released extensively by activated immune cells, resulting in a cytokine storm[136], Another well. Recognized feature of aging immunity is chronic subclinical systemic inflammation, also known as inflammation. Inflammation is a key pathogenic mechanism in Covid-19; hence, inflammation has been estimated to contribute to the poorer outcome in elderly patients with Covid-19[137]

Increased ACE2 receptor protein expression in specific organs was associated with specific organ failures, as shown by clinical criteria in SARS patients[138], ACE2 levels in the blood have been found to be higher in men than in women, as well as in patients with diabetes or cardiovascular disease[139,140].

3-2 Assessment of ABO blood group to severity.

Table 3.3 shows frequency and distribution of ABO blood group for patients, in the current study.

Table 3.3 frequency and distribution of ABO blood group for the patients in this study.

ABO	Frequency	Percent
A+	31	34.4
B+	15	16.7
AB+	12	13.3
O+	25	27.8
A-	3	3.3
B-	2	2.2
O-	2	2.2
Total	90	100.0

Table 3.4 and figure 3.2 shown ABO blood group comparison and distribution between mild, moderate and severe patients with COVID-19.

Table 3.4 Distribution of ABO blood group between categories.

ABO	Cases			Total	P value
	Mild	Moderate	Severe		
A+	4 (12.9%)	9 (29.02%)	18 (58.06%)	31	0.206* 0.131** 0.808***
B+	6 (40%)	4 (26.6%)	5 (33.3%)	15	
AB+	3 (25%)	3 (25%)	6 (50%)	12	
O+	4 (16%)	10 (40%)	11 (44%)	25	
A-	1 (33%)	0	2 (66.6%)	3	
B-	2 (100%)	0	0	2	
O-	1 (50%)	0	1 (50%)	2	
Total	21	26	43	90	

*Mean comparison of mild with moderate
** Mean comparison of mild with severe
*** Mean comparison of moderate with sever

When comparison between these group was found no significant different between ABO and the severity of COVID-19 infection in patients who enrolled in this study.

Besides age and Sex, ABO blood groups may also serve as potential factor for COVID-19. In this study, the overall distribution of the four phenotypes (A, B, AB, and O) showed a no significant variation between groups patients,

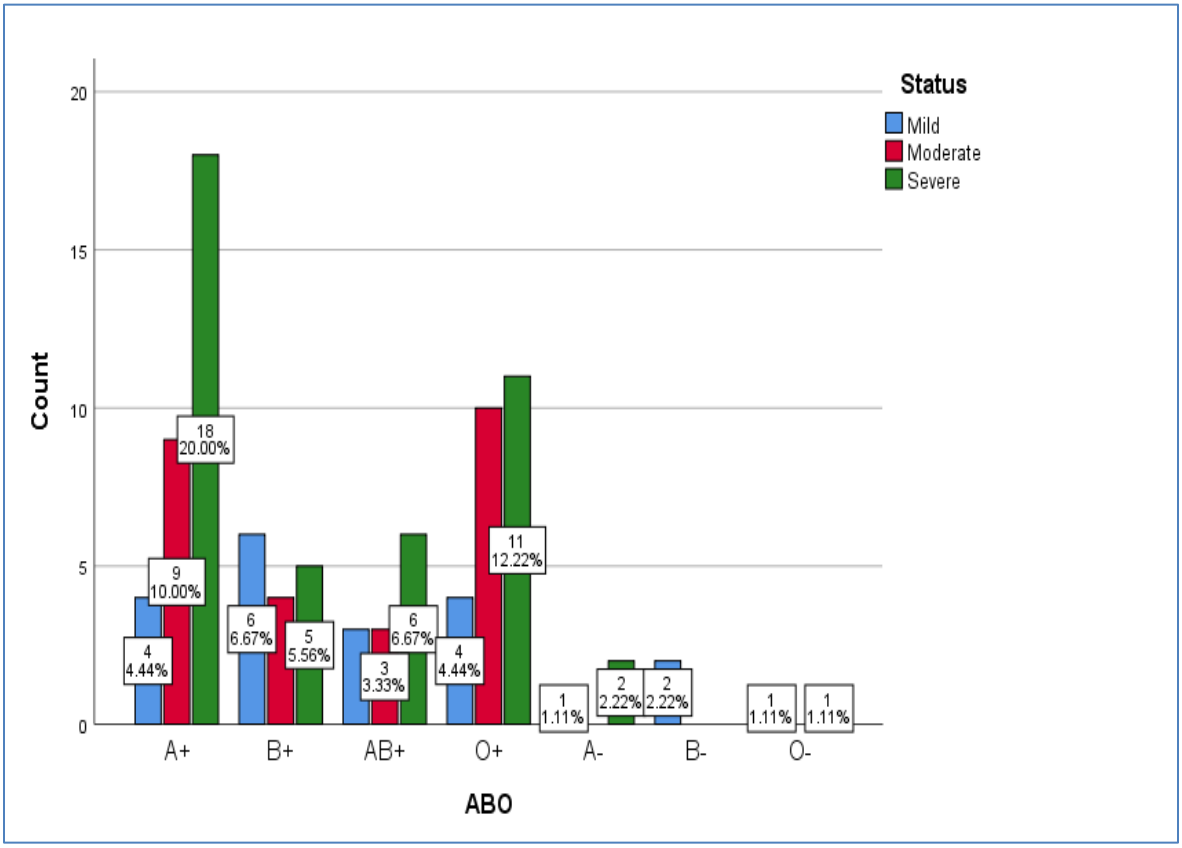


Figure 3.2 Distribution of ABO blood group between three patients categories.

where p-value (0.206) between mild and moderate, p-value 0.808 between moderate and severe, p-value (0.131) between mild and severe in statistic. Higher percentage was (A^+) but these weak statistically non-significant may be small sample size.

In summary, the blood group of individuals might not be one of the risk factors for COVID-19, but it was related to the clinical characteristics of patients with COVID-19.

In other study done in Iraq, they found that individuals with blood group AB, A were higher severity in COVID-19[141]. However, the protective potential of group O phenotype against evolution of COVID-19 was suggested. Recent

study have also depicted the significance of group O in lowering COVID-19 risk, They also found a relation among groups A, B, or AB in increasing the risk of infection [142].

Angiotensin-converting enzyme 2 (ACE2) has been reported to be the SARS-CoV receptor, and the receptor binding domain is presented on the S proteins of the coronaviruses[143]. They investigated whether ABO antibodies could stop the interaction between the SARS-CoV receptor and ACE2, and therefore adhesion of S protein and ACE2 can be inhibited by anti-A natural antibody. The anti-A and anti-B natural antibodies being produced in individuals with blood group O could potentially block viral adhesion to cells, which could explain their lower risk of infection[96]. Another recent study give an explanation for the higher risk for blood group A, may lack of these antibodies [144].

Individuals with blood group O have a lower angiotensin-converting enzyme (ACE) level, while blood group A has positive association within ACE activity[145], ACE is an enzyme that activates angiotensin; the lower level of this enzyme can thus reduce the risk of hypertension[145], which is a COVID-19 risk factor[146]. This is another proposed mechanism for developing more severe COVID-19 disease in blood group A and less severe disease in blood group O[145].

People with blood group O have also higher interleukin 6 (IL-6) levels[147], IL-6 is a proinflammatory cytokine which can be produced by many cells and plays an important role in cell defense in the acute phase [148], However, studies showed that IL-6 is associated with COVID-19 severity, as it can be part of a cytokine storm[149,150,151].

3-3 Association of D-Dimer, Serum Ferritin, C-Reactive Protein (CRP), and Lactate Dehydrogenase with COVID-19 severity .

3-3-1 Comparison of Plasma D-Dimer level between patients with COVID-19 categories

The Table 3.5 show the significant difference of D-dimer level among the mild , moderate and sever groups . the sever group record the highest level of D-dimer (1956.2 ng/ml) , figure 3.3.

Table 3.5 Comparisons of Plasma D. dimer level for the patients categories

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
D-dimer (ng/ml)	223.25±73.14	623.36±447.65	1956.28±958.51	0.0.058*
				0.000**
				0.000***

Data represented as mean ± SD: standard deviation,
***= p.value (mild +moderate),**
****=p.value (mild +severe)**
*****= p.value (moderate+ severe)**

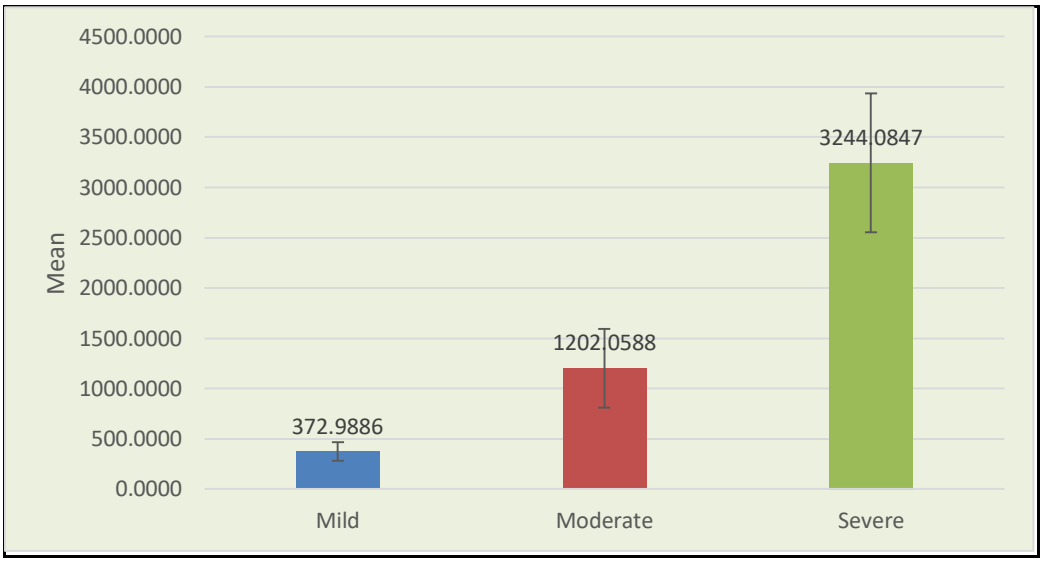


Figure 3.3 Comparisons of Plasma D. dimer level between the patient's categories

In present results, serum levels D-dimer was found to be tend toward an increase with the severity of the disease. This results are in agreement with earlier similar findings, and this evidence would imply that routine hemostasis tests may be additional useful tools for improving early diagnosis, and even more importantly the gradual progression of disease severity was mirrored by increasing values of D-dimer[152].

D-dimer elevation upon admission was common and was associated with both increased disease severity and mortality. D-dimers are one of the fragments produced when plasmin cleaves fibrin to break down clots. The assays are routinely used as part of a diagnostic algorithm to exclude the diagnosis of thrombosis[153].

The increase of D dimer may be an indirect manifestation of inflammatory reaction, as inflammatory cytokines could cause the imbalance of coagulation and

fibrinolysis in the alveoli, which may activate the fibrinolysis system, and then increase the level of D-dimer[154,155].

However, any pathologic or non-pathologic process that increases fibrin production or breakdown also increases plasma D-dimer levels. while D-dimer elevation has been observed in articles describing the clinical features of COVID-19, [156].

Level of D-dimer and disease severity stratified by the area of affected lungs on chest CT, oxygenation index, as well as clinical staging according to the interim guideline, the relationship between elevated D-dimer levels and the severity of COVID-19 disease was investigated in this study. The level of D-dimer was significantly higher in patients with severe COVID-19, so it has been shown that COVID-19 is linked to hemostatic problems by having significantly higher D-dimer levels[157].

Other previous studies have found similar results to the current study. For example, Ye et al., (2020) found an increase in D-dimer in individuals suffering from severe COVID-19 infection [158], and Litao Zhang et al., (2020) found that the cut-off value for D-dimer in severe cases who died was (2.0 g/ml)[157]

3.3.2 Comparison of Serum ferritin level between patients with COVID-19 categories

The current results in in table 3.6 and figure 3.4, found that serum ferritin level a significantly higher of ferritin level, between three group of patients with COVID-19 that enrolled in this study were P-value > 0.05 between three groups.

Table 3.6 Comparison of Serum ferritin level between patients with COVID-19 categories

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
Ferritin (ng/ml)	41.36±23.13	394.68±261.85	624.48±285.05	0.000* 0.000** 0.000***

Data represented as mean ± SD: standard deviation,
***= p. value (mild +moderate),**
****=p .value (mild +severe)**
*****= p .value (moderate+ severe)**

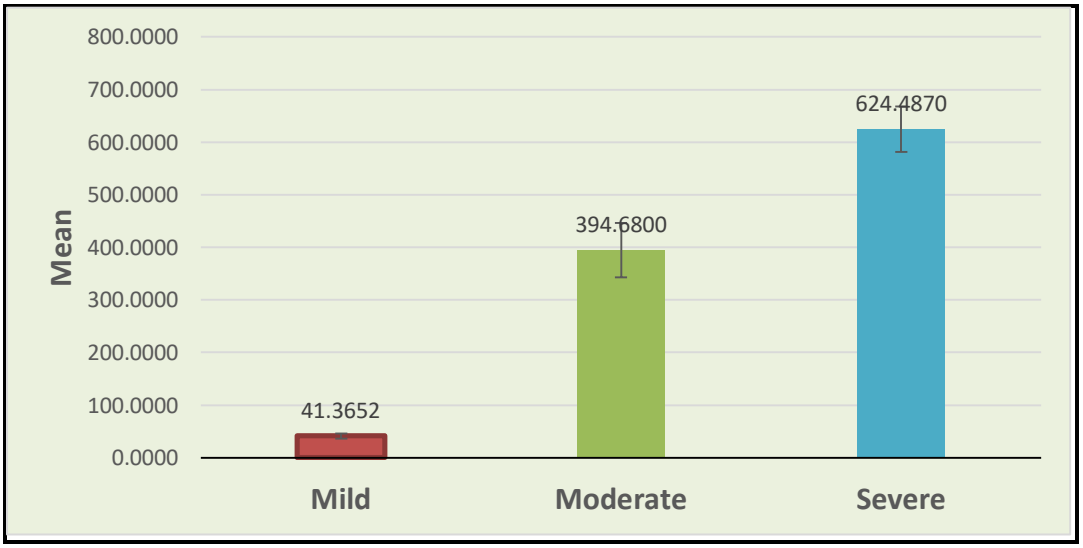


Figure 3.4 Comparisons of ferritin levels between patients categories

The current study found that serum ferritin level was significantly higher in severe cases and it was found that ferritin levels tend to increase with disease severity. From this it was concluded that hyperferritinemia is an independent risk factor in COVID-19 patients and that it can also predict disease severity.

According to new research, people with COVID-19 have higher ferritin levels as a result of the inflammatory process. Hyperferritinemia is now recognized

as a characteristic for an acute-phase reaction that doctors use to evaluate treatment response. In contrast, current research, reveals that greater level of ferritin could be observed during the acute-phase reaction in addition to the important role in the inflammatory reaction when a cytokine storm develops[48].

The result of this study was showed the same results of a study done by Firdevs Tugba Bozkurt *et al.*, (2021) which revealed that the level of ferritin in the groups including severe patients was significantly greater[159].

In this results concluded that the serum ferritin itself or combined with CRP may provide better results in predicting to severity disease.

3-3-3 C - Reactive Protein

The present study revealed that C-reactive protein was significant association between mild and moderate were p-value (0.04)< 0.05, and higher significant association between mild and moderate to severe were P-value 0.000 < 0.05, as the figure 3.5.

Table 3.7 Comparisons of serum C - reactive protein (CRP) between the patients categories

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
CRP (mg/L)	15.01±22.91	39.65±32.24	102.25±50.41	0.041* 0.000** 0.000***

Data represented as mean ± SD: standard deviation,
***= p. value (mild +moderate),**
****=p .value (mild +severe)**
*****= p. value (moderate+ severe)**

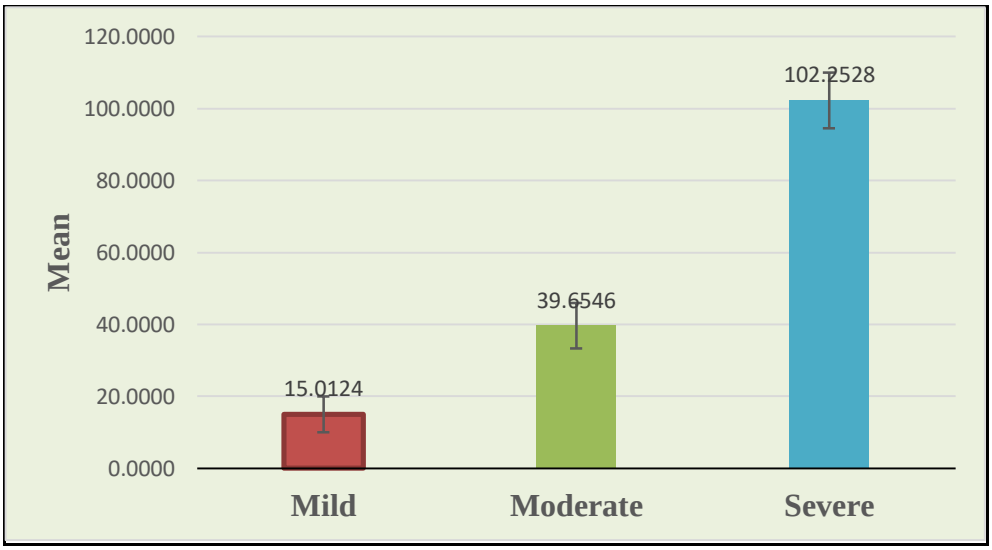


Figure 3.5 Comparison of Serum CR protein levels for patients categories

The normal concentration of CRP in the blood is less than 10 mg/l; nevertheless, Within 6-8 hours, the level rises rapidly and peaks 48 hours after the initiation of the disease, It has a half-life of approximately 19 hours, and as the inflammatory steps go away and the patient heals, its concentration decreases, CRP prefers to attach to phosphocholine, which is created in large quantities on the surface of damaged cells. This binding stimulates the immune system's classical complement pathway and regulates phagocytic activity to eliminate pathogens and damaged cells from the body. High CRP levels may be linked to an overproduction of inflammatory cytokines in COVID-19 individuals with severe symptoms. Despite the fact that cytokines kill microorganisms, an overactive immune system can injure lung tissue in COVID-19 patients, CRP production is triggered by inflammatory cytokines and tissue damage[160].

The results of the current study showed a significant elevation of CRP in patients with severe COVID-19 rather from the mild and moderate, this result was

agreed with a study done by Guan WJ *et al.*, (2020) that showed severe cases demonstrated an obvious increase in the levels of CRP[161].

3-3-4 Lactate dehydrogenase

In Table 3.8 the mean±SD of LDH of patients infected with COVID-19 in three groups. When compared mild group with moderate revealed significant difference in serum levels of LDH, and then compared mild, moderate to severe revealed positive significantly in serum levels LDH were P-value 0.000 < 0.05 as figure 3.6.

Table 3.8 Comparisons of serum Lactate dehydrogenase for the between patients categories

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
LDH (U/I)	466.10±147.09	740.44±295.72	1227.27±603.50	0.05* 0.000** 0.000***

Data represented as mean ± SD: standard deviation,
***= p. value (mild +moderate),**
****=p. value (mild +severe)**
*****= p. value (moderate+ severe)**

Several biomarkers are now being investigated for their possible role in predicting prognosis in COVID-19 patients. Lactate dehydrogenase (LDH) is another important biomarker, especially because higher LDH levels have previously been linked to poorer outcomes in people suffering from several viral diseases[162].

Early evidence from COVID-19 patients reveals that LDH concentrations differ significantly between those with severe illness and those who do not[163].

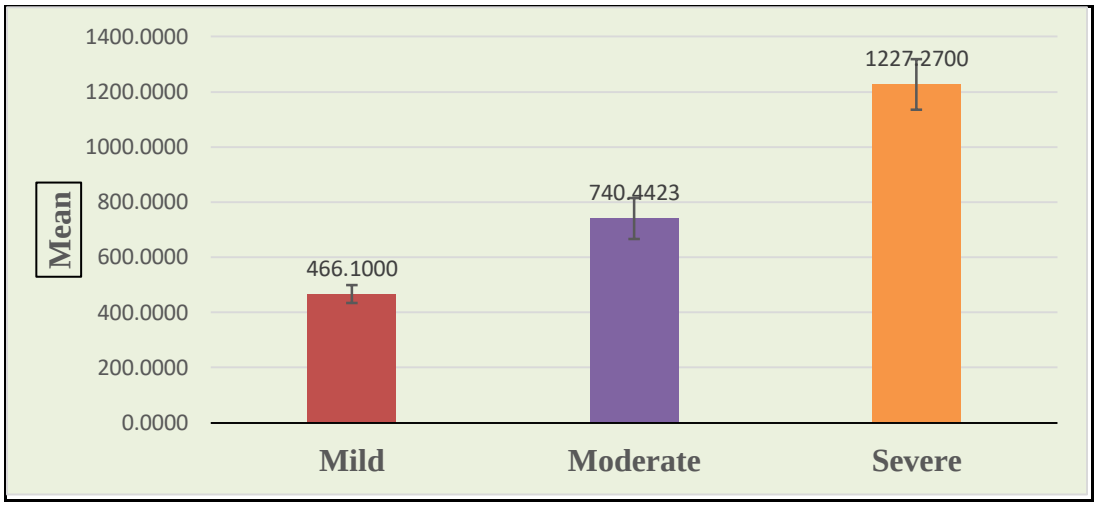


Figure 3.6 Comparison of serum LDH activity for patients categories

The results of the current study showed there was higher significant difference of LDH between mild group and moderate to severe group, so it was agree with [164] and previous studies[163].

The researchers suggest that the reason for the high activity of the enzyme lactate dehydrogenase is the increase in the anaerobic glycosylation pathway due to the severe oxygen deficiency resulting from the lung infection with COVID- 19

3-4 Assessment of Complete Blood Count with COVID-19 severity

Complete blood cell count for all patients with COVID-19 (n=90) was done in the current study, as shown in Table 3.9. The levels of the white blood cell (WBC) in three group revealed no significant differences between mild and moderate group, positive significant different between moderate and severe and significant different between mild and severe as show in figure 3.7.

Table 3.9 Comparison of complete blood count for patients groups

Parameters	Mild mean±SD	Moderate mean±SD	Severe mean±SD	P-value
WBCs 10^9/L	8.21±4.04	10.25±6.10	↑ 13.81±6.18	0.228* 0.000** 0.015***
NEU (%)	57.40±16.74	73.86±15.91	↑ 83.16±15.23	0.001* 0.000** 0.021***
LYM (%)	30.39±11.24	20.25±14.76	↓ 9.59±6.82	0.002* 0.000** 0.000***
HCT (%)	36.90±5.59	37.13±6.43	↔ 37.25±4.89	0.889* 0.813** 0.927***
HgB (g/dl)	11.98±2.04	12.26±2.41	↔ 12.24±1.67	0.639* 0.63** 0.972***
MCV (fl)	80.06±11.16	85.92±9.20	↑ 89.97±12.38	0.084* 0.002** 0.154***
PLT **10^9 /L**	280.65±62.87	251.08±111.11	↓ 213.76±94.36	0.296* 0.01** 0.12***
MPV (units)	8.65±.85	9.49±1.52	↔43.51±103.33	0.795* 0.32** 0.447***

*Mean comparison of mild with moderate
** Mean comparison of mild with severe
*** Mean comparison of moderate with sever

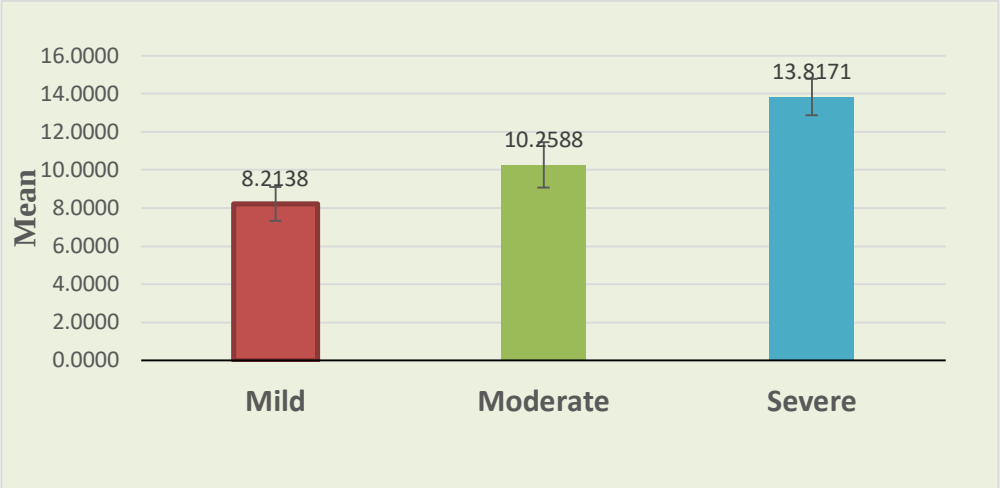


Figure 3.7 Comparison of WBC parameter for patients groups

Neutrophil (NEU), lymphocyte (LYM), in Table 3.9, show significantly defer when compares between three groups as figure 3.8, 3.9 respectively, but no significant differences between groups patients with hemoglobin(Hb), Hematocrit (HCT), Mean platelet volume (MPV), but Mean cell volume (MCV), platelets (PLT), while significant differences in severe when compare with mild.

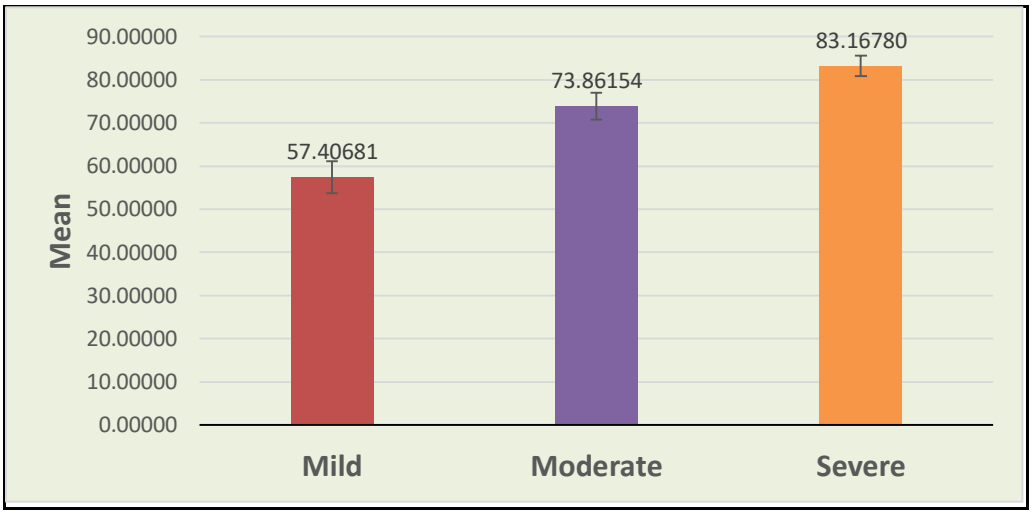


Figure 3.8 Comparison of Neutrophil (NEU) for patients groups

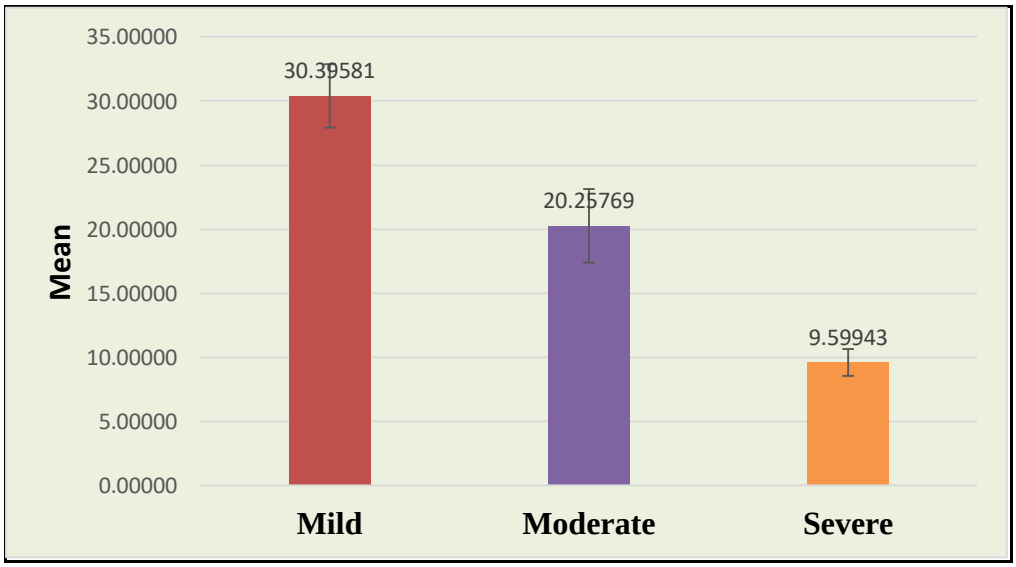


Figure 3.9 Comparison of lymphocyte (LYM) for patients groups

COVID-19 has the potential to affect different ways of organ systems in its host. According to study results, hematological profiles differ during SARS-CoV-2 infection. So the present study was designed to evaluate different cells in patients who were included.

Patients who received Intensive care unit (ICU) had numerous laboratory abnormalities. The CBC abnormalities for COVD-19 infection may be associated to with cellular immune deficiency, kidney injury, coagulation activation, hepatic injury, myocardia injury and The significant linked between patients' clinical outcome and increase of neutrophil counts was a physiologically and clinically significant finding of our study and the reasons for this are several[165].

Patients with COVID-19, particularly those with pneumonia, can have a normal leukocyte count (4–11x109/L), a low leukocyte count, or a high leukocyte count. This may make it easier to follow the progression of the disease and make treatment decisions. Some research on COVID-19 patients found that they had greater white blood cell and neutrophil counts, as well as a lower lymphocyte count, while others found that determining the neutrophil-to-lymphocyte ratio can be utilized as a biomarker to predict infection outcome[166]. When patients with mild diseases, the white blood cell (WBC) count and peripheral blood lymphocytes are normally or slightly decreased in the initial stages of COVID-19 illness, but their levels may vary as the disease progresses[167].

In contrast to prior research, Hong-Yi Zheng et al., (2020) discovered that the leukocytes count in patients with mild and severe COVID-19 stayed remained within acceptable levels[168].

Neutrophils play a role in the early stages of antiviral defense, so that in the presence of pathogenic agents and tissue injury, neutrophil numbers normally increase. Neutrophils, however , become cytotoxic during severe pneumonia due to degranulation and lysis[169].

These laboratory abnormalities are similar to those previously observed in patients with MERS-CoV and SARS-CoV infection, Neutrophilia may be related to cytokine storm induced by virus invasion, coagulation activation could have been related to sustained inflammatory response, and acute kidney injury could have been related to direct effects of the virus, hypoxia, and shock, the pathologic mechanisms may be associated with the death of patients[170].

In COVID-19 patients, as with other coronaviruses and viral infections, relative lympopenia is a prevalent symptom. In these patients, the total number of lymphocytes, T-helper (TCD4+) cells, T-cytotoxic (TCD8+) cells, natural killer cells (NK cells), and B cells decreases.

The decrease in CD8+ T cells is the most reflective, it is a significant indicator of SARS CoV-2 infection and aids in sickness progression during COVID-19 pneumonia[166]. Lower T helper (CD4+) concentrations have also been related to the severity of COVID-19, according to a study by Qin *et al.* [171].

Because lymphocytes express ACE2 and Cluster differentiation 147 (CD147) on their membranes, SARS-CoV-2 is thought to infect them, causing lymphocyte lysis and lymphopenia.

According of a second hypothesis is that increased cytokine activity first leads atrophy of secondary lymphatic organs, such as the spleen, and affects lymphocyte turnover, and then promotes apoptosis, causing in lymphopenia[172], Lymphopenia is not specific for prognosis of the disease or as a predictor for severity[173]. The neutrophil to lymphocyte ratio (NLR) has also been shown to be an accurate predictor of sickness severity. However, when comparing severely infected individuals to patients non-severe infected, discovered a considerable decline in granulocytes [174].

A study of Yuan *et al*., (2020) revealed had a higher total leukocyte count in patients with severe COVID-19[175].

The second set of CBC contained platelets' counts, which indicated that mild thrombocytopenia was present in in both survivors and non-survivors, However, while platelets' counts had a stable tendency toward normalization in survivors, platelets level irreversibly decreased during the second week on progression the diseases in perhaps due to the formation of micro-thrombotic events in tiny vessels as a result of ingestion[176].

Platelets are involved in inflammatory signals as well as the immune response to infections, By combining thrombotic and immune recruitment roles, platelets may help focus hemostasis and immune responses against potential infectious agents to prevent microbial invasion. Platelets interact directly with viruses via a variety of receptors[177], Due to the high risk of thrombosis, heparin prophylaxis has been recommended for all adolescents and adults hospitalized with Covid-19 Heparin. Induced thrombocytopenia (HIT), a well-known clinical entity occurring 5–14 days after heparin exposure, is an important differential for patients presenting with thrombocytopenia after hospitalization [137].

3-5 Allelic and Genotypic Frequencies of TLR7 Gene

The DNA extraction procedure was performed on all samples of blood from patients . After Agarose gel electrophoresis. All of the samples gave intact genomic DNA, and then use PCR for amplification TLR7 gene, The PCR product is shown in figure 3.10.

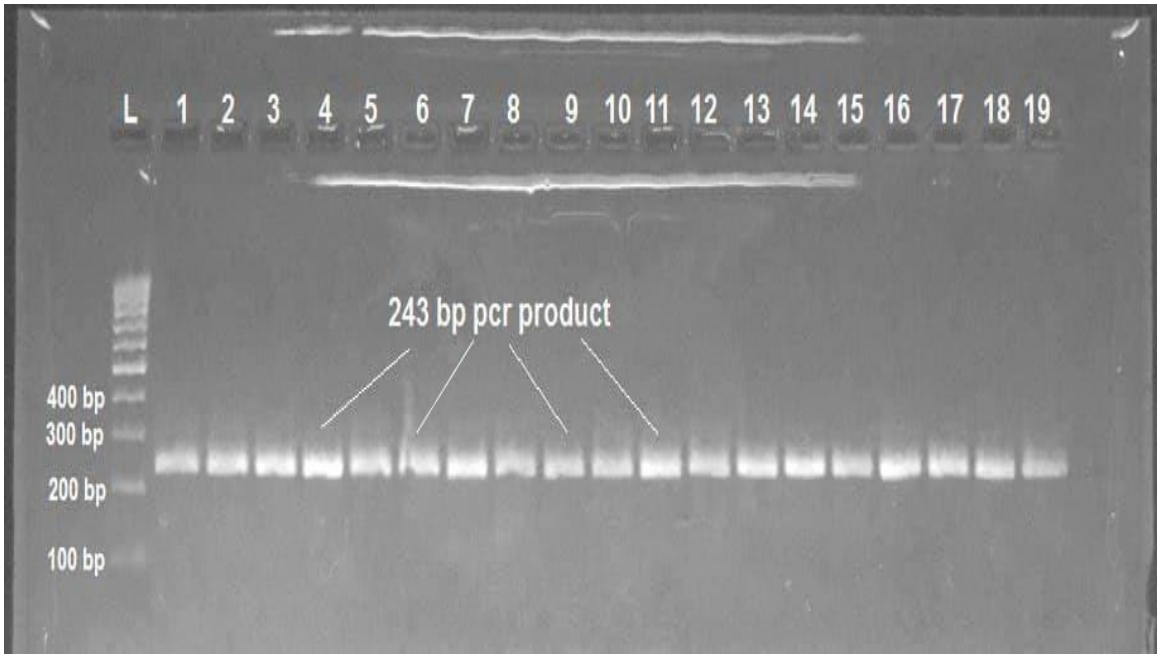


Figure 3.10 Agarose gel electrophoresis for PCR product.

After that use technique PCR-RFLP to determine rs179008 (A>T) polymorphisms of the TLR7 gene. is show in figure 3.11,

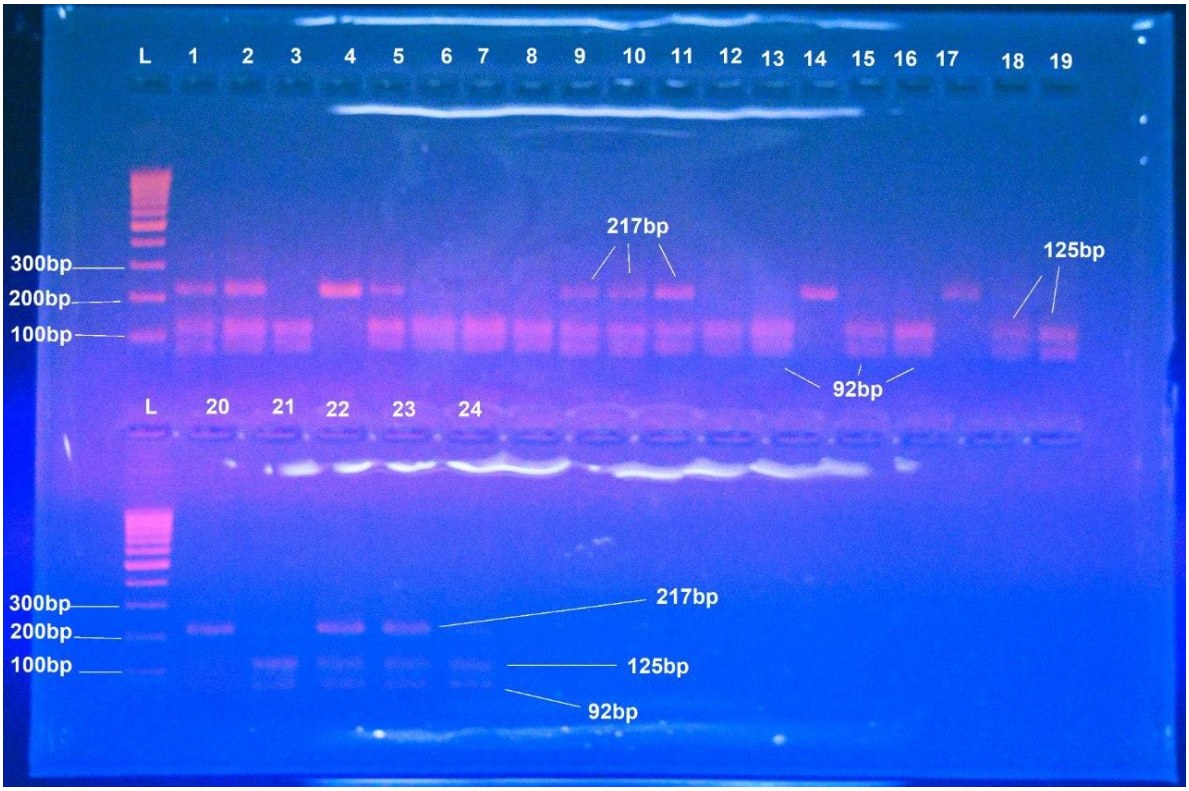


Figure 3.11 Genotyping of rs179008 polymorphism by PCR-RFLP, lanes L 100pb DNA ladder; lanse 3,6,7,8,12,13,15,16,18,19,21 and 24 AA genotype; lanes 1,5,9,10,11,22 and 23 AT genotype; lanes 4, 14,17 and 20 TT genotype

3-5-1 Frequencies of TLR7 rs179008 Alleles and Genotypes

In currant study distribution and frequency of TLR7 SNPs (rs179008) which have allelic (A, T) and genotypic (AA, AT, TT). As shown in table 3.10

Table 3.10 Distributions genotype of TLR7 in patients

	Frequency	Percent
AT	24	26.7
AA	60	66.7
TT	6	6.7
Total	90	100.0

These genotype distributions in three groups mild, moderate, and severe patients are illustrated in table 3.11, figure 3.12.

Table 3.11 Distributions genotype in categories.

count	Cases			Total
	Mild	Moderate	Severe	
AT	9	4	11	24
AA	12	18	30	60
TT	0	4	2	6
Total	21	26	43	90

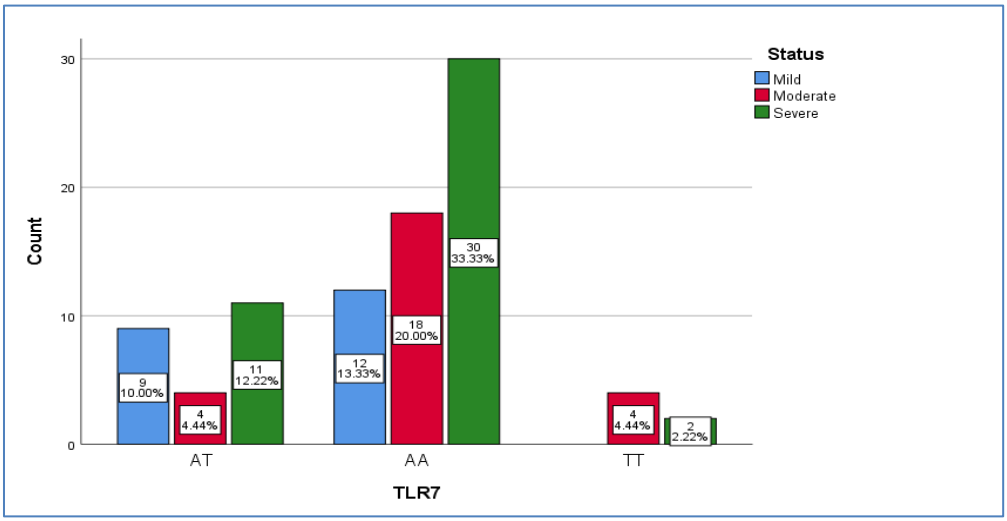


Figure 3.12 Distribution allele and genotypes for TLR7 (A/T) gene for patients categories

3.5.2 Comparison Genotype Distribution and Allele Frequency of rs179008

Comparison allele frequency of the rs179008 SNP (A>T) variant between mild to (moderate and severe) are shown in Tables (3.12).

Table 3.12Comparison of allele frequency of the rs179008 for patients groups.

	mild		Moderate, Severe		OR (95% CI)	P-value
Allele	**Count**	**Proportion**	**Count**	**Proportion**		
A	33	0.79	111	0.8	1.121 (0.480-2.620)	0.79152
T	9	0.21	27	0.2	0.892 (0.382-2.084)	

There was a non-significant difference association between the distribution of the allele (A>T) in mild group and (moderate, severe) group, as when compared mild to moderate group are show in Table 3.13, and when compared it to severe group as Table 3.14. Therefore, based on odds ratio (OR) calculations, none of the alleles can act as a protective or as a risk factor for the disease.

Table 3.13 Comparison allele frequency of the rs179008 for mild with moderate group

	mild		Moderate		OR (95% CI)	P-value
Allele	**Count**	**Proportion**	**Count**	**Proportion**		
A	33	0.79	40	0.77	0.909 (0.341-2.421)	0.84872
T	9	0.21	12	0.23	1.100 (0.413-2.929)	

Table 3.14 Comparison allele frequency of the rs179008 for mild with sever group

	mild		Severe		OR (95% CI)	P-value
Allele	**Count**	**Proportion**	**Count**	**Proportion**		
A	33	0.79	71	0.83	1.291 (0.512-3.252)	0.58741
T	9	0.21	15	0.17	0.775 (0.308-1.951)	

According to Table 3.15 when Comparison between mild and (Moderate, severe) groups for genotype and the behavior of allele with dominant, codominant, recessive or over dominant there was no association.

Table 3.15 Comparison of (A/T) SNP genotypes and alleles frequencies between mild and (Moderate, severe) groups

Model	Genotype	mild	Moderate,severe	OR (95% CI)	P-value
Codominant	A/A	12 (57.1%)	48 (69.6%)	1.00	0.421
	A/T	9 (42.9%)	15 (21.7%)	0.42 (0.15-1.18)	
	T/T	0 (0%)	6 (8.7%)	0.2 (0.016-5.66)	
Dominant	A/A	12 (57.1%)	48 (69.6%)	1.00	0.3
	A/T-T/T	9 (42.9%)	21 (30.4%)	0.58 (0.21-1.59)	
Recessive	A/A-A/T	21 (100%)	63 (91.3%)	1.00	0.32
	T/T	0 (0%)	6 (8.7%)	0.227 (0.012-4.2)	
Overdominant	A/A-T/T	12 (57.1%)	54 (78.3%)	1.00	0.063
	A/T	9 (42.9%)	15 (21.7%)	0.37 (0.13-1.04)	

OR: Odds ratio; CI: confidence interval;

In addition, there was significant difference in genotype frequency in model overdominant (AT) between mild and moderate groups is shown in Table 3.16, this patients act as protective and observe no significant difference when compared between mild and severe group is shown in Table 3.17.

Table 3.16 Comparison of (A/T) SNP genotypes and alleles frequencies between mild and Moderate group

Model	Genotype	Mild	Moderate	OR (95% CI)	P-value
Codominant	A/A	12 (57.1%)	18 (69.2%)	1.00	0.24
	A/T	9 (42.9%)	4 (15.4%)	0.30 (0.07-1.18)	
	T/T	0 (0%)	4 (15.4%)	0.164 (0.008-3.33)	
Dominant	A/A	12 (57.1%)	18 (69.2%)	1.00	0.39
	A/T-T/T	9 (42.9%)	8 (30.8%)	0.59 (0.18-1.97)	
Recessive	A/A-A/T	21 (100%)	22 (84.6%)	1.00	0.025
	T/T	0 (0%)	4 (15.4%)	NA (0.00-NA)	
Overdominant	A/A-T/T	12 (57.1%)	22 (84.6%)	1.00	0.035
	A/T	9 (42.9%)	4 (15.4%)	0.24 (0.06-0.96)	

Table 3.17 Comparison of (A/T) SNP genotypes and alleles frequencies between mild and severe group

Model	Genotype	Mild	Severe	OR (95% CI)	P-value
Codominant	A/A	12 (57.1%)	30 (69.8%)	1.00	0.65
	A/T	9 (42.9%)	11 (25.6%)	0.49 (0.16-1.48)	
	T/T	0 (0%)	2 (4.7%)	0.49 (0.02-10.9)	
Dominant	A/A	12 (57.1%)	30 (69.8%)	1.00	0.32
	A/T-T/T	9 (42.9%)	13 (30.2%)	0.58 (0.20-1.70)	
Recessive	A/A-A/T	21 (100%)	41 (95.3%)	1.00	0.54
	T/T	0 (0%)	2 (4.7%)	0.386 (0.018-8.41)	
Overdominant	A/A-T/T	12 (57.1%)	32 (74.4%)	1.00	0.17
	A/T	9 (42.9%)	11 (25.6%)	0.46 (0.15-1.38)	

This study focuses on the association between SNPs rs179008 and COVID-19 severity For instance, TLR7 rs179008 polymorphism showed a strong association with the development of bronchial asthma[178]

The genetic background of human populations can influence the susceptibility and outcome of infectious diseases. TLRs have a dual role in in confronting COVID-19 infection. TLRs play an important role in recognition of viral particles and initiation of the innate immune system with secretion of pro-inflammatory cytokines, although it can also harm the host due to persistent inflammation and tissue destruction via activation of inflammasome and production of IL-1β, which induces IL-6 leading to hyperactivation of the immune system which can contribute to acute lung injury[179]. Reports suggested that the variations within the host's genome play a role in COVID-19 disease progression[180]. (In this study TLR7 rs179008 polymorphism overdominant A/T was represent less effect than other genotype (AA, TT) against COVID-19 infection this was similar to study in HCV patients[181] and in HIV patients)[182]. Additionally, The substitution of A to T at the position of TLR7 rs179008 leads to an amino acid change from glutamine to leucine at position 11 of the protein-altering the TLR7 processing and cause an altered immune response[178]. However, our results were dissimilar to those of Mosaad et al. which showed no significant association between TLR7 rs179008 polymorphism and chronic HCV infection, in the genetic association studies of infectious diseases[183].

These conflicting findings are frequent. These discrepancies could be due to virus genetic heterogeneity, differences in the exposure rate to infectious agents, and various pathogen-induced immune responses, in addition to the interaction with environmental factors.

The present study should be validated with a bigger sample size in populations of other regions of Iraq, to better understand the role of TLR7 rs179008A/T polymorphism in susceptibility to disease progression COVID-19 infection further studies are needed.

Conclusions

&

Recommendations

5.1 Conclusions

1- The ABO blood group and gender factors had no association with COVID-19 infection in patients enrolled in the present study.

2- The level of D-dimer, ferritin, CRP, LDH recorded a significant elevation in patients' plasma and serum with severe COVID-19 infection.

3- The disease of COVID-19 can cause a variety of hematologic manifestations, such as neutrophilia, elevation of total white blood cells count, with a recorded relative lymphocytopenia in their percentage which is due to the inflammatory response against the infection.

4- There was association of genotyping (AT) for TLR7 rs179008 polymorphism represent less effected then other genotype (AA, TT).
5- Age revealed a significant elevation in patients with severe COVID-19 infection.

Recommendations: -

1- Study another gene polymorphism in this gene and gene expression.

2- Study the same SNP in other regions of Iraq with other ethnic and with a large sample size, to better understand the role of TLR7 rs179008A/T polymorphism in susceptibility to disease progression COVID-19 infection further studies are needed.

3- we recommend that the serum ferritin itself or combined with CRP may provide better results in predicting to severity disease.

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الخلاصة:-

أحدث تهديد للصحة العالمية هو التفشي المستمر لمرض تنفسي حديثاً سمية كوفيد -19. كوفيد - 19_ اكتشف في ديسمبر 2019. سرعان ما تبين انه بسبب فيروس كورونا المرتبط هيكلياً بالفيروس الذي تم تحديده سابقاً الذي سبب متلازمة الضائقة التنفسية الحادة (SARS) .

انتشار فيروس كورونا شكل تحديا خطيرا للصحة العالمية والبحوث والطب . وترتبط اختلاف قابلية الإصابة بفيروس كورونا وتطور المرض بخلفية وراثية مثل تعدد الاشكال Toll-Like Receptor7 وفصائل الدم ممكن التي ترتبط مع شدة الإصابة بالمرض هذه الدراسة صممت لتتحقق من التأثير الجيني وفصائل الدم على شدة COVID 19 . كما ذكران التغاير الجيني للنيكلوتيدات المفردة (SNP) في العديد من الجينات فد يكون عامل وراثياً يساهم في شدة المرض. يقع الجين TLR7 على كروموسوم XP22.2 الذي يمثل رسبتر لتمييز الأنماط .موجودة على العصيات داخل الخلايا والتي تنتج مناعة مضادة للفيروسات من خلال التعرف على الحامض النووي (SSRNA). TLR7 له القدرة على منع تكاثر الفيروس واطلاق السايتوكين .

فصائل الدم (ABO) انها تتكون من ثلاث الاليلات O,B,A وجميع هذه الاليلات يتم تشفيرها بواسطة (ABO Gene) . انها موجودة على سطح الكريات الدم الحمراء وتتحد مع الاجسام المضادة الموجودة في بلازما الدم .

هذه الدراسة تتضمن 90 مريض مصاب بفيروس كورونا ومقسمين الى ثلاث مجاميع بالاستناد الى منظمة الصحة العالمية وهي الحالات الخفيفة ، المتوسطة ، الشديدة تم جمع العينات من محافظة ديالى في العراق خلال فترة ما بين نوفمبر 2021 الى فبراير 2022 وتم سؤال جميع المشاركين عن طبيعة وشدة المرض وفترة ظهور الاعراض والتي يكون عادة حمى ، اسهال ، فقدان الذوق والشم ، رشح ، اعياء ، خمول ، صداع ، ضيق بالتنفس ، سعال ، التهاب الحلق والم عضلي في استبيان موحد اجابوا على الأسئلة حول عدوى فيروس كورونا.

تم قياس البارامترات الكيميائية مثلFerritin level ، D.dimer، CRP،بواسطة جهاز AFIAS اما LDH activityتم قياس بجهاز السبكتروفوتوميتر . اما صورة الدم كاملة تم قياسها بواسطة

جهاز MINDARY BC .3000Puls ، تم استخدام تقنية PCR-RFLP لتحديد RS179008 لجنين TLP7 .اثبت تقديرات وزيادة في مستوى هذه المؤشرات الحيويه (CRP, Ferritin، D.dimer، LDH) مع وجود فروق معنوية بين المرضى في المجموعات الثلاث .

كما اثبت انه لا توجد فروق معنوية لكريات الدم البيضاء (WBC)ضمن المجاميع الخفيفة والمعتدلة حيث (O.228) .P-value مع وجود فروق معنوية بين المجاميع المتوسطة والشديدة (P.value 0.015) والخفيفة والشديدة حيث (0.000) P-value كما اثبتت النتائج لهذه الدراسة ارتفاع في عدد العدلات وانخفاض في عدد لمفاويات .

كما تبين انه لا توجد فروق معنوية في مستويات (هيموغلوبين ، والهيماتوكريت ومعدل حجم الصفائح الدموية حيث P-value <0.05 . ومن جانب اخر لا توجد علاقة بين فصائل الدم وشدة المرض بين المجاميع الثلاث .

اما التحليل الجيني قد اثبت انه لا توجد علاقة معنوية ما بين الاليلات و الأنماط الوراثية Codominance، Dominance ,Recessive حيث (value>0.05-P) باستثناء التركيب الجيني AT في Overdominance ما بين المجاميع الخفيفة والمتوسطة حيث يمثل اقل تأثير من باقي الانماط الوراثية (AA, TT) ضد المرض بالاعتماد على ((0.024) P-value , (OR) .

تم الاستنتاج من هذه الدراسة الحالية ان بعض المؤشرات الحيوية ترفع عند الإصابة بفيروس كورونا وكذلك تأثير الجين TLR7 على شدة المرض مع عدم تأثير فصائل الدم على شدة المرض .

كما توجد علاقة بين المؤشرات الحيوية وتطور المرض لذلك يجب اجراء هذه التحاليل المختبرية للتمييز بين الحالات الخفيفة والشديدة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل – كلية الطب

تأثير التغاير الجيني لجين *TLR7* وبعض البارامترات على شدة الاصابة بفايروس كورونا

رسالة

مقدمة الى مجلس كلية الطب في جامعة بابل وهي جزء من متطلبات نيل درجة الماجستير في الكيمياء الحياتية السريرية

من قبل

زينب حاتم ديوان

بكلوريوس كلية العلوم جامعة ديالى

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2022

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