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College of Medicine
Department of Microbiology

Studying the association of serum levels of IL-1 alpha, IL-1 beta, Nuclear Factor kappa B and IL-6 with hematological and biochemical parameters in SARS-CoV-2 patients

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

By

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صدق الله العلي العظيم

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We, the examining committee, certify that we have read the M.Sc. thesis entitled:

“Studying the association of serum levels of IL-1 alpha, IL-1 beta, Nuclear Factor kappa B and IL-6 with hematological and biochemicals parameters in SARS-CoV-2 patients”

We have examined the student (Ismael Raheem Jabbar) in its content, find it adequate as a thesis for the degree of Master in Medical Microbiology and Immunology.

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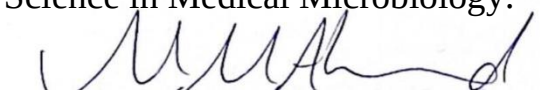

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DEDICATION

To Al-Imam Al-Munthadher as the hoped saviour...

To Al-Imam Al-Hussein and his supporters as a big "no" that nourishes life with rejection until now...

To the beats of my heart, my father and mother, as a tree that gives me all its shadow, support and care, without them I would not be what I am now...

To my family as a solid wall on which I rely, especially my twin brother, who supports me whenever it gets hard and difficult ...

To the great martyrs as truth messages to Allah ...

To the health staffs who saved many victims of the deadly Corona epidemic,

To the truth that I look for, which is a window that overlooks nothing but eternity and immortality together...

I give this humble effort ... with my sincere love for them.

Ismael Raheem

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Ismael Raheem

Summary

Corona virus disease 2019 (COVID-19) is an infectious disease caused by a newly discovered coronavirus, SARS-CoV-2. The virus infects the cells along the airways of the lung, by attaching to angiotensin converting enzyme 2 receptor (ACE-2). The disease considered a major global health problem affecting, large number of the population around the world. Covid-19 disease is characterized by cytokine storm that is the major cause of disease manifestation and complications. Better understanding the key players this storm would feed in into better formulation of treatment protocols. The aim of this study was to study cytokine candidates, namely IL-1 alpha, IL-1 beta, Nuclear Factor Kappa beta and IL-6 levels, as well as other hematological and chemical parameters in SARS-CoV-2 patients. For this purpose, this cross-sectional study which included 65 Covid-19 patients (32 male and 33 female). All patients were admitted to Al-Amal Specialized Hospital for Communicable diseases, Al-Hakeem Hospital and Al Sader General Hospital in the period extending from December 2020 to February 2021. Their ages ranged between sixteen to ninety years old. The control groups included 23 healthy people (13 male and 10 female) with the same ages and gender of the patients were randomly selected from the local community. The control group was used in the cytokine study for setting up a local reference value. The demographic and clinical data were collected from patients through interview questionnaire. The patients were classified based on SpO_2 percentage into mild/moderate group (> 90-94%) and severe/critical group (<90 %). Accordingly, 22 patients were mild/moderate and 43 patients severe/critical. Sera and whole blood were collected from each participant noting that the sera were used to determine serum IL-6, IL-1α, IL-1β and NF-κB levels as well as biochemical parameter for all samples, while whole blood was used for determination of complete blood count and for ESR. The ELISA was used to measure the

concentration of serum Interleukins and the data were statistically analysed by software SPSS version 26.

The results of the study showed the sever/critical disease was seen in older age people (mean age 62.58 years). Therefore, the older age is considered a risk factor for developing Severe/critical form of Covid-19. The lymphocyte percent was significantly lower in severe/critical patients compared with mild/moderate group (p = 0.004). While, the ESR was significantly increased in severe/critical patients (p = 0.016). On the other hand, there were a significant association between biochemical marker (urea, albumin, ALT, AST, ALP, LDH, CRP, ferritin and D-dimer) and Covid-19 severity (p-value 0.001, 0.000, 0.016, 0.014, 0.012, 0.005, 0.003, 0.031, 0.001 respectively). There was a significant positive correlation between serum IL-1α levels with neutrophil (p = 0.010). Likely there was a significant positive correlation between serum IL-6 levels and creatinine level (p = 0.012). Whereas, the SpO_2 percentage, lymphocyte and serum albumin were negatively correlated with IL-6 level (p = 0.002, 0.015, 0.034 respectively). In addition, the NF-κB had a negative correlation with RBC counts and haemoglobin levels (p = 0.034, 0.005 respectively). Finally, there was a positive correlation between the level of IL-1β with LDH and AST levels (p = 0.054, 0.049 respectively).

In conclusion, an increased risk of Covid-19 was associated with older age, comorbidities including; hypertension (increased systolic blood pressure) higher blood glucose level as well as decrease lymphocyte. Serum IL-6 level had a positive linear correlation with creatinine level and negative liner correlation with lymphocyte and albumin. Accordingly, IL-6 may have direct adverse effect on kidney and lung tissues in addition to bone marrow and the liver. The increased of IL-6 levels may be due to SARS-CoV-2, as a relationship was found between NF-κB and IL-6, while there is study indicating existence of relationship between ORF7a of SARS-CoV-2 and NF-κB which in turn responsible for upregulation

of IL-6. The IL-1α serum level had a positive linear correlation with neutrophil and negative liner correlation with lymphocyte and albumin. Thus, IL-1α has a proinflammatory effect. The IL-1β had a positive linear correlation with AST and LDH level. These results may indicate that IL-1β is associated with tissue damage. On the other hand, the NF-κB level had a negative linear correlation with white blood cells and haemoglobin. These results may indicate that NF-κB has direct adverse effect on RBC counts and hemoglobin levels.

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List of Abbreviations

Code	Words
ACE2	Angiotensin converting enzyme 2
AEC-II	Type II alveolar epithelial cells
AKI	Acute kidney injury
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ARDS	Acute respiratory disease syndrome
AST	Aspartate aminotransferase
CBC	Complete blood count
CFR	Case fatality rate
COVID-19	Corona virus disease 2019
CRP	C-reactive protein
CRS	Cytokine release syndrome
DAMPs	Damage-associated molecular patterns
DIC	Disseminated intravascular coagulation
DPP-4	Dipeptidyl peptidase-4
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophge colony stimulating factor

HRP	**Horseradish peroxidase**
ICTV	**International committee on taxonomy of viruses**
ICU	**Intensive care unit**
IgG	**Immunoglobulin G**
IgM	**Immunoglobulin M**
IKBs	**Inhibitor of NF-κB protein**
IKK	**Inhibitor of NF-κB Kinase**
IL-1α	**Interleukin-1 alpha**
IL-1β	**Interleukin-1 beta**
IL-6	**Interleukin-6**
LDH	**Lactic dehydrogenase**
MERS	**Middle east respiratory syndrome**
NAAT	**Nucleic acid amplification test**
NF-κB	**Nuclear factor kappa beta**
ORF7a	**Open reading frame 7a**
PAMPs	**Pathogen-associated molecular patterns**
PBMCs	**Peripheral blood mononuclear cells**
PRRs	**Pattern recognition receptors**
RBC	**Red blood cells**
RBD	**Receptor binding domain**
RT-PCR	**Real-time reverse transcriptase polymerase chain reaction**
SARS	**Severe acute respiratory disease syndrome**
SARS-CoV-2	**Severe acute respiratory disease syndrome 2**
SD	**Standard deviation**
SPSS	**Statistical package for the social Sciences**
TBIL	**Total bilirubin**
Th1 cell	**T-helper 1 cell**
TLRs	**Toll-like receptors**
TMB	**3,3',5,5'-Tetramethylbenzidine**
WBC	**White blood cells**
WHO	**World health organization**

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CHAPTER ONE

Introduction

and

Literature Review

1.1. Introduction

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This disease was reported for first time at the end of December 2019 in Wuhan city (Hubei province) of China. This disease displayed particular clinical signs in the respiratory system such as increasing occurrence of pneumonia of unknown aetiology **(Helmy *et al*., 2020)**. Despite many intensive attempts to contain the disease in China, the disease became pandemic and spread to all countries around the world **(Adhikari *et al.*, 2020)**. SARS-CoV-2 is novel enveloped, positive-sense, single-strand RNA beta coronavirus, with a typical crown-like appearance under an electron microscope due to the presence of glycoprotein spikes on its envelope **(Gennaro *et al*., 2020)**. Phylogenetic analysis suggests that SARS-CoV-2 might have emerged from the zoonotic cycle and rapidly spread by human to human transmission, However, the exact source of SARS-CoV-2 has not been identified yet **(Andersen *et al*., 2020)**. The transmission of virus occurs via close contact with an infected individual that produces respiratory droplets while coughing or sneezing within a distance of about 2 meters. In same way, the SARS-CoV-2 is highly infectious and asymptomatic patients may also have considered the source of infection **(Meyerowitz *et al*., 2021)**.

According to WHO and other sources fever, dry cough and tiredness are the most common symptoms in mild or moderate COVID-19 patients, while sore throat, diarrhoea, nausea or vomiting, headache, conjunctivitis, rash on skin and discoloration of fingers or toes are less common symptoms and recovered spontaneously with treatment without required hospitalization **(Dhamad and Abdal Rhida, 2020)**. On the other hand, the most common symptoms in severe Covid-19 patients are fever, cough, dyspnea and hypoxia, while the critical patients have respiratory failure from acute respiratory disease syndrome (ARDS), shock, and/or multi-organ dysfunction **(Rahman *et al*., 2021)**. Many of

severe or critical patients had to been admitted to the intensive care unit (ICU) and the mortality rate for patients in the ICU is 25% which can be even higher in the elderly people and most deaths attributed to severe inflammation and embolic complications **(Wang *et al*., 2020)**.

Several studies reported changes in the hematological and biochemical parameters, including decreased lymphocyte count, increased neutrophil count, higher D-dimer status, increased inflammatory indices such as CRP, ESR and LDH in more COVID-19 patients **(Bairwa *et al*., 2021)**.

On the early observations in Covid-19 pathogenesis is the presence of cytokines storms. Cytokines storms characterized by increase in the serum levels of several proinflammatory cytokines. Hypercytokinemia leading to injury of alveolar epithelial cells, vascular endothelial cells, and multiple organs damage. Therefore, cytokines and their receptors, as well as cytokine-dependent intracellular signaling pathways can be targeted by potential therapies aimed to relieve the heavy burden of cytokine storm **(Pelaia *et al*., 2020)**. Higher serum levels of pro-inflammatory cytokines (TNF-a, IL-1, IL-2 and IL-6) and chemokine (IL-8) have been observed in many patients with severe COVID-19 compared with individuals with mild disease **(Ghazavi *et al*., 2021)**. However, their roles in Covid-19 associated pathology are not fully understood. For instance IL-6 suggested to be the most driver of immune dysregulation and ARDS in COVID-19 **(Sabaka *et al*., 2021)**, however, blockage of IL-6 receptor has been limited therapeutic value in Covid-19 patients **(Guimarães *et al*., 2021)**.

Objective:

1. To perform hematological and biochemical analyses on blood samples.
2. To measure the serum levels of IL-6, IL-1α, IL-1β and NF-κB in the COVID-19 patients.

3. To study the possible correlations between the levels of IL-6, IL-1α, IL-1β and NF-κB with different hematological and biochemical markers to detect disease severity.

The Aim of Study:

This study was designed to determine the association of serum levels of IL-1 alpha, IL-1 beta and Nuclear Factor Kappa beta on IL-6 levels, as well as other hematological and chemical parameters in SARS-CoV-2 patients.

Medical significant (Rationale):

This is to investigating and finding other molecules playing roles in SARS-CoV-2 associated cytokine storms that offer new therapeutic targets for the treatment of patients infected with SARS-CoV-2.

1.2. Literature Review

1.2.1. SARS-CoV-2 Overview

The SARS-CoV-2 that caused COVID-19 have constituting a medical emergency and a global crisis rapidly, from have first emerging in December 2019. However, on 11 March 2020, it was declaring an pandemic by the World Health Organization **(WHO, 2020)**. Coronaviruses represent a large family of viruses, some of which having previous causing severe human diseases such as; Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) **(Wenzel, 2020)**.

SARS-CoV-2 is a novel genus of coronavirus that has not being previously identified in humans. Phylogenetic analysis, suggested that SARS-CoV-2 might have emerging from the zoonotic cycle, and rapidly spread by human to human transmission **(Chan *et al*., 2020)**. However, the exact source of SARS-CoV-2 has not been identified yet. Transmission among humans occurs via close contact with an infected individual that produces respiratory droplets while coughing or sneezing within a range of about 6 feet **(Ghinai *et al*., 2020)**. Infected individuals have been reported with common clinical symptoms involving fever, non-productive cough, myalgia, shortness of breath, as well as normal or decreased leukocyte counts **(Zhang *et al*., 2020)**. In addition, severe cases of infection cause pneumonia, severe acute respiratory syndrome, kidney failure, and death **(Xiong *et al*., 2020)**.

There is no specific treatment available for SARS-CoV-2 and the current treatment relies on supportive care of the infected patients **(Centre for Disease Control and Prevention, 2020)**. The control measure of pandemic to reducing the SARS-CoV-2 virus spread by encouraging people to stay at home, maintain a physical distance of at least 2 meters, and adhere to infection prevention and control techniques **(WHO, 2020)**.

1.2.2. The virus

1.2.2.1. Structural and molecular features of SARS-CoV-2

SARS-CoV-2 has a crown-like appearance under an electron microscope due to the presence of spike glycoproteins on the envelope. The virus particle sizes ranging from 70 to 90 nm observed under a wide variety of intracellular organelles, most specifically in vesicles **(Park *et al*., 2020)**. The viral particle is pleomorphic and possesses non-segmented, single-stranded, positive-sense ribonucleic acid (ssRNA+) as its genome. A coronavirus contains four structural base proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Among them, the S protein which mediates interaction with cell surface ACE2 receptor and plays the most important role in viral attachment, fusion, and entry **(Rahman *et al*., 2021)**. The viral membrane glycoprotein (M) and envelope (E) of SARS-CoV-2 are embedded in host membrane-derived lipid bilayer encapsulating the helical nucleocapsid comprising viral RNA (Figure.1.1). The M and E proteins are required for virus morphogenesis, assembly, and budding **(Kumar *et al*., 2019)**. SARS-CoV-2 have 30 kb genome RNA, is large enough to produce a positive sense to be read directly by ribosomes in the cell. The genome is coated with an N protein, which forms a helical nucleocapsid. As viruses cannot make their own lipids, they use the host's lipids for replication and morphogenesis. The N protein plays a crucial role in the morphogenesis phase of the viral life cycle during virion formation **(Rahman *et al*., 2021)**.

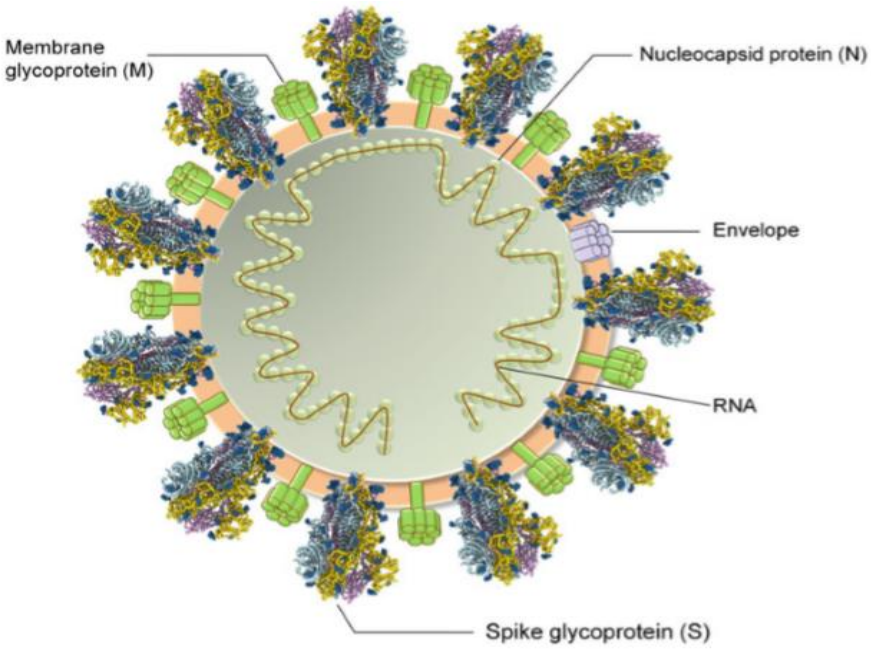

Figure 1.1 Structure of SARS-CoV-2. (Kumar et al., 2019).

1.2.2.2. Entry and replication of SARS-CoV-2 in host cells:

Entry of coronaviruses into host target cells depends on the binding of spike glycoprotein to the cellular receptor and priming of S protein by host cell proteases. Like SARS-CoV, SARS-CoV-2 uses the ACE2 receptor for internalization and entry to the cell **(Kumar *et al*., 2019)**. Similar to SARS-CoV, the extra pulmonary spread of SARS-CoV-2 may be seen due to the wide spread tissue expression of the ACE2 receptor. In addition, the spike protein of SARS-CoV-2 exhibits 10-20 times higher affinity to human ACE2 receptor as compared to that of SARS-CoV. The heightened affinity for a prevalent cellular receptor may be a factor that increases transmission **(Wrapp *et al*., 2020)**. Binding of spike protein to the ACE2 receptor results in conformational changes in spike protein that leads to the fusion of viral envelop protein with host cell membrane following entry via endosomal pathway. This event is followed by the release of viral RNA into the host cytoplasm that undergoes translation and generates replicase polyproteins pp1a and pp1b that further cleaved by virus encoded proteinases into small proteins. Assembly of virion takes place via interaction of viral RNA and protein at endoplasmic reticulum and Golgi complex. These

virions are subsequently released out of the cells via vesicles by budding (Figure. 1.2) **(Kumar *et al*., 2019)**.

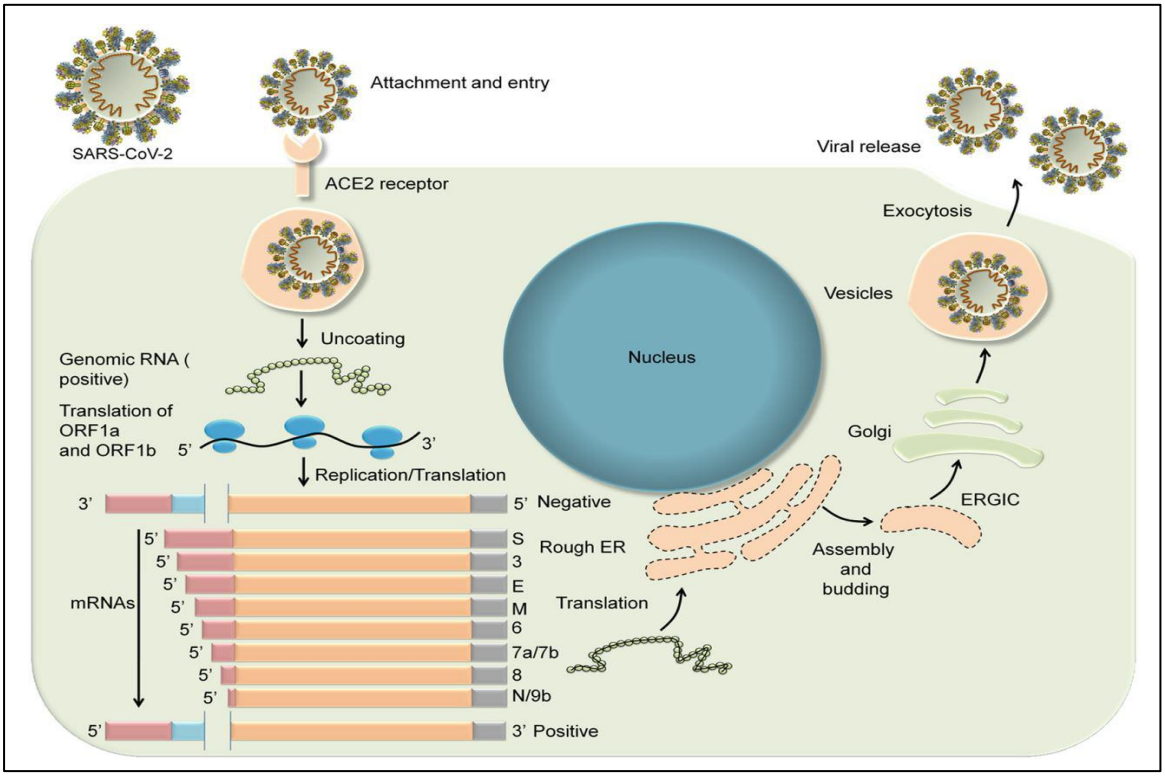

Figure 1.2: Entry and replication of SARS-CoV-2 in host cells (Kumar et al., 2019).

1.2.3. Epidemiology of SARS-CoV2

In December 2019, China reported an outbreak of pneumonia of unknown causes in Wuhan, the capital city of Hubei province. Most of the early cases were epidemiologically linked to the Huanan seafood wholesale market where aquatic animals and live animals were sold. The COVID-19 epidemic was declared a public health emergency of international concern by the World Health Organization on January 30, 2020, The WHO used the term 2019 novel coronavirus to refer to a coronavirus that affected the lower respiratory tract of patients with pneumonia in Wuhan, China on 29 December 2019. The WHO announced that the official name of the 2019 novel coronavirus is COVID-19, and the current reference name for the virus is SARS-CoV-2 **(WHO, 2020; Chowdhury and Oommen, 2020)**. The COVID-19 has been found to have

higher levels of transmissibility and pandemic risk than the SARS-CoV and MERS. Studies indicated that the spread of COVID-19 was relatively quick and reported that it had spread to all countries and effected most people around the world **(Adhikari *et al.*, 2020)**. The COVID-19 pandemic has created a public health crisis, infected millions of people, and caused a significant number of deaths. SARS-CoV-2 transmits from person to person through several routes, mainly via respiratory droplets, which makes it difficult to contain its spread into the community **(Rahman *et al*., 2021)**.

1.2.4. Pathogenesis of and clinical finding of SARS-CoV-2

The pathogenic mechanism that produces pneumonia seems to be particularly complex. The viral infection is capable of producing an excessive immune reaction in the host **(Gennaro *et al.*, 2020)**. In some cases, a reaction takes place, which as a whole is labelled a cytokine storm. The effect is extensive tissue damage. The protagonist of this storm is interleukin 6 (IL-6). IL-6 is produced by activated leukocytes and acts on a large number of cells and tissues. IL-6 is implicated into the pathogenesis of the cytokine release syndrome (CRS) that is an acute systemic inflammatory syndrome characterized by fever and multiple organ dysfunction **(Rose-John, 2018)**. The virus might pass through the mucous membranes, especially nasal and larynx mucosa, then enters the lungs through the respiratory tract. Then the virus would attack the targeting organs that express ACE-2, such as the lungs, heart, renal system and gastrointestinal tract. The virus begins a second attack, causing the patient's condition to aggravate around 7 to 14 days after onset. B lymphocyte reduction may occur early in the disease, which may affect antibody production in the patient. Besides, the inflammatory factors associated with diseases mainly containing IL-6 were significantly increased, which also contributed to the aggravation of the disease around 2 to 10 days after onset **(Gennaro *et al.*, 2020)**.

The Incubation period for COVID-19 within 14 days following exposure. Median incubation period being 4 days **(Lauer *et al*., 2020)**. It has been reported that the clinical symptoms of confirmed COVID-19 patients were varied from mild flu-like symptoms to very severe respiratory symptoms and even respiratory and kidney failures and death **(Sohrabi *et al*., 2020)**. People of all ages are susceptible to COVID-19 infection. Children and adolescents under 18 years represent under 2% of the confirmed COVID-19 cases **(Dong *et al*., 2020)**. According to WHO and other sources fever, dry cough and tiredness are the most common symptoms while sore throat, diarrhea, nausea or vomiting, headache, conjunctivitis, rash on skin and discoloration of fingers or toes are less common symptoms of COVID-19 patients **(Dhamad and Abdal Rhida, 2020; WHO, 2021)**. Similar to other upper airway viral infections, such as; common cold or flu, the loss of smell is a frequent symptom in COVID-19 patients. However, a sudden, severe, and isolated loss of smell and/or taste may also be present in COVID-19 patients who are otherwise asymptomatic **(Mullol *et al*., 2020)**. The disease manifestations in the infected patients range from asymptomatic infection to mild pneumonia to moderate pneumonia (hypoxia requiring hospitalization, and critical illness (leading to invasive mechanical ventilation, multiorgan dysfunction and possibly death, as shown in (Table 1-1) **(WHO, 2020)**. The risk of death depends on age, underlying comorbidities and severity of the disease, increasing up to 49% in critically ill patients **(Wu and McGoogan, 2020)**.

The most common complications that develop in COVID-19 are bilateral pneumonia which may progress to ARDS, sepsis and septic shock, acute kidney injury (AKI) and others such as acute cardiac injury, coagulopathy, rhabdomyolysis, hyponatremia, acidosis and neurological complications. Complications are more in severe disease compared to non-severe disease **(Wu and McGoogan, 2020)**.

Table (1-1): Criteria for clinical severity of confirmed COVID-19 pneumonia (WHO, 2020).

Classification of Covid-19 severity	Clinical Severity and Findings	Outcome of disease
Mild disease	Fever, with or without cough, no dyspnea, no gasping, no chronic disease. other non-specific symptoms, such as sore throat, nasal congestion, headache, diarrhea, loss of smell or loss of taste.	Without evidence of viral pneumonia or hypoxia.
Moderate disease	Fever, cough, dyspnea, fast breathing but no signs of severe pneumonia, including $SpO_2 \geq 90\%$ on room air	Pneumonia
Severe disease	Clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or $SpO_2 < 90\%$ on room air.	Severe pneumonia
Critical disease	Patient has any of the following: respiratory failure, needs mechanical assistance, shock and "extra pulmonary" organ failure, intensive care unit is needed.	Acute respiratory disease syndrome (ARDS), Sepsis and Septic shock

1.2.5. Risk factors of SARS-CoV-2

1.2.5.1. Aging

Aging is considered a process that causes degenerative changes at the cellular level and sometimes leads to various diseases that can be autoimmune, infectious, or inflammatory. Older people are the highest risk group by severe COVID-19 illness, could be because of the increased likelihood that a person will have comorbidities in older age, many of which are linked to poor Covid-19 outcomes. Another critical factor for the disease to be severe is the immune

response, which is not as strong compared to young people **(Pitones-Rubio *et al*., 2020)**. Preliminary reports have shown that older people were at a higher risk of COVID-19 complications with higher rates of hospitalisation, intensive care unit admissions, intubation, and death. Currently, it is unclear whether chronological age is an independent risk factor for severe COVID-19, or simply that risk factors are more common among older adults. Also, the mechanisms through which older age may predispose to poorer prognosis have yet to be elucidated. Several hypotheses have been proposed as to why older people might be more susceptible to severe COVID-19 infection, including a weaker immune response, obesity, age-related decline in respiratory function, frailty and multimorbidity **(Ho *et al*., 2020)**.

1.2.5.2. Hypertension and cardiovascular disease

Hypertension is considered the main risk factor for cardiovascular disease, and it's among the main comorbidities in COVID-19 patients **(Zhou *et al*., 2020)**. There is increasing understanding that severe COVID-19 causes considerable vascular abnormalities including widespread microthrombotic and macrothrombotic events, renal and cardiac failure. The association of hypertension with its potential microvascular disease, with more severe disease and poor outcomes from COVID-19, is therefore an important consideration **(Cook, 2020)**. Recent understanding of the role of immune dysregulation in hypertension can provide a possible correlation between immune dysregulation and a more severe course of COVID-19. Rapid deterioration in COVID-19 patients is associated with a pro-inflammatory cytokine storm. Accordingly, an increase in systemic IL-2, IL-6, and IL-7, granulocyte colony-stimulating factor (G-CSF) and TNF-a has been observed in patients with COVID-19 **(Huang *et al*., 2020)**. Interestingly, the same cytokines have been associated with the development of hypertension in experimental and clinical observational, as well as interventional studies. For example, IL-6 which appears to be strongly linked

to clinical outcomes of COVID-19, is one of the key cytokines regulating immune-inflammatory responses in hypertension. On the other hand, the loss of lymphocytes is one of the key features of COVID-19, and a recent study in the UK Biobank population demonstrated that among white blood cells, hypertension is causally associated with lymphocytes. Moreover, it was shown that CD4+ T-cell, and in particular CD8+ T-cell cells are dysregulated in hypertension which are prone to overproduction of cytokines but are less efficient in antiviral defense. These immune mechanisms also contribute significantly to accelerated end-organ damage **(Kreutz *et al*., 2020)**.

1.2.5.3. Diabetes mellitus

Diabetes mellitus is a chronic disease determined by loss of control of glucose homeostasis that can affect the organs of the body. The proposed mechanisms to understanding the association between diabetes mellitus and Covid-19 include alterations in vascular, cellular, and host repair processes **(Pitones-Rubio *et al*., 2020)**. Dipeptidyl peptidase-4 (DPP-4) it was the possible increased susceptibility to infections. Furthermore, because of the high affinity between human DPP-4 and the spike receptor-binding domain of SARS-CoV-2, it was suspected that corona virus, might be able to use the DPP-4 enzyme as a functional receptor to gain entry into the host. However, the diabetic patients are at a higher risk for COVID-19 infections due to that expression DPP-4 on the different cell surfaces such as Macrophages, natural killer cells, B cells, and T cells. This protein is multifunctional and involved in cytokine production, DNA synthesis, signalling activation, and cell proliferation. As a presence of low-grade chronic inflammation in diabetes, the NF-κB pathway is almost always excited. Elevated NF-κB pathway expression in diabetes affects the immune system in such patients. Diabetic patients could also suffer from high levels of T-helper1 and T-helper17, which can explode the inflammatory cascade in an uncontrolled manner **(Chandrasekaran and Fernandes, 2020)**.

1.2.6. Classification, origin and animals' reservoir of SARS-CoV-2

According to the International Committee on Taxonomy of Viruses, coronaviruses are classified under the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* **(Fung & Liu, 2019)**. Corona represents crown-like spikes on the outer surface of the virus; thus, it was named as a coronavirus. This virus was reported to be a member of the group B coronaviruses. The novel virus was named as Wuhan coronavirus or 2019 novel coronavirus (2019-nCov) by the Chinese researchers. The International Committee on Taxonomy of Viruses (ICTV) named the virus as SARS-CoV-2 and the disease as COVID-19 **(Cui *et al*., 2019)**.

As many early cases of COVID-19 were linked to the Huanan market in Wuhan, it is possible that an animal source was present at this location. Compared with the sequences of coronaviruses found in wildlife, SARS-CoV-2 shares 96.2% homology with BatCoV-RaTG13 in bats and approximately 90% homology with coronavirus in pangolins. Consequently, a likely route of viral transmission involves the enlargement of the reproductive scale by SARS-CoV-2 derived from bats infecting one or more intermediate hosts, such as pangolins **(Peng *et al*., 2021)**. This clearly shows that the SARS-CoV-2 spike protein optimized for binding to human-like ACE-2 is the result of natural selection. It is possible that a progenitor of SARS-CoV-2 jumped into humans, through adaptation during undetected human-to-human transmission. Once acquired, these adaptations would enable the pandemic to take off and produce a sufficiently large cluster of cases to trigger the surveillance system that detected it **(Andersen *et al*., 2020)**.

1.2.7. Transmission of SARS-CoV-2

The SARS-CoV-2 is spread predominantly via virus-containing droplets through sneezing, coughing, or when people interact with each other for some

time in close proximity, usually within one metre. These droplets can then be inhaled or land on surfaces where they can be detectable for up to four hours on copper, up to 24 hours on cardboard and up to two to three days on plastic and stainless steel **(van Doremalen *et al*., 2020)**. Other people may come into contact with these droplets and get infected when they touch their nose, mouth or eyes **(Deng *et al*., 2020)**.

SARS-CoV-2 RNA has also been detected in other biological samples, including the urine and feces of some patients. One study found viable SARS-CoV-2 in the urine of one patient **(Sun *et al*., 2020)**. Other studies have cultured SARS-CoV-2 from stool specimens **(Xiao *et al.*, 2020)**. The transmission of SARS-CoV-2 through feces or urine uncertain. However, recently evidence supporting the possibility of a fecally mediated route of transmission has been accumulating **(Guo *et al*., 2021)**. Some studies have reported detection of SARS-CoV-2 RNA, in either plasma or serum, and the virus can replicate in blood cells. However, the role of blood-borne transmission remains uncertain and low viral titters in plasma and serum suggest that the risk of transmission through this route may be low **(Chang *et al*., 2020)**. Perinatal transmission of SARS-CoV-2 may occur during pregnancy or during labor and delivery. Intrauterine transmission occurs when SARS-CoV-2 crosses the placenta to infect the fetus. Several studies have proposed criteria for intrauterine transmission **(Shah *et al*., 2020)**. Data regarding possible intrauterine transmission during the first and early second-trimester infections are extremely limited, most congenital infections have occurred following maternal infection late in gestation **(Zhu *et al*., 2020)**.

1.2.8. Host immune response to SARS-CoV2

1.2.8.1. Humoral immune response

Humoral immune response is an antibody-mediated immune response. T helper cells assist B cells to differentiate into plasma cells, which in return

produces antibodies specific to a viral antigen. In order to limit infection, an antibody which is of neutralizing nature is efficient in fully blocking the virus from entering into host cells and hence plays a very intense protective role at a later stage of infection and also prevents relapse of cases in the future **(Kumar *et al*., 2020)**. In case of SARS-CoV, the antibody profile of this virus produces IgM and IgG and at a later phase sero-conversion has been observed which is mediated by the helper T cells. IgM disappears at the end of week 12 whereas IgG has been found to last for a longer time pointing out to the probability of IgG being a potent protector Ab during the infection. Current evidence strongly indicates that Th1 type response is key to the successful control of SARS-CoV and MERS-CoV and probably true for SARS-CoV-2 as well **(Yong *et al*., 2019)**. In study involved 254 samples from 188 COVID-19 cases revealed Antibodies against SARS-CoV-2 spike and RBD declined moderately over 8 months, comparable to several other reports. Memory B cells against SARS-CoV-2 spike actually increased between 1 month and 8 months after infection. Memory CD8+ T cells and memory CD4+ T cells declined with an initial half-life of 3 to 5 months **(Dan *et al*., 2021)**.

1.2.8.2. Cellular immune response

Cellular immune response is a mechanism of adaptive immunity. Cellular immunity in contrast to the humoral immune response can be seen inside the infected cells, which is mediated by T-lymphocytes. T-helper cells direct the overall adaptive immune response while cytotoxic T cells play a vital role in clearance and killing of viral infected cells. For any effective vaccine advancement, cellular immunity provided by T cells is very much essential as shown by the mouse model experiment on MERS-CoV and SARS-CoV, where in their reports suggested that the lack of T cells resulted in no viral clearance in infected mice, hence explaining the importance of T cells in viral infection **(Yong *et al*., 2019)**. When investigated 14 out of 23 SARS-recovered patients, post 6 years of infection, it was reported that distinct T cell memory responded to the S

library of peptide of SARS-CoV **(Channappanavar *et al.*, 2014)**. Similar findings of distinct CD8+ T cells were seen during the case of MERS-CoV clearance in a mouse model too **(Wirblich *et al.*, 2017)**. Hence, this information can be useful in case of SARS-CoV-2 as well. However, in case of SARS-CoV-2 recent reports suggest that the peripheral blood mononuclear cells (PBMCs) of SARS-CoV-2 infected individuals have shown efficient reduction in the CD8+ and CD4+ T cell counts, which may result in compromised T memory cell generation and persistence in SARS-CoV-2 survivors **(Kumar *et al.*, 2020)**.

1.2.8.3. Cytokine storm syndrome in SARS-CoV-2

cytokine storm syndrome is a form of systemic inflammatory response syndrome that can be triggered by a variety of factors such as infections, therapies, cancers and autoimmune conditions **(Fajgenbaum and June, 2020)**. It occurs when large numbers of white blood cells are activated and release inflammatory cytokines, which in turn activate yet more white blood cells. COVID-19-associated cytokine storm is associated with higher risk for fatal outcome. the major factors of death due to SARS-CoV-2 infection, which is acute respiratory distress syndrome. ARDS has a significant relation with cytokine storm syndrome because, during ARDS, the immune effector cells have been shown to release huge amounts of chemokines and proinflammatory cytokines, which result in a fatal uncontrolled systemic inflammatory response **(Tang *et al.*, 2020)**.

SARS-CoV-2 binds to alveolar epithelial cells. mainly alveolar epithelial type 2 cells through the ACE2 receptor. Destruction of epithelial cells and the increase of cell permeability leading to release of the virus. SARS-CoV-2 activates the innate immune system; macrophages and other innate immune cells not only capture the virus but also release a large number of cytokines and chemokines, including IL-6 **(Qin *et al.*, 2020)**. Adaptive immunity is also activated by antigen-presenting cells (mainly dendritic cells). T and B cells not

only play an antiviral role but also directly or indirectly promote the secretion of inflammatory cytokines. In addition, under the stimulation of inflammatory factors, a large number of inflammatory exudates and erythrocytes enter the alveoli, resulting in dyspnoea and even respiratory failure. (Figure 1.3). The first gross examination autopsy report of a COVID-19 death reported that a bronzed appearance of both lungs and a large amount of grey-white viscous liquid overflow could be seen after incision **(Zhang *et al.*, 2020)**. Lately, drugs targeting IL-18, IL-1, IL-6, and Interferon-gamma have been found effective in treating cytokine storm syndrome in other viral infections for the treatment and therefore may be used for the treatment of the COVID-19 patients for reducing the severity **(Sonja *et al.*, 2020)**.

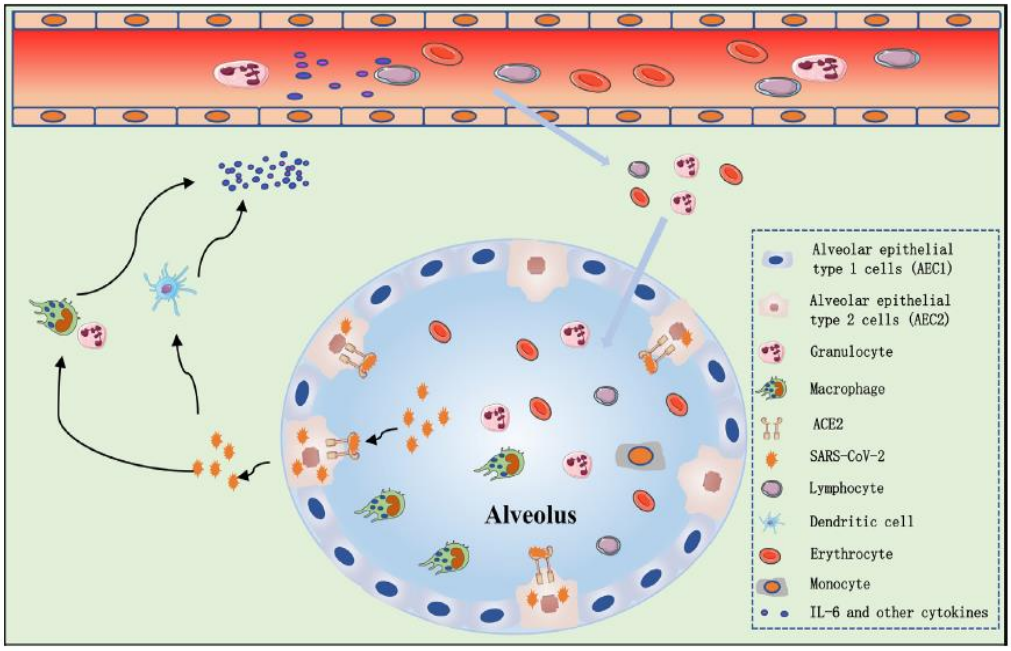

Figure (1.3): Possible mechanism of cytokine release syndrome in severe COVID-19 patients (Zhang *et al.*, 2020).

1.2.8.4. Interlukin-6 (IL-6)

IL-6 is an essential cytokine that transmits defence signals from a pathogen invasion or tissue damage site to stimulate acute phase reactions, immune responses, hematopoiesis and various internal organs to prepare for host defence **(Narazaki and Kishimoto, 2018)**. IL-6 can be produced by almost all stromal cells and immune system cells, including B-lymphocytes, T-lymphocytes, macrophages, monocytes, dendritic cells, mast cells, and other non-lymphocytic cells such as fibroblasts, endothelial cells, keratinocytes, glomerular mesangial cells and tumour cells **(Jones and Jenkins, 2018)**. In the early stage of infectious inflammation, IL-6 is produced by monocytes and macrophages stimulated by Toll-like receptors (TLRs). This acute IL-6 expression plays a central role in host defence by stimulating various cell populations **(Zhang *et al.*, 2020)**. A lot of patients affected by COVID-19 develop a fulminant and damaging immune reaction sustained by cytokines leading to alveolar infiltration by macrophages and monocytes. IL-6 is one of the main mediators of inflammatory and immune response initiated by infection or injury and increased levels of IL-6 are found in more than one half of patients with COVID-19. Levels of IL-6 seem to be associated with inflammatory response, respiratory failure, needing for mechanical ventilation and/or intubation and mortality in COVID-19 patients. In a meta-analysis including nine studies (total 1426 patients) reporting on IL-6 and outcome in COVID-19, mean IL-6 levels were more than three times higher in patients with complicated COVID-19 compared with those with non-complicated disease, and IL-6 levels were associated with mortality risk **(Grifoni *et al*., 2020)**.

Expression of IL-6 is regulated by various mechanisms. Nuclear Factor κB is a main transcriptional factor commonly activated by TLRs-mediated signals and pro-inflammatory cytokines including TNFα, IL-1β and IL-17. Activation of cell surface and intracellular TLRs in monocytes and macrophages induces

mRNA-transcription of IL-6 and other pro-inflammatory cytokines, such as TNFα and IL-1-β, via nuclear factor-kappa **(Narazaki & Kishimoto, 2018)**.

1.2.8.5. Interlukin-1 Alpha (IL-1α)

IL-1α are proinflammatory cytokines produced by monocytes and macrophages. IL-1 production may be induced by the presence of microbial pathogens, bacterial lipopolysaccharides, or other cytokines. However, IL-1α remains intracellular within monocytes and macrophages and is rarely found outside these cells. IL-1α can be released after cell death and can help attract inflammatory cells to areas where cells and tissues are being killed or damaged **(Mantovani *et al*., 2019)**. SARS-CoV-2 infection causes local and systemic inflammation mediated by pro-inflammatory cytokines and tissue damage that can lead to patient death. The SARS-CoV-2 will cause epithelial damage leading to the release of IL-1α that will recruit neutrophils and monocytes to the site of infection and induce IL-1β in monocyte/macrophages **(Van De Veerdonk and Netea, 2020)**. There is elevated IL-1α levels in patients with severe COVID-19, and these were strongly associated with lung injury **(Liu *et al*., 2020)**. IL-1 levels are related to the virulence of the process, and significantly higher serum levels have been observed in SARS-CoV-2 cases with severe symptoms than in mild cases or in those infected with the 2003 SARS-CoV or 2012 MERS coronavirus **(Qin *et al*., 2020)**. IL-1 has a broad spectrum of biological activities and participates in both innate and acquired immunity. In infections, IL-1 induces gene expression and synthesis of several cytokines/chemokines in both macrophages and mast cells. IL-1 activated by SARS-CoV-2 stimulates the secretion of IL-6 and TNF, a pro-inflammatory complex that can lead to cytokine storm and be deleterious both in the lung and systemically **(Conti *et al*., 2020)**.

1.2.8.6. Interlukin-1 Beta (IL-1β)

IL-1β is a proinflammatory cytokine that is central for host responses to infection **(Garlanda *et al*., 2013)**. IL-1β is produced as an inactive precursor called pro-IL-1β mainly by inflammatory cells of myeloid lineage. Pro-IL-1β is rapidly induced upon exposure of inflammatory cells to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that bind to pattern recognition receptors (PRRs) to upregulate proinflammatory gene expression **(Takeuchi & Akira, 2010)**. SARS-CoV-2 appears to act on the activation and maturation of IL-1β, which in turn activates other proinflammatory cytokines, such as IL-6 and TNF-α. Hence, IL-1β forms part of the cytokine storm produced by coronavirus infections **(Nalumansi *et al*., 2020)**. Moreover, the SARS-CoV-2 will induce pro-IL1β in monocyte/macrophages which in turn will induce more IL-1 that will recruit and activate more innate immune cells, these effects are primarily induced by IL-1 and IL-6, cytokines which are involved in the elevation of hepatic acute phase proteins and fever **(Van De Veerdonk & Netea, 2020)**. Elevated levels of the antagonistic receptor of IL-1 (IL-1Ra) in severe cases of COVID-19, and this marker has been associated with increased viral load, loss of pulmonary function, lung damage, and mortality risk **(Yang *et al*., 2020)**. Most COVID-19 patients with severe symptoms have elevated levels of IL-1β, which has been associated with SARS, hypercoagulation, and disseminated intravascular coagulation. For this reason, some therapeutic strategies have used the inhibition of IL-1 in an attempt to avoid the cytokine storm **(Nalumansi *et al*., 2020)**.

1.2.8.7. Nuclear Factors kappa-beta (NF-κB)

NF-κB is an ancient protein transcription factor, is a master regulator of the inflammatory and immune responses. In response to foreign stimuli, NF-kB recruits adaptive and innate immune cells such as macrophages and neutrophils

to the infection site and triggers inflammation **(Liu *et al*., 2017)**. NF-κB is a complex system of proteins present inactive in the cytoplasm along with inhibitory proteins that are known as inhibitors of NF-κB (IκBs). Upon stimuli, phosphorylation of IκBs by IκB kinase (IKK) leads to nuclear translocation of NF-κB, binding to their cognate DNA and activates transcription of a wide variety of genes involved in host immunity, inflammation, cell proliferation and apoptosis **(Hariharan *et al*., 2021)**. The activated NF-κB transcription factors promote the gene expression of wide variety of cytokines (e.g., IL-1, IL-2, IL-6, IL-12, TNF-α and GM-CSF), chemokines (e.g., IL-8, MIP-1, MCP1, and RANTES) and acute phase proteins (e.g., Serum Amyloid) Thus NF-κB serves as a 'rapid acting' primary transcription factor which can regulate various cellular responses such as host's early innate immune response to infection, and also associated with chronic inflammatory states, viral infections, septic shock syndrome and multi-organ failure **(Zhang *et al*., 2017)**.

One of the major pathways through which beta coronavirus causes hyperactivation of NF-κB is via the myeloid differentiation primary response 88 (MyD88) pathway through PPRs (Figure. 1.4) **(Birra *et al*., 2020)**. This leads to induction of a variety of cytokines including IL-6, TNF-α and chemokines **(Hirano & Murakami, 2020)**. This was shown as a major factor in the higher case-fatality rates associated with SARS and MERS, when compared to COVID-19 **(De Wit *et al*., 2016)**.

The possible reason for this cytokine storm in COVID-19 patients might be the elevated level of anti-viral inflammatory cytokines (IL-10, IL-6, IL-2, IFN-γ and TNF-α) which were attenuated and diversified in SARS and MERS infections, respectively **(Zhang *et al*., 2020)**. The involvement of IL-6, the activation of NF-κB by elevated level of TNF-α create a degenerative feedback loop through Protein Kinase B and resulted in lung failure. In addition, previous reports suggested that the inflammatory cytokines TNF-α and IL1β induces the

Granulocyte-Colony stimulating factor (G-CSF) via NF-κB pathway. These elevated levels of cytokine G-CSF and GM-CSF found in the cytokine storm of SARS-CoV2 induce the accelerated inflammation process in COVID-19 patients **(Zhu *et al.*, 2020)**.

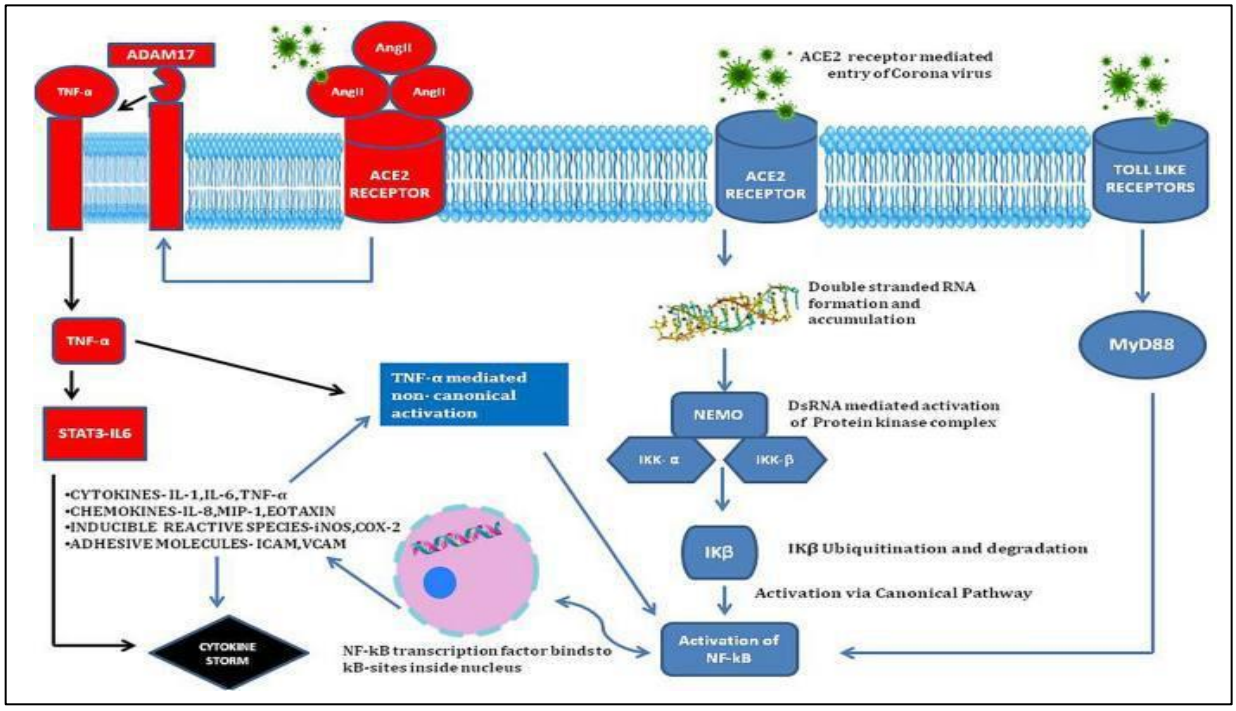

Figure (1.4): The mechanism of action of NF-κB in COVID-19 via various pathways leading to cytokine storm syndrome (Hirano & Murakami, 2020).

1.2.9. Laboratory Diagnosis of Sars-CoV-2:

Early diagnosis is the key for prompt management of cases and control of the spread of the virus. Currently, the laboratory diagnosis of SARS-CoV-2 is based on nucleic acid amplification tests (NAAT) like real-time reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, nucleic acid sequencing may be done for the identification of mutation in the genome of SARS-CoV-2. The development of serological assays and point of care molecular test will further intensify the diagnostic modalities of SARS-CoV-2 **(Padhi *et al.*, 2019)** .

1.2.9.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a plate-based method that has been used for detecting and quantifying soluble substances such as proteins and antibodies in clinic and research laboratories. It includes; direct and indirect formats. The indirect ELISA, the most popular and more sensitive than the direct ELISA, an antigen (e.g., a recombinant protein (N protein) of SARS-CoV-2 virus) is coated onto the inner surface of 96-well polystyrene plates. A diluted patient's plasma which may have anti-SARS-CoV-2 IgG/IgM is added to the wells. The plate is incubated for one hour to allow the antibodies to interact with coated antigens. After washing the plate to eliminate unspecific interactions, a conjugated antibody with a reported enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) is added to form sandwich complexes. These complexes are detected and quantified by adding a substrate (e.g., 3,3′,5,5′-tetramethylbenzidine) that is utilized by the report enzyme and leads to change in the reaction colour. The colour is detected and measured by a plate reader. ELISA is relatively fast (2–5 h) and cheap compared to rRT-PCR. It has been reported that ELISA results were 50% (IgG) and 81% (IgM) for patients on day zero and became 81% (IgG) and 100% (IgM) on day five of SARS-CoV-2 infection **(Dhamad and Abdal Rhida, 2020)**. Another study showed that using ELISA to detect IgM and IgG on day four of symptom onsite revealed a sensitivity of 77.3% and specificity of 100% for IgM while those were 83.3% and 95% respectively for IgG **(Xiang *et al*., 2020)**.

1.2.9.2. Laboratory screening tests

Laboratory screening tests based on assessing the biological and chemical factors in blood would be helpful for better determination of COVID-19 infected patients, although they do not have higher specificity and sensitivity. For this purpose, the complete blood count, as well as blood biochemistry measurement, should be carried out for each patient. According to published results, the

different common laboratory abnormalities which have been found in SARS-CoV2 cases are as follows: low count of white cells (leukopenia; lower than 1,000), a low percentage of lymphocytes (lymphopenia), decreased level of albumin (hypoalbuminemia), increased levels of C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR). Moreover, there are elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in infected patients due to the abnormal function of the liver. Increased levels of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) due to myocardial abnormality have been reported, as well. Moreover, a higher level of D-dimer was also detected in patients with severe conditions **(Tahmasebi *et al*., 2020)**.

CHAPTER TWO

Materials and Methods

2. Material and Methods

2.1. Materials:

2.1.1. Equipment and Instruments:

In the present study, the following equipment and instruments were used (Table 2.1 and Table 2.2).

Table (2-1): Equipment and instruments with their manufacturing company and country of origin

Equipment and Instruments	Manufacturing company	Country
Autoclave	Hirayama HVE-50	Japan
Centrifuge	Hettich	Germany
I phone camera	Apple	USA
ELISA devices (washer & reader)	Biokit ELx800	USA
Freezer	Panasonic	Korea
Haematology analyser	Sysmex XP-300	Japan
Incubator	Memmert	Germany
Compact multiparametric immunoanalyzer	BioMérieux	France
Refrigerator	Panasonic	Korea
automated clinical chemistry analyzer	FUJ DRI-CHEM NX500	Japan
Water distillatory	GFL	Germany

Table (2-2): Equipment and instruments with their country of origin

Equipment and instruments	Country
Cold medical box	China
EDTA tube	China
Eppendorf tube (0.5 ml & 1.5 ml)	China
Filter paper	China
Gel tube	China
Sodium citrate tube	China
ESR tube	China
Gloves	China
Face mask	China
Micropipettes (different size)	Japan
Tips (yellow & blue)	China

2.1.2. Biochemical kits:

Table (2-3): Biochemical kits used in the study

Biochemical kit	Manufacturing company	Country
Creatinine	FUJ DRI-CHEM NX500	Japan
Urea	FUJ DRI-CHEM NX500	Japan
TBIL	FUJ DRI-CHEM NX500	Japan
Albumin	FUJ DRI-CHEM NX500	Japan
ALT	FUJ DRI-CHEM NX500	Japan
AST	FUJ DRI-CHEM NX500	Japan
ALP	FUJ DRI-CHEM NX500	Japan
LDH	FUJ DRI-CHEM NX500	Japan
CRP	LORNE LABRATORIES	United Kingdom
D-dimer	BioMérieux	France
Ferritin	BioMérieux	France

2.1.3. ELISA kit:

Table (2-4): ELISA kits used in the study

ELISA Kit	Manufacturing company	Country
Human IL-6	ELK	China
Human IL-1α	ELK	China
Human IL-1b	ELK	China
Human NFkB	ELK	China

2.1.3.1. ELISA kit content of human IL-6:

Table (2-5): ELISA kit for detection IL-6

Components	Format
1. Pre-coated microplate wells	8×12
2. Standard (lyophilized)	2
3. Standard diluent buffer	20 ml
4. Biotinylated antibody (100×)	120 µl
5. Biotinylated antibody diluent	12 ml
6. Streptavidin-HRP (100×)	120 µl
7. Enzyme conjugate horseradish peroxidase (HRP) diluent	12 ml
8. Wash buffer (25×) concentrated	20 ml
9. TMB substrate solution	9 ml
10. Stop reagent	6 ml
11. Plate covers	4
12. Instruction manual	1

2.1.3.2. ELISA kit content of human IL-1α:

Table (2-6): ELISA kit for detection IL-1α

Components	Format
1. Pre-coated microplate wells	8×12
2. Standard (lyophilized)	2
3. Standard diluent buffer	20 ml
4. Biotinylated antibody (100×)	120 µl
5. Biotinylated antibody diluent	12 ml
6. Streptavidin-HRP (100×)	120 µl
7. Enzyme conjugate horseradish peroxidase (HRP) diluent	12 ml
8. Wash buffer (25×) concentrated	20 ml
9. TMB substrate solution	9 ml
10. Stop reagent	6 ml
11. Plate covers	4
12. Instruction manual	1

2.1.3.3. ELISA kit content of human IL-1β:

Table (2-7): ELISA kit for detection IL-1β

Components	Format
1. Pre-coated microplate wells	8×12
2. Standard (lyophilized)	2
3. Standard diluent buffer	20 ml
4. Biotinylated antibody (100×)	120 µl
5. Biotinylated antibody diluent	12 ml

6. Streptavidin-HRP (100×)	120 µl
7. Enzyme conjugate horseradish peroxidase (HRP) diluent	12 ml
8. Wash buffer (25×) concentrated	20 ml
9. TMB substrate solution	9 ml
10. Stop reagent	6 ml
11. Plate covers	4
12. Instruction manual	1

2.1.3.4 ELISA kit content of human NF-κB:

Table (2-8): ELISA kit for detection NF-κB

Components	Format
1. Pre-coated microplate wells	8×12
2. Standard (lyophilized)	2
3. Standard diluent buffer	20 ml
4. Biotinylated antibody (100×)	120 µl
5. Biotinylated antibody diluent	12 ml
6. Streptavidin-HRP (100×)	120 µl
7. Enzyme conjugate horseradish peroxidase (HRP) diluent	12 ml
8. Wash buffer (25×) concentrated	20 ml
9. TMB substrate solution	9 ml
10. Stop reagent	6 ml
11. Plate covers	4
12. Instruction manual	1

2.2. Methods:

2.2.1. Subjects:

This study included samples from almost all Covid-19 patients were admitted into three specialized centres, in Al Najaf Province, namely: Al-Amal Specialized Hospital for Communicable diseases, ward of COVID-19 care in Al-Hakeem Hospital and ward of COVID-19 care in Al Sader General Hospital in the period extending from December 2020 to February 2021. The diagnosis of all patients were by specific SARS-CoV-2 primer in RT-PCR according to world health organization **(WHO, 2020)**. In addition, this study included samples from healthy people with the same ages and gender of the patients who were randomly selected from the local community. The samples from the healthy people were used as controls in the cytokines levels analyses.

2.2.2. Inclusion and Exclusion criteria:

2.2.2.1. Inclusion criteria: Those include both severe/critical and mild/moderate cases of Covid-19 patients.

2.2.2.2. Exclusion criteria: Patients on Tocilizumab (Actemra), Anakinra and other Autoimmune diseases including:

1- Systemic lupus erythematosus.
2- Inflammatory bowel disease.
3- Rheumatoid arthritis.

2.2.3. Study Design:

This is a cross-sectional study which involved covid-19 patients of both sexes and of different age groups. In addition to healthy people who are age- and gender-matched with patients were used as control in the studying the cytokine levels for comparisons. A representative flow diagram for the study design is shown in figure 2.1.

2.2.4. Ethics and Scientific Approval:

The study protocol was approved by the ethical committee of College of Medicine, University of Karbala and the relevant ethical committee in the Najaf Health Directorate. In addition, People permissions were verbally obtained from patients and their relatives before samples collections. All samples were collected under health instructions and following the protocol safety.

2.2.5. Questionnaire:

A specific questionnaire was designed to record the demographic and clinical data of the COVID-19 patients. The questionnaires were covered socio-demographic and observed data that included the sex, age, clinical symptoms, hypertension and cardiovascular disease, diabetes mellitus, and smoking (Appendix I and Appendix II).

2.2.6. Blood samples collection:

Approximately 7 ml of venous blood were drawn from each participant which were obtained by disinfecting antecubital fossa with 70% alcohol and then make vein puncture by disposal syringes after applying a tourniquet. Two ml of blood were dispensed into EDTA tube for the haematological tests, and

Two ml of blood were dispensed into sodium citrate tube for D-dimer. One ml of blood was dispensed into ESR tube for Erythrocyte sedimentation rate. Two ml of blood was dispensed into plan tube and allowed to clot then serum was separated by centrifugation at 3000 round per minutes (RPM) for 15 minutes and used for biochemical test. Then the serum was transferred to Eppendorf tube and stored in freeze (-20°C) to be used for immunological assays.

2.2.7. Complete blood count (CBC):

Blood specimens were collected in EDTA tubes, samples were shaken up, then examined as soon as possible in Sysmex XP-300 differential automated hematology analyzer (Sysmex, Japan), to count blood cells.

2.2.8. Erythrocyte sedimentation rate (ESR):

Erythrocyte sedimentation rate was measured by ESR fast detector method.

2.2.9. Biochemical tests:

There are different biochemical materials were detected in this study, consequently, C-reactive protein (CRP) was detected by immunoturbidimetry method. While D-dimer and ferritin were measured using MINI VIDAS Compact multiparametric immunoanalyzer. Other biochemical test such as: (Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), total bilirubin (TBIL), Lactate dehydrogenase (LDH), albumin, urea and serum creatinine) were measured using FUJ DRI-CHEM NX500 automated clinical chemistry Analyzer.

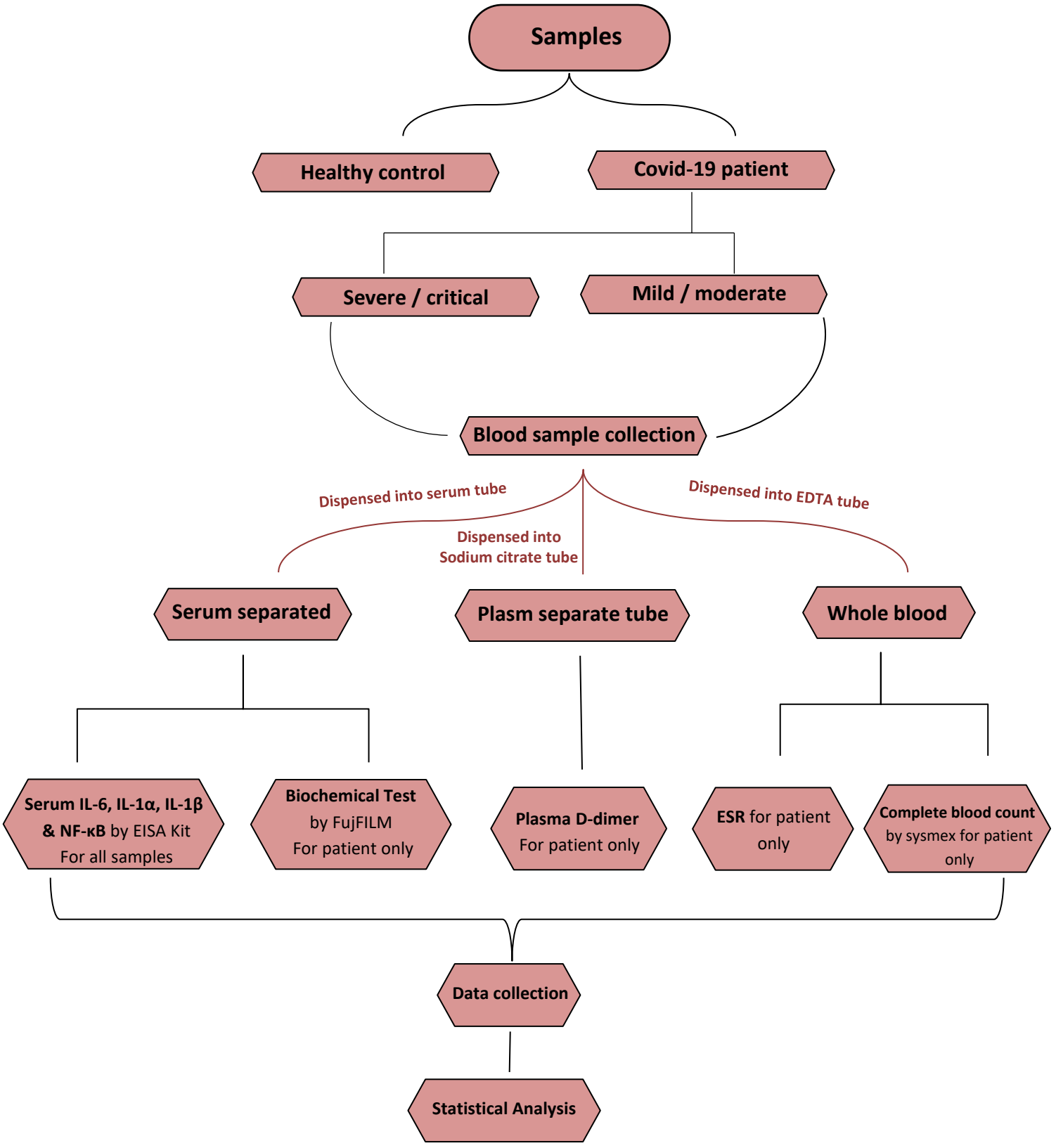

Figure 2.1: A flow chart illustrating the study design.

2.2.10. ELISA:

2.2.10.1. Measuring the concentration of serum IL-6 in patient's blood:

Serum sample were analysed to determine the total IL-6 concentration by BioTek ELx800 automated immunoassay analyzer (BioTek, USA) using ELK IL-6 ELISA kit (LOT NO. 16773671244), in Al-Rawan specialized laboratory in Najaf.

2.2.10.1.1. The principle of the test:

In this experiment, kit instructions and protocol followed and applied sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL-6. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IL-6. Next, Avidin conjugated to Horse radish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain IL-6, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change were measured spectrophotometrically at a wavelength of 450 nm. The concentration of IL-6 in the samples was determined by comparing the optical density (OD) of the samples to the standard curve.

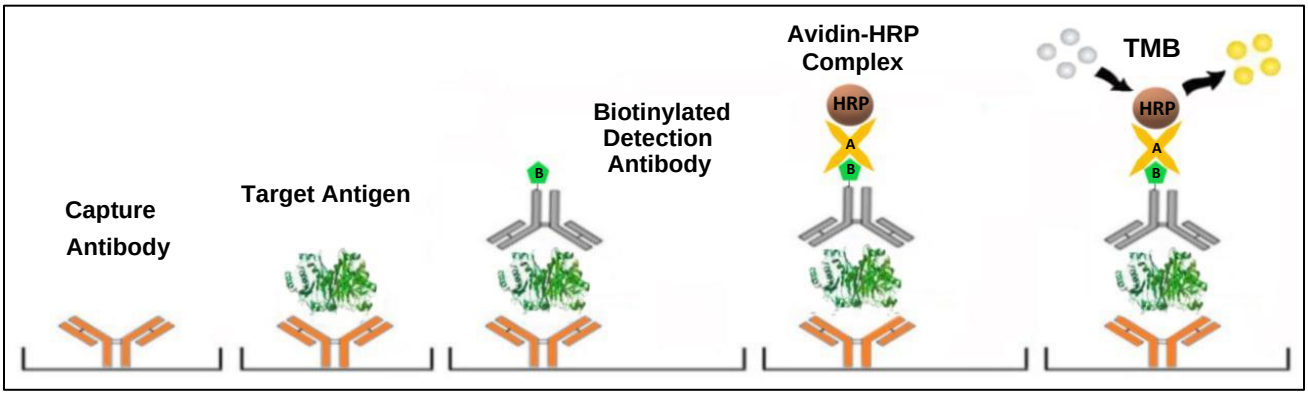

Figure 2.2: The principle of Sandwich-ELISA

2.2.10.1.2. Procedure of the test:

1. Sample preparation and Incubation: the wells for diluted standard, blank and sample was prepared. seven wells for standard and one well for blank. 100 µL of diluted standard, blank and samples were transferred to micro plate wells and covered with the plate sealer and allowed to incubate for 2 hours at 37°C.

2. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

3. Biotinylated Detection Ab incubation: 100 µl of biotinylated detection Ab was added into each micro plate wells and left and incubate for 1 hour at 37°C.

4. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

5. Conjugate incubation: 100 µL of Streptavidin-HRP conjugate was added into each micro plate wells and left and incubate for 1 hour at 37°C.

6. Washing: automatic wash was done 5 times with 500 µL of wash buffer.

7. Substrate incubation: 90 µl of TMB substrate solution was added into each micro plate wells, covered with the plate sealer and incubated for 20 minutes at 37°C.

8. Stopping: 50 µl of stop solution was added into each micro plate wells.

9. photometric measurement was done at 450 nm within 30 min of adding stop solution.

2.2.10.1.3. Reading results:

Average of the duplicate readings for each standard, control and samples and subtract the average zero standard optical density. Construct a standard curve with the human IL6 concentration on the x-axis and absorbance on the y-axis, and a best fit curve was drawn through the points on the graph.

2.2.10.2. Measuring the concentration of serum IL-1α in patient's blood:

Total IL-1α concentration was measured by BioTek ELx800 automated immunoassay analyzer (BioTek, USA) using ELK ELISA kit (LOT NO. 16773673401), in Al-Rawan specialized laboratory in Najaf.

2.2.10.2.1. Principle of Test:

In this experiment, kit instructions and protocol followed and applied sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL-1α. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IL-1α. Next, Avidin conjugated to Horse radish Peroxidase (HRP) was added to each microplate well and incubated.

After TMB substrate solution was added, only those wells that contain IL-1α, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change were measured spectrophotometrically at a wavelength of 450 nm. The concentration of IL-1α in the samples was then determined by comparing the OD of the samples to the standard curve.

2.2.10.2.2. Procedure of the test:

1. Sample preparation and Incubation: the wells for diluted standard, blank and sample was prepared. seven wells for standard and one well for blank. 100 µL of diluted standard, blank and samples were transferred to micro plate wells and covered with the plate sealer and allowed to incubate for 2 hours at 37°C.

2. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

3. Biotinylated Detection Ab incubation: 100 µl of biotinylated detection Ab was added into each micro plate wells and left and incubate for 1 hour at 37°C.

4. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

5. Conjugate incubation: 100 µL of Streptavidin-HRP conjugate was added into each micro plate wells and left and incubate for 1 hour at 37°C.

6. Washing: automatic wash was done 5 times with 500 µL of wash buffer.

7. Substrate incubation: 90 µl of TMB substrate solution was added into each micro plate wells, covered with the plate sealer and incubated for 20 minutes at 37°C.

8. Stopping: 50 µl of stop solution was added into each micro plate wells.

9. photometric measurement was done at 450 nm within 30 min of adding stop solution.

2.2.10.2.3. Reading results:

Average of the duplicate readings for each standard, control and samples and subtract the average zero standard optical density. Construct a standard curve with the human IL-1α concentration on the x-axis and absorbance on the y-axis, and a best fit curve was drawn through the points on the graph.

2.2.10.3. Measuring the concentration of serum IL-1β in patient's blood:

Total IL-1β concentration was measured by BioTek ELx800 automated immunoassay analyzer (BioTek, USA) using ELK ELISA kit (LOT NO. 16773671426), in Al-Rawan specialized laboratory in Najaf.

2.2.10.3.1. Principle of test:

In this experiment, kit instructions and protocol followed and applied sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL-1β. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IL-1β. Next, Avidin conjugated to Horse radish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain IL-1β, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change were measured

spectrophotometrically at a wavelength of 450 nm. The concentration of IL-1β in the samples was then determined by comparing the OD of the samples to the standard curve.

2.2.10.3.2. Procedure of the test:

1. Sample preparation and Incubation: the wells for diluted standard, blank and sample was prepared. seven wells for standard and one well for blank. 100 µL of diluted standard, blank and samples were transferred to micro plate wells and covered with the plate sealer and allowed to incubate for 2 hours at 37°C.

2. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

3. Biotinylated Detection Ab incubation: 100 µl of biotinylated detection Ab was added into each micro plate wells and left and incubate for 1 hour at 37°C.

4. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

5. Conjugate incubation: 100 µL of Streptavidin-HRP conjugate was added into each micro plate wells and left and incubate for 1 hour at 37°C.

6. Washing: automatic wash was done 5 times with 500 µL of wash buffer.

7. Substrate incubation: 90 µl of TMB substrate solution was added into each micro plate wells, covered with the plate sealer and incubated for 20 minutes at 37°C.

8. Stopping: 50 µl of stop solution was added into each micro plate wells.

9. photometric measurement was done at 450 nm within 30 min of adding stop solution.

2.2.10.3.3. Reading results:

Average of the duplicate readings for each standard, control and samples and subtract the average zero standard optical density. Construct a standard curve with the human IL-1β concentration on the x-axis and absorbance on the y-axis, and a best fit curve was drawn through the points on the graph.

2.2.10.4. Measuring the concentration of serum NF-κB in patient's blood:

Serum was analysed to determine the total NF-κB concentration by BioTek ELx800 automated immunoassay analyzer (BioTek, USA) using ELK NF-κB ELISA kit (LOT NO. 16773673324), in Al-Rawan specialized laboratory in Najaf.

2.2.10.4.1. Principle of test:

In this experiment, kit instructions and protocol followed and applied sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to NF-κB. Standards and samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to NF-κB. Next, Avidin conjugated to Horse radish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain NF-κB, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change were measured spectrophotometrically at a wavelength of 450 nm. The concentration of NF-κB in the samples was then determined by comparing the OD of the samples to the standard curve.

2.2.10.4.2. Procedure of the test:

1. Sample preparation and Incubation: the wells for diluted standard, blank and sample was prepared. seven wells for standard and one well for blank. 100 µL of diluted standard, blank and samples were transferred to micro plate wells and covered with the plate sealer and allowed to incubate for 2 hours at 37°C.

2. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

3. Biotinylated Detection Ab incubation: 100 µl of biotinylated detection Ab was added into each micro plate wells and left and incubate for 1 hour at 37°C.

4. Washing: automatic wash was done 3 times with 500 µL of wash buffer.

5. Conjugate incubation: 100 µL of Streptavidin-HRP conjugate was added into each micro plate wells and left and incubate for 1 hour at 37°C.

6. Washing: automatic wash was done 5 times with 500 µL of wash buffer.

7. Substrate incubation: 90 µl of TMB substrate solution was added into each micro plate wells, covered with the plate sealer and incubated for 20 minutes at 37°C.

8. Stopping: 50 µl of stop solution was added into each micro plate wells.

9. photometric measurement was done at 450 nm within 30 min of adding stop solution.

2.2.10.4.3. Reading results:

Average of the duplicate readings for each standard, control and samples and subtract the average zero standard optical density. Construct a

standard curve with the human NF-κB concentration on the x-axis and absorbance on the y-axis, and a best fit curve was drawn through the points on the graph.

2.2.11. Statistical Analysis:

Data was introduced into a specific software statistical package for the social sciences (SPSS) version 26 for windows (GraphPad Software, San Diego, California, USA) to do statistical analysis, while the figures constructed was by EXEL program of Microsoft Office 2016.

The results were expressed as mean ± SD. comparisons between two means were performed using *t*-test, ANOVA was used to compare among mean. A p-value of <0.05 was considered to indicate the statistical significance and highly significant if p-value <0.001. In addition, the pearson correlation was used to explain the relation between IL-6, IL-1α, IL-1β and NF-κB levels with different hematological and biochemical parameters.

CHAPTER THREE

Results

3. Results

3.1. Demographic and clinical characteristic of Covid-19 patients:

A total of 65 Covid-19 patients were included in this study. The baseline and clinical characteristics of them showed in Table (3-1). Those patients were classified into mild/moderate patients and severe/critical patients based on the guideline of WHO. Among them, 32 (49.2 %) were male and 33 (50.7 %) were female. The most common symptoms in mild/moderate cases were cough (n =18 ,81.8 %) followed by headache (n = 14, 63.6 %). On the other hand, the most common symptoms in severe/critical cases were cough (n = 41, 95.3 %) followed by respiratory distress (n = 37, 86.04 %). All patients were admitted to the hospitals.

Table (3-1): The clinical characteristic of 65 COVID-19 patients

Total number (n = 65)		**Mild / Moderate (n = 22) Severe / Critical (n = 43)**	
Gender	Male N (32)	**Percentage (%)**	
		Mild/Moderate	10 (45.45)
		Severe/Critical	22 (51.16)
	Female N (33)	Mild/Moderate	12 (54.54)
		Severe/Critical	21 (48.83)
Vital signs		**Mean ± S.D.**	
SpO_2		Mild/Moderate	0.959 ± 0.025
		Severe/Critical	0.867 ± 0.072
Pulse rate		Mild/Moderate	90.59 ± 16.936
		Severe/Critical	93.60 ± 19.733
Clinical symptoms		**Percentage (%)**	
Fever		Mild/Moderate	5 (22.7)
		Severe/Critical	16 (37.2)
Cough		Mild/Moderate	18 (81.8)
		Severe/Critical	41 (95.3)
Respiratory distress		Mild/Moderate	2 (9.09)
		Severe/Critical	37 (86.04)

Headache	Mild/Moderate	14 (63.6)
	Severe/Critical	27 (62.7)
Diarrhea	Mild/Moderate	3 (13.63)
	Severe/Critical	4 (9.30)

Abbreviations: SpO_2: oxygen saturation, S.D. Standard Deviation.

Table (3-2) showed the relationship between Age, blood pressure and blood glucose with severity of Covid-19. The mean age was 42.27 years for mild/moderate group and 62.58 years for severe/critical group and the age range of total patients was between 16 to 90 years. The age was significantly higher in severe/critical group in comparison to mild/moderate group (p = 0.000). Additionally, the severe/critical group were more likely to have high blood pressure (systolic blood pressure) and higher blood glucose level which have a significant association with disease severity (p = 0.022, p = 0.000 respectively).

Table (3-2): The relationship between Age, blood pressure and blood glucose with COVID-19 according to degree of disease severity

Total number (n = 65)		**Mild / Moderate (n = 22) Severe / Critical (n = 43) Mean ± S.D.**		**P-value**
Age according to severity	16 - 30 years	Mild/Moderate	42.27 ± 20.136	0.000**
	30 - 50 years	Severe/Critical	62.58 ± 17.409	
	50 - 90 years			
Blood pressure	- Systolic	Mild/Moderate	118.18 ± 17.898	0.022*
		Severe/Critical	130.93 ± 21.910	
	- Diastolic	Mild/Moderate	71.36 ± 12.834	0.470
		Severe/Critical	73.49 ± 10.208	
Blood glucose		Mild/Moderate	134.27 ± 68.919	0.000**
		Severe/Critical	229.65 ± 103.92	

Abbreviations: S.D. Standard Deviation, *: significant difference (P≤0.05), **: highly Significant (P<0.001).

3.2. Hematological parameters in COVID-19 patients:

In table (3-3) the hematological parameter was shown. The RBC count were within normal range in both mild/moderate group and severe/critical group and there was no significant relationship between Covid-19 severity and RBC count (p = 0.119). The WBC count was normal in mild/moderate group but slightly increased in severe/critical group and this increase was statistically significant (p = 0.010).

While neutrophil percentage was increased in both groups of patients, and it was significantly higher in severe/critical group (p = 0.010). Furthermore, the lymphocyte percentage was normal in mild/moderate patients but decreased significantly in severe/critical group in comparison to mild/moderate group (p = 0.004). The same trend was seen with monocyte percentage, where it was decreased significantly within the severe/critical group (p = 0.022).

Hemoglobin was slightly decreased in severe/critical patients compared to mild/moderate group. However, the decrease did not reach statical significant (p = 0.144). On the other hand, the platelet count was normal in both mild/moderate and severe/critical groups and statistically not significant (p = 0.790).

There was a significant association between Covid-19 severity and ESR, where ESR was significantly higher in severe/critical patients than mild/moderate group (p = 0.016).

Table (3-3): Hematological parameters of patients with COVID-19 according to degree of disease severity

Haematological parameter	Normal range	Mild/Moderate Mean ± S.D. (n = 22)	Severe/critical Mean ± S.D. (n = 43)	P-value
Red blood cells 10^{12}/L	4.5 - 6.5	4.492 ± 0.626	4.115 ± 1.020	0.119
White blood cells 10^{9}/L	4 - 10.5	8.030 ± 5.211	12.174 ± 6.316	0.010*

Neutrophil %	39.3 - 73.7	75.250 ± 19.132	86.160 ± 10.072	0.004*
Lymphocyte %	16 - 43.3	17.305 ± 9.410	9.751 ± 7.600	0.001**
Monocyte %	2.8 - 10.2	6.832 ± 4.425	4.742 ± 2.720	0.022*
Haemoglobin, g/dL	13.1 - 16.7	12.864 ± 2.098	10.947 ± 2.483	0.144
Platelet 10^3/uL	155 - 450	223.77 ± 85.178	231.93 ± 129.12	0.790
ESR, mm/h	0 - 30	37.36 ± 27.676	61.94 ± 32.464	0.016*

Abbreviations: ESR: erythrocyte sedimentation rate, a P-value indicate differences between mild/moderate and severe/critical, S.D. Standard Deviation, *: significant difference (P≤0.05), **: highly Significant (P<0.001).

3.3. Biochemical parameters in COVID-19 patients:

Table (3-4) showed biochemical parameter of COVID-19 patients. The renal function tests creatinine and urea were within normal range in mild/moderate group and slightly increased of serum creatinine in severe/critical patients but it not statistically significant (p = 0.136). While the urea is significantly higher in severe/critical group (p = 0.001).

The TBIL was slightly increased in both groups of patients and is statistically significant (p = 0.012). Whereas, the serum albumin was within normal range in mild/moderate group but, it's decreased in severe/critical patients and was highly associated with disease severity (p = 0.000).

The liver enzyme ALT was increased in both groups of patients and compared with mild/moderate group; the level was significantly higher in severe/critical group (p = 0.016). Likely, there was elevated above reference value of AST in both groups of patients and significantly increased in severe/critical group (p = 0.014).

On the other hand, the ALP was slightly elevated in mild/moderate patients Whereas, in severe/critical group the level was significantly higher (p = 0.012).

In both groups of patients, the mean level of LDH and CRP were higher than normal range. However, in severe/critical patients were more elevated comparison

to mild/moderate group and highly associated with disease severity (p = 0.005, p = 0.003 respectively).

The mean level of coagulation marker (D-dimer) was elevated in both groups of patients but, in severe/critical group was significantly higher (p = 0.001). whereas the mean level of ferritin was slightly increased in mild/moderate patients and significantly higher in sever/critical group (p = 0.031).

Table (3-4): Biochemical parameters of patients with COVID-19 according to degree of disease severity

Biochemical parameter	Normal range	Mild/Moderate Mean ± SD (n = 22)	Severe/critical Mean ± SD (n = 43)	P-value
Creatinine, mg/dL	0.1 - 1.3	0.766 ± 0.388	1.296 ± 1.615	0.136
Urea, mg/dL	15 - 45	37.800 ± 27.837	76.470 ± 48.971	0.001**
TBIL, mg/dL	0 - 1	0.551 ± 0.922	0.887 ± 0.557	0.012*
Albumin, g/L	3.5 - 5	4.105 ± 0.753	2.736 ± 0.428	0.000**
ALT, U/L	3 - 40	34.634 ± 16.484	57.547 ± 45.978	0.016*
AST, U/L	5 - 40	35.998 ± 17.00	55.776 ± 40.957	0.014*
ALP, U/L	40 - 130	126.59 ± 91.404	246.50 ± 209.60	0.012*
LDH, U/L	109 - 245	309.32 ± 169.10	600.03 ± 256.62	0.005*
CRP, mg/L	0 - 6	29.267 ± 24.118	70.027 ± 52.821	0.003*
D-dimer, ng/mL	0 - 500	855.83 ± 749.58	2829.13 ± 2588.31	0.001**
Ferritin, ng/mL	13 - 350	353.91 ± 348.28	646.40 ± 399.15	0.031*

Abbreviations: TBIL: total bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, CRP: C-reactive protein, a P-value indicate differences between mild/moderate and severe/critical, S.D. Standard Deviation, *: significant difference (P≤0.05), **: highly Significant (P<0.001).

3.4. Immunological study:

3.4.1 Association between cytokines serum levels and severity of Covid-19:

The cytokines serum levels of COVID-19 patients were shown in Table (3-5). In both groups of patients, the three cytokines (IL-6, IL-1α and IL-1β) were elevated.

However, the increased of IL-6 serum level was much more in severe/critical patients compared with mild/moderate group and this increase was statistically significant (p = 0.033). The serum level of IL-1β was higher in severe/critical patients, but did not reach statistical significance (p = 0.083).

On the other hand, the serum level of IL-1α was slightly increased in severe/critical patients compared with mild/moderate group and however, this was statistically insignificant (p = 0.451). In same way, the NF-κB was elevated in severe/critical patients compared with mild/moderate group but it's statistically not significant (p = 0.201).

Table (3-5): Cytokines serum levels of patients with COVID-19 according to degree of disease severity

Interleukins	Mild/Moderate Mean ± S.D. (n = 22)	Severe/critical Mean ± S.D. (n = 43)	P-value
IL-6 pg/ml	15.755 ± 20.462	113.75 ± 209.50	0.033*
IL-1α pg/ml	11.702 ± 20.703	15.554 ± 18.652	0.451
IL-1β pg/ml	367.17 ± 124.07	367.17 ± 180.15	0.083
NF-κB ng/ml	0 .068 ± 0.022	0.162 ± 0.340	0.201

Abbreviations: IL-6: interlukein-6, IL-1α: interlukein-1 alpha, IL-1β: interlukein-1 beta, NF-κB: nuclear factor kappa beta, a P-value indicate differences between mild/moderate and severe/critical, S.D. Standard Deviation, *: significant difference (P≤0.05).

3.4.2. The correlation between immunological and hematological parameters:

As shown in Table (3-6) the RBC was negatively correlated with NF-κB (p = 0.034; R= - 0.264). This indicates that NF-κB has an inverse relationship with the red blood cells counts.

There was negative correlation between hemoglobin level and NF-κB level (p = 0.005; R = - 0.348). which meaning direct association of NF-κB elevated level on haemoglobin level.

In the same way, there was a negative correlation between serum level of both IL-6 and IL-1α with lymphocyte percentage (p = 0.015, R= - 0.300; p = 0.040; R= - 0.256 respectively). This indicates that both interleukins may have a direct effect on lymphocyte percentage.

Conversely, IL-1α serum levels have shown to positively correlated with blood neutrophil percentage (p = 0.010; R= 0.317). This indicates that IL-1α may has a direct impact on neutrophil percentage.

Table (3-6): Correlation between interleukins markers of patients with COVID-19 and haematological parameters

Haematological parameter	NF-κB ng/ml	IL-1α pg/ml	IL-6 pg/ml	IL-1β pg/ml
Red blood cells 10^{12} /L	R= - 0.264* P= 0.034	R= - 0.085 P= 0.502	R= - 0.190 P= 0.130	R= - 0.079 P= 0.532
Hemoglobin, g/dL	R= - 0.348** P= 0.005	R= - 0.179 P= 0.154	R= - 0.212 P= 0.091	R= - 0.224 P= 0.073
Lymphocyte %	R= - 0.147 P= 0.244	R= - 0.256* P= 0.040	R= - 0.300* P= 0.015	R= 0.141 P= 0.263
Neutrophil %	R= 0.094 P= 0.457	R= 0.317* P= 0.010	R= 0.090 P= 0.477	R= 0. 031 P= 0.807
Monocyte %	R= - 0.025 P= 0.846	R= - 0. 231 P= 0. 064	R= 0.020 P= 0.876	R= - 0.098 P= 0.435
White blood cells 10^9/L	R= - 0.017 P= 0.892	R= - 0.043 P= 0.736	R= 0.072 P= 0.567	R= 0.039 P= 0.758
Platelet 10^3/uL	R= - 0.138 P= 0.274	R= - 0.116 P= 0.359	R= - 0.177 P= 0.158	R= - 0.016 P= 0.902
ESR, mm/h	R= - 0.190 P= 0.192	R= 0.138 P= 0.346	R= - 0.019 P= 0.895	R= 0.115 P= 0.430

Abbreviations: ESR: erythrocyte sedimentation rate, R: Pearson correlation between different parameter, a P-value indicate significant correlation between different parameter, *: significant difference (P≤0.05), **: highly Significant (P<0.005).

3.4.3. The correlation between immunological and biochemical parameters:

There was a positive correlation between IL-6 level and creatinine level (p = 0.012; R = 0.309), as shown in Table (3-7). This indicates that IL-6 may has a direct effect on creatinine level.

On the other hand, there was negative correlation between the level of albumin and increased levels of both IL-6 and IL-1α (p = 0.034; R= - 0.301; p = 0.039; R= - 0.259 respectively). This means that both cytokines may have a direct impact on the albumin level.

The increase in the level of ALP was positively correlated with increased level of IL-1α (p = 0.024; R= 0.304). This indicates that of IL-1α may has a direct effect on the level of alkaline phosphatase.

In the same way, the increased level of AST was positively correlated with increased level of IL-1β (p = 0.049; R = 0.255). This indicates that IL-1β may has a direct impact on AST level.

There was positive correlation between the level of IL-1β and LDH level (p = 0.054; R = 0.320). This indicate that IL-1β may has a direct effect on the LDH.

Table (3-7): Correlation between interleukin markers of patients with COVID-19 and biochemical parameters

Biochemical parameter	IL-6 pg/ml	IL-1α pg/ml	IL-1β pg/ml	NF-κB ng/ml
Creatinine, mg/dL	R= 0.309* P= 0.012	R= - 0.072 P= 0.571	R= 0.023 P= 0.856	R= 0.220 P= 0.078
Albumin, g/L	R= - 0.301* P= 0.034	R= - 0.259* P= 0.039	R= 0.164 P= 0.195	R= - 0.116 P= 0.362
ALP, U/L	R= - 0.146 P= 0.286	R= 0.304* P= 0.024	R= - 0.023 P= 0.868	R= 0.087 P= 0.530
AST, U/L	R= - 0.085 P= 0.514	R= 0.170 P= 0.190	R= 0.255* P= 0.049	R= - 0.128 P= 0.326
LDH, U/L	R= 0.188	R= 0.024	R= 0.320*	R= - 0.216

	P= 0.266	P= 0.889	P= 0.054	P= 0.198
Urea, mg/dL	R= 0.117 P= 0.353	R= 0.089 P= 0.482	R= - 0.044 P= 0.730	R= 0.047 P= 0.713
TBIL, mg/dL	R= - 0.004 P= 0.976	R= - 0.069 P= 0.588	R= - 0.069 P= 0.588	R= 0.042 P= 0.744
ALT, U/L	R= - 0.126 P= 0.334	R= 0.240 P= 0.063	R= - 0.046 P= 0.762	R= - 0.037 P= 0.777
CRP, mg/L	R= - 0.014 P= 0.925	R= - 0.014 P= 0.923	R= 0.154 P= 0.292	R= - 0.118 P= 0.420
D-dimer, ng/mL	R= 0.082 P= 0.516	R= 0.052 P= 0.681	R= 0.093 P= 0.459	R= - 0.094 P= 0.456
Ferritin, ng/mL	R= 0.047 P= 0.586	R= 0.072 P= 0.603	R= - 0.217 P= 0.114	R= 0.066 P= 0.633

Abbreviations: TBIL: total bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, CRP: C-reactive protein, R: Pearson correlation between different parameter, a P-value indicate significance of correlation between different parameter, *: significant difference (P≤0.05), **: highly Significant (P<0.005).

3.5. Cytokines levels in Covid-19 patients and healthy control:

There was a significant difference between Covid-19 patients and healthy controls in immunological parameters: IL-6, IL-1α, IL-1β and NF-κB (p = 0.000) (as shown in table 3.8).

Table (3-8): Difference in cytokine levels between Covid-19 patients and healthy controls

Immunological parameter	Covid-19 patients Mean ± S.D. (n = 65)	Healthy control Mean ± S.D. (n = 23)	T-test	P-value
IL-6 pg/ml	80.587 ± 176.42	1.869 ± 1.014	3.683	0.000**
IL-1α pg/ml	14.250 ± 19.295	3.796 ± 0.898	5.954	0.000**
IL-1β pg/ml	389.84 ± 147.55	159.98 ± 32.68	21.300	0.000**
NF-κB ng/ml	0.130 ± 0.279	0.060 ± 0.022	3.774	0.000**

Abbreviations: IL-6: interlukein-6, IL-1α: interlukein-1 alpha, IL-1β: interlukein-1 beta, NF-κB: nuclear factor kappa beta, a P-value indicate differences between mild/moderate and severe/critical, S.D. Standard Deviation, **: highly Significant (P<0.001).

3.6. Correlation between IL-6 and others cytokines:

There was a positive correlation between NF-κB level with IL-6 level (p = 0.009; R = 0.320). This indicate that NF-κB has a direct impact on the IL-6 level.

Table (3-9): Correlation of cytokines levels with IL-6 level in Covid-19 patients

		IL-1α pg/ml	IL-1β pg/ml	NF-κB ng/ml
IL-6 pg/ml	Pearson correlation (R)	- 0.027	- 0.073	0.320**
	Significant (*p*-value)	0.833	0.566	0.009

Abbreviations: R: Pearson correlation between different parameter, a P-value indicate significance of correlation between different parameter, **: highly Significant (P<0.001).

CHAPTER FOUR

Discussion

Conclusion & Recommendation

4.1 Discussion

This study included 65 Covid-patients confirmed by SARS-COV-2-specific RT-PCR on Nasopharyngeal swabs (or in some occasion on Oropharyngeal swab specimen). The patients were admitted to Al-Amal Specialized Hospital for communicable diseases or Al-Hakeem Hospital or Al-Sader Hospital in the period extending from December 2020 to February 2021. Among them 33 (50.7 %) were female and 32 (49.2 %) were male. The mean age was 55.71 years and the age range was from 16 to 90 years. The patients were classified based on SPO_2 percentage in to mild/moderate group (> 90-94%) and severe/critical group (<90 %) **(WHO, 2021)**. Accordingly, 22 patients were mild/moderate and 43 patients severe/critical. The clinical characteristics in addition to the hematological and biochemical parameters were compared between mild/moderate and severe/critical patients.

This study found that the severe/critical disease was seen in older age people (mean age 62.58 years) (*p*-value 0.000). This indicate the older age is a risk factor to develop sever/critical disease. This results are in line with Gallo Marin who found the age above 55 years associated with increased disease severity and/or mortality and it is thus considered a key factor in the proposed clinical severity risk scores **(Gallo Marin *et al*., 2021)**. Further, Grasselli reported in a retrospective cohort study of 1591 patients in Italy critically ill with COVID-19, the majority were older men, and median age was 63 years, the researcher also suggested that male sex is a variable that is independently associated with COVID-19 severity **(Grasselli *et al*., 2020)**. Older peoples are linked to reduced immune reaction, more underlying comorbidities, and limited organ reserve **(Ho *et al*., 2020)**.

The present study also found comorbidities such as hypertension (systolic blood pressure) and diabetes mellitus associated with the severity of COVID-19 (*p*-value 0.022, *p*-value 0.000 respectively).These results are similar to those of de

Lucena in China who reported that diabetes, hypertension and other cardiovascular diseases are strongly related to a higher risk of mortality or disease's severity among COVID-19 patients **(de Lucena *et al*., 2020)**. The effect of comorbidities may be related to the imbalance of ACE2 and the cytokine storm induced by metabolic disorders **(Chen *et al*., 2020)**. Diabetics patients have marked hyperglycemia which leads to inflammatory as well as coagulation imbalance, which potentiates the replication of the virus also there may be an imbalance of various inflammatory cytokines in COVID-19 which may be more severe in hypertensive patients. The possible link between the Renin-angiotensin-aldosterone system and COVID-19 has also been suggested. The spike protein (S-protein) of SARS-CoV-2 can bind to ACE2 receptors, this allows the virus to enter the host cells. The viral and receptor complex is then endocytosed. This eventually leads to a lower number of the receptors and a higher level of Angiotensin II **(Ramphul *et al*., 2021)**.

The current study found that RBC count are within normal range in both mild/moderate group and severe/critical group and there was no significant relationship between Covid-19 severity and red blood cells count (*p*-value 0.119). These result are comparable with previous study **(Ding *et al*., 2021)** who noted no significant differences between mild/moderate and severe/life-threating Covid-19 patients with respect to RBC count.

The present study found that WBC count and neutrophil percent were increased in severe/critical patients and this increase was statistically significant (*p*-value 0.010, *p*-value 0.004 respectively). These results are consistent with study of Taj who reported significant increase of WBC count (*p*-value 0.004) and neutrophil percent (*p*-value 0.001) in patients with severe and critical disease compared to the patients with mild and moderate **(Taj *et al*., 2021)**. Leukocytosis, neutrophilia which might be due to inflammatory response, have a significant association with the

disease severity **(Borges *et al*., 2020)**. These findings explain that increased of neutrophil may be correlated with the concomitant bacterial infection in severe cases **(Feldman and Anderson, 2021)**.

This study reported that decrease of lymphocyte and monocyte percentage in severe/critical group were significantly lower compared with mild/moderate group (*p*-value 0.004, *p*-value 0.022 respectively). These results are comparable with Terpos in Washington USA, who found lymphopenia was more significantly decreased in critically ill patients with COVID-19 ($p < 0.005$) **(Terpos *et al*., 2020)**. In addition Vanderbeke showed that most notable drop of monocytes cell count in severe/critical Covid-19 patients comparing to mild/moderate **(Vanderbeke *et al*., 2021)**. Further, Wu found that increased risk of ARDS during the disease course was significantly associated with decreased lymphocytes (*p*-value < 0.001) and increased neutrophils (*p*-value < 0.001) **(Wu *et al*., 2020)**. In SARS-CoV-2, the underlying mechanisms of reduced lymphocyte and monocyte have not yet been delineated. It was recently suggested that several factors may contribute to COVID-19 associated lymphopenia. It has been shown that lymphocytes express the ACE2 receptor on their surface; thus SARS-CoV-2 may directly infect those cells and ultimately lead to their lysis **(Terpos *et al*., 2020)**. On the other hand, the monocytes also express ACE2 receptor, making them potentially susceptible to SARS-CoV-2 infection. Moreover, ACE2 expression is reduced in the circulating population of monocyte/macrophages. This may explain direct infection of these cells by SARS-CoV-2 **(Pence, 2020)**. Furthermore, the cytokine storm is characterized by markedly increased levels of interleukins (mostly IL-6, IL-2, IL-7, granulocyte colony stimulating factor, interferon-γ inducible protein 10, MCP-1, MIP1-a) and TNF-alpha, which may promote lymphocyte apoptosis **(Terpos *et al*., 2020)**.

In current study, the hemoglobin and platelet counts showed no significant relationship with disease severity (*p*-value 0.144, *p*-value 0.790 respectively). These results are similar to the previous study of Taj who found hemoglobin, and platelet count in patients showed no association with the severity of COVID-19 **(Taj *et al*., 2021)**. The hemoglobin showed slightly decreased in severe/critical groups of patients.

The present study found significant association between Covid-19 severity and ESR where significantly higher ESR reported in severe/critical patients compared to mild/moderate group (*p*-value 0.016). This result agreed with Ghahramani who showed in meta-analysis, significant increase of ESR in severe COVID-19 patients. The increased in ESR could be attributed to the inflammatory process caused by SARS-CoV2 virus **(Ghahramani *et al*., 2020)**.

In this study the renal function tests creatinine and urea were within normal range in mild/moderate group and slightly increase of serum creatinine in severe/critical patients but it not statistically significant (*p*-value 0.136). While the urea is significantly higher in severe/critical group (*p*-value 0.001). These results are in line with study of Wu who reported the value of renal dysfunction indices (urea [$p < .001$]) in COVID-19 patients with ARDS compared with patients without ARDS. Whereas, the serum creatinine related to the development of ARDS in severe/critical patients but not associated with death **(Wu *et al*., 2020)**.

Kidney damage caused by COVID-19 typically manifests as tubular damage with obvious urinalysis abnormalities. Impaired glomerular filtration also occurs, usually manifested by increased blood creatinine and urea nitrogen levels **(Han and Ye, 2021)**. The kidney damage caused by SARS-CoV-2 is expected to be multifactorial; it can directly infect kidney podocytes and proximal tubular cells and based on ACE2 pathway it can lead to acute tubular necrosis, protein leakage in

Bowman's capsule, collapsing glomerulopathy and mitochondrial impairment. The SARS-CoV-2-driven dysregulation of the immune responses including cytokine storm, macrophage activation syndrome, and lymphopenia can be other causes of the acute kidney injury (AKI). Endothelial dysfunction, hypercoagulability, rhabdomyolysis, and sepsis are other potential mechanisms of AKI. Moreover, lower oxygen delivery to kidney may cause an ischemic injury **(Ahmadian *et al*., 2021)**.

The current study showed the total bilirubin was slightly increased in both groups of patients and were statistically significant (*p*-value 0.012). Whereas, the serum albumin was significantly decreased in severe/critical patients and highly associated with disease severity (*p*-value 0.000). The present study also showed the liver enzyme ALT was increased in both groups of patients and compared with mild/moderate group; the ALT was significantly higher in severe/critical group (*p*-value 0.016). Also, there was elevated above reference value of AST in both groups of patients and significantly increased in severe/critical group (*p*-value 0.014). These finding are agree with Xu who showed significantly higher levels of ALT ($p = 0.015$), AST ($p < 0.001$), TBIL ($p = 0.026$), but significantly lower albumin ($p < 0.001$) were commonly observed in severe patients, compared with non-severe patients **(Xu *et al*., 2021)**.

This study also showed the ALP was significantly higher in severe/critical group (*p*-value 0.012). These results are consistent with study recorded by Kumar who found ALP levels among different severity groups of COVID-19 was statistically significant (*p*-value < 0.05) **(Kumar *et al*., 2020)**.

These findings explain liver injury over the course of hospitalization caused by SARS-CoV-2, as the ACE2 receptor expressed in epithelial cells of the bile duct and liver, and providing an easy access point for SARS-CoV-2 to bind directly to

ACE2 in cholangiocytes and disrupt liver function. Therefore, elevation of the liver enzyme levels in patients with severe COVID-19, indicating some degree of liver impairment caused by the virus. Additionally, the drug-induced liver injury has been noted as another cause of some of the liver abnormalities seen with COVID-19. Also, the systemic inflammatory response is thought to be another potential cause for liver damage in patients infected with SARS-CoV-2. In severe/critical COVID-19 patients the cytokines are released in greater amount (Cytokine storms) which causes multiple tissues and organs injury and death **(Clark *et al*., 2021)**.

The present study found a significant association between Covid-19 severity and inflammatory markers LDH in addition to CRP. Both markers were elevated in severe/critical patients comparison to mild/moderate group and highly significant with disease severity (*p*-value 0.005, *p*-value 0.003 respectively). Also, the coagulation marker (D-dimer) was significantly higher in in severe/critical patients (*p*-value 0.001). The elevation of these markers considered as risk factors for ARDS in COVID-19 patients **(Poggiali *et al*., 2020)**. These results are similar to the study of Ghahramani who reported that inflammatory markers (CRP, LDH), and coagulation function tests (D-dimer), were positively associated with the COVID-19 severity **(Ghahramani *et al*., 2020)**.

Further, the results also agrees with study of Zhang who showed in retrospective analyzed of 1099 patients confirmed by SARS-COV-2-specific RT-PCR, from more than 550 hospitals in China, and found the non-survivors had a significantly higher D-dimer (median, 2.12 μg/mL) than that of survivors (median, 0.61 μg/mL) **(Zhang *et al.*, 2020)**.

These findings are suggested that increased levels of LDH and CRP might represent an expression of lung damage and might reflect the respiratory distress consequent to the abnormal inflammation status in COVID-19 patients **(Poggiali *et***

***al*., 2020)**. Whereas, the elevation of D-dimer as a results of hyper-coagulopathy state occurred in patients with Covid-19, which might be attributed to several factors. First; SARS-CoV-2 may activate the innate immune system to clear the virus. But an excessive immune response can cause an inflammatory storm, which destroy microcirculation, activate the coagulation system and lead to disseminated intravascular coagulation (DIC). Second; the hypoxia found in severe Covid-19 can stimulate thrombosis through not only increasing blood viscosity, but also a hypoxia-inducible transcription factor dependent signaling pathway. Third, the severe and critical patients, were more likely to have older ages, underlying comorbidities, long-term hospitalization, and invasive treatment, which were all risk factors of thrombosis. As evidence, the lung organ dissection of critical patient with Covid-19 have reported occlusion and microthrombosis formation in pulmonary small vessels **(Zhang *et al*., 2020)**.

The results of the study revealed that the mean level ferritin was slightly elevated in mild/moderate patients and significantly higher in severe/critical group ($p = 0.031$). These result are similar to the previous study **(Cheng *et al*., 2020)** who noted in meta-analysis involved 10614 COVID-19-confirmed patients, the ferritin level was significantly increased in severe patients compared with the level in non-severe patients ($p < 0.001$). Also, non-survivors had a significantly higher ferritin level compared with the survivors ($p < 0.001$).

These findings suggested that there were several reasons for elevated level of ferritin. First; ferritin is an iron-storing protein, its serum level reflects the normal iron level. In relation, iron is a crucial micronutrient for both human cells and pathogens. To restrain the pathogen's use of it, the natural immune response may limit iron turnover during infections to disturbing the replication of the virus. However, this mechanism can also lead to anemia, which reduces oxygen delivery

to tissues and results in multi-organ failure. Second; the clinical course of severe COVID-19 patients, may be predictive of an inflammatory reaction, which is characterized by elevated ferritin levels and presence of a cytokine storm. During the cytokine storm in COVID-19, many inflammatory cytokines are rapidly produced, including IL-6, TNF-α, IL-1β, IL-12, and IFN-γ, which stimulate hepatocytes, Kupffer cells, and macrophages to secrete ferritin **(Bozkurt *et al.*, 2021)**.

This study found increase in the levels of serum cytokines (IL-6, IL-1α and IL-1β) in COVID-19 patients. The three cytokines were elevated in both groups of patients. However, the increased of IL-6 serum level was much more in severe/critical patients compared with mild/moderate group and this increase was statistically significant (*p*-value 0.033). On the other hand, the serum level of IL-1β was higher in severe/critical patients, but did not reach statistical significance (*p*-value 0.083). These results are in line with study of Chen who reported low expression of inflammatory cytokines in patients with mild to moderate disease (patients admitted to hospital who survived and did not require ICU admission). While, the patients with severe to critical disease (those who died or required ICU admission) had highly elevated pro-inflammatory cytokines including (IL-1α, IL-1β, IL-6, IL-18 and TNF-a) **(Chen *et al.*, 2020)**.

Moreover, Ghazavi mentioned that higher serum levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-2 and IL-6) have been observed in many patients with severe COVID-19 compared to individuals with mild disease. Therefore, imbalance in the T-helper-cell (Th1/Th2/Th17) and regulatory T-cells (Tregs) is suggested to contribute in the pathogenesis of COVID 19 **(Ghazavi *et al.*, 2021)**.

These findings explain that SARS-CoV-2 binds ACE2 receptors on the alveolar epithelial cells. The virus then activates the innate and adaptive immune

systems, resulting in cytokines storms characterized by uncontrolled release of a large number of cytokines, including (IL-6, IL1, IL-10 and TNF-a). In addition, vascular permeability is increased by these pro-inflammatory factors, resulting in a large amount of fluid and blood cells entering the alveoli, resulting in dyspnea and even respiratory failure **(Zhang *et al.*, 2020)**.

The present study found the NF-κB was elevated in severe/critical patients. These findings are consistent with previous study **(Carcaterra and Caruso, 2020)** who reported that hyper-activation of the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathway drives ARDS leading to multiple organ failure, one of the most frequent causes of death has been implicated in the pathogenesis of the severe/critical COVID-19.

This explains that COVID-19 activation of transcription factor, NF-κB in various cells such as macrophages of lung, liver, kidney, central nervous system, gastrointestinal system and cardiovascular system leads to production of IL-1, IL-2, IL-6, IL-12, TNF-α, GM-CSF, and various chemokines. The sensitized NF-κB in elderly and in patients with metabolic syndrome makes this groups of population susceptible to COVID-19 and their worse complications, including higher mortality **(Hariharan *et al*., 2021)**.

The main target of SARS-CoV-2 is type II Alveolar epithelial (AEC-II) cell and the NF-κB activation is a key driver of severe ARDS in COVID-19 patients. The first colonization of the upper airways is a necessary but not sufficient condition for the development of COVID-19 syndrome, because of an initial immuno-escape strategy of the virus from the innate immune response. suggest that the greater affinity of SARS-CoV-2 for ACE2 relatively to other coronaviruses enables it to more efficiently reach the main target (AEC-II) cells which though represent only

10% of all alveolar cells, play a fundamental role in the mediation of inflammatory responses and ultimately in the remodeling of the lung and its homeostasis. The infection of these cells subsequently leads to the activation and amplification of the inflammatory process in other cell types, including type I Alveolar epithelial (AEC-I) and the endothelium, during which the activation of the NF-κB pathway is amplified by an excessive transcription mediated by the virus **(Carcaterra and Caruso, 2020)**.

The results of the study revealed that the RBC count was negatively correlated with NF-κB (p = 0.034; R= - 0.264). Likely, there was negative correlation between hemoglobin level and NF-κB level (p = 0.005; R= - 0.348). which mean direct impact of NF-κB on the RBC count and hemoglobin level.

NF-κB triggers endothelial cells activation and makes the endothelium more susceptible to apoptosis, but protective, anti-inflammatory genes help regulate activation and apoptosis. imbalance between NF-kB and its protective gene, NF-κB inhibitor-α (IkB-α), induces endothelial cells activation, followed by endothelial cells apoptosis and necrosis. Therefore, a possible sequence in the pathogenesis of endothelial cells dysfunction is activation of NF-κB, followed by the release of proteins by activated endothelial cells and morphologic derangement. The interactions among the alterations of pathophysiology (e.g., cytokine storm, pro-inflammation, pro-coagulation, vasodilatation, increased vascular permeability, barrier disturbance, and acute phase response) eventually lead to irreversible endothelial injury and result in clinically relevant complications, such as COVID-19 associated endotheliitis, coagulation, and thrombus **(Zhang *et al*., 2021)**.

This study found that the lymphocyte percentage was negatively correlated with increased serum levels of both IL-6 and IL-1α (p = 0.015; R = - 0.300, p = 0.040; R = - 0.256). These results agree with Jiang who found that counts of

lymphocyte subsets (T cells, B cells and NK cells) were all negatively correlated with IL-6 level ($p < 0.05$) **(Jiang *et al*., 2021)**. Further, Fathi and Rezaei reported that the increased of proinflammatory cytokines play a critical role in the induction of lymphopenia. Moreover, the synergic action of most inflammatory cytokines such as IL-1, IFN-γ, and IL-6 was confirmed for inhibition of T-cell proliferation. Hyper-/ proinflammatory cytokines provide an inverse correlation between the induction of granulopoiesis and lymphopoiesis in the bone marrow of patients with SARS-CoV2 infection. The increased number of monocytes and granulocytes produces more and more inflammatory cytokines and this detrimental positive feedback makes the patient's condition worse. Accordingly, hyper-cytokinemia influences the lymphopenia and hence is incapable to defend against SARS-CoV-2 infection **(Fathi and Rezaei, 2020)**.

The exact mechanism of lymphopenia in severe COVID-19 patients remains still unclear. It may be suggested that lymphocytes were directly invaded by virus infection or indirectly damaged by cytokine storm which induced by immune response. And a substantial decrease in lymphocytes revealed that the immune cells may be consumed by the viruses and the body's cellular immune function may be restrained. It suggested that COVID-19 infection can lead to immune dysfunction through affecting the subsets of T cells **(Yang *et al*., 2021)**.

Moreover, Jafarzadeh showed there was a powerful association between IL-6 level and disease severity. Using various mouse models, it has been indicated that IL-6 suppresses lymphopoiesis via direct effects on hematopoietic stem/progenitor cells. There was an inverse association between serum IL-6 concentrations and absolute blood count of lymphocytes in COVID-19 patients. The absolute blood number of lymphocytes increased within the 24 hours following treatment with Tocilizumab, a monoclonal antibody against IL-6 receptor. Accordingly, the

blocking of IL-6 may have the capacity to attenuate the lymphocyte reduction in COVID-19 patients **(Jafarzadeh *et al*., 2021)**.

The present study found a positive correlation between the blood neutrophil percentage and increased serum level of IL-1α (p = 0.010; R = 0.317). These results are comparable with study of Nalumansi who found elevated IL-1α levels in patients with severe COVID-19, and these were strongly associated with lung injury. IL-1 levels are related to the virulence of the process, and significantly higher serum levels have been observed in SARS-CoV-2 cases with severe symptoms than in mild cases or in those infected with the 2003 SARS-CoV or 2012 MERS coronavirus **(Nalumansi *et al*., 2020)**.

Borges showed that cytokines play a relevant function in immunopathology during COVID-19 infections. The host-viral interactions are established via host identification of pathogen-associated molecular patterns (PAMPs) of the virus. This identification occurs through host pattern recognition receptors (PRRs) manifested on innate immune cells (e.g., neutrophils, dendritic cells, epithelial cells, and macrophages), and the recognition of PAMPs and viral danger-associated molecular patterns (DAMPs) by conserved PRRs marks the first line of defense against pathogens, involving toll-like receptors (TLRs). TLR stimulation activates the NF-κB signaling cascade, causing the production of inflammatory markers from monocytes (IL-1, TNF-α, and IL-6) to control viral infections **(Borges *et al.*, 2020)**.

This current study found that increased level of creatinine was positively correlated with increased level of IL-6 (p = 0.012; R = 0.309). And negative correlation between serum albumin level and increased of both IL-6 and IL-1α levels (p = 0.034, R = - 0.301; p = 0.039; R= - 0.259 respectively). This indicates that IL-1α may has a direct effect on albumin level. Whereas, the level of IL-6 may have a direct impact on both creatinine and albumin levels. These finding are agreed with

Liu who found that IL-6 were positively correlated with creatinine (p <0.001; R = 0.355). The researcher also mentioned that there was a negative correlation between serum albumin and increased level of IL-6 (p <0.001; R = - 467) **(Liu *et al*., 2020)**. Moreover, Xu refer that the severity of the disease was associated with a decrease level of serum albumin, which might be related to an increased in the levels of IL-6 and IL-1α **(Xu *et al*., 2021)**. The reducing of albumin serum concentration in patients with severe COVID-19-infection are mainly due to reduction in albumin synthesis because of reduced food intake especially during mechanical ventilation **(Ramadori, 2021)**.

SARS-CoV-2 driven dysregulation of the immune responses (cytokine storm), that can influence the kidney injury in COVID-19 patients. Meanwhile, IL-6 induces renal endothelial cells to secrete pro-inflammatory cytokine and chemokine that induces kidney vascular permeability and playing a part in microcirculatory dysfunction. The pro-inflammatory cytokines can also induce capillary leak syndrome and the production of thrombosis, which may result in DIC. Additionally, cell death and tissue damage can occur due to the presence of high levels of circulating cytokines, together (disturbances of vascular hemostasis, and cytokine-induced injuries) leading to multi-organ failure in kidney **(Ahmadian *et al*., 2021)**.

The present study found the increased level of ALP was positively correlated with increased level of IL-1α (p = 0.024; R = 0.304). While, the increased level of AST was positively correlated with increased level of IL-1β (p = 0.049; R = 0.255). This indicates that there was a direct effect of IL-1α on the level of ALP and direct impact of IL-1β on the AST level. These results are consistent with Portincasa who reported that during COVID-19 progression, the liver could be involved either as a direct target of the SARS-CoV-2 and secondary to the complex pathways of

systemic alterations promoted by the viral infection, mainly including inflammation and cytokine release (including IL-1, IL-6, IL-10), immune response, altered coagulation, hepatic ischemia and hypoxia, and sepsis-related abnormalities **(Portincasa *et al*., 2020)**.

This explains that the liver damage is more likely to occur in patients with more severe disease, in whom concomitant alterations of liver function tests are more likely. Aggravating factors include ischemic/hypoxic liver injury, and immunologic, inflammatory and toxic mechanisms promoted by systemic sepsis. Viral inclusions seem to be absent in the liver, but this possibility deserves further investigations, because of potential viral RNA translocation from intestine though portal blood **(Portincasa *et al*., 2020)**.

The results of the study revealed that there was positive correlation between the level of IL-1β and lactate dehydrogenase level (p = 0.054; R = 0.320). These results disagree with Liu who mentioned there was no significant correlation between LDH level and IL-1β (p = 255 ; R = - 0.065) **(Liu *et al*., 2020)**.

LDH is a major predictor of cytokine storm in COVID-19 disease because it is associated with metabolic acidosis. In viral infections, conditions of tissue hypoxia prevail when pyruvate is converted to lactic acid and accumulates. Lactic acidosis induces the monocytes, and macrophages to produce IL-1β and trigger the inflammasome to activate inflammatory responses. In homeostatic response to the acidosis, the LDH level is increased. Therefore, LDH is a marker of tissue damage caused by viraemia and dysregulated immune response because of tissue deterioration progresses, the LDH increases. It reflects the degree of various pathophysiological processes, and therefore it can predict the progression or regression of disease **(Rowaiye *et al*., 2020)**.

The results showed that the levels of immunological parameters: (IL-6, IL-1α, IL-1β and NF-κB) were significantly elevated in Covid-19 patients compared with healthy control (*p*-value 0.000). The increase of these cytokines as a result of excessive immune inflammatory reaction against SARS-CoV-2. These results are agreed with study recorded by Kathim who showed the highest level of IL-6 was found in COVID-19 patients comparing with healthy control (p <0.01) **(Kathim *et al*., 2021).** On the other hand, Rabaan showed the high concentrations of IL-1β, IFN-γ and monocyte chemoattractant protein (MCP-1) in patients with COVID-19 **(Rabaan *et al*., 2021)**. Further, Yousif showed serum concentrations of TNF-α and IL-1α in different groups of COVID-19 patients were evaluated **(Yousif *et al*., 2021)**. Moreover, Buszko showed that IL-6, IL-8 and TNF, and to a lesser extent, IL-1β, were elevated in Covid-19 patients at the time of hospitalization, and their concentrations correlated with disease outcome and mortality **(Buszko *et al*., 2020)**. However, these results are in contrast with study carried out by Chen who demonstrated an increase in the IL-6 levels, but the concentration of TNF-α and IL-1 remained unaltered in severely affected patients **(Chen *et al*., 2020)**

These finding explain that SARS-CoV-2 elicits an innate immune response and causes an immediate rise in the neutrophils and other immune cells along with a marked reduction in the T cells (CD4+ and CD8+). However, the reduction of T cells along with the enhanced production of IL-6 and IL-8 has been reported as a remarkable characteristic of SARS-CoV-2 infection. The inflammatory cytokines are produced in abundance, eliciting a cytokine storm. This dysregulated and excessive cytokine production leads to pathological symptoms such as severe pneumonia, acute lung injury, ARDS and multiple organ damage. Moreover, the presumptive assumptions of the causative reason for multiple organ damage and

mortality during SARS-CoV-2 infection have been closely linked to cytokine storm **(Rabaan *et al*., 2021)**.

This current study found that NF-κB level was positively correlated with IL-6 level (p = 0.009; R = 0.320). This indicate that NF-κB has a direct impact on the IL-6 level. These results are in line with Su who found that the ORF7a protein of SARS-CoV-2 activates the NF-κB signaling and promotes major proinflammatory cytokine productions IL-6 ($p < 0.01$) **(Su *et al*., 2021)**.

These results explains that in COVID-19 patients, highly expressed of proinflammatory cytokines further stimulates NF-κB. SARS-CoV-2 seems to activate NF-κB and produces proinflammatory cytokines, which is correlated with COVID-19 pathogenesis. The ORF7a protein of SARS-CoV-2 was the most potent NF-κB inducer and thus proinflammatory cytokine producer. Indeed, NF-κB is activated in SARS-CoV-2 infected cells. However, underlying mechanisms for viral modulation of NF-κB functions are still unclear **(Su *et al*., 2021)**.

4.2. Conclusion:

- The severity of Covid-19 is associated with older age and comorbidities including; hypertension (increased systolic blood pressure) as well as higher blood glucose level.
- The increased white blood cells count, neutrophil percentage, erythrocyte sedimentation rate and decreased lymphocyte percentage are linked to the severity of the disease.
- The severity of Covid-19 is also associated with increased of urea, ALT, AST, ALP, LDH, CRP, D-dimer, ferritin and decreased of albumin.
- Serum IL-6 levels had a positive linear correlation with creatinine levels and negative liner correlation with lymphocyte and albumin. Accordingly, IL-6 may have direct adverse effect on kidney tissue in addition to bone marrow and the liver.
- The increased of IL-6 levels may be due to SARS-CoV-2, as a relationship was found between NF-κB and IL-6, while there is study indicating existence of relationship between ORF7a of SARS-CoV-2 and NF-κB which in turn responsible for upregulation of IL-6.
- D-dimer serum levels were not associated with IL-6, this reducing the value of this cytokine in predicting the mortality associated with the disease.
- Serum IL-1α levels had a positive linear correlation with neutrophil and ALP level. While, had a negative liner correlation with lymphocyte and albumin. Thus, IL-1α has a proinflammatory effect.
- Serum IL-1β levels had a positive linear correlation with AST and LDH level, this meaning that IL-1β is responsible of tissue damage.

- Serum NF-κB levels had a negative linear correlation with red blood cells and hemoglobin. may indicate that NF-κB has direct adverse effect on RBC counts and hemoglobin levels.

4.3. Recommendation:

- The use of the IL-1β in determining the severity of the disease because it is more relevant than the other cytokines.
- Since the study showed that age was related to the severity of the disease, we recommended that the priority in health care and vaccination should be given to this age group.
- Monitoring of the lymphocyte could estimate the prognosis in clinical practice for severe Covid-19 patients.
- The dynamic monitoring of these biochemical parameters (ALT, AST, Albumin, LDH, CRP, and D-dimer) could estimate the prognosis in clinical practice for severe Covid-19 patients.
- Study with larger sample size to confirm the results of this study is recommended.

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Appendix

Appendix I: Covid-19 patients' questionnaires

COVID-19 QUESTIONNAIRE

File Number: . . . . . .Date: . . . . . .

Patient Name: . . . . . Age: . . . . . .year

Gender male ☐ female ☐ Address: . . . . . .

Clinical signs and Comorbidities:

Hypertension: ☐ Diabetes mellitus: ☐ Headache: ☐

Other chronic disease: ☐ Respiratory distress: ☐

Diarrhoea: ☐ Loss of smell: ☐

Spo2: ☐ Pulse rate: ☐ Fever: ☐

Haematological parameters:

1- WBC counts ……. 2- Neutrophil counts ……. 3- Lymphocyte …….

4- Haemoglobin …… 5- Platelets counts …….. 6- Monocyte counts …….

Biochemical parameter:

1- AST ……. 2- ALT ……. 3- ALP …… 4- TBIL ……

5- ALB ……. 6- Urea ……. 7- Serum creatinine ……..

Serological parameter:

1- D-dimer ……. 2- Ferritin …….

3- CRP …… 4- LDH ……

Immunological Parameter:

Serum IL-1α ……

Serum IL-1β ……

Serum NF-κB ……....

Serum IL-6 ……

Appendix II: Healthy controls questionnaires

Healthy controls QUESTIONNAIRE

File Number: . . . . . .Date: . . . . . .

Patient Name: . . . . . Age: . . . . . .year

Gender male ☐ female ☐ Address: . . . . . .

Clinical signs and Comorbidities:

Hypertension: ☐ Diabetes mellitus: ☐ Headache: ☐

Other chronic disease: ☐ Respiratory distress: ☐

Diarrhoea: ☐ Loss of smell: ☐ Fever: ☐

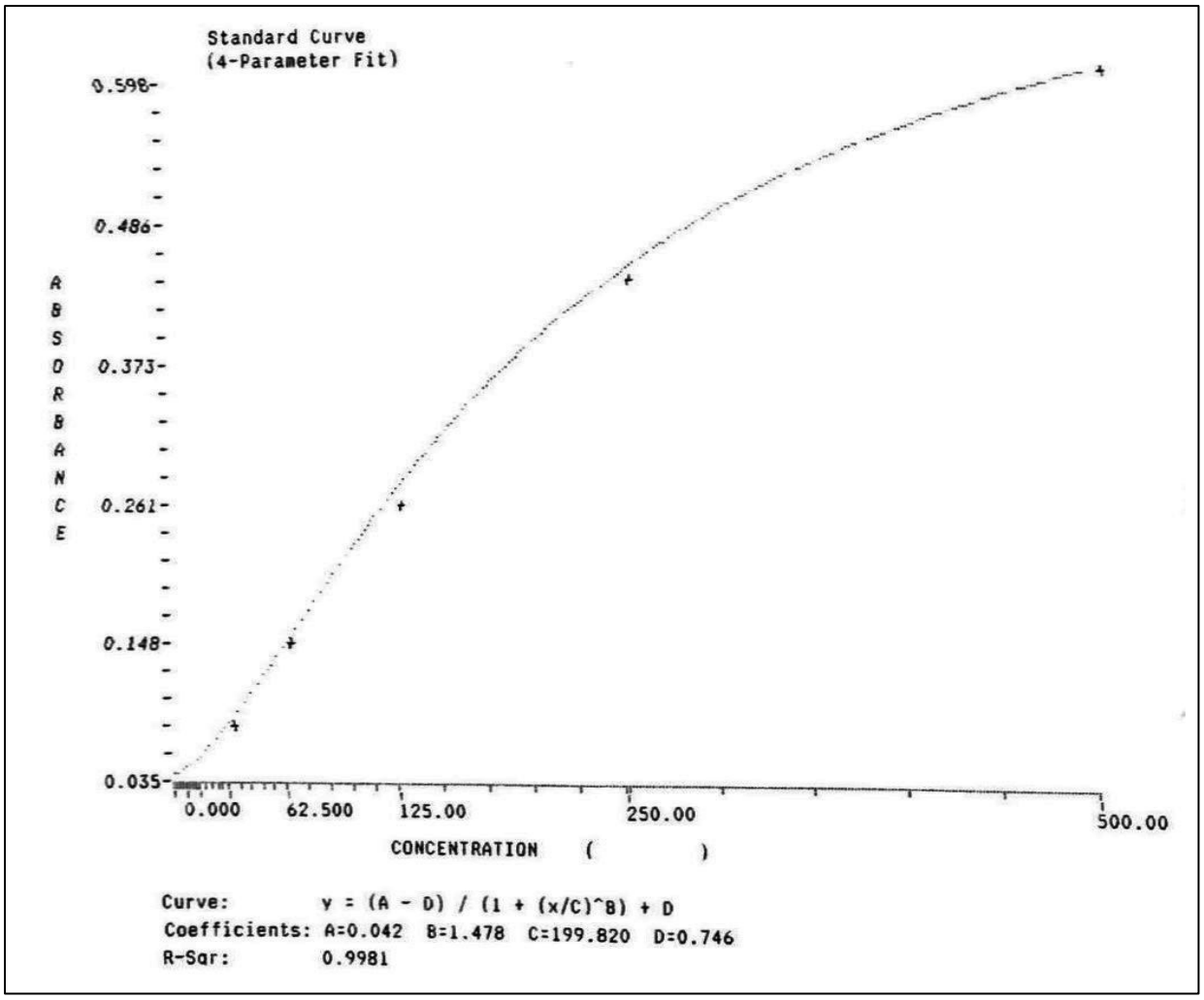
Immunological Parameter:

Serum IL-1α ……

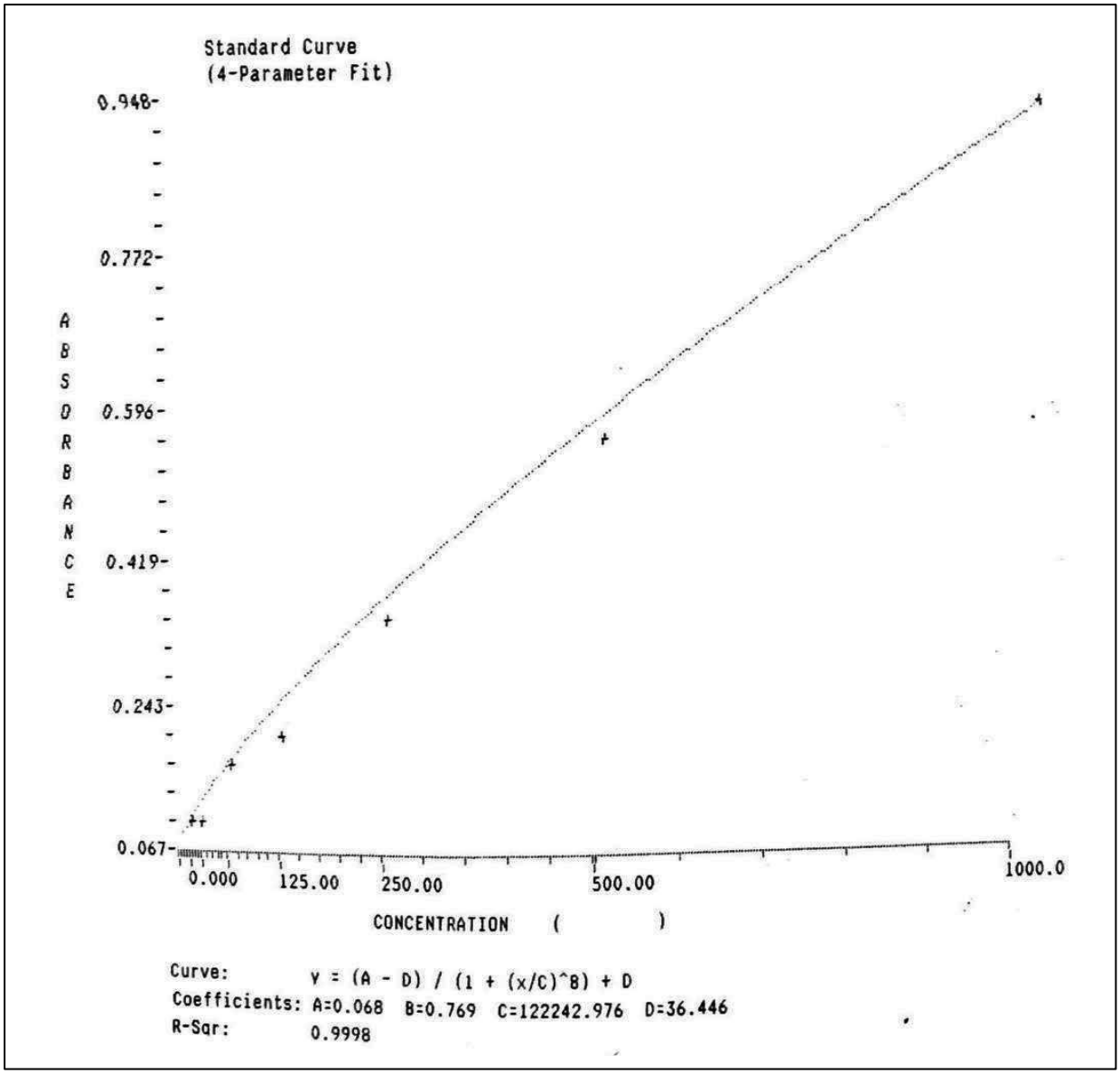
Serum IL-1β ……

Serum NF-κB ……....

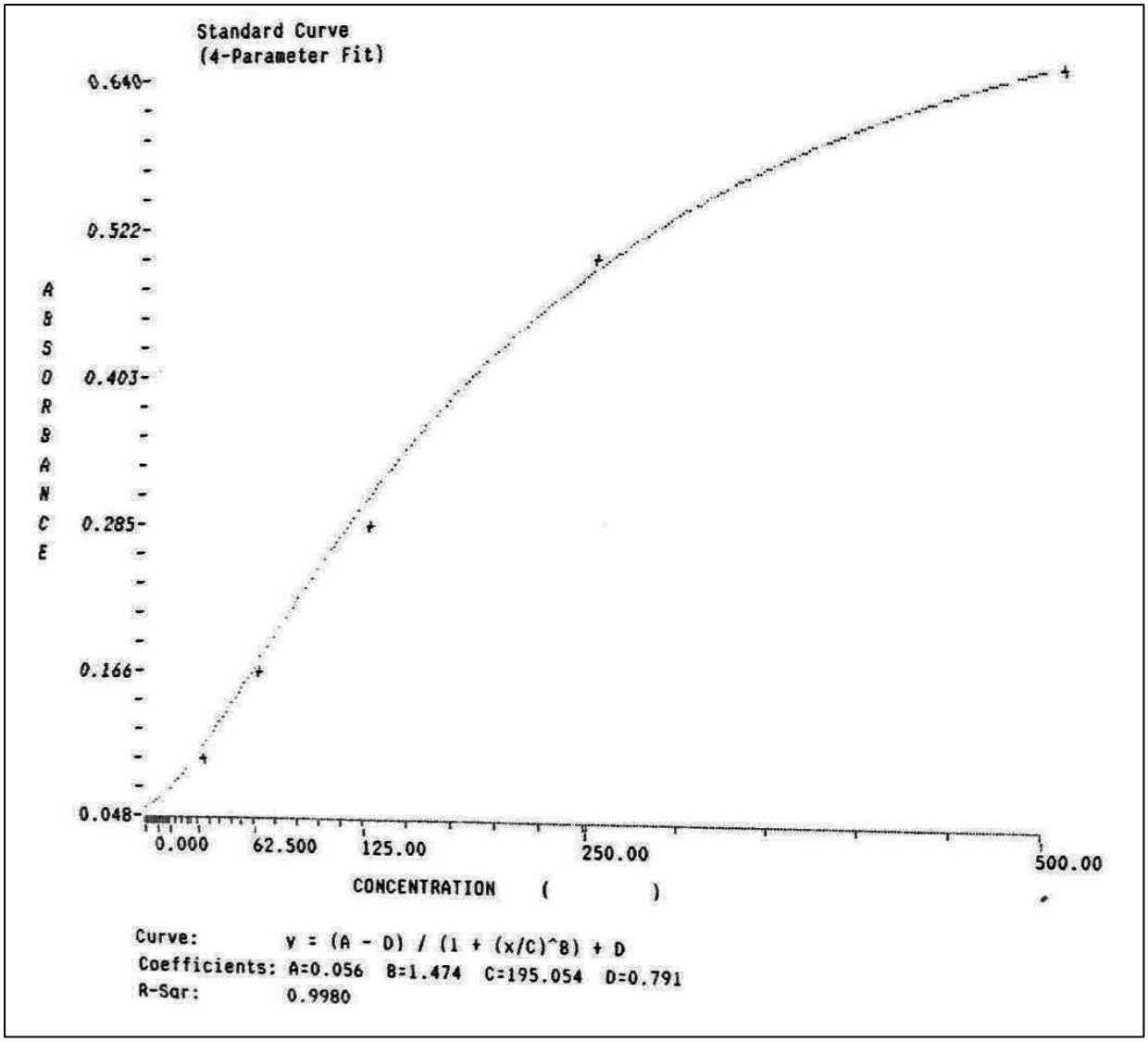
Serum IL-6 …….



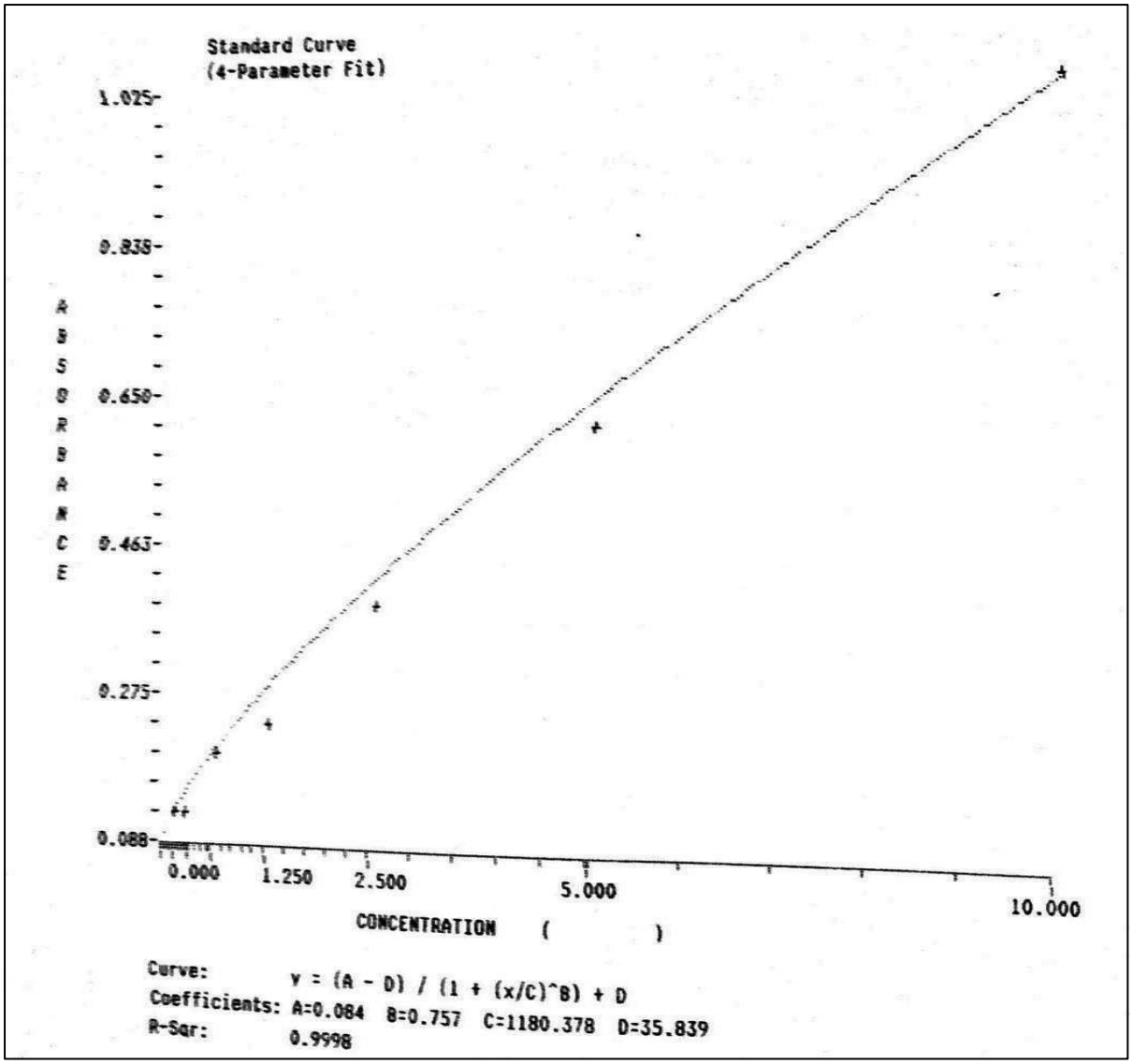
Appendix III: Interleukin IL-1α curve of ELISA technique



Appendix IV: Interleukin IL-1β curve of ELISA technique



Appendix V: NF-κB curve of ELISA technique



Appendix VI: IL-6 curve of ELISA technique

Appendix VII: ELISA device

الخلاصة

Covid-19 هو مرض معد يسببه فيروس كورونا المكتشف حديثًا (SARS-CoV-2) يصيب الفيروس الخلايا الموجودة على طول الممرات الهوائية للرئة، عن طريق الارتباط بمستقبلات الإنزيم المحول للأنجيوتنسين ٢. يعتبر المرض مشكلة صحية عالمية كبرى، وقد أصاب على عدد كبير من السكان في جميع أنحاء العالم. يتسم مرض Covid-19 بظاهرة العصف السايتوكيني التي تعد السبب الرئيسي لظهور مضاعفات المرض. من الأفضل فهم اللاعب الأساسي في هذه العاصفة أن يساعد على ايجاد بروتوكولات مناسبة للعلاج. كان الهدف من هذه الدراسة هو دراسة البروتينات المناعية، وهي؛ انترلوكين ١ الفا، انترلوكين ١ بيتا، انترلوكين ٦ والعامل النووي كابا بيتا. ولهذا الغرض، شملت هذه الدراسة المقطعية ٦٥ مريضًا بفيروس كوفيد -١٩ (٣٢ ذكرًا و٣٣ أنثى) راقدين في مستشفى الأمل التخصصي للأمراض الأنتقالية وردهة العزل في مستشفى الحكيم العام بالأضافة الى مستشفى الصدر العام في الفترة الممتدة من شهر كانون الاول ٢٠٢٠ إلى شهر شباط ٢٠٢١. تراوحت أعمار المرضى بين ١٦ الى ٩٠ سنة. اضافة الى ذلك تم اخذ مجموعة مقارنة ٢٣ شخصًا سليمًا (١٣ ذكورًا و١٠ إناث) من نفس اعمار وجنس المرضى تم اختيارهم عشوائيًا من المجتمع المحلي. تم استخدام مجموعة المقارنة في دراسة السيتوكينات لمعرفة قيمها الطبيعية. تم جمع البيانات الديموغرافية والسريرية من المرضى من خلال استبيان. تم تصنيف المرضى بناءً على النسبة المئوية لتشبع الاوكسجين (SpO_2) إلى مجموعة خفيفة / متوسطة نسبة الاوكسجين لديهم تتراوح من (٩٠-٩٤ %) ومجموعة شديدة / حرجة نسبة الاوكسجين لديهم اقل من (٩٠٪) وفقًا لذلك، كان مجموع المرضى ٢٢ مريضًا خفيفًا / متوسطا و٤٣ مريضًا شديدًا / حرجًا.

تم جمع عينات الدم والأمصال من كل مريض حيث تم استخدام المصل لتحديد مستويات IL-6 و IL-1α و IL-1β و NF-κB في المرضى وكذلك الاختبارات البيوكيميائية، بينما تم استخدام الدم الكامل للتحديد تعداد الدم الكامل ومعدل ترسيب كريات الدم الحمراء. تم استخدام جهاز ELISA للكشف عن تركيز الانترلوكينات وتم تحليل البيانات إحصائيًا بواسطة برنامج SPSS الإصدار ٢٦.

أظهرت نتائج الدراسة أن حالات المرض الشديدة والحرجة كانت شائعة لدى كبار السن (متوسط العمر ٦٢,٥٨ سنة). لذلك يعتبر التقدم في السن عامل خطر لتطوير الاعراض الشديدة والحرجة من Covid-19. كانت نسبة الخلايا الليمفاوية أقل بشكل ملحوظ في الحالات الشديدة والحرجة مقارنة مع الحالات الخفيفة والمتوسطة (p-value 0.004). بينما زادت نسبة ESR بشكل ملحوظ في المرضى الذين يعانون من اعراض شديدة وحرجة

(p-value 0.016). من ناحية أخرى، كان هناك ارتباط بين (Urea، Albumin، ALT، AST، ALP، LDH، CRP، Ferritin و D-dimer) وشدة امراضية Covid-19 (p-value 0.001, 0.000, 0.016, 0.001, 0.031, 0.003, 0.005, 0.012, 0.014 على التوالي). كان هناك ارتباط إيجابي معنوي بين مستويات IL-1α والعدلات في الدم (p-value 0.010). وكذلك هناك ارتباط إيجابي معنوي بين مستويات IL-6 في الدم ومستوى الكرياتينين (p-value 0.012). في حين أن النسبة المئوية لتشبع الاوكسجين في الدم SpO_2 ، الخلايا الليمفاوية والبومين المصل كانت مرتبطة سلبًا بمستوى IL-6 (p-value 0.002, 0.015, 0.034 على التوالي). بالإضافة إلى ذلك ، كان لـ NF-κB علاقة سلبية مع كريات الدم الحمراء والهيموغلوبين (p-value 0.034, 0.005 على التوالي). أخيرًا، كان هناك ارتباط إيجابي بين مستوى IL-1β مع مستويات LDH و AST (p-value 0.054, 0.049 على التوالي).

تم الاستنتاج، أن ارتباط زيادة خطر الإصابة بـ Covid-19 مع تقدم العمر، ارتفاع ضغط الدم الانقباضي ومستوى سكر الكلوكوز في الدم، بالإضافة الى انخفاض الخلايا الليمفاوية. ترتبط شدة مرض Covid-19 أيضًا بزيادة اليوريا و ALT و AST و LDH و CRP و D-dimer و ferritin وانخفاض الألبومين، بالاضافة الى ذلك كان لمستوى IL-6 علاقة موجبة مع مستوى الكرياتينين وارتباط سلبي مع الخلايا الليمفاوية والألبومين. وفقًا لذلك، قد يكون لـ IL-6 تأثير سلبي مباشر على أنسجة الكلى بالإضافة إلى نخاع العظام والكبد. بينما كان لـ IL-1α علاقة موجبة مع العدلات وارتباط سلبي مع الخلايا الليمفاوية والألبومين. وهكذا، فإن IL-1α له تأثير مضاد للالتهابات. كان لـ IL1β علاقة موجبة مع مستوى AST و LDH. قد تشير هذه النتائج إلى أن IL1βمرتبط بتلف الأنسجة. من ناحية أخرى، كان لمستوى NF-κB ارتباط سلبي مع كريات الدم الحمراء والهيموجلوبين. قد تشير هذه النتائج إلى أن NF-κB له تأثيرسلبي على عدد كريات الدم الحمر ونسبة الهيموجلوبين.

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رسالة مقدمة إلى
مجلس كلية الطب جامعة كربلاء
كجزء من متطلبات نيل شهادة الماجستير في الأحياء المجهرية الطبية

من قبل
إسماعيل رحيم جبار
بكالوريوس كلية الطب البيطري/ جامعة الكوفة (٢٠١٤)

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