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The Role of PAF, ICAM-1, VCAM-1 and Membrane Attack Complex on Immunothrombosis in Patients with COVID-19 in Kerbala Province

A thesis

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{وَقُلْ رَبِّ زِدْنِي عِلْمًا}

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The Role PAF, ICAM-1, VCAM-1 and Membrane Attack Complex on Immunothrombosis in Patients with COVID-19 in Kerbala Province

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DEDICATION

To my brother Ibrahim who gave his soul for the country….

To all the martyrs who saved Iraq with their pure blood….

To my parents and family who are my support in life….

And I do not forget…. My Supervisors that gave all the support and gave the fruit of their effort to me….

To all of the above- mentioned I dedicate my theses….

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Summary

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

COVID-19 is characterized by the presence of an immunothrombosis, which is manifested by an increase in the levels of some inflammatory and biochemical parameters as well as alterations in the blood parameters.

The study aimed to investigate and evaluate the role of ICAM-1, VCAM-1, PAF and membrane attack complex (MAC) along with other hematological, biochemical, and coagulation parameters in the immunothrombosis that develops in COVID-19 patients.

In order to achieve this goal, 82 participants were enrolled in this study who had COVID-19 confirmed by real-time-PCR on a nasopharyngeal swab specimen. Patients were treated in Imam Al-Hussein Medical City in Kerbala province during the period from October 2021 to May 2022.

The patients were divided into three groups (mild n = 27, severe n = 27 and critical n = 28). The mean age was 59.9 years, and the age range was between 25 and 85 years. Among them, 44 (53.7%) were male and 38 (46.3%) were female.

A significant mortality rate (17%) occurred among older patients in the severe and critical groups, while no mortality occurred among mild patients, who were mostly younger in age. These results indicated that older patients were at a higher risk for mortality.

The levels of ICAM-1, VCAM-1 and MAC were significantly increased with disease progression ($p < 0.001$; $p = 0.002$*; $p = 0.047$*, respectively). While, the level of platelet activating factor (PAF) was increased in the mild patients and stabilized within a limited range in the severe and critical patients ($p = 0.447$).

D-dimer was the only coagulation parameter that was significantly increased with disease severity in this study ($p < 0.001$), and most mortality cases associated with high D-dimer levels, whereas no significant changes occurred in prothrombin time (PT), partial thromboplastin time (PTT) or platelet count ($p = 0.053$, $p = 0.259$, $p = 0.422$, respectively).

The C-reactive protein (CRP) level was significantly elevated with disease severity ($p < 0.001$). That was an indicator of the activation of the inflammatory process during COVID-19.

The lymphocyte percentage was significantly decreased ($p < 0.001$), while neutrophil percentage was significantly increased ($p < 0.001$) with disease severity.

In conclusion, the data indicated that all studied immunological parameters (ICAM-1, VCAM-1, MAC, and PAF) significantly correlated with the occurrence of inflammatory immunothrombosis in COVID-19 and the immunothrombosis was significantly associated with disease severity and increased mortality.

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List of Abbreviations

Code	Words
ACE2	Angiotensin Converting Enzyme 2
APC	Activated Protein C
APCs	Antigen Presenting Cells
ARDS	Acute Respiratory Distress Syndrome
AT	Antithrombin
C3aR	C3a Receptor
C5R1	C5 Receptor 1
CAMs	Cell Adhesion Molecules
CBC	Complete Blood Count
CD	Cluster Differentiation

Code	Words
CD4 + Tcell	**Cluster Differentiation 4 + T Cell**
CD8 + Tcell	**Cluster Differentiation 4 + T Cell**
COVID-19	**Corona Virus Disease-2019**
CRP	**C-Reactive Protein**
CT	**Computerized Tomography**
CTLs	**Cytotoxic T-Lymphocytes**
CVD	**Cardiovascular Disease**
DIC	**Disseminated Intravascular Coagulation**
ECs	**Endothelial Cells**
EDTA	**Ethylenediamine Tetraacetic Acid**
ERGIC	**Endoplasmic Reticulum Golgi Intermediated Compartment**
FB	**Factor B**
FH	**Factor H**
FKN	**Fractalkine**
G- CSF	**Granulocyte Colony- Stimulating Factor**
GFR	**Glomerular Filtration Rate**
GM- CSF	**Granulocyte- Macrophage – Colony Stimulating Factor**
GP	**Glycoprotein**
H3	**Histone 3**
H4	**Histone 4**
Hb	**Hemoglobin**
HE	**Hemagglutinin – Esterase**
HLA	**Human Leukocyte Antigen**
ICAM-1	**Intercellular Adhesion Molecule-1**
ICU	**Intensive Care Unit**

Code	Words
IFN- α	Interferon - Alpha
IFN- β	Interferon – Beta
IFNAR	Infection – Induced Type 1 Interferon Receptor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgSF	Immunoglobulin Super Family
IL	Interleukine
JAK	Janus Kinase
LA	Left Atrium
LAA	Left Atrial Appendage
LAMP – 3	Lysosomal Associated Membrane Protein – 3
LFA-1	Lymphocyte Function-Associated Antigen-1
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannose Binding Lectin
MERS	Middle East Respiratory Syndrome
MHC	Major Histocompatibility Complex
MIP1a	Macrophage Inflammatory Protein 1a
MPO	Myeloperoxidase
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NP	Nasopharyngeal
NK	Natural Killer Cell
NKGA2	Natural Killer Cell Receptor G2A
OD	Optical Density

Code	Words
PAF	**Platelet Activating Factor**
PAI-1	**Plasminogen Activator Inhibitor**
PAMPs	**Pathogen Associated Molecular Pattern Molecules**
PLTs	**Pletelets**
PSGL-1	**P- Selectin Glycoprotein Ligand – 1**
PT	**Prothrombin Time**
PTT	**Partial Thromboplastin Time**
RBD	**Receptor – Binding Domain**
RNA	**Ribonucleic Acid**
RT- PCR	**Reverse Transcription - Polymerase Chain Reaction**
SARS-CoV-2	**Severe Acute Respiratory Syndrome Corona Virus -2**
SD	**Standard Deviation**
SPSS	**Statistical Package for the Social Sciences**
STAT1	**Signaling transducer and activator of transcription 1**
TEM	**Trans-Endothelial Migration**
TF	**Tissue Factor**
TFPI	**Tissue Factor Pathway Inhibitor**
TH cell	**T- Helper Cell**
TNF	**Tumor Necrosis Factor**
TRAP	**Thrombin Receptor Activating Peptide**
VCAM-1	**Vascular Adhesion Molecule-1**
VLA-4	**Very Late Antigen – 4**
VTE	**Venous Thrombo-Embolism**
VWF	**Von Wellibrande Factor**

Chapter One
Introduction

1.1 Introduction

The novel disease, termed coronavirus disease 2019 (COVID-19), that is the most significant pandemic in the past century remains a significant threat to public health. As of March 15, 2022, COVID-19 has taken nearly 6 million lives and infected more than 535 million people, and the number of cases continues to increase worldwide (WHO, 2022).

Even though lung infections are the most common symptom of coronavirus disease (COVID-19), the infection is frequently made worse by coagulopathy and thrombo-embolic events can be observed in a number of affected individuals (Allegra *et al*, 2020). Dehydration, acute inflammatory conditions, prolonged immobilization due to illness, diabetes, obesity, or hypertension, prior ischemic stroke, peripheral artery disease, and other conditions are frequently present in COVID-19 hospitalized subjects and may increase the risk of thrombo-embolic events. However, there are still other possible causes that can be found, such as increased synthesis of adhesion molecules that might cause endothelial activation and vascular inflammation, stimulation of complement, and increased platelet activation (Allegra *et al.*, 2020).

COVID-19 induces a systemic inflammatory response that involves the dysregulation and misexpression of several inflammatory cells. The activation and recruitment of inflammatory cells requires the expression of some types of inflammatory mediators such as adhesion molecules (intercellular adhesion

molecule 1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]), cytokines (for example, platelet activating factor [PAF]), and chemokines (Wang *et al.*, 2020). Pathological evidence of venous thrombo-embolism, direct viral infection of the endothelial cells, and diffuse endothelial inflammation have been reported in recent studies such as (Varga *et al.*, 2020). It was discovered that critical illness is related to markers of coagulation activation, specifically higher D-dimer and fibrinogen levels. On the other hand, relatively minimal alterations were identified in prothrombin time and platelet counts. In addition, a series of autopsies conducted on deceased COVID-19 patients described many instances of thrombosis. According to these data, vascular microthrombotic disease is likely the predominant contributor to mortality in critically ill COVID-19 patients (Wichmann *et al.*, 2020).

The mechanisms underlying increased thrombotic events are not fully understood; however, mounting evidence suggests that endothelial and platelet activation that leads to thrombosis (Immunothrombosis) plays a critical role. In light of the fact that viral inclusions have been identified in endothelial cells, it has been proposed that damage and activation of endothelial cells could be the driving force behind platelet activation and the resultant coagulopathy. Therefore, understanding the involvement of platelets in COVID-19 critical sickness is essential for both comprehending the biology of SARS-CoV-2 infection and locating potential therapy methods (Ackermann *et al.*, 2020).

Recent articles have reported the presence of severe endothelial injury and widespread pulmonary micro-thromboses along with increased angiogenesis in the lungs of deceased patients who had been infected with COVID-19. These results support other recent publications from various centers reported the presence of increased coagulation markers and microthromboses in lungs and other organs of patients with COVID-19 (Munster V, *et al.,* 2020). Recently, platelet activation and aggregation have been reported in patients with severe COVID-19. Platelet-activating factor, or PAF for short, is a cytokine that is known for being the most potent trigger of platelet aggregation. PAF is also a potent phospholipid activator and a mediator of many leukocyte functions, including platelet aggregation and degranulation, inflammation, and anaphylaxis. In addition to this, it plays a role in the alterations of vascular permeability, the oxidative burst, the chemotaxis of leukocytes, as well as the enhancement of arachidonic acid metabolism in phagocytes (Benveniste *et al.,* 1972).

Platelet-activating factor (PAF) is produced by many different types of cells, but it is most prevalent in cells that play an important role in the host's immune response. These cells include platelets, endothelial cells, neutrophils, monocytes, and macrophages. PAF is continuously produced by these cells, albeit in low quantities, and production is controlled by the activity of PAF acetyl hydrolases. PAF production occurs in low quantities and it is produced in greater quantities by inflammatory cells in response to specific stimuli such as COVID-19, and its biological actions bear similarities with COVID-19 disease manifestations (Zimmerman *et al.,* 2002). Recent research has shown that platelets, by releasing PAF into the bloodstream, are responsible for activating perivascular mast cells, which ultimately results in inflammation. In addition to this, mast cell degranulation that accompanied by interstitial edema and immunothrombosis was recently reported in the alveolar septa of COVID-19 patients who had passed away. Mast cells are a significant source of PAF and are abundant in the lungs, where they may contribute to the development of COVID-19 (Theoharides *et al.,* 2020).

Immunothrombosis is also thought to be caused by endothelial activation and elevated expression of serum endothelial cell adhesion molecules such as (ICAM-1and VCAM-1) in COVID-19 patients, as of now becoming clear that endothelial cells actively and reactively participate in hemostasis and immune and inflammatory reactions (Galley *et al.*, 2004).

Recent studies have found pathological evidence of venous thromboembolism, direct viral infection of endothelial cells, and widespread endothelial inflammation. As a result, it is important to investigate the expression of endothelial cell adhesion molecules in COVID-19 (Varga *et al.*, 2020).

Another mechanism for immunothrombosis formation is caused by the activation of the complement system, which is also detected in coronavirus disease19 (COVID-19). This cascade is comprised of over fifty plasma proteins that sense and respond to invading pathogens (Ghebrehiwet, 2016). This component of the innate immune response connects the innate and adaptive immune responses. Activation can happen through one of three pathways: the classical, lectin, or alternative pathway. These different routes ultimately converge at the same location, which is the source of C5 convertase as well as a number of anaphylatoxins. C5 convertase is responsible for the production of membrane attack complex (MAC) that induces the formation of cytotoxic membrane channels, which ultimately results in the death of cells; yet, even sublytic doses have important immunomodulatory effects (Tegla *et al.*, 2011). Excessive and unregulated complement activation may promote the formation of a systemic pro-inflammatory,

pro-oxidant, and pro-coagulant state characterized by multi-organ dysfunction and an increased risk of severe clinical outcomes. (Noris *et al.*, 2013; Santiesteban-Lores *et al.*, 2021). The complement system is a central mediator of innate immune defense and it, together with the coagulation system, helps the peri-and intravascular elimination of invading microorganisms in a process termed immuno-thrombosis. Some studies have suggested that COVID-19 pathophysiology involves dysregulation of microvascular thromboinflammatory pathways, the most prominent of which is the complement system (Chauhan *et al.*, 2020).

1.2 The Aim of Study

The study aimed to investigate and evaluate the role of PAF, ICAM-1, VCAM-1 and MAC in immunothrombosis that associated with severe and critical COVID-19 cases in comparison with mild cases.

1.3 Objectives

1. In order to determine the levels of (PAF, ICAM-1, VCAM-1, and MAC) in the serum of severe and critical COVID-19 patients in comparison with mild cases.

2. To investigate the possible correlations that exist between the levels of these markers and a variety of other hematological, biochemical, and coagulation parameters.

Chapter Two
Literature Review

2. Literature review

2.1 COVID-19 Disease

Coronavirus Disease (COVID-19) pandemic, occurred in Wuhan, Hubei Province, China at December 2019. The disease which was caused by a new ß-coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread (Wang *et al.*, 2020). It is primarily manifests its symptoms in the respiratory system, just like most other human coronaviruses. Although the majority of people infected with SARS-CoV-2 only exhibit mild symptoms, approximately 5-16 % of those infected with the virus require intensive care and more than half of these patients rapidly deteriorate into a life-threatening state of respiratory dysfunction that requires the assistance of mechanical ventilation. Hypoxia, pneumonia, disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS), and multiorgan failure are common symptoms seen in patients suffering from severe or critical infections (Fig. 2-1). Several lines of evidence have shown that immuno-pathological damage may be responsible for the deterioration of COVID-19 (Huang *et al.*, 2020).

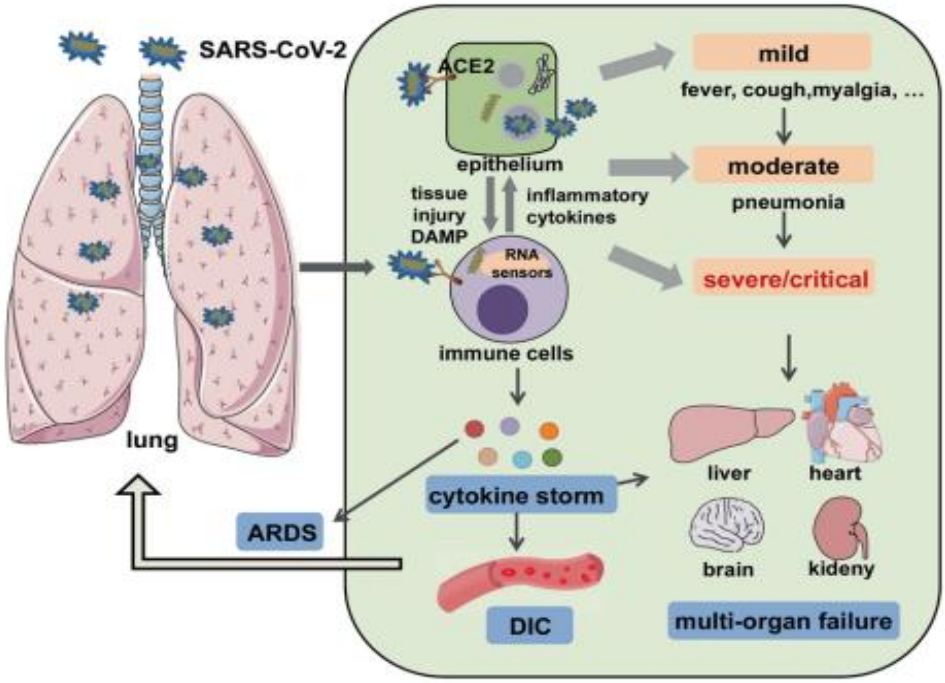

Figure (2-1): Clinical Manifestations of COVID-19 (Fu L *et al*.,2022).

2.1.1 Structure of SARS-CoV-2:

SARS-CoV-2 is a pleomorphic to spherical encapsulated particle. The size is between 80 and 120 nm in diameter. The greatest size has been seen to be as little as 50 nm and as high as 200 nm (Jung *et al.*, 2022). There are four kinds of primary structural proteins observed in the coronaviruses: the nucleocapsid (N), envelope (E), membrane (M) and spike (S) proteins, that are encoded within the viral genome (Table 1). The virion envelope can be shown in thin sections as inner and outer shells separated by a transparent gap. The virion envelope includes phospholipids, glycolipids, cholesterol, di-and triglycerides, and free fatty acids. The genome RNA is complexed with the basic nucleocapsid (N) protein, which forms a helical capsid within the viral membrane. The enclosed glycoproteins are responsible for host cell adhesion (Shaikh *et al.*, 2020).

Table (2-1): SARS-CoV-2 Proteins and their Functions (Shaikh *et al.*, 2020):

Structural proteins	**Functions of proteins**
Spike protein (S)	Mediate virus attachment to the host cell receptors
Membrane protein (M)	Nutrient transport, envelope formation, and shape determination
Envelope protein (E)	Interferes with host immune response
Nucleocapsid protein (N)	Binds with RNA genome and makes up nucleocapsid
Hemagglutinin-esterase (HE)	Binds sialic acids on surface glycoprotein

2.1.2 Classification of SARS-CoV-2

SARS-CoV-2 is a member of the *Coronavirinae* subfamily of the *Coronaviridae* family and the *Nidovirales* order. *Coronaviridae* are further classified into four genera based on phylogeny:

1. *Alpha (α) coronavirus*.

2. *Beta (β) coronavirus*.

3. *Gamma (γ) coronavirus.*

4. *Delta (δ)coronavirus*.

α and *β corona viruses* are found in mammals, whereas *γ* and *δ corona viruses* are primarily found in birds (Otieno *et al.*, 2022).

2.1.3 The Transmission of COVID-19

Many wild and domestic animals, including cattle, cats, camels, and bats, may act as hosts for coronaviruses. The exceptions are SARS and MERS, which are mostly transmitted through intimate contact with sick individuals by respiratory droplets from sneezing or coughing. Early COVID-19 patients were reported to have some link to the Hunan Seafood Market in Wuhan, China, indicating that these early infections were caused via animal-to-human transfer. However, subsequent cases were documented among medical staff and others who had not visited Wuhan or had no exposure to that market, which was interpreted as evidence of human-to-human transmission (Liu *et al*., 2020).

There are three primary ways that respiratory viruses are spread:

1. Contact transmission occurs when a person comes into close proximity to an infected person or touches a contaminated surface.
2. By the spread of respiratory droplets, that get infected with the virus when they come into contact with an infected individual.
3. By smaller droplets and particles airborne transmission.

According to infection control recommendations, the majority of respiratory virus transmission happens through the close contact of large contaminated droplets generated by coughing, sneezing, and breathing. Social distance has become the cornerstone of public health guidance as a result of this insight, however there is disagreement on the ideal safe distance between individuals to limit transmission, with the WHO recommending 1 meter (Medicine, 2020).

2.1.4 Attachment and Entry of SARS-CoV-2

There are several stages required to start and finish the COVID-19 infection cycle:

1. Recognition and adhering to the cellular receptor(s).
2. Alterations to the S protein's structure and proteolysis.
3. Fusion to cellular membrane.
4. Endocytosis, the process by which the virus enters the host cells.

Researchers have confirmed early in the pandemic that the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein interacts to the ACE2 receptor, a well-known protein that is present on the surface of the majority of human throat and lung cells. SARS-CoV, the virus that causes severe acute respiratory syndrome, also docks at this receptor. SARS-CoV-2, on the other hand, binds to ACE2 an estimated 2-4 times stronger than SARS-CoV-1 because various modifications in the RBD solidify its virus-binding hotspots (Pillay, 2020).

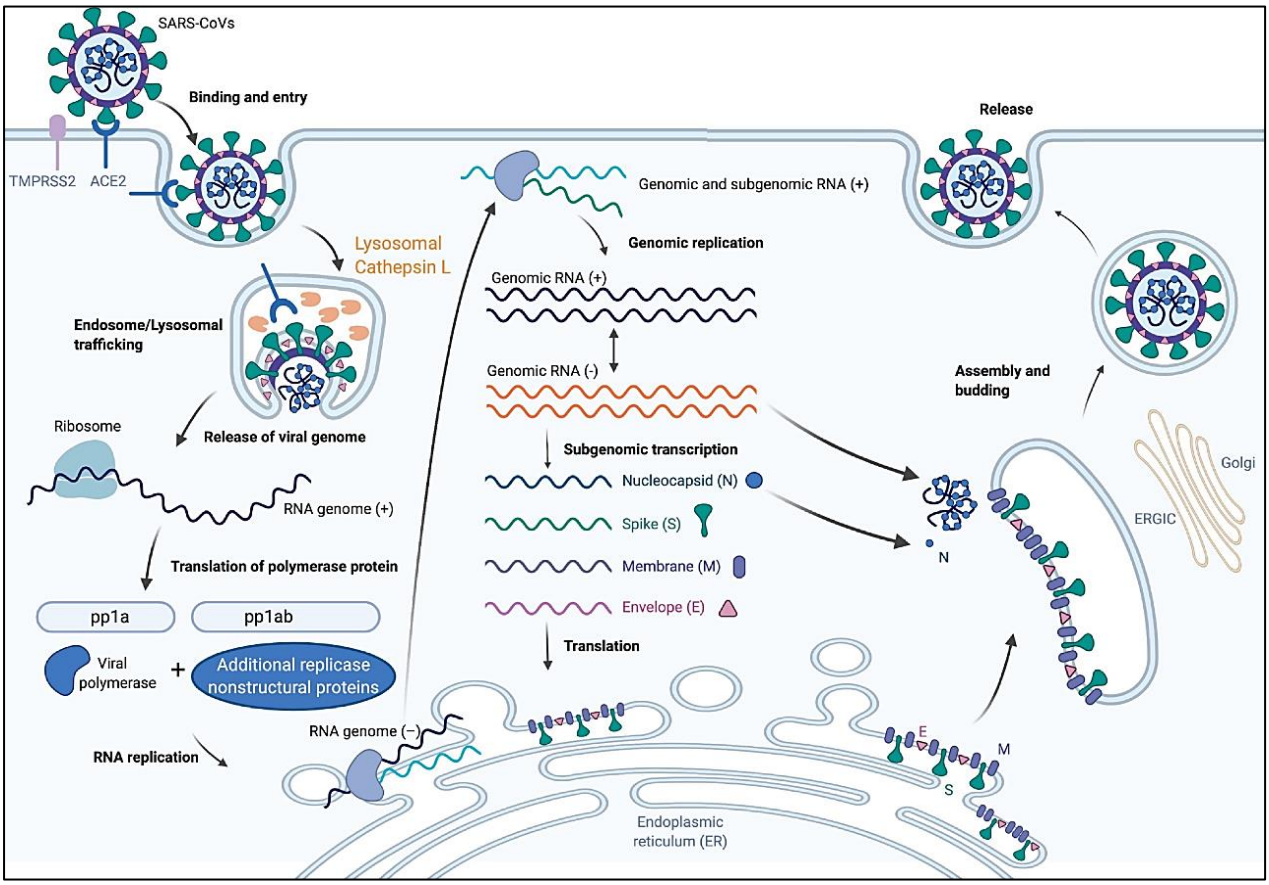

Figure 2-2: Life Cycle of SARS-CoV-2 (Harrison *et al.*, 2020).

2.1.5 Pathogenesis of COVID-19

Coronavirus envelope spike glycoprotein (S protein) has been identified as a key factor in virus entrance into host cells. For SARS-CoV-2, it interacts to its cellular ACE2 receptor. The entrance of the virus into cells was once thought to be conducted by direct membrane fusion between the virus and the plasma membrane (Li *et al.*, 2020).

This key photolytic cleavage event happened at position (S2′) of the SARS-CoV-2 S protein, which facilitated membrane fusion and viral infectivity. SARS-CoV-2 entrance was also mediated by clathrin-dependent and -independent endocytosis. The viral RNA genome is released into the cytoplasm once the virus enters the cells and is translated into two poly proteins and structural proteins, following which the viral genome begins to multiply (Li *et al.*, 2020).

The freshly created envelope glycoproteins are introduced into the endoplasmic reticulum or Golgi membrane, and the nucleocapsid is generated by combining genomic RNA and nucleocapsid protein. Virus particles then germinate in the endoplasmic Reticulum-Golgi intermediate compartment (ERGIC). Finally, the virus-containing vesicles fuse with the plasma membrane to release the virus. (De Wit *et al.*, 2016).

2.2 Antigen Presentation in Coronavirus Infection

As the virus penetrates the cells, its antigen is delivered to antigen presentation cells (APC), which are an important component of the body's anti-viral response. In humans, the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) presents antigenic peptides, which are then recognized by cytotoxic T lymphocytes that are specific for the virus. Therefore, appreciation of SARS-CoV-2 antigen presentation will aid in our understanding of COVID-19 pathophysiology. However, there is still no report on it, and the only information

we have comes from earlier studies on SARS-CoV-2 and MERS-CoV-2. MHC I molecules are primarily responsible for the antigen presentation of SARS-CoV-2, while MHC II also plays a role (Li *et al.*, 2020).

2.3 Humoral and Cellular Immunity

After antigen presentation, the body's humoral and cellular response is triggered, which is mediated by B and T cells that are specifically reactive to certain viruses. The anti-SARS-CoV-2 virus antibody profile is identical to that of common acute viral infections, with a normal pattern of IgM and IgG production. IgG antibodies specific for SARS may remain for a long period whereas IgM antibodies decline by the end of week 12, suggesting that IgG may predominantly perform a protective function **(Li *et al.*, 2020)**. In comparison to humoral responses, there have been more studies on coronavirus cellular immunity. According to the most recent data, the number of total T lymphocytes is dramatically decreased in the peripheral blood of SARS-CoV-2 infected individuals (Xu *et al.*, 2020).

2.4 The Proinflammatory Environments in COVID-19

The pathophysiology of COVID-19 is connected to an inflammatory response. The early phase in the pathogenesis of COVID-19 is the invasion of SARS-CoV-2 into target cells via its S proteins in the virus's outer lipid layer. SARS-CoV-2-expressing pathogen-associated molecular pattern molecules (PAMPs) activate a wide range of adaptive and innate immune cells after entering host cells, especially respiratory epithelial cells. This activation causes the release of inflammatory cytokines as well as the type I interferons (IFN) IFN- α and IFN-β, resulting in proinflammatory environment (Hoffmann*etal.*, 2020;Petrey*etal.*,2021).

2.4.1 Cytokine Storm in COVID-19

Cytokine storm is a pathogenic hyper-inflammatory condition caused by an sudden rise in particular circulating pro-inflammatory cytokine levels, which results in excessive systemic inflammation, aggravating viral pathogenesis and leading to ARDS, sepsis, and multi-organ failure. In severe SARS-CoV-2 infection, the total T cell counts as well as T cell count were all significantly lower than that in more moderate cases (Chen *et al.*, 2020).

2.4.2 Mechanisms of Cytokine Storm in COVID-19

After infecting respiratory epithelial cells, SARS-CoV2 causes an immunological response that includes the generation of inflammatory cytokines and a mild interferon (IFN) response. Next, macrophages and neutrophils infiltrate the lung tissue, which causes a cytokine storm (Hussman, 2020). SARS-CoV2 in particular has the ability to quickly activate pathogenic Th1 cells and cause the release of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor GM-CSF and IL-6. Tumor necrosis factor (TNF), IL-6, and other cytokines are produced in high amounts by CD14+ CD16+ inflammatory monocytes that have been further activated by GM-CSF. CD8+ and CD4+ T cells are the most dominant cells that take part in the immune responses to an infection with SARS-CoV-2 (Huang *et al.*, 2020; Grifoni *et al.*, 2020). Other types of immune cells, such as B cells, macrophages, neutrophils, and natural killer cells, are also involved in the immune responses to the infection caused by SARS-CoV-2. Excessive cytokines attract monocytes, neutrophils, and macrophages to the site of the lesion, where these immune cells not only clear virus particles but may also induce organ failure. Significantly, these cytokines also stimulate immune cells, resulting in increased cytokine production. Despite the fact that the particular features and methods of the cytokine storm in COVID-19 remain unclear, researchers believe it is linked to a disordered immune response to eliminate the

virus (Ye Q *et al.*, 2020). The immune response to SARS-CoV-2 can be broken down into two distinct phases. In the first phase, known as the incubation phase, recruited cells and released cytokines work together to combat the SARS-CoV-2 infection. The immune response eradicates the virus in the majority of individuals, the immune response recedes and patients recover. However, when the immune response fails to combat SARSCoV-2, the second phase begins. During this phase, an overactive immune response occurs to compensate for the failure of target clearance, manifesting clinically as a cytokine storm (Lin *et al.*, 2021).

2.5 Immunothrombosis in COVID-19

According to some studies, COVID-19 frequently triggers thrombosis episodes, and the prevalence of these episodes is greater in more severe cases. Recent researches that were published has suggested that thrombosis is an important factor that contributes to the pathogenesis of COVID-19, particularly respiratory dysfunction. Interestingly, inflammation makes patients more likely to develop thrombosis, and vice versa, thrombosis is associated with inflammation. This association is a process that is sometimes referred to as immunothrombosis (Tang *et al.*, 2020).

2.5.1 Thrombosis

Thrombosis is defined as the formation of a blood clot (thrombus) within a blood vessel. This process is generally considered pathologic except in the case of traumatic injury, where thrombus formation may prevent loss of blood volume and protect against systemic infection. In 1856, the German pathologist Rudolf Virchow postulated that a triad of conditions predispose to thrombus formation; these three factors include endothelial cell injury, hypercoagulability and abnormalities in blood flow (Esmon CT, 2009).

2.5.2 Endothelial Cell Injury

Any inflammatory process, including injury, surgery, or infection, can damage the endothelial lining of the vessel wall. The primary mechanism involves the introduction of tissue factor into the coagulation system of the blood. Alterations in gene expression in the endothelium, which can be brought on by inflammatory and other stimuli (such as high cholesterol levels), can produce a pro-thrombotic state. When this happens, the integral membrane protein thrombomodulin, which is expressed on the surface of endothelial cells and is a crucial modulator of thrombin activity, is downregulated by the endothelial cells. The final result is a continuous activation of thrombin and decreased production of tissue factor inhibitor and protein C, that intensifies the pro-thrombotic condition (Kumar *et al.*, 2015).

2.5.3 Hypercoagulability

Thrombophilia or hypercoagulability is caused by, for instance, autoimmune diseases or genetic deficiencies. According to recent research, white blood cells are critical to deep vein thrombosis and mediate a number of pro-thrombotic actions. (Swystun *et al.*, 2016).

2.5.4 Abnormalities in Blood Flow

Blood flow can become disrupted for a number of reasons, including the following: blood flow that stops flowing past the site of an injury; venous stasis, which can occur as a result of heart failure; or prolonged periods of sedentary behavior, such as sitting for an extended period of time while traveling by airplane. Additionally, atrial fibrillation can cause blood to pool in the left atrium (LA) or the left atrial appendage (LAA), which increases the risk of developing a thromboembolism. An increased risk of thrombosis may be caused by cancers or malignancies such as leukemia. This increased risk may be caused by possible activation of the coagulation system by cancer cells or secretion of procoagulant

substances (paraneoplastic syndrome), by external compression on a blood vessel when a solid tumor is present, or (less frequently) by extension into the vasculature of the body (for example, renal cell cancers extending into the renal veins). Additionally, cancer treatments such as radiation therapy and chemotherapy frequently cause additional hypercoagulability. (Mchedlishvili , 1998).

2.5.5 Immunothrombosis

The term "immunothrombosis" was first coined by Engelmann and Massberg, who were referring to an intrinsic effector pathway of innate immunity that is activated in response to pathogens and injured cells. The purpose of this pathway is to inhibit the growth and survival of invading pathogens (Engelmann *et al.*, 2013). Immunothrombosis is predominantly induced by neutrophils and monocytes, and it is helped by microthrombi development in tiny vessels. During the formation of microthrombi, endothelial cells that have been exposed to microorganisms take on a pro-adhesive phenotype. In conclusion, immunothrombosis can be seen as a good travascular immunity mechanism. However, uncontrolled immunothrombosis results in dysregulated activation of the coagulation cascade, which causes the development of microthrombus and inflammation. These two conditions feed off of one another to eventually lead to thrombosis (thromboinflammation) and disseminated intravascular coagulation (Fuchs, *et al.*, 2010).

2.6 Neutrophil Extracellular Traps (NETs) in COVID-19

After a pathogen invades the body, neutrophils, as important members of the innate immune cell population, are drawn to the site of inflammation or infection, where they trigger other types of immune cells and remove infections. An autopsy specimen taken from a died COVID-19 patient demonstrated the presence of neutrophil infiltration in the lung tissues. An increased number of peripheral blood neutrophils is thought to be an early sign of SARS-CoV-2 infection, and it is linked to severe respiratory dysfunction and poor clinical outcomes (Barnes *et al.*, 2020).

Brinkmann and colleagues discovered in 2004 that circulating neutrophils develop web-like structures known as neutrophil extracellular traps (NETs) in response to bacterial endotoxins and inflammatory cytokines or medicines. These NETs are generated from intracellular components that are produced by active neutrophils. Neutrophil elastase (NE), myeloperoxidase (MPO), histones, defensins, calprotectin matrix metalloproteinase-9, and cathepsin G are some of these components (Brinkmann *et al.*, 2004). NETs are an innate immune system component whose primary job is to capture and even destroy bacteria. However, several research has revealed that NET activities are a double-edged sword. This is because, in addition to microbicidal function, NETs have been associated to a range of tissue damage and have been implicated in the course of sepsis and influenza pneumonia. During immunothrombosis, neutrophils and monocytes facilitate the activation of coagulation that is caused by inflammation by releasing tissue factor and extracellular nucleosomes and degrading endogenous anticoagulants, respectively (Von Bruhl *et al.*, 2012). It is crucial to note that tissue factor decorates neutrophil extracellular traps (NETs), as shown in sepsis and, more recently, in coronavirus disease 2019 (COVID-19)-related immunothrombosis. NETosis by neutrophils also increases coagulation system activity by elevating fibrin deposition. Histones found in NETs, particularly histones H3 and H4, can increase thrombin production in a dose-dependent manner not only through lowering thrombomodulin-mediated protein C activation but also by directly activating platelets. Finally, NETs can bind plasma proteins including von Willebrand factor (VWF) and fibronectin that are necessary for platelet adhesion and thrombus formation. Platelets themselves also contribute to this process in a number of ways including: increasing the number of immune cells that express tissue factor, directly binding to neutrophils and NETs, releasing damage-associated molecular patterns that encourage tissue factor expression within thrombi, and directly binding to microorganisms for presentation to innate immune cells (Fuchs *et al.*, 2010).

2.7 Relationships between Inflammation and Thrombosis in COVID-19

It has been demonstrated that the immune response plays an active role in the formation of thrombi inside blood vessels, particularly in micro-vessels. The process, defined as immunothrombosis, accurately describes the complex network between the innate immune system and the coagulation system. Immunothrombosis can help contain an infection by making pathogens easier to detect, contain, and destroy. Consideration has been given to immunothrombosis in SARS-CoV-2 infection because of the similar mechanisms found in COVID-19 and sepsis caused by bacteria. More complex interactions between inflammation and thrombosis, including endothelial cells (ECs), coagulation (activated tissue factor (TF), platelets, and neutrophils), anticoagulation [impaired antithrombin (AT), activated protein C (APC), and tissue factor pathway inhibitor (TFPI) systems], and decreased fibrinolysis (Engelmann *et al.,* 2013).

2.8 Endothelial Activation in COVID-19

Endothelial cells (ECs) are responsible for maintaining the equilibrium between the coagulation and anticoagulation systems of the blood. They do this by expressing a number of mediators that that prevent platelet activation and suppress coagulation and thrombus formation, whereas activation of endothelial cells or injury to these cells can lead to platelet activation, thrombosis, and inflammation. The interaction of SARS-CoV-2 receptor with angiotensin-converting enzyme 2 (ACE2) that expressed by endothelial cells possibly mediates endothelial activation (Krüger-Genge *et al.,* 2019). Proinflammatory cytokines activate endothelial cells, and activated dysfunctional cells may contribute to the pathogenesis of thrombosis by altering the expression of pro-and antithrombotic factors (Fong *et al.,* 2015; Wang *et al.,* 2019).

2.8.1 Leukocyte Adhesion and Migration through Activated Endothelial Cells

Leukocytes adhesion and migration is a dynamic process that is controlled by specific receptors of adhesion molecule and counter-receptors expressed on leukocytes, endothelial cells, and particular areas of coagulation proteins and extracellular matrix. The uncoordinated activation and adhesion of leukocytes causes damage to the tissue, which has been associated to a number of inflammatory illnesses such as asthma, arthritis, acute lung injury, and ischemia reperfusion injury (Pilewski *et al.,* 1993; Gorski *et al.,* 1994). Anti-inflammatory research has placed a lot of emphasis on disrupting the leukocyte-endothelial cell cascade, and its therapeutic potential is now being thoroughly investigated. If one has a greater grasp of the cellular and molecular processes of leukocyte contacts, as well as their involvement in both healthy and pathological cell and tissue function, it will be simpler to identify novel methods to the creation of therapeutic treatments (Oppenheimer *et al*, 1996).

2.9 Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are a group of cell surface glycoproteins that are involved in the binding of cells with other cells or with the extracellular matrix (ECM), in a process called cell adhesion. Fundamentally, CAMs aid cell adhesion to one another and to their surroundings, and they play a key role in both inflammatory and cancerous disorders (Samanta *et al.,* 2015).

Cell adhesion molecules are generally divided into: integrins, selectins, cadherins, and members of the immunoglobulin superfamily (IgSF) including nectins and mucins. Apart from structural differences, cell adhesion molecules also bind to different ligands. Integrins typically bind to the extracellular matrix, while selectins, cadherins, and IgSF members are associated with cell-cell adhesion. However, immune cell integrins also bind to soluble ligands and ligands on other cells (Samanta *et al.,* 2015).

2.9.1 The Importance of Adhesion Molecules

It has been found that adhesive interactions between cells and cells with the extracellular matrix play an important role in a variety of tissue functions, including organ morphogenesis, embryonic development, wound healing, inflammatory responses, tumor metastasis, blood coagulation, and immune surveillance (Hynes *et al.*, 1992). According to recent studies, one of the adhesion molecules' most crucial roles, is to translate biochemical information from the extracellular environment into specific cell functional responses by activating intracellular signaling pathways. This function, in addition to fixing cells in particular locations within tissues and controlling their movement, is demonstrated by adhesion molecules (Crockett-Torabi *et al.*, 1995). Along with immunological and inflammatory processes, it is important for leukocytes to attach with extracellular matrix and vascular endothelial cells. This attachment is depending on a series of cellular sticky processes. At least three separate families of adhesion molecules are involved in leukocyte attachment and motility. These families include the selectins, integrins, and certain members of an immunoglobulin superfamily such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules-1 (ICAM-1). Coordination of their function and expression is crucial for the recruitment of leukocytes from the circulation to the tissue since each is involved in a separate phase of leukocyte emigration through the endothelium. Selectins and their counter-receptors are important in the first interaction of leukocytes with the endothelium. Beside this, the integrins and ICAMs coordinate the next adhesive interactions, such as cell adhesive interactions with the extracellular matrix and transendothelial migration (Gorski *et al.*,1994; Pilewski *et al.*,1993).

2.9.2 Vascular Cell Adhesion Molecule-1 (VCAM-1)

VCAM-1 is an inducible glycoprotein that is primarily expressed in endothelial cells. VCAM-1 was identified as an endothelial cell surface glycoprotein for the first time in 1989 (Rice *et al.*, 1989). The expression of VCAM-1 is triggered not only by pro-inflammatory cytokines like TNF, but also by oxidized low density lipoprotein, high glucose concentration, shear stress and toll-like receptor agonists. VCAM-1 is expressed on the surface of other cells, such as tissue macrophages, dendritic cells, bone marrow fibroblasts, myoblasts, oocytes, Kupffer cells, Sertoli cells, and cancer cells, when there is a high level of inflammation and when there are chronic conditions present in certain diseases (Van *et al.*,1945; Cook-Mills *et al.*, 2011).

2.9.3 The Roles of VCAM-1 in Inflammation and in Immune Response

In most cases, the production of TNF by immune cells—including macrophages, T lymphocytes, and natural killer cells—is what kicks off the inflammatory response. In turn, TNF- causes a cascade of different cell adhesion molecules, such as selectins, ICAM-1, and VCAM-1, to become activated. This allows leukocyte adhesion molecules to recruit a subset of leukocytes at sites of inflammation (Pober *et al.*, 2002). Among these adhesion molecules, VCAM-1 is a major regulator of leukocyte adhesion and transendothelial migration through interaction with very late antigen-4, VLA-4 (also known as α4β1 integrin). VLA-4, which is found on leukocytes, binds to VCAM-1, which is found on the surface of endothelial cells. Following this, signaling pathways within activated endothelial cells become activated, which in turn makes it possible for leukocytes to migrate through the endothelium. VLA-4 and VCAM-1 play an important role in recruiting of leukocyte during inflammation (Cerutti *et al.*, 2017).

2.9.4 Intercellular Adhesion Molecule-1 (ICAM-1)

ICAM-1 is a glycoprotein that is found on the surface of immune, endothelial (EC), and epithelial cells. It is expressed at a low level at rest in these cell types, but its expression level increases in response to inflammatory stimulation. In leukocyte trans-endothelial migration (TEM), ICAM-1 binds to lymphocyte function-associated antigen (LFA)-1 that expressed on leukocyte and thus ICAM-1 regulates leukocyte rolling and adhesive interactions with the vessel wall, and guides leukocyte crossing of the endothelial (Gorina *et al.*, 2014). Recent functional studies have identified several new roles for ICAM-1 in innate and adaptive immune responses in inflammation, epithelial injury-resolution responses, and tumorigenesis. As a result, the intercellular adhesion molecule 1 (ICAM-1) has emerged as a master regulator of many essential tissue functions, both at the beginning of pathologic conditions and at their end. Since quite some time ago, ICAM-1 has been of clinical and therapeutic interest. It is possible that the clinical value of ICAM-1 could be reevaluated in the future with a better understanding of the various roles it plays in health and disease as well as new mechanistic insights into the function of the protein. This would allow for the development of improved therapeutic strategies (Yacyshyn *et al.*, 2007).

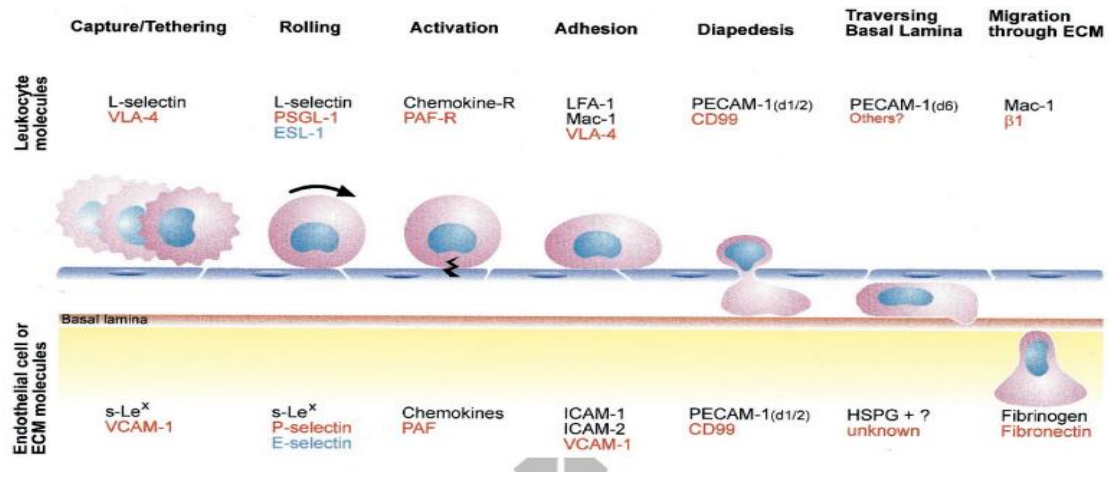

Figure 2-3: The roles of (ICAM-1 and VCAM-1) and other adhesion molecules in leukocyte–endothelial interaction (Muller *et al.,* 2020).

2.9.5 VCAM-1 and ICAM-1 in COVID-19

COVID-19 induces a systemic inflammatory reaction characterized by dysregulation and misexpression of several inflammatory cytokines. Many kinds of inflammatory mediators are required for the recruitment and activation of inflammatory cells. Examples for these inflammatory mediators are: cytokines (interleukin-1 [IL-1], IL-6, and IL-18), chemokines (fractalkine [FKN]), and adhesion molecules (ICAM-1 and VCAM-1) (Tong *et al.*, 2021).

Recent investigations have found pathological evidence of venous thromboembolism, direct viral infection of endothelial cells, and widespread endothelial inflammations. Hence, it is important to look at the expression of ICAM-1 and VCAM-1 molecules in COVID-19. And this suggests that these molecules could be used as biomarkers to measure the severity of the disease or the patient's recovery from it (Smith *et al.*, 2021).

2.10 The Role of Complement System in Immunothrombosis

2.10.1 The Complement System

The complement system is a crucial part of the innate immune response and functions as a bridge between the innate immune system and the acquired immune system. It is composed of a variety of proteins that are mostly, but not completely, generated in the liver, and their inactive precursors may be detected in the plasma and on the cell surfaces. By a coordinated sequential enzyme cascade, the complement system mediates responses to inflammatory stimuli and, in turn, facilitates the clearance of foreign cells through pathogen detection, opsonization, and lysis (Schifferli *et al.*,1986). Additionally, complement has anti-inflammatory properties. It attaches to immune complexes and apoptotic cells and helps remove them from the bloodstream and injured tissues. The word "complement" comes

from the fact that IgG and IgM antibodies activate complement proteins, which work in conjunction with them. There are many precursor complement proteins that are active when there is inflammation. The complement system is more complex than numerous other enzymatic pathways because it needs the synthesis of sequentially activated protein fragments that are not covalently linked to one another. The rapid dissociation of these complexes (and loss of enzymatic activity) is a crucial component of the elegant control of complement activity. These go on to become convertases and cleave components for the subsequent enzymatic complex in the cascade (Davies *et al.*,1994; Mevorach *et al.*,1998).

2.10.2 The Complement System in COVID-19

Although the role of complement in the acute respiratory distress syndrome caused by influenza, respiratory syncytial, and the previous SARS-CoVs is well established (Gralinski *et al.*, 2018), its contribution to COVID-19 is still unclear. Diao *et al.,* (2020) have found that acute renal failure associated with tubular necrosis and abundant complement deposition develops in a significant percentage of patients with severe COVID-19, which suggests that complement plays a pathogenic role, and Gao *et al.*, (2020) have reported in a preprint increased serum levels of C5a in a small series of patients with severe COVID-19. SARS-CoV-2 activates extracellular and/or intracellular complement pathways directly or indirectly. SARS-CoV-2 envelope proteins activate the lectin pathway by binding to mannose-binding lectin (MBL). SARS-CoV-2-specific antibodies and C1q, on the other hand, activate the classical pathway. When SARS-CoV-2 competes with C3b-regulatory factor H (FH) for binding to heparan sulfate, the inhibitory effects of factor H on C3 are removed, allowing the alternative route to remain activated. Production of intracellular complement factor B (FB) and C3 is induced by infection-induced type I interferon receptor (IFNAR) signaling through the Janus kinase (JAK)-signaling transducer and activator of transcription 1 (STAT1) pathway in type II pneumocytes (Fletcher *et al.*, 2020). This seeds an intracellular

C3 convertase and causes cleavage-mediated activation of intracellular C3. The activation of C3 fragments produced by type II pneumocytes bind to and activate nearby immune cells' corresponding receptors (C3a-C3aR and C3b-CD46). The activation of complement in the capillaries by SARS-CoV-2 enhances C5a production. C5a enhances leukocyte and neutrophil activation, adherence to endothelial cells, generation of proinflammatory cytokines, and/or development of local neutrophil extracellular traps (NETs). Endothelial cells exposed to SARS-CoV-2 upregulate C5a receptor 1 (C5aR1) expression and become sensitive to pathological C5a activation and insertion of the membrane assault complex (MAC), resulting in endothelial cell death and loss of thromboresistance (Fletcher *et al.*, 2020). Simultaneously, complement causes platelet activation (mainly through the action of the MAC) and coagulation cascade activation (mostly by the action of MBL-associated serine protease 1 (MASP1) or MASP2), supporting detrimental thrombus formation. The creation of a transmembrane channel, which leads to the lysis and death of infected cells, is brought about by the insertion of a MAC into the cell membrane of infected cells or directly onto pathogens. When inserted into endothelial cells, MAC is capable of not only activating platelets but also inducing endothelial secretion of von willebrand factor (VWF) and causing damage to endothelial cells. When these normal defenses against pathogens are overactive, they cause excessive endothelial damage, which can serve as thrombosis foci. Individual components of the complement are cause thrombosis. For instance, C5a can upregulate the activity of plasminogen activator inhibitor-1(PAI-1) and tissue factor (TF), and it can also activate neutrophils, which leads to increased production of IL-6 and IL-8 and also promotes the formation of NETs (Fletcher *et al.*, 2020). Increased levels of mannose-binding protein-associated serine protease 2 (MASP2) are found in COVID-19. This enzyme which is a key enzyme in the lectin pathway of complement activation may contribute to the formation of blood clots by activating C2 and C4, which, in turn, boost the performance of thrombin, fibrinogen, and factor XIII. It is likely that activation of the complement system will

amplify the COVID-19 thrombotic phenotype; therefore, future research should focus on explanation the particular components of the complement system that are involved as well as the effect of modulation (Chauhan *et al*; 2020).

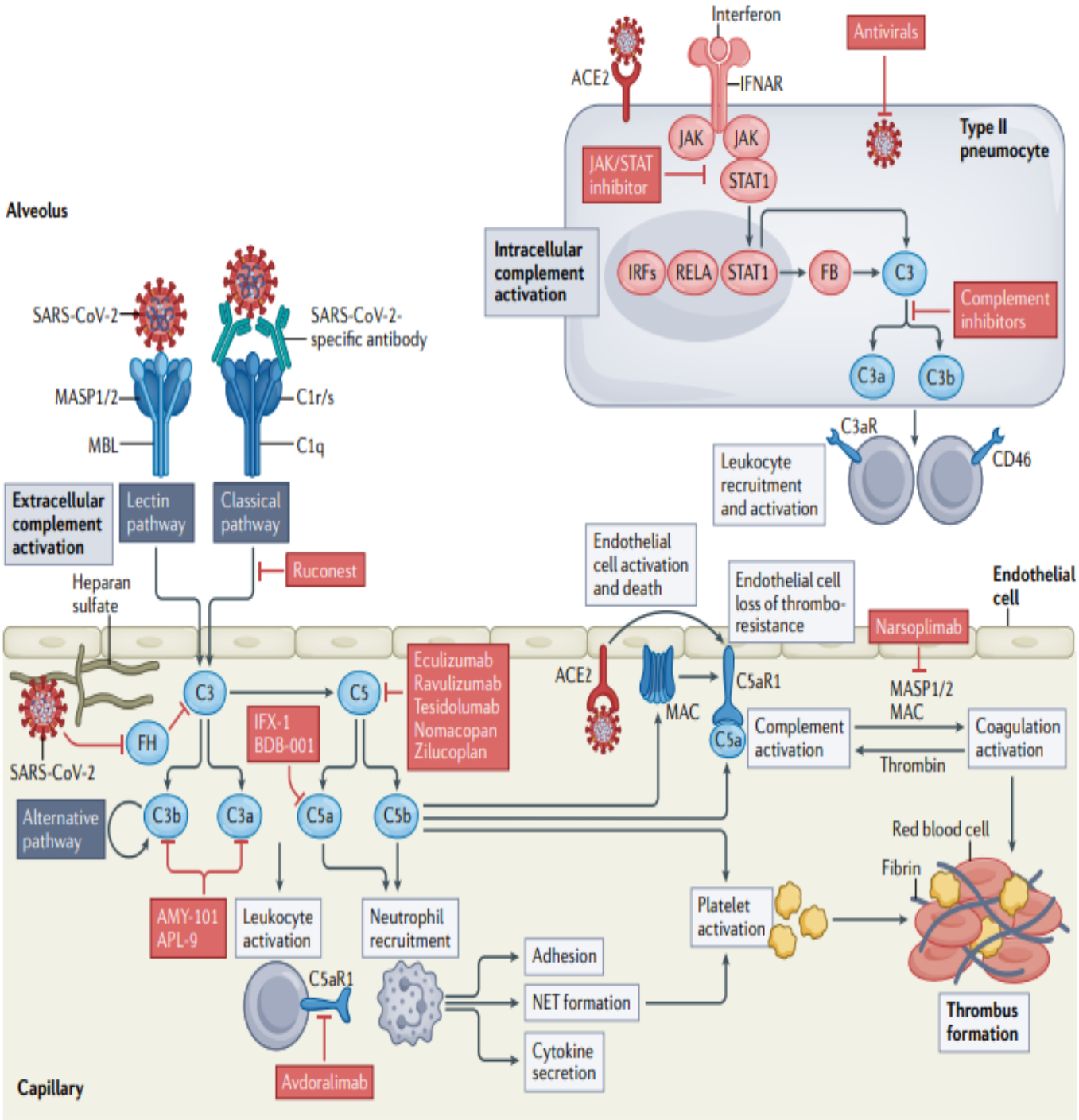

Figure 2-4: Schematic Summary of the Currently Known Contributions of Complement to COVID-19 (Noris *et al*., 2020).

2.10.3 The Relationship between Complement System and Immunothrombosis

A frequent comorbidity in the clinical manifestation of severe COVID-19 pneumonia is the increased incidence of thrombotic events, such as stroke and myocardial infarction, as well as generalized microvascular dysfunction that is linked to shunt and microvascular thrombi (Poor *et al.*, 2020; Magro *et al.*, 2020). These findings, along with earlier research on SARS-CoV and MERS-CoV, suggest that the pathophysiology of COVID-19 involves dysregulation of microvascular thromboinflammatory pathways, the most prominent of which is the complement cascade (Chauhan *et al.*, 2020). The thromboinflammatory response is a complicated process that requires integration at multiple levels between the complement cascade, the coagulation pathway, and the platelet pathway (Figure 2). For instance, C5a and MAC cause the expression of tissue factor to be produced, MAC has direct effects on the activation of thrombin and platelets, and C5a amplifies the effects of prothrombosis by inducing the production of IL-6, IL-8, and tumor necrosis factor-α (TNF-α). These factors stimulate endothelial activation and thrombin formation. In addition, thrombin can cleave and activate C3 and C5, thereby perpetuating the cycle of inflammation and coagulation (Hill *et al.*, 2013).

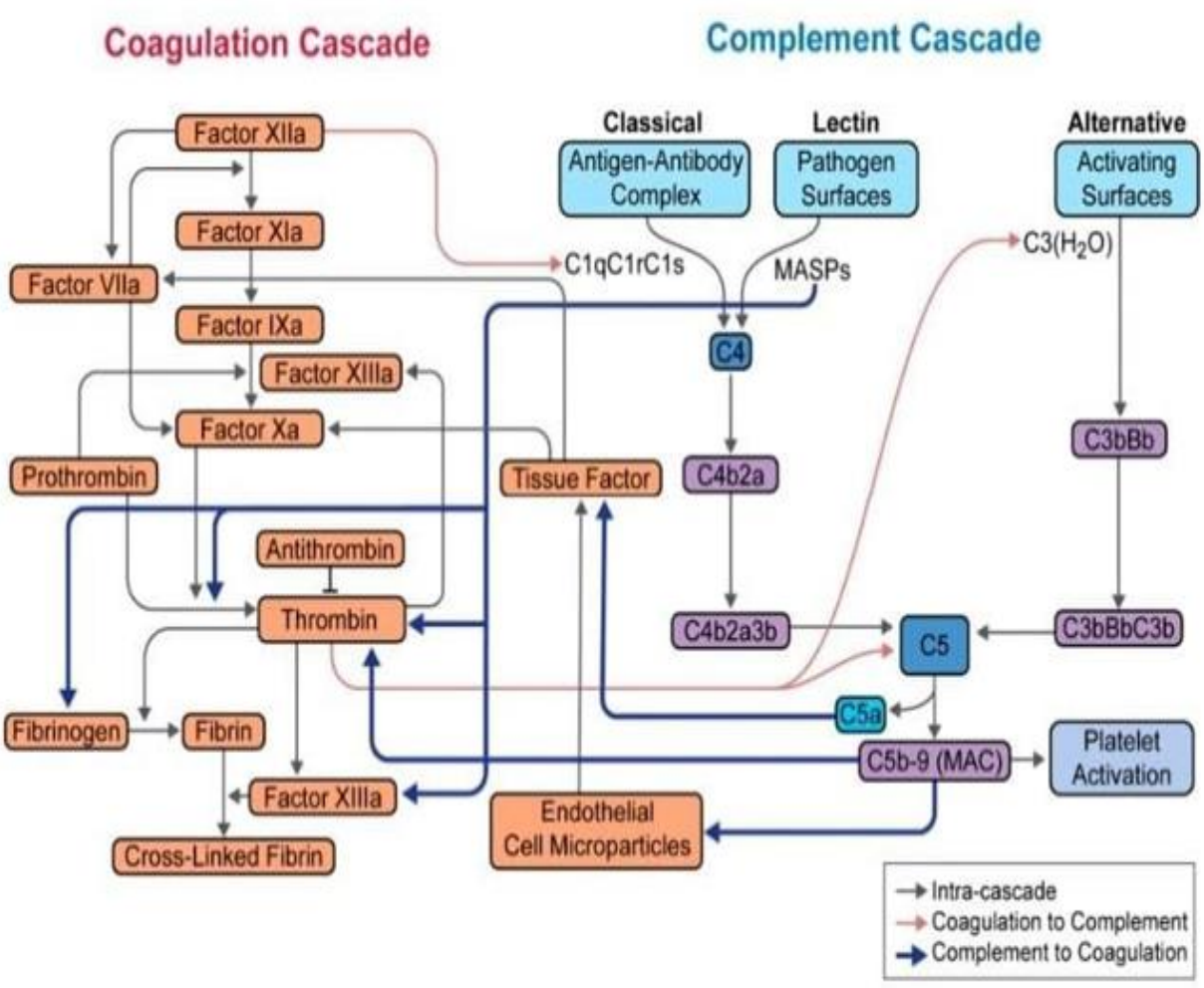

Figure 2-5: Integration between the Complement and Coagulation Cascades (Hill *et al*., 2013).

2.10.4 Terminal Complement Complex (TCC)

An effector of the immune system, the terminal complement complex (TCC), also known as the membrane attack complex (MAC), is a protein complex that is typically formed on the surface of pathogen cell membranes as a result of the activation of the host's complement system. Deposition of MAC on the surface of infected cells occurs as a result of complement activation that is mediated by antibodies. The formation of pores, which ultimately result in the lysis and death of target cells, is caused by the assembly of the MAC, which in turn causes a disruption of the cell membrane (Janeway *et al*., 2001).

2.10.5 Structure and Function of MAC

The membrane attack complex (MAC) is made up of a complex of five proteins: four complement proteins (C5b, C6, C7, and C8) that bind to the outer surface of the plasma membrane, and many copies of a fifth protein (C9) that hook up to one another and form a ring within the membrane. C6-C9 all contain a common membrane attack complex/perforin (MACPF) domain. A pore is created in the membrane by the ring structure that is formed by C9, and this pore allows molecules to freely diffuse into and out of the cell. If a sufficient number of pores are created, the cell will lose its ability to survive. In the event that the pre-MAC complexes of C5b-7, C5b-8, or MAC are unable to insert themselves into a membrane, they have the potential to form inactive complexes with Protein S. (sC5b-7, sC5b-8 and sMAC). These fluid phase complexes do not bind to the cell membranes and are eventually scavenged by the complement regulators clusterin and vitronectin (Hadders, *et al.*, 2012).

2.10.6 MAC in COVID-19 Associated Immunothrombosis

Patients with COVID-19 who have a viral infection of their endothelial cells caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can cause vascular changes and elevate the formation of MAC, and which may be used as a biomarker to measure the severity of the disease or how well the patient is recovering from it.

2.11 Prothrombotic Phenotype in COVID-19

Despite the fact that COVID-19 infection is linked with a wide range of symptoms, the appearance of alveolar capillary micro-thrombi is a common observation in COVID-19 patients and appears as a result of significant endothelial damage with endothelial cell membrane breakdown **(Ackermann *et al.*, 2022).** These findings strongly suggest the presence of a COVID-19-associated

coagulopathy, which may lead to thrombosis, multi-organ damage, and death due to its severity and mortality. The pathophysiological processes causing arterial and venous thrombosis during COVID-19 illness are still unclear, and several features of platelet sensitivity to SARS-CoV-2 are notably contrast. However, there is consensus on the existence of prothrombotic anomalies, and platelets have been demonstrated to play a significant role. Platelets have long been thought to be important players in hemostasis. Indeed, platelets are extensively involved in host defense in the event of infections because, together with other immune cells and the coagulation process, they operate as modulators and effectors of immune cells other than clot formation (Yeaman *et al.*, 2010).

Platelets can respond to infections mostly by stimulating the release of neutrophil extracellular traps (NETs) and indirectly disposing of them. Platelets serve as a connection between host defense and thromboinflammation due to their capacity to stimulate macrophages, attract and activate neutrophils, and actively engage in intravascular thrombosis. In reality, inflammation can generate hemostatic changes that lead to thrombosis, and thrombosis can enhance inflammation, creating a feedback loop that increases tissue damage and thrombotic consequences (Liverani *et al.*, 2018).

During COVID-19 pathogenesis, the increased cytokines production is important in more influencing the systemic hemodynamic irregularities and cardiovascular diseases (CVD). In this context, platelets secrete a variety of chemokines, pro-inflammatory cytokines, and growth factors that significantly contribute to thromboinflammation, which is responsible for infection complications and severity in organ failure. Of course, platelets must interact with other circulating cells like macrophages and monocytes, and each of them must interact with endothelial cells in the cross-talk between thrombosis and inflammation (Taus *et al.*, 2020). According to reports on patients who died from COVID-19, extensive platelet-fibrin clot formation in the pulmonary

microvasculature was discovered in 80–100% of the lungs and other organs examined. It has also been established that marked inflammation is a typical clinicopathological feature that worsens the prognosis for COVID-19. Patients with COVID-19 were shown to have much higher levels of circulating platelet-neutrophil, -monocyte, and -T-cell aggregates than healthy controls, and platelets themselves exhibit hyperreactivity, which contributes to the pathophysiology of COVID-19 (Acanfora *et al.,* 2021).

2.11.1 Platelet Activation

Platelets, which are tiny anucleated cells secreted by megakaryocytes, are known to have an impact on not just vascular hemostasis but also immunological response, tumor growth, and other inflammatory processes (Ghasemzadeh *et al.,* 2013). In particular, during sepsis, platelets may induce endothelial dysfunction, the production of NETs, and the generation of micro-thrombi, aggravating coagulation and inflammation. Additionally, it has been established that platelet hyperactivation plays a role in promoting the interaction of platelets with inflammatory cells and endothelial cells, which in turn promotes the cascade reaction between inflammation and coagulation. This is supported by the ability of antiplatelet therapy during sepsis to reduce the uncontrolled inflammation, coagulation, and damage to organ function and improve patient prognosis. Platelets have three types of granules (alpha, dense, and lysosomes), and activation of these granules leads in the production of chemicals capable of modulating aggregation and thrombus formation (Li, Z. *et al.,* 2011).

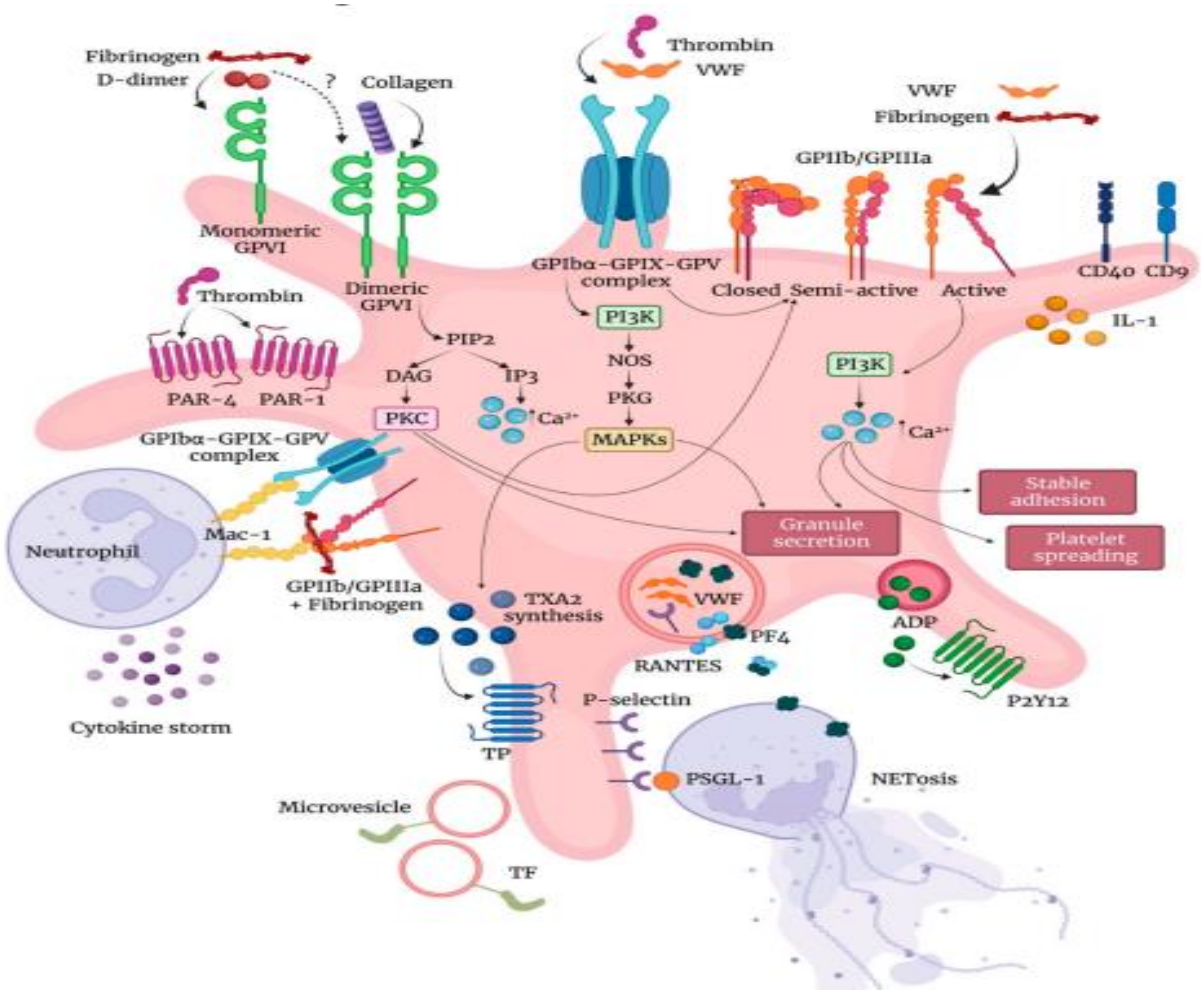

Figure 2-6: Platelet Activation in COVID-19 (Ferrer *et al.,* 2021).

The binding of soluble fibrinogen to platelet integrins activates platelets and promotes clot formation. Integrin IIb3, which is involved in platelet spreading, is one of the most essential platelet integrins to which fibrinogen binds. Integrin IIb3 is normally inactive and has a modest affinity for ligands in resting platelets. Following platelet activation, the structure of integrin IIb3 changes, resulting in a receptor with a greater affinity for ligands and allowing for further signaling processes (Xu *et al.,* 2016). Glycoprotein (GP) VI is the primary platelet receptor for collagen that is exposed during endothelial damage. GPVI dimerization is essential for collagen binding, however only monomeric GPVI can bind fibrin (ogen) and D-dimer. Figure (2-6) depicts the activation of more relevant fibrin (ogen)- and D-dimer-induced signaling pathways involved in platelet activation

observed in COVID-19. Activation of these signaling pathways causes platelet aggregation and activation, as well as conformational shape change and subsequently clot formation and clot retraction (Varga *et al.*, 2018). In comparison with controls, platelets from hospitalized stable COVID-19 patients show enhanced levels of the platelet activation markers P-selectin and lysosomal-associated membrane protein 3 (LAMP-3), and significantly higher expression of the transmembrane integrins GPIIb/GPIIIa complex, GPIbα, GPIX, CD9, and CD40. The authors demonstrate in the same study that after stimulation with thrombin receptor activating peptide (TRAP), platelets respond with increased expression of the collagen receptor GPVI (Bongiovanni *et al.*, 2021). Following platelet activation, the expression of P-Selectin, a 120 kDA transmembrane protein, on the platelet surface regulates neutrophil-platelet, platelet-platelet, and monocyte-platelet interactions, becoming a driver for neutrophil integrin activation, NET formation, and tissue factor expression. Upregulation of the integrins GPIIb (CD41) and GPIIIa (CD61), as well as the von Willebrand factor (VWF) receptor subunits GPIb and GPIX, which control platelet-leukocyte interactions, may contribute to the inflammatory response in COVID-19 patients (Hottz *et al.*, 2020).

2.11.2 Platelets and Immunothrombosis

SARS-CoV-2 infection causes immunothrombosis, a process in which the interaction of activated neutrophils, monocytes, the coagulation cascade, and platelets results in the production of intravascular clots from tiny to large capillaries. Platelets' ability to release various potent cytokines and chemokines, in addition to their well-known role in hemostasis, has elevated these small cells from simple cell fragments to critical modulators in the blood, including their inflammatory functions, which have a large influence on the immune response during infectious diseases (Koupenova *et al.*, 2018). Platelets express a variety of immunoreceptors, making them sentinels capable of detecting intravascular infections. Platelets trigger immune cells to guarantee infection clearance, even though platelets can

directly inhibit pathogen development by releasing antimicrobial chemicals. However, abnormal platelet activation causes inflammation and thrombotic consequences. Platelet-neutrophil interaction enhances neutrophil recruitment into inflammatory areas in inflammatory situations. PSGL-1, a neutrophil-expressed glycoprotein ligand that binds to platelet P-selectin, facilitates neutrophil-platelet interaction (Hamburger *et al.*, 1990). In sepsis-induced models of acute lung damage, lowering circulating platelets or blocking the P-selectin-mediated platelet interaction with neutrophils has been demonstrated to drastically decrease neutrophil recruitment and vascular permeability, enhance gas exchange, and prolong life. The 2-integrin macrophage antigen-1 (Mac-1, CD11b/CD18) binding to GPIb on the surface of platelets and the simultaneous binding of CD11b/CD18 on neutrophils and fibrinogen GPIIb/GPIIIa on platelets strengthen neutrophil adherence to platelets (Hidari *et al.*, 1997).

2.11.3 Platelet Activating Factor (PAF)

Platelet activating factor, also known as PAF, is a naturally occurring active phospholipid that is secreted by inflammatory cells, platelets, and endothelial cells. Because PAF has not received as much attention as other biochemical mediators and pathways, it is simple to ignore its potential contribution to pathogenesis. On the other hand, there is a growing interest in PAF as a significant mediator of normal physiological function, and research into it is still ongoing. On the other hand, pathogenic effects can be brought about by PAF pathways that are dysfunctional or improperly regulated.

2.11.4 Functions of PAF

PAF plays a significant role in the activation of the thrombotic cascade as well as inflammatory reactions. It accomplishes this by activating a receptor on target cells, which then leads to the production of a variety of inflammatory mediators such as prostaglandins, cytokines, and other types of inflammatory

mediators. There is evidence that the condition can also affect other body systems, including the cardiovascular system, the nervous system, and the respiratory system (Imaizumi *et al.,* 1995). The followings are some of its function:

Liver: PAF enhances glycogenolysis by the liver. It induces breakdown of glycogen by hemodynamic effects instead of enzymatic regulation (Buxton *et al.,* 1986).

Heart: PAF may affect the heart directly or indirectly. Directly, it can change heart rate and contractility. Indirectly, it can induce endothelial cells or inflammatory cells to release thromboxane A2 (TXA2) or TNF-alpha production, which results in the same alteration. These molecules can cause coronary artery constrictions and can even contribute to arrhythmias (Evangelou *et al.,* 1994; Feuerstein *et al.,* 1997).

Kidneys: The primary source of PAF in the body is the kidneys. A nephric patients and animals undergone bilateral nephrectomy have undetectable levels of PAF in the blood. The glomerulus in the nephron contains the most PAF receptors. In vivo, it is difficult to measure PAF renal effects due to PAF's systemic hemodynamic effects. PAF lowers cardiac output, circulation volume, and blood pressure. Leading to low glomerular filtration rate (GFR), urine flow and renal plasma flow along with high renal vasculature resistance. Also along the nephron there is an elevation in potassium and sodium reabsorption (Schlondorff *et al.,* 1986).

Lungs: In the lungs, PAF enhances the release of leukotrienes D4 and C4. This results in fluid loss from the microvasculature, which may be a crucial event in allergic or inflammatory diseases development. PAF may also leads to severe bronchoconstriction (Voelkel *et al.,* 1982).

2.11.5 Platelet Activating Factor Pathogenesis

Although PAF promotes a natural inflammatory response to allergens and infectious processes, researchers have hypothesized that PAF-mediated physiological actions could become pathogenic in the presence of excessive activity or dysregulation. The presence of inflammation and leukocytes stimulates the PAF production cycle (Zimmerman *et al.*, 2002). The danger of pathogenicity develops when PAF synthesis or termination cycles are disrupted as a result of illness or individual genetic variation in physiology. Because PAF induces localized inflammation, it acts as a "beacon" for additional granulocyte, monocyte, and macrophage recruitment. The migration of leukocytes to the damaged location feeds the PAF production cycle even further, resulting in a rise that can overcome intrinsic regulatory systems designed to suppress PAF (Zimmerman *et al.*, 2002).

Allergic stimuli and infection are powerful inducers of PAF-mediated changes in vascular permeability, which cause localized swelling, edema, hypotension, and cytokine release. Pathogenicity can develop when these reactions in key organ systems are exacerbated. Examples include the respiratory system, where increased vascular permeability increases pulmonary edema and infiltration, impairing pulmonary function (Zimmerman *et al.*, 2002).

2.11.6 PAF in COVID-19 Related Acute Respiratory Distress Syndrome (ARDS)

Among the first clinically documented COVID-19 symptoms was acute pneumonia, which resulted in Acute Respiratory Distress Syndrome (ARDS) and required mechanical ventilation. Whereas the mechanism of PAF in ARDS caused by COVID-19 is unknown, multiple research have looked at PAF as an initiator of acute lung damage and ARDS in the area of bacterial sepsis (Prescott *et al.*, 2000). According to in vitro studies, exogenously given PAF enhances vascular permeability and pulmonary edema in animal models. Furthermore, in comparable

animal models, inhibiting PAF activity lowers the development of pulmonary edema (Zimmerman *et al.*, 2002). The significance of these findings for human patients are unclear. Prior researches has also looked at the nature of PAF acetylhydrolase deficiency and its link to asthmatic symptoms (Miwa *et al.*, 1988). Acetylhydrolase is an enzyme responsible for stopping PAF activity by recycling PAF back into its intermediate precursor. Researchers studied PAF acetylhydrolase deficiency in Japanese children due to autosomal recessive genetics and discovered that the incidence of serum PAF acetylhydrolase deficiency was much greater in those who had severe asthma symptoms. Furthermore, PAF has been demonstrated to enhance mucus production, bronchial inflammation and bronchoconstriction in asthmatic patients (Miwa *et al.*, 1988; Hsieh *et al.*, 1993).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Materials

3.1.1 Equipment and Instruments

The following equipment were used in the current study. See (table 3.1 and 3.2).

Table 3-1: Instruments and Equipment and their Manufacturing Country and Company

Instruments and equipment	Manufacturing Company	Country
ELISA Devices (washer & reader)	Bio kit ELx800	U.S.A
Biochemical Analyzer	Cobas C111	Germany
Freezer	Panasonic	Korea
Haematology analyser	Sysmex XN 350	Japan
Incubator	Memmert	Germany
Optical Coagulion Analyzer	Wondfo	Germany
Refrigerator	Panasonic	Korea
Water bath	GFL	Germany
Water distillatory	GFL	Germany

Table 3-2: Tools and their Origin Country

Tools	Country
Cold medical box	China
Clot Activator and Gel Tube	China
Cylinders (250,500 ml)	Germany
EDTA tube	China
Eppendorf tube (0.5 ml & 1.5 ml)	China
Filter paper	China
Flasks (various size)	China
Disposable Gloves	China
Micropipettes (various size)	Japan
Tips (Blue & Yellow)	China

3.1.2. ELISA Kits

In the current study, four ELISA kits were used as shown in table (3-3).

Table 3-3: The Study's ELISA kits

ELISA Kit	Manufacturing Company	Country
Human platelet activating factor	Bioassay technology Laboratory (BT LAB)	China
Human intercellular-adhesion molecule-1		
Human vascular cell adhesion molecule		
Human membrane attack complex		

3.1.2.1. The Contents of ELISA Kits of Human (ICAM-1, VCAM-1, MAC, PAF)

All contents of the four kits (PAF, MAC, ICAM-1 and VCAM-1) were the same except the standard solutions and the biotinylated human antibodies that differ according to the kits.

Table (3-4): ELISA Kits Contents of Human (ICAM-1, VCAM-1, MAC, PAF)

Components	Quantity / Kit
1. ICAM-1 Standard Solution (6400ng/ml) VCAM-1 Standard Solution (240ng/ml) MAC Standard Solution (4800ng/ml) PAF Standard Solution (64ng/ml)	0.5ml x1
2. Pre-coated ELISA Plate	12 * 8 well strips x1
3. Standard Diluent	3ml x1
4. Streptavidin-HRP	6ml x1
5. Stop Solution	6ml x1
6. Substrate Solution A	6ml x1
7. Substrate Solution B	6ml x1
8. Wash Buffer Concentrate (25x)	20ml x1
9. Biotinylated human ICAM-1 Antibody Biotinylated human VCAM-1 Antibody Biotinylated human MAC Antibody Biotinylated human PAF Antibody	1ml x1
10. User Instruction	1
11. Plate Sealer	2 pics
12. Zipper bag	1 pic

3.2 Subjects and Study Design

3.2.1. Subjects

The focus of this research was the correlation of ICAM-1, VCAM-1, MAC and PAF levels with immunothrombosis in COVID-19 patients treated at Imam AL-Hussein Medical City in Kerbala province during the time period extending from October 2021 to May 2022.

In accordance with the WHO, real-time PCR and CT scans were used to diagnose COVID-19 in all patients.

A total of 82 patients with COVID-19 were admitted to the hospital, divided of 44 males and 38 females. Their ages ranged between 25 to 85 years old.

These patients divided into three groups: mild (27 patients: 14 males and 13 females); severe (27 patients: 14 males and 13 females); and critical (28 patients:17 males and 11 females). Patients were chosen at random from the local community, taking into consideration their ages and sex.

3.2.2. Inclusion and Exclusion Criteria:

3.2.2.1. Inclusion criteria

1. Patients with completed data.

2. Patients with positive PCR for COVID-19.

3.2.2.2. Exclusion criteria

1. Patients with uncompleted data.

2. Patients on Tocilizumab (Actemra).

3. patients with immunological disorders.

3.2.3 Sample Collection

Blood samples were taken from all participants and each sample is divided into three parts:

Part one- placed in EDTA tube for hematological tests Complete blood count (CBC).

Part two- transferred into sodium citrate tube to perform (D-dimer, PT, PTT).

Part three – in a gel tube for chemical tests (C. reactive protein, B. urea, S.creatinine) and immunological tests (ICAM-1 and VCAM-1, MAC and PAF).

3.2.4 Patients Classification According to Disease Severity

The patients clinically classified according to some features that approved by World Health Organization (WHO,2022):

1. Mild Illness: dry cough, mild fever, nasal congestion, sore throat, headache, muscle pain and malaise.

2. Moderate Illness: cough, shortness of breath and tachycardia.

3. Severe Illness: severe pneumonia, acute respiratory distress syndrome, sepsis and SpO2 < 94%.

4. Critical Illness: respiratory failure, septic shock, and/or multiple organ dysfunction.

Note: - in the current study the mild and moderate cases regarded as one group.

3.2.5 The Study Design

This is a cross-sectional study that included 82 patients with COVID-19, who were categorized as having either mild, severe, or critical symptoms as in figure (3-1).

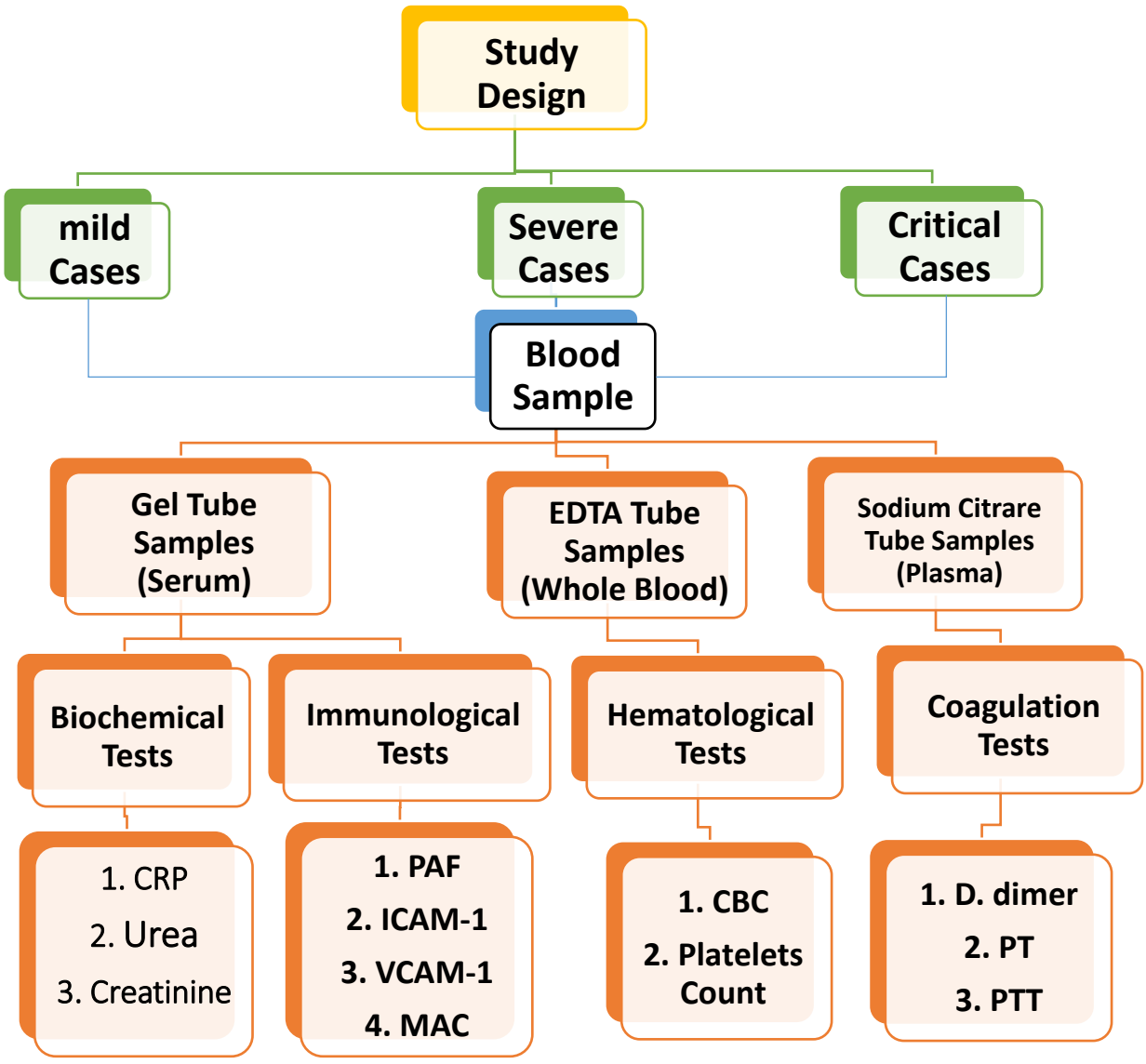

Figure (3-1): The Study Design.

3.3 Ethical and scientific approval

The ethical side was obtained from the Kerbala Health Department. In addition, prior to taking the sample, verbal consent from the participants in the study was obtained before the procedure was carried out.

3.4 Data Collection

The medical records of all COVID-19 patients who had positive results from a real-time RT-PCR test for SARS-CoV-2 were examined. During the hospitalization period, both demographic data and laboratory parameters were collected. All data was reviewed and verified by a group of licensed medical professionals.

An interview and/or a questionnaire were used to collect demographic and clinical data from patients. The patients themselves or their relatives participated in the interviews.

3.5 Questionnaire

In order to collect data from patients who had COVID-19, a questionnaire prepared to get information for COVID-19 patients in Imam AL-Hussein Medical City. Under the supervision of a consultant this questionnaire was designed, by taking into consideration both international and local standards.

3.6 Methods

3.6.1 Measurement of human hematological parameters (CBC)

An automated hematology analyzer device was used to determine the human complete blood count (CBC). The analysis was performed on Sysmex XP-300 (Japan) automated assay.

3.6.1.1 Assay procedures

1. All reagents were placed at room temperature at least 24 hours prior to testing.

2. At least 1 ml whole blood samples were taken from patients and collected in EDTA tubes and mixed well for 10 minute.

3. The instrument was turned on and waited for it to be ready.

4. After the device had been ready, the mixed sample putted under sample probe, and in that condition, the start switch was pressed. The analysis started.

5. The status display indicated (Aspirating). When sample aspiration completed, status display (Aspirating) changed to (Running).

6. When (Running) was displayed, the sample was removed safely.

7. The analysis results for all parameters were displayed about 60 seconds after starting the analysis.

3.6.2 **Measurement of Human C-reactive Protein (CRP)**

Measurement of CRP was done by full automated the Roche cobas C 111 that is an in vitro diagnostic test system designed to quantitatively determine the C-reactive protein (CRP) in human capillary whole blood and serum by photometric measurement.

3.6.2.1 Test Principle

Particle enhanced immunoturbidimetric assay. Human CRP agglutinares with latex particles coated with monoclonal anti-CRP antibodies. The aggregates are determined turbidimetrically.

3.6.2.2 Procedures

1. Sample type: serum or EDTA K2/K3 and lithium heparin anticoagulated plasma.

2. Sample volume: 4 µl.

3. Pipetting parameters:

   a. R1 (Buffer with bovine serum albumin; preservatives) 150 µl.

   b. Sample 2 µl + 10 µl diluent (H_2O).

   c. SR (Latex particles with anti-CRP (mouse) in glycine buffer; immunoglobulins (mouse); preservative 48 µl + 14 µl diluent (H_2O).

4. Storage and stability: 2 weeks in 15 – 25 C˚ or 3 weeks in 2-8 C˚.

5. Measuring mode: Absorbance end point calculation mode.

6. Wave length A: 552 nm.

7. Unite: mg/l.

8. Reaction time: 10 minute.

3.6.3. Measurement of Human Blood Urea (BUN)

Measurement of Urea/BUN was done by full automated the Roche cobas C 111 that is an in vitro diagnostic test system designed to quantitatively determine Urea/BUN in human capillary whole blood, serum and urine by photometric measurement.

3.6.3.1 Test Principle

Urea is hydrolyzed by urease to form ammonium and carbonate.

$$\text{Urea} + 2\ H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + CO3^{2-}.$$

In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD+ for each mole of urea hydrolyzed.

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O.$$

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen and is measured photometrically.

3.6.3.2 Procedures

1. Sample type: serum or EDTA K2/K3 and lithium heparin anticoagulated plasma.

2. Sample volume: 4 µl.

3. Pipetting parameters:

   a. R1 (NaCl 9 %) 10 µl + 90 µl.

   b. Sample 2 µl

   c. R2 TRIS buffer: 220 mmol/L, pH 8.6; 2-oxoglutarate: 73 mmol/L;

   NADH: 2.5 mmol/L; ADP: 6.5 mmol/L; bean): ≥ 300 µkat/L; GLDH (bovine liver): ≥ 80 µkat/L; preservative; nonreactive stabilizers 38 µl + 110µl (H2O).

4. Stability and storage: 2-8 C˚ , See expiration date on cobas c 111 pack label.

5. Measuring mode: Absorbance end point calculation mode.

6. Wave length A: 700/340 nm.

7. Unite: mg/dl.

8. Reaction time: 10 minute.

3.6.4 Measurement of Human Creatinine (CREJ2)

Measurement of creatinine was done by full automated the Roche Cobas c 111 that is an in vitro diagnostic test system designed to quantitatively determine creatinine in human capillary whole blood, serum and urine by photometric measurement.

3.6.4.1 Test principle

This kinetic colorimetric assay is based on the Jaffé method. In alkaline solution, creatinine forms a yellow-orange complex with picrate. The rate of dye formation is proportional to the creatinine concentration in the specimen. The assay uses "rate-blanking" to minimize interference by bilirubin. To correct for non-specific reaction caused by serum/plasma pseudo-creatinine chromogens, including proteins and ketones, the results for serum or plasma are corrected by -26 µmol/L (-0.3 mg/dL). Creatinine + picric acid $\xrightarrow{\text{Alkaline pH}}$ yellow-orange complex

3.6.4.2 Procedures

1. Sample type: serum or EDTA K2/K3 and lithium heparin anticoagulated plasma.

2. Sample volume: 4 µl.

3. Pipetting parameters:

a. R1 (Potassium hydroxide: 900 mmol/L; phosphate: 135 mmol/L; pH ≥ 13.5; preservative; stabilizer) 13 µl + 77 µl diluent (H2O).

b. Sample 10 µl .

c. R2 (Picric acid:38 mmol/L; pH 6.5;non-reactive buffer): 17 µl+30 µl (H2O).

4. Stability and storage: Until the printed expiration date on pack label at 2-8 °C.

5. Measuring mode: Absorbance end point calculation mode.

6. Wave length A: 570/505 nm.

7. Unite: mg/dl.

8. Reaction time: 10 minute.

3.6.5 Measurement of Human D-dimer Level

Quantitative immunological tests for the detection of d-dimer in heparinized venous blood done by the Cobas c 111 instrument.

3.6.5.1 Test Principle

The test consists two monoclonal antibodies against fibrin breakdown products with the d-dimer structural feature. One of the antibodies is gold-labeled, while the other is biotinylated. In the blood, the antibodies form a sandwich combination with the d-dimer. After the erythrocytes have been removed from the sample, the plasma passes through the detecting zone, where the gold-labeled d dimer sandwich complexes gather and the positive signal is presented as a crimson line (the signal line). Excessive gold-labeled antibodies accumulate along the control line, indicating that the test was successful. The signal line's strength rises in proportion to the concentration of d dimers. The instrument's optical system detects the two lines and measures the strength of the signal line. The embedded software translates the signal strength to a quantifiable value, which is shown on the screen.

3.6.5.2 Procedures

1. Sample type: heparinized venous or sodium citrate whole blood.

2. Sample volume: 150 µl.

3. Reagents: Biotinylated mouse monoclonal anti-d-dimer antibodies ≥ 1.0 µg

Gold-labelled mouse monoclonal anti-d-dimer antibodies ≥ 1.0 µg

Buffer and non-reactive components ≥ 2.8 mg

4. Stability and storage: Until the printed expiration date on cobas c 111 pack label at 2-8 °C.

5. Measuring mode: Absorbance end point calculation mode.

6. Unite: ng/ml. 7. Reaction time: 15 minute.

3.6.6 Measurement of Human Prothrombin Time (PT)

The prothrombin time reagent kit (clotting) is intended to be used along with optical coagulation analyzer (Model No.: OCG-102) to provide quantitative measurement of prothrombin time (PT) and international normalized ratio (INR) in citrated venous whole blood.

3.6.6.1 Test principle

The test is performed by inserting a test strip into the optical coagulation analyzer (Model No.: OCG-102). The instrument contains attest chamber which worms the test strip to the required temperature. The test strip contains a rotating, spoked wheel that draws the sample into the reaction well after it is applied to the sample receptacle. The spokes rotate across the path of a light beam and mix the liquid sample clots, the is picked up by spokes, interrupting the path of light beam that is detected by the instrument. An internal timer measures the elapsed time between the start of the test and the clot formation.

3.6.6.2 Procedures

1. Fresh venous whole blood was collected in trisodium citrate anticoagulant tube and mixed gently by inverting it for several times.

2. The instrument powered on and test strip removed from it was foil pouch and then inserted into the instrument as instructed on the display screen.

3. The instrument heated the test strip to the required temperature.

4. 20 µl of sample was loaded into the sample well.

5. The esults appeared on the main screen within a few minutes and the used test strip was removed from the instrument.

3.6. 7. Measurement of Human Serum (ICAM-1, VCAM-1, MAC, and PAF) Levels:

A Bioassay Technology Laboratory (BT LAB, China) ELISA kits were used in the analysis of serum to determine the human serum (PAF, MAC, ICAM-1 and VCAM-1) concentration. The analysis was performed on a BioTek ELx50 automated immunoassay analyzer (BioTek, USA) (Cat.No E1156Hu).

3.6. 7.1 The Principle of the Test:

These kits are Enzyme-Linked Immunosorbent Assays (ELISA). The plates have been pre-coated with human (ICAM-1, VCAM-1, MAC, and PAF) antibodies. (ICAM-1, VCAM-1, MAC, and PAF) present in the samples are added and bind to antibodies coated on the wells. Next biotinylated human (PAF, MAC, ICAM-1 and VCAM-1) antibodies are added and bind to (PAF, MAC, ICAM-1 and VCAM-1) in the samples. After that Streptavidin-HRP is added and binds to the Biotinylated antibodies. After incubation unbound Streptavidin-HRP has been washed away during a washing step. Substrate solutions are then added and color develops in proportion to the amount of human (ICAM-1, VCAM-1, MAC, and PAF). The reactions were terminated by addition of acidic stop solutions and absorbance measured at 450 nm.

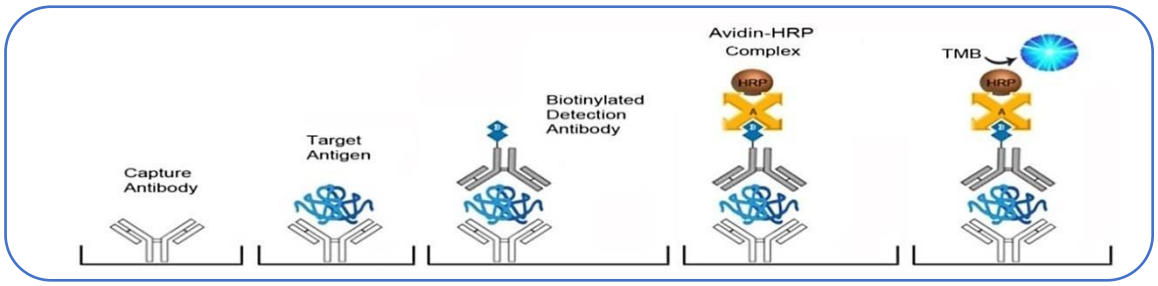

Figure 2.1: Principle of Sandwich ELISA

3.6.7.2. Calculation of Results

A standard curve is created by plotting the mean OD of each standard on the vertical (Y) axis versus the focus on the horizontal (X) axis and plotting a best-fit curve through the points on the graph. These calculations did by the Excel program and the fit line is determined through regression analysis.

3.6.7.3 Assays Procedures

All procedures of the four kits (ICAM-1, VCAM-1, MAC, and PAF) are the same except the fourth step in which the anti-antibody differs according to the test.

1. As directed, all samples, standard solutions, and reagents were prepared. Before use, all reagents were brought to room temperature. The experiment was carried out at room temperature.

2. The number of assay's strips is calculated, then were placed in the appropriate number of frames. The strips were kept at 2 to 8 °C.

3. 50µl of standard was added to standard wells. Note: antibody didn't add to standard well because the standard solution already contains antibody that has been biotinylated.

4. 40µl of sample was added to sample wells and then 10µl (anti-PAF antibody, anti- MAC antibody, anti- ICAM-1 antibody and anti- VCAM-1antibody) were added to sample wells, then 50µl of streptavidin-HRP were added to sample wells and standard wells (Not blank control well). After well mixing, the plate was covered with a sealer and then incubated for 60 minutes at 37°C.

5. The sealer was removed and the plate washed 5 times with wash buffer. For each washing, the wells were submerged in at least 0.35 ml of wash buffer for a period ranging from 30 seconds to 1 minute.

6. 50µl of substrate solution A was added to each well and then 50µl of substrate solution B was added to each well. Next the plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

7. 50µl of Stop Solution is added to each well and the blue color changed into yellow immediately.

8. The optical density (OD value) of each well was determined immediately using a microplate reader (450 nm within 10 minutes) after adding the stop solution.

3.6.7.4 Statistical Analysis

The data was loaded into a Specific Software Statistical Package for the Social Sciences (SPSS) version 21 for Windows computer for statistical analysis. The results were presented as mean standard deviation (mean ± SD). When a *p*-value was less than 0.05, it was deemed statistically significant; a *p*-value less than 0.001 was considered highly significant. In addition, the Pearson correlation (*r*- value) used to explain the relation among ICAM-1, VCAM-1, MAC and PAF levels with hematological, biochemical and coagulation parameters.

Chapter Four

Results & Discussions

4. Results and Discussions

4.1 Demographic Data of the Studied Groups

As shown in table (4-1), a total of 82 patients with COVID–19 determined by SARS-COV-2 specific real-time PCR on nasopharyngeal (NP) swab specimens were included in the current study. The patients were either hospitalized or received care at Imam Al-Hussein Medical City in Kerbala from October 2021 to May 2022.

According to SpO_2 percentage and respiratory rate (RR), COVID-19 patients were classified into mild ($SpO_2 \geq 94$, $RR \leq 22$), severe ($SpO_2 \leq 93$, $RR \geq 30$) and critical which were the same as severe but required mechanical support such as ventilator and Continuous positive airway pressure (CPAP) therapy. Computerized topography (CT) percentage was taken into consideration, and it was elevated with the severity of disease. As a result, the following patient groups were chosen (n=27 mild, n=27 severe, and n=28 critical). There were 44 (53.7%) males and 38 (46.3%) females among them. The average age was 59.9 years, with a range of 25 to 85 years.

Vaccination with the Corona virus vaccine was inversely proportional to disease severity, with more people vaccinated in mild cases than in severe and critical cases.

As for smoking, it increased the severity of the disease, as it was noted that the number of smokers was more in severe and critical cases. Similarly, diabetes mellitus, hypertension, renal failure and heart failure all these chronic diseases related with more COVID-19 severity according to the current study.

Table (4-1): Demographic Data of the Studied Groups:

Total number			**82**	
Age			**Mean (59.9) year** **Average (25-85) year**	
		Mild (N=27)	**Severe (N=27)**	**Critical (N=28)**
Sex	**Male No.(%)**	**14 (51.9%)**	**14 (51.9%)**	**17 (60.7%)**
	Female No.(%)	**13 (48.1%)**	**13 (48.1%)**	**11 (39.3%)**
SpO_2 % Mean ± SD		**96%** ± 2	**89%** ± 4	81% ± 3
Respiratory Rate (RR) Mean ± SD		**22.1** ± 5.4	**36.3** ± 6.1	**40.8** ± 9.1
Computerized Topography (CT%)		**12%** ± 4	**39%** ± 14	**57%** ± 11
Vaccination %		**34%**	**22%**	**10%**
Smoking %		**6%**	**13%**	**27%**
Diabetes %		**25%**	**46%**	**48%**
Hypertension %		**14%**	**56%**	**58%**
Renal Failure %		**8%**	**11%**	**16%**
Heart Failure %		**3%**	**9%**	**13%**

There was no significant difference between female and male patients, according to this table. This agrees with Wang *et al*, (2020) who claimed that the percentage of men and women among ICU patients and non-ICU patients was the same. Despite the fact that there were no statistically significant differences between gender and illness severity in

this specimen, male gender was discovered to be a risk factor for disease severity by Abate *et al.*, (2020) and Gebhard *et al.*, (2020) who found that the frequency of symptomatic COVID-19 was greater in males than in women.

Oxygen saturation (SpO2) and respiratory rate (RR) were significantly correlated with disease severity. The patients with severe and critical disease had a significantly higher respiratory rate as compared to mild patients, while the SpO2 of those with severe and critical disease was significantly lower as compared to those with mild disease. All patients with critical illness required mechanical ventilation. These findings were similar to those mentioned by Suresh *et al.*, (2021), in which they concluded that patients with severe and critical disease have significantly higher RR and significantly lower SpO2 than mild patients.

4.2 Age Distribution for COVID-19 Studied Patients

In table (4-2), the ages of the patients who were subjects of the study distributed into three groups; the first young group aged from 25–45 years, and they mostly occurred in mild cases and decreased in severe and critical cases. The second post-young group that aged from 46–65 years old showed a decrease in mild cases and an increase in both severe and critical cases. The third elderly group ranged in age from 66 to 85 years old, and they mostly presented in critical and severe cases, with a decrease in mild cases. This means that the severity of the disease significantly increased with aging, and the elderly were more susceptible to severe and critical diseases. This means that there was a significant correlation between patients' age and disease severity.

Table (4-2): Age Distribution for COVID-19 Studied Patients:

Age group (yrs)	Mild (N=27) No. (%)	Severe (N=27) No. (%)	Critical (N=28) No. (%)	*p*- value
(25 – 45) Young	**18** (66.6)	**7** (25.9)	**3** (10.7)	***p* = 0.267**
(46 – 65) Post young	**5** (18.56)	**8** (29.7)	**5** (17.9)	***p* = 0.134**
(66 – 85) Elderly	**4** (14.8)	**12** (44.4)	**20** (71.4)	***p* < 0.001**
Total	**27** (100)	**27** (100)	**28** (100)	

The explanation for this link is that older people frequently had low immunity and are suffering from one or more chronic conditions such as (hypertension, diabetes mellitus, smoking, etc...), resulting in increased complications and illness severity. A comparable set of data was recently published by Statsenko et al., (2022) which in their investigation, they discovered that older people had the highest percentage of critical cases. Teixera *et al.;* (2022) mentioned that the pro-inflammatory cytokines IL-1β, IL-6 and TNFα showed higher expression in the elderly patients due to an increase in the rate of cell death, which leads to the release of cytokines inside the cells, thus suggesting that aging could be considered a modifier of more severe disease.

4.3 Mortality Distribution within the Groups of COVID-19 Patients

Table (4-3) shows the number of cases in each COVID-19 patients classified groups (27mild, 27 severe and 28 critical). The numbers and percentages of mortality are showed in this table. There were no dead cases in mild group, while 3 patients are dead within severe group and they represented 3.6% from total patients' groups. The mortality number in critical group was higher more than three folds from severe group, there were 11 deaths within critical group and they represented 13.4% from total patients. The total mortality percentage was 17% and all patients who died were between (66-85) years old.

Table 4-3: Mortality distribution within the groups of COVID-19 patients:

Classified Groups	No. of Cases	Mortality distribution within age groups No. (%)			
		Young	Post young	Elderly	Total
Mild	**27**	**0**	**0**	**0**	**0**
Severe	**27**	**0**	**1** (1.2%)	**2** (2.4%)	**3** (3.6%)
Critical	**28**	**0**	**1** (1.2%)	**10** (12.2%)	**11** (13.4%)
Total	**82**	**0**	**2** (2.4%)	**12** (14.6%)	**14** (17%)

As shown in above, mortality rate increased with disease severity and the patients aging in this study because no death recorded within mild patients, while 3 deaths in severe cases and 11 deaths in critical cases were recorded. These outcomes suggested that younger individuals were more prone to have mild symptoms, and they were less prone to die, but older individuals were more likely to die and had severe symptoms. This was agreed upon by a large number of other researchers, including (Kang *et al*., 2020) which stated that elderly COVID-19 patients had a significant death rate due to a high case fatality rate and symptomatic infection rate.

4.4 Hematological Parameters in COVID-19 Patients

Table (4-4) displays the hematological parameters of subjected patients. Total white blood cell counts were not significantly elevated (p= 0.253), but the percentage of neutrophils was significantly elevated in severe and critical compared with mild COVID-19 cases ($p < 0.001$). In contrast, the percentage of lymphocytes significantly decreased with COVID-19 severity (p= 0.001). Hemoglobin (Hb) levels also significantly decreased with disease progression (p = 0.001).

Table (4-4): Hematological parameters in COVID-19 patients classified according to degree of disease severity:

Variables	Normal range	Mild Mean ± SD	Severe Mean± SD	Critical Mean ± SD	P- value
White blood cell counts x10^9/L	4.0 – 11.0	11.73 ± 4.77	13.09± 5.92	11.43± 5.12	p = 0.253
Neutrophils %	40-75 %	73% ± 12	↑80% ± 8	↑86% ± 5	$p < 0.001$
Lymphocytes %	20 – 40 %	↓18% ± 7	↓14% ± 5	↓ 8% ± 3	$p < 0.001$
Haemoglobin g/l	13.5 – 17.5	13.8 ± 1.8	↓11.5 ± 1.5	↓10.8 ± 1.8	p = 0.001

Neutrophil percentage elevation may be due to increase in inflammatory process and excessive cytokines release (cytokine storm). This explanation is similar to that mentioned by Masso *et al.*, (2022) who reported that the elevation in neutrophils associated with increased secretion of some cytokines that include IL-8, IL-6, interferon 10 (IP-10), granulocyte- macrophage colony stimulating factor (GM-CSF), IL-1b, IL-10 and TNF.

While lymphocyte percentage decreasing may be due to functional depletion of lymphocytes such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, which were involved in viral infection management. Zheng *et al.*, (2020) had found that the overall number of CTLs and NK cells was significantly reduced in SARS-CoV-2 patients. These findings implied that SRAS-CoV-2 infection was linked with cytotoxic lymphocyte functional depletion.

Hemoglobin significantly decreased in severe and critical cases in comparison with mild cases in both males and females. The explanation of this was that the inflammation in COVID-19 patients can lead to an alternation of iron hemostasis and reduced intestinal iron absorption, resulting in the reduced availability of the metal for erythropoiesis and the production of hemoglobin (anemia of inflammation). Another cause of Hb decrease was impaired renal function that may occur in severe and critical COVID-19 cases, where the kidneys were the primary organs that regulate erythropoiesis. This agreed by Bellmann-Weiler *et al.*, (2020), who reported that the elevated expression of ferritin during COVID-19 infection lead to alteration in iron homeostasis and retention within macrophages along with reduced intestinal iron absorption. This resulted in reduced metals for erythropoiesis. The patients with anemia were older and had a reduced renal function, and had significantly higher levels of inflammatory markers such as C-reactive protein or interleukine-6 (IL6).

However, another study found that there was no significant correlation between hemoglobin levels and disease severity (Taj *et al*., 2021).

4.5 Some Biochemical Parameters in COVID -19 Patients

Three biochemical parameters were shown in Table (4-5). CRP showed the most prominent elevation in the mild cases, where it significantly increased more than three times in comparison to the normal range, and it increased in severe and critical more than mild cases ($p < 0.001$). This makes it the most sensitive COVID-19 biomarker predictor, particularly for mild cases. Blood urea (BUN) level was slightly elevated in the mild cases, however, it was significantly increased in the severe and critical cases ($p < 0.001$). Serum creatinine in mild patients was within the normal range. However, S. creatinine levels were significantly elevated in severe and critical patients in comparison with mild patients ($p < 0.001$).

Table (4-5): Some Biochemical Parameters in COVID -19 Patients Classified According to Degree of Disease Severity:

Variable	Normal Range	Mild Mean ± SD	Severe Mean ± SD	Critical Mean ± SD	*p*- value
C-reactive protein (CRP) mg/l	**0 – 8**	**↑20.0 ± 9.6**	**↑47.9 ± 20.4**	**↑63.4 ± 25.6**	***p* < 0.001**
Blood urea (BUN) mg/l	**15 – 45**	**↑54.5 ± 20.2**	**↑71.3 ± 34.8**	**↑121.1 ± 59.5**	***p* < 0.001**
Serum creatinine (S.creatinine) mg/l	**0.6 - 1.2**	**1.0 ± 0.4**	**↑1.3 ± 0.6**	**↑1.9 ± 0.8**	***p* < 0.001**

C-reactive protein is an acute-phase protein of that increase following interleukin-6 secretion by macrophages and T cells. Therefore, SAR-CoV-2 infection significantly caused CRP secretion from the beginning of the disease and it more elevated with COVID-19 severity. This agreed by Azar *et al.*, (2022) who mentioned that CRP levels were generally low in viral infections, but adaptive

immunity appeared to be required for COVID-19 virus clearance, and the macrophage activation syndrome might explain the elevated serum CRP levels and contribute to illness severity. Cytokines such as (IL-6, TNF-α) stimulate hepatocyte to produce CRP during a cytokine storm that could be triggered by the process of COVID-19 pneumonia.

Blood urea and serum creatinine levels elevation during COVID-19 might indicated early injury of the kidney. One possible explanation of the high prevalence of kidney involvement was the systemic immune response to the SARS-COV-2 could be detrimental in some patients, leading to so-called a cytokine storm. Therefore, the kidney may be a susceptible target of this novel coronavirus. Werion *et al*., (2022) agreed with these results, they proved the fact that proximal tubule cells in the kidneys highly express ACE2 suggested that they could be targeted by SARS-CoV-2 at an early stage of disease. The binding affinity of SARS-CoV-2 spike glycoprotein to ACE2 was a major determinant of disease severity.

4.6 Some Coagulation Parameters in COVID -19 Patients

Table (4-6) showed some coagulation parameters in COVID-19 patients. In mild cases no one of these indices increased more than normal range, but in severe and critical cases, only D-dimer levels increased to more than the normal. Additionally, this elevation was statistically more significant ($p < 0.001$). There is no significant elevation in platelets count, prothrombin time and partial thromboplastin time (p=0.422; p=0.053, p=0. 0.256, respectively). Therefore, D-dimer was considered the single routine marker that indicated the presence of thrombosis (immunothrombosis) in COVID-19 patients during this study. All COVID-19 related mortalities in this study were associated with high D-dimer levels. This indicated that D-dimer was a significantly correlatesd with disease progression, hypercoagulability that increased the risk of venous thromboembolism (VTE) events, leading to thrombo-inflammation and even death in severe and critical conditions.

Table (4-6): Some Coagulation Parameters in COVID-19 Patients Classified According to Degree of Disease Severity:

Variable	Normal Range	Mild Mean ± SD	Severe Mean ± SD	Critical Mean ± SD	*p* – value
D. dimer ng/ml	**0-500**	**328.8 ± 114.9**	**1031.5 ± 305.2**	**2351.4 ± 1133.8**	***p* < 0.001**
Platelet count $x10^9$/L	**150 - 450**	**239.23 ± 70.92**	**260.3 ± 109.3**	**231.4± 89.2**	***p*= 0.422**
Prothrombin time (PT) Sec	**10 - 14**	**13.1 ± 1.1**	**13.1 ± 1.2**	**13.9 ± 1.4**	***p*= 0.053**
Partial thromboplastin time(PTT)	**30 - 38**	**32.3 ± 2.6**	**32.6 ± 4.1**	**33.5 ± 2.6**	***p*= 0.256**

In mild COVID-19 cases, D-dimer levels were slightly elevated in the patients but remain within high normal rang. However, D-dimer levels in severe and critical cases were significantly elevated in comparison with mild cases. These result agreed with Taj *et al*., (2021) who stated that D-dimer, ferritin and LDH levels were significantly increased in patients with critical disease.

D-dimer is a fibrin breakdown product that has a mechanistic role in COVID-19 thrombo-inflammation. As a result, D-dimer can be employed as an essential coagulation biomarker that can assist establish patient screening, therapy options, and prognosis management (Bikdeli *et al*., 2020)**.**

Patients who have D-dimer levels >1000 ng/ml have a 20-fold increased mortality risk than those who have lower D-dimer levels. Therefore, D-dimer is a possible screening test for VTE in COVID-19 patients, and changing therapeutic anticoagulant dosages based on D-dimer elevation is more useful to patients than preventive doses (Zhou *et al*., 2020).

Platelet counts showed no significant correlation with disease progression. This may be due the early and good prognosis of the COVID-19 cases in this study because the platelets (PLTs) at the time of admission were not affected and the number of PLTs was normal. This agreed with Taj *et al*., (2021), who reported that platelets count did not show statistically significant association with severity of disease. Delshad *et al*., (2020) have a consensus that COVID-19-related thrombocytopenia is a delayed event during infection and thrombocytopenia in COVID-19 patients occurs in intensive care unit (ICU) and threatens the life of severe COVID-19 cases.

Prothrombin time (PT) was normal in most COVID-19 patients with just 5% who have extended PT. Results like these agreed with Taj *et al*., (2021) who said that prothrombin time did not show significant increase with COVID-19 progression.

Partial thromboplastin time (PTT) also did not show any statistically significance with the disease severity in the current study. Huang *et al*., (2020) explained that PTT is often normal in patients with COVID-19 infection and only 6% of the patients develop prolongation of PTT, and the average duration of PTT appears to be similar in COVID-19 critically ill and non-critically ill patients, with no significant correlation to disease severity or mortality. Therefore, PTT does not appear to be a reliable indicator of disease progression in COVID-19.

4.7 Some Immunological Parameters (ICAM-1, VCAM-1, MAC and PAF) Levels in COVID -19 Patient's Groups

Table 4-7 shows the levels of four immunological parameters in the patient's serum. In mild cases, ICAM-1 adhesion molecule level increased approximately more than four folds over the normal range, and dramatically elevated with the severity of disease ($p < 0.001$). VCAM-1 and C5b-5 also significantly elevated ($p = 0.002$ and p=0.047, respectively), but their elevations were slower than ICAM-1 elevation. PAF elevation in mild cases was very quickly (faster than ICAM-1, VCAM-1 and MAC elevations), but in severe and critical cases PAF level was stable within limited range.

4.7 Some Immunological Parameters (ICAM-1, VCAM-1, MAC and PAF) Levels in COVID -19 Patient's Groups:

Variable	Normal Range	Mild Mean± SD	Severe Mean± SD	Critical Mean± SD	*p* – value
ICAM-1 ng/ml	**100 – 200**	**↑831.8 ± 307.8**	**↑1550.6 ± 673.8**	**↑1875.0 ± 626.7**	***p* < 0.001**
VCAM-1 ng/ml	**0.14 – 9.0**	**↑13.9 ± 4.8**	**↑23.0 ± 4.4**	**↑30.1 ± 13.4**	***p* = 0.002**
MAC ng/ml	**< 250**	**↑483.8 ± 201.0**	**↑759.1 ± 371.8**	**↑797.6 ± 360.8**	***p* = 0.047**
PAF ng/ml	**0 – 5**	**↑126.5 ± 49.5**	**130.5 ± 56.1**	**143.3 ± 65.3**	***p*= 0.447**

The adhesion molecule (ICAM-1) is highly increased with disease progression. This may be due to the nature of ICAM-1 that is a primary mediator of cell adhesion express in the inflammatory process, and it often used to indicate the gravity of all inflammatory conditions. Therefore, the inflammatory process that occurred by SARS-CoV-2 causes increasing in ICAM-1 expression. These results agreed by Smith *et al.*, (2021) that reported Viral infection of endothelial cells caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can cause vascular changes and elevate the expression of ICAM-1 in coronavirus disease 2019 (COVID-19) patients and may be used as a biomarker to measure disease severity or recovery. ICAM-1 also contributes in the recruitment and activation of inflammatory cells and facilitates leukocyte-endothelial binding and the migration of leukocytes across the endothelial barrier (Tong *et al.*, 2021).

The roles of VCAM-1 in COVID-19 are similar to that of ICAM-1 because they belong to the same endothelial adhesion molecules. Therefore, patients with SARS-CoV-2 infection also have elevated VCAM-1 levels. However, elevation of both ICAM-1 and VCAM-1 during COVID-19 indicates endothelial damage (often in alveolar epithelium) that is caused by excessive inflammation or cytokine storm. Data similar to these were published by Spadaro *et al*., (2021), who reported that ICAM-1 and VCAM-1 which are biomarkers of endothelial damage were significantly increased in COVID-19.

In COVID-19 patients, MAC levels were significantly elevated with the disease progression in the current study. MAC elevation in the current study indicates COVID-19 induced complement activation that mainly occurs in lungs and kidneys. This agreed by Diao *et al.,* (2022), who have found that acute renal failure associated with tubular necrosis and abundant complement deposition develops in a significant percentage of patients with severe COVID-19. The complement system activation in COVID-19 can occur either directly through the

lectin, classical and/or the alternative pathways or cause endotheliopathy (endothelial cell injury and dysfunction) and thromboinflammation (inflammation associated with coagulation and thrombosis), that activate the complement system and leads to endothelial injuries by the complement terminal component MAC that binds to cells and causes cell lysis by forming pores in plasma membrane. Therefore, high serum levels of MAC were significantly associated with higher COVID-19 severity and mortality (Ali *et al*., 2021; Zinellu *et al.*, 2021).

In mild cases PAF levels elevated very quickly, but then stabled within limited range with the disease progression. The explanation is that PAF secretion was reduced by PAF inhibitors which were obtained by taking some nutritional supplements as vitamins, minerals and some medicines. This agreed with Detopoulou *et al.,* (2021) who reported that PAF synthesis is modulated by PAF inhibitors which inactivate PAF receptors and affect PAF metabolism. These inhibitors have been found to reduce the activity of PAF regulatory enzymes and/or increase the activity of the PAF degrading enzyme. Several micronutrients (vitamin A, vitamin C, vitamin E, vitamin D, selenium, omega-3 fatty acids, and minerals), and medicines such as statins and antiviral drugs act as inhibitors for PAF synthesis.

4.8 ICAM-1 and VCAM-1 with Hematological Parameters within Mild, Severe and Critical COVID-19 Patients

Table (4-8) showed the correlation of serum levels of ICAM-1 and VCAM-1 with the hematological parameters within severity groups. ICAM-1 was not correlated with total WBC count and hemoglobin within all groups. However, ICAM-1 had a strong positive and significant correlation with low lymphocyte percentages (r =0.686, p=0.002) in mild cases, but in severe cases this correlation changed to medium negative significant correlation (r = -0.496, p=0.009), while in critical cases the correlation of ICAM-1 with diseases severity did not show statistical significance (r = -0.142, p=0.461). In contrast, ICAM-1 had a strong negative and significant correlation with high neutrophils percentages (r =-0.508, p=0.037) in mild cases, but in severe cases this correlation changed to strong positive and significant correlation (r= 0.545, p=0.003) and then became weak positive not significant in critical cases (r =0.254, p=0.182). These results may indicate differential effect of ICAM-1 on lymphocytes and leukocytes in COVID-19.

VCAM-1 did not show any significant correlation with lymphocyte and leukocyte percentages nor with any other hematological parameters within the severity groups.

Table (4-8) Correlation of Serum Levels of ICAM-1 and VCAM-1 with Hematological Parameters in Mild, Severe and Critical COVID-19 Patient's Groups:

Variables		ICAM-1 Mean ± SD (831.8 ± 307.8) ng/ml	Correlation	VCAM-1 Mean ± SD (13.9 ± 4.8) ng/ml	Correlation
Mild	White blood cell counts ,x10^9/L	11.73 ± 4.77	r = 0.012 p = 0.592	11.73 ± 4.77	r = -0.285 p = 0.266
	Neutrophils%	73% ± 12	r = -0.508* p = 0.037*	73% ± 12	r = -0.001 p = 0.996
	Lymphocytes%	18% ± 7	r = 0.686* p = 0.002*	18% ± 7	r = 0.205 p = 0.429
	Hemoglobin g/l	13.3 ± 1.6	r= 0.129 p = 0.619	13.3 ± 1.6	r = 0.129 p = 0.619
Variables		**ICAM-1 Mean ± SD (1550.6 ±673.8) ng/ml**	**Correlation**	**VCAM-1 Mean ± SD (23.0 ± 4.4) ng/ml**	**Correlation**
Severe	White blood cell counts ,x10^9/L	13.1 ± 5.92	r = 0.184 p = 0.365	13.1 ± 5.92	r = 0.349 p = 0.080
	Neutrophils%	80 % ± 8	r = 0.545 p = 0.003	80 % ± 8	r = -0.092 p = 0.653
	Lymphocytes%	14 % ± 5	r = -0.496 p = 0.009	14 % ± 5	r = 0.166 p= 0.415
	Hemoglobin g/l	11.5 ± 1.5	r = 0.015 p = 0.941	11.5 ± 1.5	r = -0.024 p = 0.903
Variables		**ICAM-1 Mean ± SD (1875.0 ±626.7) ng/ml**	**Correlation**	**VCAM-1 Mean ± SD (30.1 ± 13.4) ng/ml**	**Correlation**
Critical	White blood cell counts ,x109/L	11.4 ± 5.1	r= -0.070 p = 0.716	11.4 ± 5.1	r = 0.177 p = 0.358
	Neutrophils%	86 % ± 5	r= 0.254 p = 0.182	86 % ± 5	r = 0.106 p = 0.582
	Lymphocytes%	8 % ± 3	r = -0.142 p = 0.461	8 % ± 3	r = -0.154 p = 0.422
	Hemoglobin g/l	10.8 ± 1.8	r = -0.085 p = 0.658	10.8 ± 1.8	r = -0.065 p = 0.737

The explanation of ICAM-1 significant correlation with neutrophil and lymphocyte percentages was that SARS-CoV-2 infection causes inflammatory process which leads to activation of many inflammatory cell including leukocytes that should bind to endothelium at inflammation site, ICAM-1 was necessary to leukocyte/endothelium interaction. Therefore, ICAM-1 significantly increased along with COVID-19 severity. These agreed with Nizamudeen *et al.*, (2021) they mentioned that ICAM-1 during SARS-CoV-2 infection binds and interacts with leukocyte markers (lymphocyte function-associated antigen or LFA-1 or αLβ2) and Macrophage-1 antigen (integrin αMβ2 or macrophage integrin or Mac-1) and this interaction is necessary to leukocyte adhesion to endothelial cells. Additionally, SARS-CoV-2 has a protein known as open reading frame7a (ORF7a) which has a structural homology with ICAM-1. A study reported that SARS-CoV2 ORF7a protein has a conserved Ig immunoglobulin-like fold containing an integrin binding site that provides a mechanistic hypothesis for SARS-CoV2's interaction with the human immune system. This suggests that the experimental investigation of ORF7a-mediated effects on immune cells such as T lymphocytes and macrophages (leukocytes) could help understand the disease further and develop effective treatments (Nizamudeen *et al.*, 2021).

4.9 Correlation of Serum Levels of MAC and PAF with Hematological Parameters in Mild, Severe and Critical COVID-19 Patients

Table (4-9) shows the correlation of serum levels of MAC and PAF with some hematological parameters in all COVID-19 patients groups. In mild and severe groups, there are no statistically significant correlations between MAC and the hematological parameters. However, a medium negative correlation occurred between MAC and hemoglobin levels in critical group and this correlation reach the statistical significance (r= 0.423, p=0.019).

In mild cases, PAF levels also did not show any statistically significant correlation with hematological parameters. Only a strong positive statistically significant correlation occurred between PAF and Hb in severe group (r= 0.513, p=0.007).

However, no statistically significance remain between PAF and Hb in critical group (r= -0.236, p=0.217).

(Table 4-9): Correlation of Serum Levels of MAC and PAF with Hematological Parameters in Mild, Severe and Critical COVID-19 Patients:

Variables		MAC (483.8 ± 201.0) ng/ml	Correlation	PAF (126.5 ± 49.5) ng/ml	Correlation
Mild	White blood cell counts x10^9/L	11.73 ± 4.77	*r* = -0.186 *p* = 0.473	11.73 ± 4.77	*r* = -0.212 *p* = 0.412
	Neutrophils%	73% ± 12	*r* = 0.154 *p* = 0.554	73% ± 12	*r* = -0.271 *p* = 0.291
	Lymphocytes%	18% ± 7	*r* = 0.031 *P* = 0.905	18% ± 7	*r* = 0.398 *P* = 0.113
	Hemoglobin g/l	13.3 ± 1.6	*r* = -0.098 *P* = 0.706	13.3 ± 1.6	*r* = 0.210 *P* = 0.416
Variables		**MAC (759.1 ± 371.8) ng/ml**	**Correlation**	**PAF (130.5 ± 56.1) ng/ml**	**Correlation**
Severe	White blood cell counts ,x10^9/L	13.1 ± 5.92	*r* = -0.129 *p* = 0.529	13.1 ± 5.92	*r* = 0.184 *p* = 0.367
	Neutrophils%	80 % ± 8	*r* = 0.297 *p* = 0.140	80 % ± 8	*r* = -0.195 *p* = 0.338
	Lymphocytes%	14 % ± 5	*r* = -0.204 *p* = 0.316	14 % ± 5	*r* = 0.051 *p* = 0.804
	Hemoglobin g/l	11.5 ± 1.5	*r* = 0.252 *p* = 0.214	11.5 ± 1.5	***r* = 0.513** ***p* = 0.007**
Variables		**MAC (797.6 ± 360.8) ng/ml**	**Correlation**	**PAF (143.3 ± 65.3) ng/ml**	**Correlation**
Critical	White blood cell counts ,x109/L	11.4 ± 5.1	*r* = -0.061 *p* = 0.751	11.4 ± 5.1	*r* = 0.154 *p* = 0.422
	Neutrophils%	86 % ± 5	*r* = 0.221 *p* = 0.249	86 % ± 5	*r* = -0.256 *p* = 0.179
	Lymphocytes%	8 % ± 3	*r* = -0.198 *p* = 0.303	8 % ± 3	*r* = 0.219 *p* = 0.252
	Hemoglobin g/l	10.8 ± 1.8	***r* = -0.431** ***p* = 0.019**	10.8 ± 1.8	*r* = -0.236 *p* = 0.217

In mild cases, where hemoglobin (Hb) levels began to decrease but remain within low normal range. While in severe and critical cases, Hb was down below of the normal range. These agreed with Bellmann *et al.*, (2020), who reported that Hb decreasing occurs because of the inflammation in COVID-19 patients that can lead to an alternation of iron hemostasis and reduced intestinal iron absorption resulting in intravenous hemolysis and this in turn triggers platelets activation. PAF that elevated in mild cases triggers platelets activation that is necessary to control hemolysis (Tomaiuolo *et al.*, 2016). This is the explanation of the significant correlation between PAF and hemoglobin levels in severe cases. However, PAF elevation begin to be stable within limited range during severe and critical cases. Therefore, there is no significant correlation remains between PAF and Hb in critical cases.

In critical cases, the significant correlation between MAC and Hb may be due to the increased rate of hemolysis that causes complement system activation leading to more increase in MAC levels. These agreed with Merle *et al.*, (2018), they reported that heme and red blood cell degradation products are triggers to complement activation, and increased MAC levels is an important evidence to complement activation**.**

4.10 Correlation of Serum Levels of MAC and PAF with Biochemical Parameters in Mild, Severe and Critical COVID-19 Patients

Table (4-10) showed that there is no significant correlation between increased serum levels of (MAC, PAF) and the biochemical parameters in the current study. However, a medium positive statistically significant correlation occurred between MAC and CRP levels in critical group (r= 0.388, p=0.037).

Table (4-10): Correlation of Serum Levels of MAC and PAF with Biochemical Parameters in Mild, Severe and Critical COVID-19 Patients:

Variables		MAC (483.8 ± 201.0)	Correlation	PAF (126.5 ± 49.5)	Correlation
Mild	C-reactive protein mg/l	20.0 ± 9.6	r = -0.197 p = 0.447	20.0 ± 9.6	r = 0.199 p = 0.442
	Blood urea mg/l	54.5 ± 20.2	r = 0.334 p = 0.189	54.5 ± 20.2	r = -0.263 p = 0.307
	S.creatinine mg/l	1.0 ± 0.4	r = 0.297 p = 0.246	1.0 ± 0.4	r = -0.112 p = 0.666
Variables		**MAC (759.1 ± 371.8)**	**Correlation**	**PAF (130.5 ± 56.1)**	**Correlation**
Severe	C-reactive protein	47.9 ± 20.4	r = -0.067 p = 0.742	47.9 ± 20.4	r = 0.232 p = 0.253
	Blood urea mg/l	71.3 ± 34.8	r = 0.541 p = 0.125	71.3 ± 34.8	r = -0.276 p = 0.170
	S.creatinine mg/l	1.3 ± 0.6	r = 0.137 p = 0.502	1.3 ± 0.6	r = -0.167 p = 0.414
Variables		**MAC (797.6 ± 360.8)**	**Correlation**	**PAF (143.3 ± 65.3)**	**Correlation**
Critical	C-reactive protein	63.4 ± 25.6	**r = 0.388** **p= 0.037**	63.4 ± 25.6	r = 0.187 p = 0.329
	Blood urea mg/l	121.1 ± 59.5	r = 0.131 p = 0.498	121.1 ± 59.5	r = 0.031 p = 0.869
	S.creatinine mg/l	1.9± 0.8	r = -0.198 p = 0.302	1.9± 0.8	r = -0.267 p = 0.160

C-reactive protein (CRP) acts as complement system activator, whereas it binds to C1q and activates the classical pathway of complement system leading to increased MAC levels (Mold *et al.*, 1999). This is the explanation of the significant correlation between MAC and CRP.

4.11. The Correlations Between Four Immunological Parameters (ICAM-1, VCAM-1, MAC and PAF) within Mild COVID-19 Patients

Table (4-11) showed the correlations among above mentioned parameters within mild group. MAC significantly correlated with ICAM-1 and VCAM-1 (r=0.451, p=0.025; r=0.413, p=0.033, respectively), and not significantly correlated with PAF. significantly correlated with ICAM-1 (r= 0.558, p=0.020), and not significantly with VCAM-1. While ICAM-1 shows no significant correlation with VCAM-1.

Table (4-11): Correlation Between Four Immunological Markers (ICAM-1, VCAM-1, MAC and PAF) within Mild COVID-19 Patients:

Variables	ICAM-1 (831.8 ± 307.8) ng/ml	VCAM-1 (13.9 ± 4.8) ng/ml	MAC (483.8 ± 201.0) ng/ml	PAF (126.5 ± 49.5) ng/ml
ICAM-1 (831.8 ± 307.8) ng/ml	r = 1 p= 0			
VCAM-1 (13.9 ± 4.8) ng/ml	r = 0.245 p= 0.342	r = 1 p= 0		
MAC (483.8 ± 201.0) ng/ml	r = 0.451 p= 0.025	r = 0.413 p= 0.033	r = 1 p= 0	
PAF (126.5 ± 49.5) ng/ml	r = 0.556 p= 0.020	r = 0.421 p= 0.091	r = 0.238 p= 0.357	r = 1 p= 0

According to above mentioned table, increased PAF triggers ICAM-1 expression. This in agreement with Hamilos *et al.*, (2018) who approved that PAF known to increase the expression of ICAM-1 in inflammatory process, so that a significant correlation appeared between PAF and ICAM.

Gregory *et al.*, (2002) mentioned that MAC as a part of complement system increases the expression of endothelial adhesion molecules including ICAM-1 and VCAM-1. This is the explanation of the significant correlation between MAC and ICAM-1, VCAM-1 in mild cases.

4.12 Correlation Between Four Immunological Markers (ICAM-1, VCAM-1, MAC and PAF) within Severe COVID-19 Patients

Table (4-12) showed the correlations among above mentioned markers within severe cases. Only MAC correlated more significantly than in mild cases with ICAM-1 and VCAM-1 (r=0.538, p=0.003; r= 0.506, p=0.008, respectively). There is no significant correlation between other markers.

Table (4-12): Correlation Between Four Immunological Markers (ICAM-1, VCAM-1, MAC and PAF) within Severe COVID-19 Patients:

Variables	ICAM-1 (**1550.6 ± 673.8**) ng/ml	VCAM-1 (**23.0 ± 4.4**) ng/ml	MAC (**759.1 ± 371.8**) ng/ml	PAF (**130.5 ± 56.1**) ng/ml
ICAM-1 (**1550.6 ± 673.8**) ng/ml	r = 1 p = 0			
VCAM-1 (**23.0 ± 4.4**) ng/ml	r = 0.013 p= 0.947	r = 1 p= 0		
MAC (**759.1 ± 371.8**) ng/ml	**r = 0.538** **p = 0.003**	**r = 0.506** **p = 0.008**	r = 1 p = 0	
PAF (**130.5 ± 56.1**) ng/ml	r = 0.077 p = 0.707	r = 0.355 p = 0.074	r = 0.101 p = 0.622	r = 1 p= 0

As mentioned previously, MAC as a part of complement system increases the expression of endothelial adhesion molecules including ICAM-1 and VCAM-1 (Gregory *et al.*, 2002). The is the explanation of a significant correlation between MAC and ICAM-1, VCAM-1 in severe cases. In addition, the increased significance may be due to increased disease severity that associated with more elevated levels of this parameters.

As previously mentioned, PAF elevation in severe and critical cases is stabilized within limited range, therefore no significant correlation appeared PAF and VCAM-1 with COVID-19 progression.

4.13 Correlation Between Four Immunological Markers (ICAM-1, VCAM-1, MAC and PAF) within Critical COVID-19 Patients

Table (4-13) showed the correlations among ICAM-1, VCAM-1, MAC and PAF within severe cases. Only MAC correlated significantly more than in mild and severe cases with ICAM-1 and VCAM-1 (r=0.604, p=0.0005; r= 0.565, p=0.006, respectively). And there are no significant correlations between other parameters.

Table (4-13): Correlation Between Four Immunological Markers (ICAM-1, VCAM-1, MAC and PAF) within Critical COVID-19 Patients:

Variables	ICAM-1 Mean ± SD (**1875.0 ± 626.7**) ng/ml	VCAM-1 Mean ± SD (**30.1 ± 13.4**) ng/ml	MAC Mean ± SD (**797.6 ± 360.8**) ng/ml	PAF Mean ± SD (**143.3 ± 65.3**) ng/ml
ICAM-1 (**1875.0 ± 626.7**) ng/ml	*r* = 1 *p*= 0			
VCAM-1 (**30.1 ± 13.4**) ng/ml	*r* = 0.339 *p*= 0.071	*r* = 1 *p*= 0		
MAC (**797.6 ± 360.8**) ng/ml	***r* = 0.604** ***p*= 0.0005**	***r* = 0.565** ***p*= 0.006**	*r* = 1 *p*= 0	
PAF (**143.3 ± 65.3**) ng/ml	*r* = 0.285 *p*= 0.133	*r* = 0. 213 *p*= 0. 133	*r* = 0.213 *p*= 0.265	*r* = 1 *p*= 0

In critical cases the most significant correlation was between MAC and ICAM-1, VCAM-1. This may be due to increased inflammatory and thrombotic events within critical cases. Therefore, high quantities of adhesion molecules that involve ICAM-1 and VCAM-1 required for activation and recruitment of more inflammatory cells.

Conclusions & Recommendations

Conclusions

1. ICAM-1, VCAM-1 and MAC can be considered as COVID-19 severity or follow up markers.
2. Steroids and micronutrients (vitamins) can decrease the activation of PAF.
3. All coagulation markers in the current study did not show significant correlations with the immunothrombosis, except for D-dimer, which showed significant correlations.
4. No significant correlation occurred between patient sex and disease severity in the current study.
5. COVID-19 can impair renal functions in severe and critical cases.

Recommendations

1. Using ICAM-1, VCAM-1, and MAC in the classification of COVID-19 severity.
2. Study the integration of (ICAM-1, VCAM-1, MAC, and PAF) with other coagulation factors that are more rapid in detecting thrombosis, such as (fibrinogen, protein z, and von Willebrand factor).
3. Searching for more about MAC's roles in immunothrombosis.
4. Study the other causes of PAF limited elevation in severe and critical cases.

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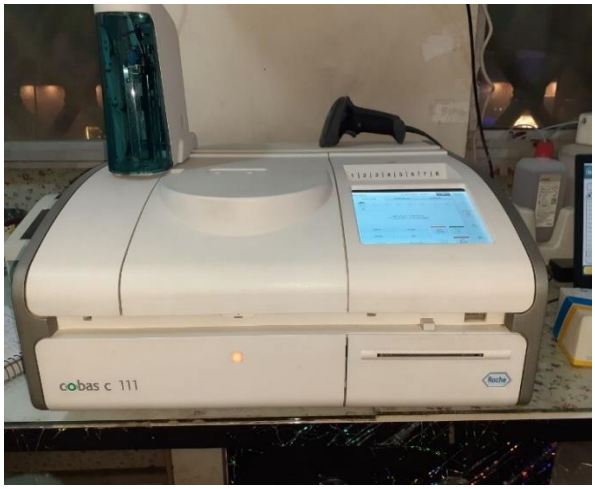
Appendix

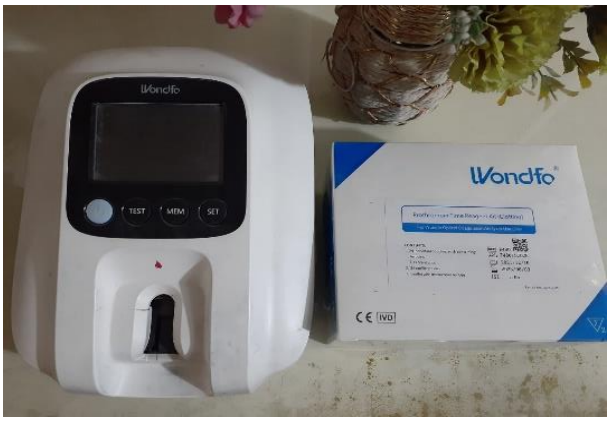
ELISA devise (Reader)



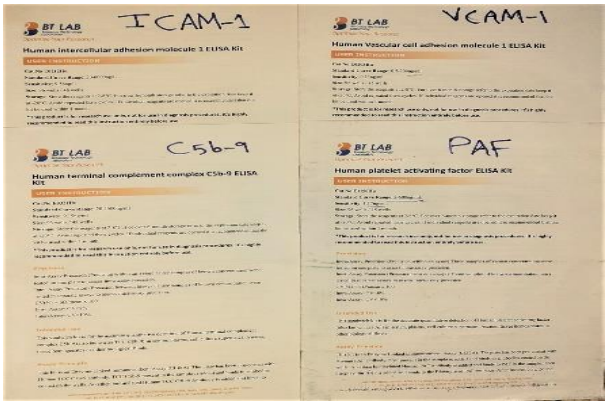
Cobas c111 device for chemical tests

Sysmex device (Hematological tests)

Pre-coated ELISA plate



Wondfo device for PT, PTT test



leaflets of four immunological tests

Questionnaire

Name: **Age:** **Date:**

Sex: **Weight:** **BMI:**

Address:

Family history:

Clinical symptoms: 1.

2.

3.

4.

Smoking ☐ **Diabetes** ☐ **Renal failure** ☐ **Heart failure** ☐

Hypertension ☐ **Asthma** ☐ **Vaccination** ☐

Vital Signs: 1. Temp:

2. Bp:

3. SpO2:

4. R.R:

Lab tests: 1.

2.

3.

4.

5.

الخلاصة

يعد كوفيد 19 (COVID-19) هو مرض معد يسبب الالتهاب الرئوي الحاد الوخيم الفايروسي (SARS-CoV-2) تم الإبلاغ عنه لأول مرة في ووهان، عاصمة هوبي، وسرعان ما تسبب في جائحة وانتشر في كل الدول تقريباً.

هدفت الدراسة إلى تقييم الدور الذي تلعبه المستويات المختلفة لكل من العامل المنشط للصفائح الدموية (PAF) وجزيئتي الالتصاق (ICAM-1, VCAM-1) والمركب النهائي لنظام المتمم المناعي (MAC) على التخثر المناعي عند المرضى المصابين بفايروس كورونا في محافظة كربلاء المقدسة.

من أجل تحقيق هذا الهدف، تم تسجيل 82 مريضًا لديهم COVID-19 وأكده فحص RT-PCR الخاص بـ SARS-COV-2 على عينة مسحة من البلعوم او الانف في هذه الدراسة. تمت الدراسة الحالية على المرضى الراقدين في مدينة الامام الحسين الطبية في كربلاء خلال الفترة من تشرين الاول – 2021 الى ايار-2022.

وتم تقسيم المرضى إلى ثلاثة مجاميع: 27 حالة خفيفة و27 حالة شديدة و28 حالة حرجة. وكان متوسط العمر 59.9 سنة والمدى العمري ما بين 25 إلى 85 سنة. من بينهم 44 (53.7٪) ذكور و38 (46.3٪) إناث.

ازدادت مستويات كل من MAC , VCAM-1, ICAM-1 بشكل ملحوظ مع تطور المرض ، بينما ازداد مستوى العامل المنشط للصفائح الدموية PAF في الاصابات الخفيفة وثم استقر مستواه ضمن نطاق محدود في الحالات الشديدة والحرجة.

كان D-dimer هو معامل التخثر الوحيد الذي ازداد بشكل ملحوظ في هذه الدراسة مع شدة المرض، ومعظم حالات الوفيات ترافقت مع مستويات D-dimer المرتفعة. بينما لم تحدث زيادة معنوية في زمن البروثرومبين PT و زمن الثرومبوبلاستين الجزئي PTT وكذلك تعداد الصفائح الدموية .

وانخفضت النسبة المئوية للخلايا الليمفاوية بشكل ملحوظ مع شدة المرض، وهذا دليل على اشتراك الاستجابة المناعية الخلوية في عدوى COVID-19 التي تسببه SAR-CoV-2 داخل الخلايا.

كان معدل الوفيات كبيراً (17٪) بين المرضى الأكبر سنًا في الحالات الشديدة والحرجة، بينما لم تحدث وفيات بين المرضى في الحالات الخفيفة والذين كانوا في الغالب أصغر سنًا. وأشارت هذه البيانات إلى أن المرضى الأكبر سنًا كانوا أكثر عرضة للوفاة.

وأشارت النتائج إلى أن جميع المعلمات المناعية (ICAM-1 وVCAM-1 و MAC و PAF) في الدراسة الحالية مرتبطة بشكل معنوي بحدوث التخثر المناعي الالتهابي في COVID-19 والتخثر المناعي مرتبط بشكل معنوي مع شدة المرض وزيادة معدل الوفيات.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية العلوم الطبية التطبيقية
قسم التحليلات المرضية

دور كل من العامل المنشط للصفائح الدموية (PAF) وجزيئتي الالتصاق (ICAM-1, VCAM-1) والمركب النهائي لنظام المتمم المناعي (MAC) على التخثر المناعي عند المرضى المصابين بفايروس كورونا في محافظة كربلاء المقدسة

رسالة مقدمة الى

مجلس كلية العلوم التطبيقية وهي جزء من متطلبات نيل شهادة الماجستير في التحليلات المرضية

كُتبت بواسطة

عبد القادر وصفي محمد طاهر جدو

بكالوريوس تحليلات مرضية/ كلية العلوم/ جامعة الكوفة 2012

بإشراف

الاستاذ الدكتور

هادي رسول حسن

2022 1444