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Calprotectin and Cystatin C Levels in Severe and Moderate Covid-19 Patients and their Correlations with Kidney Injury Molecule-1

A Thesis

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بسم الله الرحمن الرحيم

(وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا)

صَدَقَ اللهُ العَلِيُّ العَظيم

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Hawraa

Summary

Background: Coronavirus disease (Covid-19) causes severe acute respiratory syndrome, coronavirus 2 (SARS-CoV-2) was spread to hundreds of countries and it is served as global pandemic. Death occurs as a result of severe Covid-19 due to increasing hypoxemia, acute respiratory distress syndrome (ARDS), and multi-organ failure. Covid-19 affects the kidneys in approximately 3-9% of patients and its developed acute kidney damage (AKI) and it is significantly higher.

Calprotectin is a member of the calcium-binding S100 protein family and it is a heterodimeric protein, found in the cytoplasm of neutrophils and monocytes, both of which play important roles in the inflammatory response in the human body. Calprotectin used an early biomarker in coronavirus disease with bacterial co-infections and patients at risk to develop severe events. Kidney injury molecule 1 (KIM-1) is a transmembrane glycoprotein that is up-regulated in the proximal tubule after kidney injury.

The aim of this study for evaluating severity in cases of Covid- 19 patients by measuring Calprotectin and Cystatin C levels and study the correlation with Kidney injury molecule-1 and others biomarkers.

Material and Methods: The study was a cross sectional study. This study included 91 samples patients with Covid-19 include (56 male, 35 female) ranged between (22-88)years patients. The study was conducted from Al-hayat unit, Al-Hussein Teaching Hospital, Al-Hussein Medical City during the period from (Oct., 2020 to July., 2021). COVID-19 cases were divided into three groups based on disease severity (moderate and severe). The levels of bio-markers were measured for following parameter Ferritin, C-reactive protein total lactate dehydrogenase activity, creatinine, urea, (cystatin C, calprotectin, and kidney injury molecule-1) by ELISA and complete blood count test by XP-300™ Automated hematology analyzer Sysmex.

Results: According to the presented results which include 46 case of severe group and 45 case of moderate group of Covid-19 patients, there is positive correlation between Kidney injury molecule-1 and Cystatin C in severe($p< 0.01$) and moderate group ($p<0.05$) and with Calprotectin ($p<0.01$) in severe group. There was positive correlation between Cystatin C and S.Creatinine in severe group. There is no significant correlation when compared (Calprotectin Cystatin C and kidney injury molecule-1) among moderate and severe group ($p > 0.05$)

Conclusion: The obtained results illustrated that was positive correlation between Kidney Kidney injury molecule-1, Cystatin C and Calprotectin. And positive correlation between Cystatin C and S.Creatinine in severe group. There is no significant correlation in Calprotecti, Cystatin C and Kidney injury molecule-1 among moderate and severe cases of Covid-19.

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Abbreviations	Full nomenclature
ACE2	Angiotensin Converting Enzyme 2
AKI	Acute kidney injury
ALT	Alanine transaminase
Ang	Angiotensin
ARDS	Acute respiratory distress syndrome
AST	Aspartate transaminase
ATN	Acute tubular necrosis
BUN	Blood urea nitrogen
CBC	Complete Blood Count
CK	creatine-kinase
CKD	Chronic kidney disease
CLP	Calprotectin
CNS	Central nervous system
COVID-19	Coronavirus Disease 2019
CRP	C-Reactive protein
CRS	Cytokine release syndrome
CTSL	Cathepsin L
Cys C	Cystatin C
DAMP	Damage-associated molecular pattern
DIC	Disseminated intravascular coagulation
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
hCC	Human Cystatin c
ICU	Intensive care unit
IL-2,4,6,8	Interleukins
KIM-1	Kidney Injury Molecule
LDH	Lactate dehydrogenase
MERS-CoV	Middle East respiratory syndrome–related coronavirus
MOF	Multi-organs failure
NAAT	Nucleic acid amplification test
NADPH	Nicotinamide adenine dinucleotide phosphate
NGAL	Neutrophil gelatinase-associated lipocalin
NLR	Neutrophil to lymphocyte ratio
PaO_2 / FiO_2	Ratio of arterial oxygen partial pressure to fractional inspired oxygen
PAR2	Protease-activated receptor-2
PCT	Procalcitonin

PMNs	Polimorphonucleates
RDW	Red blood cell distribution width
RT-PCR	Reverse transcription polymerase chain reaction
S.Cr	Serum Creatinine
SARS-COV	Severe Acute Respiratory Syndrome by Coronavirus
SpO_2	Saturation of peripheral oxygen
TIM-1	T-cell immunoglobulin and mucin domain-1
TLR4	Toll-like receptor 4
TMPRSS2	transmembrane serine protease 2
TNF-α	Tumor necrosis factor-alpha
WBC	White blood cells
WHO	World health organization

Chapter ONE

Introduction and Review

1. Introduction

Coronavirus is one of the major viruses which primarily affecting the respiratory system in human **(Chen, Y. *et. al.*, 2020).** However, coronaviruses have been also diagnosed in animals and can cause a range of severe diseases such as gastroenteritis and pneumonia **(Hoek, *et. al.*, 2004 ; Gralinski and Menachery, 2020).** Previous coronavirus outbreaks have been reported, including severe acute respiratory syndrome (SARS-CoV) and Middle East respiratory syndrome (MERS-CoV), which is described as a significant public health threat **(Rothan and Byrareddy, 2020).**

Recently, novel coronaviruses emerge periodically in different areas around the world. Severe acute respiratory syndrome coronavirus (SARS-CoV) occurred in 2002, which reportedly infected 8422 people and caused 916 deaths worldwide during the epidemic. Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in 2012, bringing a total of 1401 MERS-CoV infections, and 543 (~39%) of which died **(WHO, 2020).** On Feb.13th, 2021, there were 107,838,255 confirmed cases worldwide with 2,373,398 confirmed deaths **(WHO, 2021)**.

1.1. Covid-19 Virus

Coronaviruses (CoVs) are enveloped viruses with a single-stranded, positive-sense RNA genome, which is the largest discovered genome of RNA virus **(Su, *et. al.*, 2020).** Before the appearance of SARS-CoV-2, six types of human coronavirus (HCOV) have been discovered by researchers, including HCoV-229E, HCoV-HKU1, HCoV-NL63, HCoV-OC43, severe acute respiratory syndrome (SARS) CoV and Middle East Respiratory Syndrome (MERS) CoV **(Huang, *et. al.*, 2018).** There are currently 7 CoVs (including SARS-CoV-2) that can cause human respiratory diseases, but to date, only SARS-CoV, MERS-CoV, and SARSCoV-2 have caused a large outbreak with high mortality. World Health Organization (WHO) has classified Covid-19 as a

β CoV of group 2B **(Hui, *et. al.*, 2020).** Ten genome sequences of Covid-19 obtained from a total of nine patients exhibited 99.98% sequence identity **(Lu, *et. al.*, 2020).** Another study showed there was 99.8–99.9% nucleotide identity in isolates from five patients and the sequence results revealed the presence of a new beta-CoV strain**.** The genetic sequence of the Covid-19 showed more than 80% identity to SARS-CoV and 50% to the MERS-CoV **(Ren, *et. al.*, 2020 ; Lu, *et. al.*, 2020),** both SARS-CoV and MERS-CoV originate in bats **(Cui, *et. al.*,2019).** Thus, the evidence from the phylogenetic analysis indicates that the Covid-19 belongs to the genus beta coronavirus, which includes SARS-CoV, that infects humans, bats, and wild animals **(Zhu, *et. al.*, 2020).**

Based on the genetic sequence identity and the phylogenetic reports, Covid-19 is sufficiently different from SARS-CoV and it can thus be considered as a new beta coronavirus that infects humans. The Covid-19 most likely developed from bat origin coronaviruses. In a preliminary report, complete viral genome analysis revealed that the virus shared 88% sequence identity to two bat-derived SARS-like coronaviruses, but more distant from SARS coronavirus **(Lu, *et. al.*, 2020)**. Hence, the virus was temporarily called 2019 novel coronavirus (2019-nCoV). Coronavirus is an enveloped and single-stranded ribonucleic acid named for its solar corona-like appearance due to 9–12-nm-long surface spikes **(Wang, Q. *et. al.*, 2020)**.

There are four major structural proteins encoded by the coronaviral genome on the envelope, one of which is the spike protein (S) that binds to angiotensin-converting enzyme 2 receptor and mediates subsequent fusion between the envelope and host cell membranes to aid viral entry into the host cell **(Kirchdoerfer, *et. al.*, 2016 ; Xu, X. *et. al.*, 2020)**. On Feb. 11th, 2020, the Coronavirus Study Group of the International Committee on Taxonomy of Viruses finally designated it as SARS coronavirus 2 based on phylogeny, taxonomy, and established practice **(Gorbalenya, *et. al.*, 2020)**. Shortly

thereafter, the WHO named the disease caused by this coronavirus Covid-19 **(WHO, 2020).** On the basis of current data, it seems that SARS coronavirus 2 might be initially hosted by bats and might have been transmitted to humans by means of pangolin **(Lam, *et. al.*, 2020)** or other wild animals **(Lu, *et. al.*, 2020 ; Zhang, L. *et. al.*, 2020)** sold at the Huanan Seafood Market but subsequently spread by means of human to- human transmission.

1.1.2. Viral Transmission and Spread

Based on the large number of infected people that were exposed to the wet animal market in Wuhan City where live animals are routinely sold, it is suggested that this is the likely zoonotic origin of the Covid-19. Efforts have been made to search for a reservoir host or intermediate carriers from which the infection may have spread to humans. Initial reports identified two species of snakes that could be a possible reservoir of the Covid-19.

Genomic sequence analysis of Covid-19 showed 88% identity with two bat-derived SARS like coronaviruses **(Wan *et. al.*, 2020)**, indicating that mammals are the most likely link between Covid-19 and humans. Several reports have suggested that person-to-person transmission is a likely route for spreading Covid-19 infection. This is supported by cases that occurred within families and among people who did not visit the wet animal market in Wuhan **(Carlos, *et. al.*, 2020 ; Wu, P. *et. al.*, 2020)**. Person-to-person transmission occurs primarily via direct contact or through droplets spread by coughing or sneezing from an infected individual. In a small study conducted on women in their third trimester who were confirmed to be infected with the coronavirus, there was no evidence that there is transmission from mother to child. However, all pregnant mothers underwent cesarean sections, so it remains unclear whether transmission can occur during vaginal birth. The binding of a receptor expressed by host cells is the first step of viral infection followed by fusion with the cell membrane. Thus, it has been reported that human-to-human

transmissions of SARS-CoV occurs by the binding between the receptor-binding domain of virus spikes and the cellular receptor which has been identified as angiotensin- converting enzyme 2 (ACE2) receptor **(Jaimes, *et. al.*, 2020)**. Importantly, the sequence of the receptor-binding domain of Covid-19 spikes is similar to that of SARS-CoV. This data strongly suggests that entry into the host cells is most likely via the ACE2 receptor **(Wan, *et. al.*, 2020)** .

1.1.3. Covid-19 Pathophysiology

1.1.3.1. Virus Entry and Spread

Primary viral replication is presumed to occur in mucosal epithelium of upper respiratory tract (nasal cavity and pharynx), with further multiplication in lower respiratory tract and gastrointestinal mucosa **(Xiao, *et. al.*, 2020)**, giving rise to a mild viremia. Few infections are controlled at this point and remain asymptomatic. Some patients have also exhibited non-respiratory symptoms such as acute liver and heart injury, kidney failure, diarrhea **(Cheng, *et. al.*, 2020)**, implying multiple organ involvement. Angiotensin Converting Enzyme 2 is broadly expressed in nasal mucosa, bronchus, lung, heart, esophagus, kidney, stomach, bladder, and ileum, and these human organs are all vulnerable to SARS-CoV-2 **(Zou, *et. al.*, 2020)**. Recently, potential pathogenicity of the SARS-CoV-2 to testicular tissues has also been proposed by clinicians, implying fertility concerns in young patients **(Fan, *et. al.*, 2020)**. The postulated pathogenesis of SARS-CoV-2 infection is graphed in Figure (1-1) **(Jin, *et al.*, 2020).**

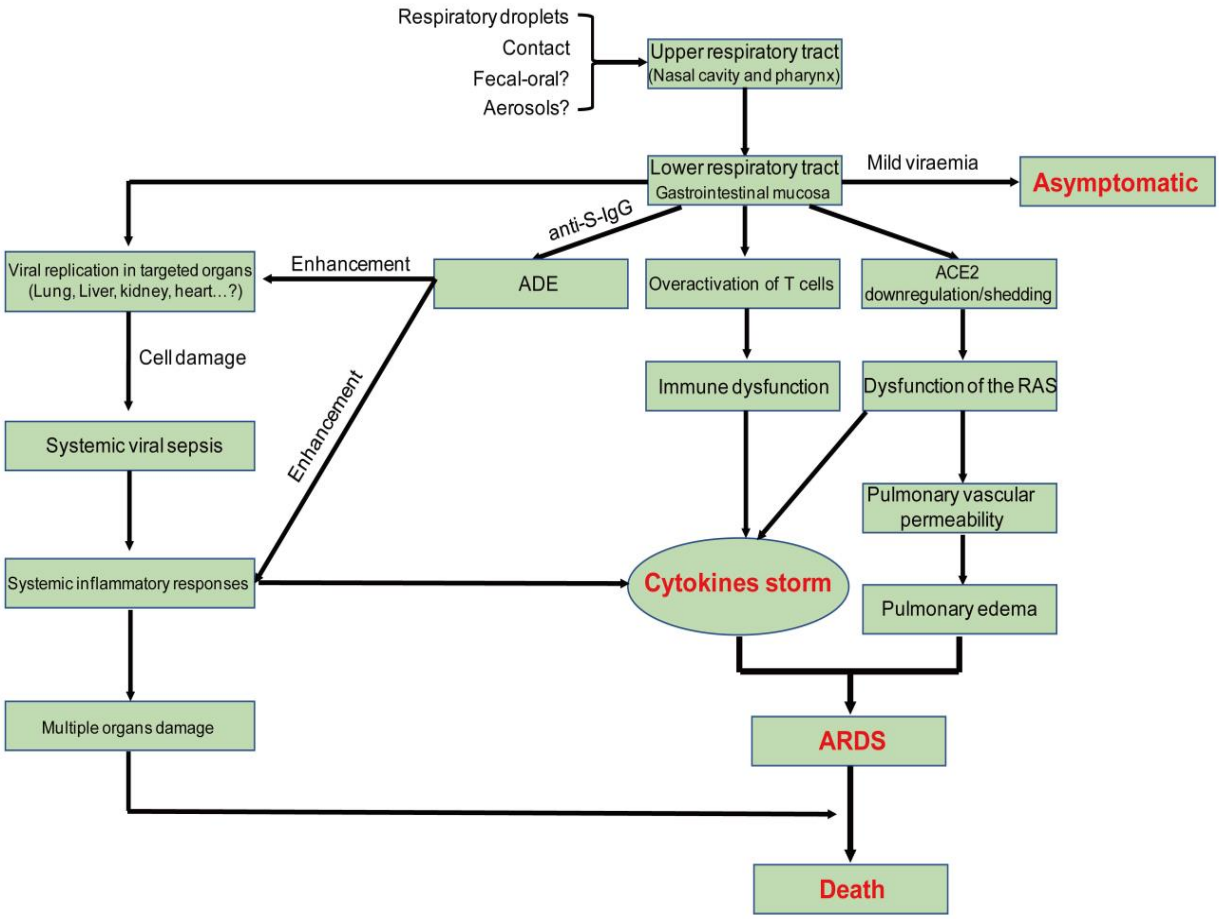


Figure (1-1): Postulated pathogenesis of SARS-CoV-2 infection. Antibody-dependent enhancement (ADE); ACE2: angiotensin-converting enzyme 2; RAS: renin-angiotensin system; ARDS: acute respiratory distress syndrome. Red words represent the important turning points in SARS-CoV-2 infection.

1.1.3.2. Pathological Findings

The first report **(Xu, Z. *et. al.*, 2020)** of pathological findings from a severe Covid-19 showed pulmonary bilateral use alveolar damage with cellular fibromyxoid exudates. The right lung showed evident desquamation of pneumocytes and hyaline membrane formation, indicating acute respiratory distress syndrome. The left lung tissue displayed pulmonary edema with hyaline membrane formation, suggestive of early-phase acute respiratory distress syndrome. Interstitial mononuclear inflammatory infiltrates, dominated by lymphocytes, could be observed in both lungs. Multinucleated syncytial

cells with atypical enlarged pneumocytes characterized by large nuclei, amphophilic granular cytoplasm, and prominent nucleoli were identified in the intra-alveolar spaces, indicating viral cytopathic-like changes. These pulmonary pathological findings extremely resemble those seen in SARS **(Ding, *et. al.*, 2003)** and MERS **(Ng, *et. al.*, 2016)**. Moderate microvascular steatosis and mild lobular and portal activity were observed in liver biopsy specimens, which might be caused by either SARS-CoV-2 infection or drug use. In addition, only a few interstitial mononuclear inflammatory infiltrates were found in the heart tissue, which means that SARS-CoV-2 might not directly impair the heart **(Xu, Z. *et. al.*, 2020)**. Massive mucus secretion in both lungs was found in death cases with Covid-19, which was different from SARS and MERS **(Liu, X. *et. al.*, 2020)**.

1.1.4. Clinical Symptom Spectrum

The period from the onset of Covid-19 symptoms to death ranged from 6 to 41 days with a median of 14 days. This period is dependent on the age of the patient and status of the patient's immune system. It was shorter among patients> 70-years old compared with those under the age of 70 **(Wang, W. *et. al.*, 2020)**. The most common symptoms at onset of Covid-19 illness are fever, cough, and fatigue, while other symptoms include sputum production, headache, haemoptysis, diarrhoea, dyspnea, and lymphopenia **(Wang, W. *et. al.*, 2020)**. Clinical features revealed by a chest CT scan presented as pneumonia, however, there were abnormal features such as RNAaemia, acute respiratory distress syndrome, acute cardiac injury, and incidence of grand-glass opacities that led to death **(Huang, *et. al.*, 2020)**. In some cases, the multiple peripheral ground-glass opacities were observed in subpleural regions of both lungs **(Lei, *et. al.*, 2020)** that likely induced both systemic and localized immune response that led to increased inflammation. Regrettably, treatment of some cases with interferon inhalation showed no clinical effect and instead

appeared to worsen the condition by progressing pulmonary opacities **(Lei, *et. al.*, 2020)**.

It is important to note that there are similarities in the symptoms between Covid-19 and earlier beta coronavirus such as fever, dry cough, dyspnea, and bilateral ground-glass opacities on chest CT scans **(Huang, *et. al.*, 2020)**. However, Covid-19 showed some unique clinical features that include the targeting of the lower airway as evident by upper respiratory tract symptoms like rhinorrhea, sneezing, and sore throat **(Assiri, *et. al.*, 2013)**. In addition, based on results from chest radiographs upon admission, some of the cases show an infiltrate in the upper lobe of the lung that is associated with increasing dyspnea with hypoxemia **(Phan, *et. al.*, 2020)** .

Importantly, whereas patients infected with Covid-19 developed gastrointestinal symptoms like diarrhea, a low percentage of MERS-CoV or SARS-CoV patients experienced similar GI distress. Therefore, it is important to test fecal and urine samples to exclude a potential alternative route of transmission, specifically through health care workers, patients etc. **(Assiri, *et. al.*, 2013)**.

1.1.5. Diagnosis of Covid-19

Clinical features of Covid-19 include dry cough, fever, diarrhea, vomiting, and myalgia. Individuals with multiple comorbidities are prone to severe infection and may also present with acute kidney injury (AKI) and features of ARDS **(Chen, N. *et. al.*, 2020)**. Extensive laboratory tests should be requested for patients with suspected infection. Patients may present with an elevated C-reactive protein, erythrocyte sedimentation rate, lactate dehydrogenase, creatinine, and a prolonged prothrombin time **(Wang, D. *et. al.*, 2020)**. Full genome sequencing and phylogenetic analysis on fluid from bronchoalveolar lavage can confirm Covid-2019 infection **(Zhu, *et. al.*, 2020)**.

National Institutes of Health concluded an clinical classification for Covid-19 disease **(American Academy of Pediatrics, 2019)**

A. **Asymptomatic or presymptomatic infection:** People who test positive for SARS-CoV-2 but have no symptoms.
B. **Mild illness:** People who have any of various signs and symptoms (e.g., fever, cough, sore throat, malaise, headache, muscle pain) without shortness of breath, dyspnea, or abnormal imaging.
C. **Moderate illness :** People who have evidence of lower respiratory disease by clinical assessment or imaging and an oxygen saturation (SpO_2) >93% on room air at sea level.
D. **Severe illness :** People who have respiratory frequency >30 breaths per minute, $SpO_2 \leq 93\%$ on room air at sea level, ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300, or lung infiltrates >50%.
E. **Critical illness:** People who have respiratory failure, septic shock, and/or multiple organ dysfunctions.

1.1.5.1. Nucleic Acid Amplification Test

Nucleic acid amplification test **(**NAAT) is the technology of choice to make a diagnosis of an active Covid-19 infection. Use of real time polymerase chain reaction (RT-PCR) assay to detect SARS-CoV-2 RNA from the upper respiratory tract is the preferred initial diagnostic test **(Patel and Jernigan, 2020).** Other NAAT techniques, such as Loop-mediated isothermal amplification (LAMP)-based **(Carter, *et. al.*, 2020)** and clustered regularly interspaced short palindromic repeats (CRISPR-based) assays has also been developed **(Yan, *et. al.*, 2020)** .

The NAAT assays targets the SARS-CoV-2 nucleocapsid (N), envelope (E), and spike (S) genes, and regions in the first open reading frame

(*orf1a* and *orf1b*), and the RNA-dependent RNA polymerase (RdRp) gene **(Chu, *et. al.*, 2020)**. When performing in-house assays, ideally two independent gene targets should be utilized as the performance of single target assays may be affected by viral mutations. When using commercially available assays ensure a means to keep track of possible performance variation due to cumulating genetic mutations **(WHO, 2020).**

1.1.5.2. Serology of Covid-19.

Members of the coronavirus family have four structural proteins: the spike (S), membrane (M]), envelope (E), and nucleocapsid (N) proteins. Two of these proteins appear to be important antigenic sites for the development of serological assays to detect Covid-19. Serological methods have focused on detecting serum antibodies against S proteins from the coronavirus spike **(Chan, *et. al.*, 2009)**.

The coronavirus envelope spike is responsible for receptor binding and fusion and determines host tropism and transmission capability **(Cui, *et. al.*, 2019; Lu, *et. al.*, 2020)**. S proteins are determined by the S gene and are functionally divided into two subunits (S1 and S2). The S1 domain is responsible for receptor binding while the S2 domain is responsible for fusion. SARS-CoV and SARS-CoV-2 bind to human angiotensin-converting enzyme 2 recptor, which is found on human respiratory cells, renal cells, and gastrointestinal cells **(Liu, Z. *et. al.*, 2020)**. The other protein that appears to be an important antigenic site for the development of serological assays to detect Covid-19 is the N protein, which is a structural component of the helical nucleocapsid. The N protein plays an important role in viral pathogenesis, replication, and RNA packaging. Antibodies to the N protein are frequently detected in Covid-19 patients **(Liu, Z. *et. al.*, 2020)**, suggesting that the N protein may be one of the immunodominant antigens in the early diagnosis of Covid-19 **(Guo, . *et. al.*, 2020)**. As mentioned above, rapid lateral flow assays for antibodies (IgM and

IgG) produced during Covid-19 have been developed **(Li, Z. *et. al.*, 2020)**. Seroconversion occurred after 7 days of symptomatic infection in 50% of patients (14 days in all) but was not followed by a rapid decline in viral load **(Wolfel, *et. al.*, 2020)**. Serological methods, when available, will play an important role in the epidemiology of Covid-19 and in determining the immune status of asymptomatic patients but are unlikely to play any role in screening or for the diagnosis of early infections **(Wolfel, *et. al.*, 2020)**. However, serology may be useful for confirming the diagnosis of Covid-19 **(Zhang, W. *et. al.*, 2020)** .

1.1.6. Biochemical Profiles Alterations in Covid-19

According to the severity of the disease, the clinical course of Covid-19 can be classified into three stages, namely early infection, pulmonary phase, and hyper-inflammation phase, each one characterized by specific biochemical alterations, Figure (1-2) **(Siddiqi and Mehra, 2020)**.

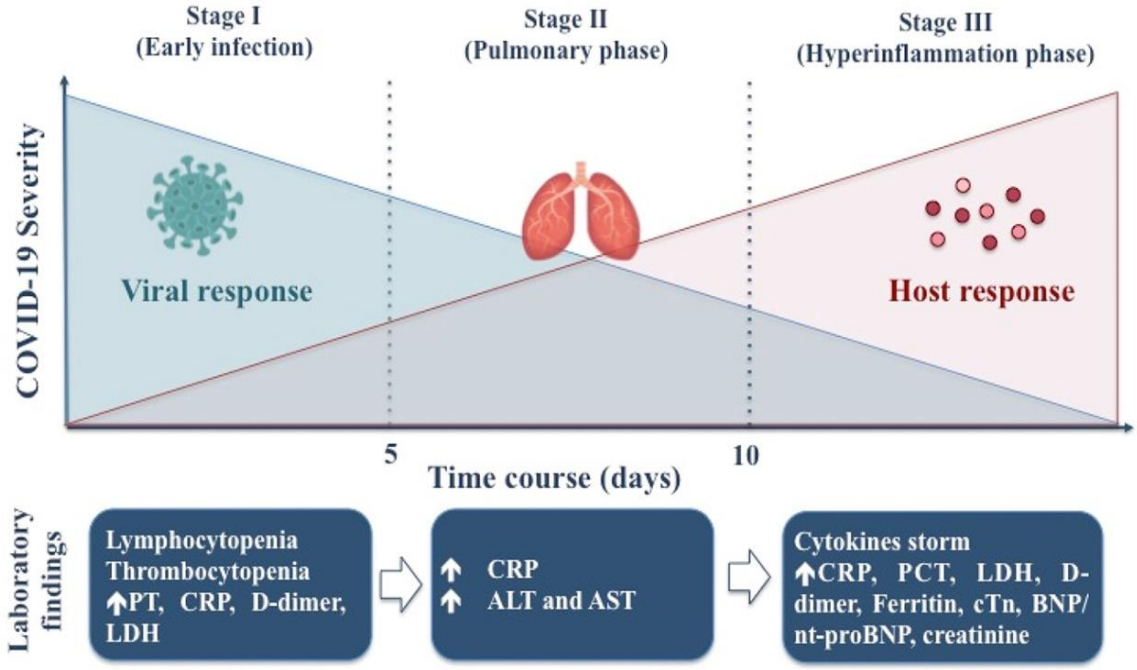


Figure (1-2): Disease progression and main laboratory findings(Siddiqi and Mehra, 2020).

The first stage occurs at the time of the infiltration of the virus in the lung parenchyma, where SARS-CoV-2 infects ciliated bronchial epithelial cells through the interaction with the ACE2. angiotensin-converting enzyme2 is a monocarboxypeptidase with a critical role in regulating the cleavage of several peptides within the renin-angiotensin system. It is highly expressed on pneumocytes in the lung **(Yang, W. *et. al.*, 2020)**. At this stage, most of the patient's present non-specific symptoms, such as dry cough and fever, associated with an initial inflammatory response due to the innate immunity, mainly monocytes and macrophages **(Akhmerov and Marban, 2020)**. At this stage, most of the patients require hospitalization. The third stage of Covid-19 is the most severe, characterized by systemic inflammation, or cytokine storm, leading to ARDS and MOF **(Xu, Z. *et. al.*, 2020)**. At this stage, patients should be admitted to an Intensive Care Unit (ICU). Several inflammatory biomarkers are significantly increased. Moreover, most of the patients present cardiac and kidney injury, which can be detected by circulating biomarkers. Also, alterations of the central nervous system (CNS) have been described **(Roe, 2020)** .

The laboratory provides critical support for the appropriate clinical management of Covid-19, from screening to diagnosis, prognosis, and monitoring **(Lippi and Plebani, 2020).**

1.1.6.1. Hematological Alterations

Several mechanisms have been proposed to explain the reduced lymphocyte levels. As showed for SARS-CoV, it has been hypothesized that the virus might directly infect lymphocytes, principally T cells, inducing depletion of CD4+ and CD8+ cells **(Guan, *et. al.*, 2020)** and, thus, suppressing the cellular immune response **(Yang, W. *et. al.*, 2020).**The evidence that lymphocytes express the ACE2 receptor on their cellular membrane supports such a

hypothesis **(Xu, H. *et. al.,* 2020)**. Additionally, the virus might directly destroy lymphatic organs. However, this hypothesis needs to be confirmed by the evidence of pathological dissection in such organs. Finally, pro-inflammatory cytokines, such as IL-6 and TNF-alpha, could induce lymphocyte deficiency **(Liao, *et. al.,* 2002)**. Leucocytosis, especially neutrophilia, is another SARS-CoV2 infection-induced alteration detectable at the CBC of Covid-19 patients **(Mo, *et. al.,* 2020)**. Some authors proposed neutrophil-to-lymphocyte ratio (NLR) as an independent risk factor for severe disease **(Liu, Y. *et. al.,* 2020)**.

Finally, thrombocytopenia has been described in Covid-19 patients and associated with the progression and prognosis of the disease **(Xu, P. *et. al.,* 2020)**. Several causes can induce platelet deficiency, such as the direct SARS-CoV2 infection of hematopoietic cells or bone marrow stromal cells, leading to hematopoietic inhibition **(Eickmann, *et. al.,* 2020)**. The lung injury could also contribute to the platelet depletion due to the activation, aggregation, and retention of platelets in the lung, and the formation of thrombus at the injured site, leading to decreased platelet production and increased consumption **(Pilaczynska, *et. al.,* 2020)**.

1.1.6.2. Coagulation Dysfunction

A hypercoagulable state, which might promote thrombotic coagulopathies such as pulmonary microthrombosis and disseminated intravascular coagulation (DIC), is a common complication of severe Covid-19 **(Chen, N. *et. al.,* 2020)**.

In addition increasing D-dimer level was related to a hazard ratio for death due to the underlying thromboembolic burden and increased mortality among those patients **(Naymagon, *et. al.,* 2020)**. Moreover, D-dimer is a reflective of high thrombotic activity and may not only be a biomarker of prothrombotic state and a hypercoagulability but may contribute in pathogenesis of acute

pulmonary dysfunction **(Berger, *et. al*., 2020)**. While the reduction in antithrombin levels in patients with Covid-19 are also appear to have an important role in patient prognosis through both arterial and venous thrombotic complications **(Liao, *et. al*., 2020 ; Christensen, *et. al*., 2020)**. Like antithrombin, a low platelet count is accompanying by fivefold increased risk of disease and mortality in Covid-19 patients and therefore should used as prognostic indicator of worsening disease during hospitalization **(Lippi, *et. al*., 2020)**.

1.1.6.3. Inflammatory Response

The hallmark of severe Covid-19 is the hyper-inflammatory host response due to the so-called "cytokine storm", defined as an uncontrolled systemic inflammatory response due to the release of large amounts of pro-inflammatory cytokines, resulting from the SARS-CoV-2 induced activation of both natural and cellular immunity.

Increased levels of several inflammatory biomarkers, including cytokines such as IL-6, IL-2, IL-7, TNF-α, interferon (IFN)-γ, monocyte chemo-attractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, granulocyte-colony stimulating factor (G-CSF), and CRP, procalcitonin (PCT), lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR) and ferritin, have been reported in Covid-19 patients. Among inflammatory biomarkers, CRP levels increase significantly at the early stage of the disease, and a positive correlation between increased CRP levels and disease severity has been described **(Wang, D. *et al*., 2020)**.

1.1.6.4. Liver Dysfunction

The alteration of hepatocytes damage biomarkers, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and albumin, is a common laboratory finding in Covid-19 patients. However, the

underlying mechanism is not fully understood. Although hepatocytes and bile duct epithelial cells express ACE2 receptor **(Chai, *et. al*., 2020)** no significant altered histopathological features have been detected in such cells from Covid-19 patients **(Xu, Z. *et. al*., 2020)**. Thus, Covid-19-related liver dysfunction could be the result of secondary liver damage due to the administration of hepatotoxic drugs, systemic inflammatory response, respiratory distress syndrome-induced hypoxia, and MOF **(Feng, *et. al*., 2020)** .

1.1.6.5. Muscle Injury

Covid-19 patients typically present increased levels of biomarkers of muscle injury, namely creatine-kinase (CK) and myoglobin **(Lippi and Plebani, 2020)** . However, the alterations of such biomarkers could be the result of several clinical conditions, including kidney dysfunction and cardiac injury, or a direct effect of the SARS-CoV-2, which can also infect cells of the muscle tissue due to the expression of the ACE-2 receptor.

1.1.6.6. Kidney Dysfunction

Several studies reported kidneys alterations, as reflected by increased serum creatinine, in Covid-19 patients. SARS-CoV-2 could directly infect kidney tubular cells, which express the ACE2 receptor on their cellular surface. Moreover, circulating mediators could interact with kidney-resident cells resulting in endothelial dysfunction, microcirculatory derangement, and tubular injury **(Joannidis, *et. al*., 2020).**

1.1.6.7. Electrolyte Imbalance

Some authors described alterations of electrolyte levels, including sodium, potassium, chloride, and calcium, in Covid-19 patients **(Huang, *et. al*., 2020)**. Specifically, hyponatremia, hypokalemia, and hypocalcemia have been associated with severe disease **(Lippi, *et. al*., 2020)**. Although

pathophysiological mechanisms underlying such alterations are not fully understood yet, some hypotheses have emerged. The interaction of SARS-Cov-2 with ACE2 receptor might reduce ACE2 expression, resulting in an increased angiotensin II, which promote potassium excretion, leading to hypokalemia **(Chen, D. *et. al*., 2020)**. Also, gastrointestinal impairment, characterized by diarrhea, could contribute to electrolyte disequilibrium **(Pan, L. *et. al*., 2020).**

1.1.7. Kidney and Covid-19

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) primarily affects the lungs, however, this virus can also affect other organs such as intestine, kidney, heart, and brain **(Rodriguez, *et. al.*, 2020)**. Kidney dysfunctions are also observed in a large proportion of Covid-19 patients **(Li, Z. *et. al*., 2020)**.

Subsequently, AKI incidence in Covid-19 patient was found to be up to 15% in comparison to an earlier suggested range of 3%–9% potentiating the evidence that AKI is common and that the virus can specifically damage the kidneys **(Gabarre, *et. al.*, 2020)**. The incidence of AKI could be up to 25% among critically-ill Covid-19 patients with underlying comorbidities **(Gabarre, *et. al.*, 2020)**. An autopsy study also showed the virus tropism to the kidney **(Puelles, *et. al.*, 2020)**.

The exact mechanism of SARS-CoV-2 associated renal damage is not fully known. Studies showed that the cellular components required for virus entry such as ACE2, cellular transmembrane serine protease 2 (TMPRSS2), and cathepsin L (CTSL) are highly expressed in kidneys **(Puelles, *et. al.*, 2020)**. Expressions of ACE2 RNA in the small intestine, duodenum and kidneys were found much higher (around 100-fold) than the lung **(Li, Z. *et. al.*, 2020)**. Furthermore, the co-expression of ACE2 and TMPRSS is reported to be

relatively high in the proximal straight tubule cells and podocytes, suggesting favorable condition for localization of the SARS-CoV-2 in kidneys **(Pan, X. *et. al.*, 2020)**.

Studies reporting albuminuria and hematuria in the Covid-19 patients along with the detection of viral RNA from the urine samples further support the potential tropism of the SARS-CoV-2 for the renal tissues **(Li, Z. *et. al.*, 2020 ; Guan, *et. al.*, 2020)**. The cytokine storm associated along with the direct cytopathic effect of SARS-CoV-2 is suggested as the probable cause of kidney dysfunction **(Naicker, *et. al.*, 2020)**.

Moreover, the AKI in response to cytokine storm might occur due to renal inflammation, increased vascular permeability, cardiomyopathy and volume depletion leading to cardiorenal syndrome-1 **(Ronco, *et. al.*, 2020)**. Additionally, injuries of the renal tubules related to the hypoperfusion in response to cytokine storm may also be partly responsible for the kidney injury **(Naicker, *et. al.*, 2020)**. The computed tomography of the kidneys revealed a reduction in the density suggesting the renal inflammation and oedema **(Li, Z. *et. al.*, 2020)**. Those Covid-19 patients suffering from the chronic kidney disease and other comorbidities are reported to be at higher risk of a severe form of the disease and they are advised to take extra preventive measures to avoid the exposure of SARS-CoV-2 **(Henry, *et. al.*, 2020)**. A higher number of comorbidities was also found to be associated with this virus tropism for kidney **(Puelles, *et. al.*, 2020)**. Patients with renal injury are at high risk of developing Covid-19 and AKI is an important risk factor for Covid-19 patients **(Naicker, *et. al.*, 2020)**. Furthermore, renal impairment is one of the complications of SARS-CoV-2 infection. A large prospective study demonstrated that kidney impairment is more prevalent among hospitalized Covid-19 patients and is also associated with increased in-hospital death.

Of the hospitalized patients, 43.9-75.4% had evidence of abnormal kidney function, and 5.1–10.5% of them presented AKI with an excess mortality rate **(Pei, *et. al.*, 2020)**. Postmortem evaluations detected viral antigen in tubular epithelial cells and revealed severe acute tubular injury **(Su, *et. al.*, 2020).** Moreover, preexisting chronic kidney disease (CKD) could reasonably act as an ominous clinical predictor and associate with high mortality **(Pei, *et. al.*, 2020)**. Accumulating evidence has suggested that patients with chronic comorbidities were more likely to develop into severe cases **(Chen, T. *et. al.*, 2020)**. Data from multiple cohorts and meta-analyses have listed aging, hypertension, diabetes, and cardiovascular disease as adverse prognostic markers for Covid-19 patients **(Pan, L. *et. al.*, 2020)**, which were also commonly seen in CKD **(Guan, *et. al.*, 2020)**. While AKI is often carefully monitored in such a complicated infectious disease **(Hoste, *et. al.*, 2018)**, CKD, affecting over 10% of the general population, is often neglected **(Liu, 2013)**.

In fact, a meta-analysis by Ali *et al*. 2020 evaluating the outcome of Covid-19 subjects who developed AKI observed that those with severe AKI, defined as Kidney Disease Improving Global Outcome (KDIGO) stage III or need of acute renal replacement therapy (RRT), showed a higher mortality than those with stage I or II stage **(Ali, *et. al.*, 2020).** Early reports from China and Italy found the rate of AKI to range widely from 0.5% to 29%, with most estimates on the lower end **(Wang, D. *et. al.* 2020)** .U.S. data has been limited to critically ill patients in the ICU in a Seattle hospital showing a 19% rate of AKI **(Henry and Lippi, 2020).** Data on the risk of patients with CKD are limited and highly variable, especially in a scenario of lung-dominated infectious disease **(Arentz, *et. al.*, 2020**).

1.1.7.1. Pathogenesis of Kidney Injury

Severe Acute Respiratory Syndrome by Coronavirus2 infects the host using the ACE2, a membrane-bound peptidase, expressed more in the kidney than in

other organs (lung, heart, intestine, and endothelial cells). **(Serfozo, *et. al.*, 2020)** .While ACE converts angiotensin I (Ang I) to angiotensin II (Ang II), ACE2 degrades Ang II to angiotensin 1–7 [Ang-(1–7)]. Ang II plays a role in vasoconstriction and adrenergic stimulation, binding type 1 Ang II receptors (AT1), **(South, *et. al.*, 2020)** while Ang-(1–7) opposes the Ang II-AT1 axis through vasodilatation, and anti-inflammatory and anti-fibrotic action, mainly increasing the production of nitric oxide.

By downregulating ACE2, SARS-CoV2 determines an increase in ACE activity and a shift to overproduction of Ang II **(Chappell, *et.al*, 2014).**

1.2. Calprotectin

1.2.1. Chemistry

Calprotectin (CLP) is a heterodimeric complex formed by two binding proteins of the calcium ion, which belong to the S-100 protein family, S100A8 and S100A9, having both anti-inflammatory and anti-bacterial properties. The first applied name to CLP was major leukocyte protein L1 or 27E10 **(Ometto, *et. al.*, 2016)**. Calprotectin is a 36-kDa protein, with 2 heavy and 1 light non-covalently linked chains that binds calcium and zinc, Figure (1-3) **(Korndorfer, *et. al.*, 2007)**. CLP represents almost two-thirds of the soluble cytosolic protein content of neutrophils. CLP may also be detected at various levels in monocytes, macrophages, epithelial cells, and platelets **(Shabani, *et. al.*, 2018)** .

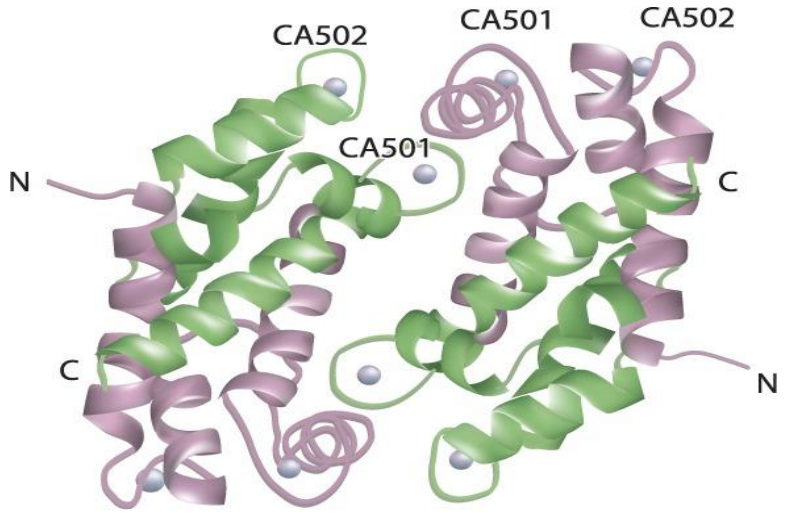


Figure (1-3): The heterotetramer structure of calprotectin (Gupta and Bamezai, 2010). S100A8 chains (upper left); S100A9 chains (upper right); CA501 and CA502 sites where Ca^{2+} ions (spheres) bind (Korndorfer, *et. al.*, 2007).

1.2.2. Physiological Roles

It is expressed as an anti-bacterial agent, mainly but not exclusively by neutrophils, when activated **(Ometto, *et. al.*, 2016).** Upon cell activation or death, CLP is released extracellularly, acts as an alarmin, or damage-associated molecular pattern (DAMP). It serves as a stimulatory ligand for the innate cell-surface receptors such as receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) **(Shabani, *et. al.*, 2018)**. However, there are data demonstrating that physiological levels of S100A9 homodimers can trigger an inflammatory response in vivo despite the capacity of RAGE and TLR4 blockade to inhibit responses in vitro **(Chen, *et. al.*, 2015)**. Calprotectin inhibits the oxidative metabolism of polymorphonuclear neutrophils in vitro, an effect that can be potentiated by the controlled activation of the protease-activated receptor-2 (PAR2) **(Sun, *et. al.*, 2013).** In particular, the survival rate was almost doubled from 33% to 65% or 63% in mice treated with S100A8 or PAR2 activating peptide, respectively, whereas 85% of the mice were treated with both PAR2 activating peptide and S100A8 survived, at a statistically significant higher rate than those treated with a single agent **(Sun, *et. al.*, 2013)**.

Calprotectin has microbicidal, cytotoxic functions via heavy-metal detoxification **(Vogl, *et. al.*, 2007).** Calprotectin plays a critical role in multiple cellular processes, including cell cycle progression, proliferation, differentiation, chemotaxis, and migration in a calcium-dependent manner, and survival, as well as in redox regulation, proteins' phosphorylation, and cytoskeletal components rearrangement **(Shabani, *et. al.*, 2018)**. The features that CLP possesses, such as its small size, easy diffusion between tissue and blood, and enzymatic degradation resistance, make it a sensitive marker of neutrophil activation anywhere in the body **(Roseth, *et. al.*, 1992)**.

1.2.3. Intracellular Functions of Calprotectin

In polimorphonucleates (PMNs), CLP is implicated in the rapid rearrangement of tubulin-dependent cytoskeleton, which allows cell migration. In the presence of calcium, S100A8 and S100A9 form heterotetramers and translocate to the cell membrane allowing tubulin polymerization **(Vogl, *et. al.*, 2004)**. The evidence that S100A9 knockout mice have reduced granulocyte migration supports the idea that CLP has a role in cytoskeleton rearrangement **(Vogl, *et. al.*, 2004).** CLP is implicated in the activation of the respiratory burst. In the presence of calcium, S100A9 binds to arachidonic acid in the cytosol and transports it to the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in the neutrophil plasma membrane through a PKC-dependent mechanism. S100A8 and S100A9 have multiple effects on NADPH oxidase. By interacting with gp91phox, p67phox, and rac-2 subunits of the NADPH oxidase complex, S100A8 and S100A9 induce the production of reactive-oxygen species, which are necessary for PMNs activity **(Kerkhoff, *et. al.*, 2005)**. Calprotetin is secreted by granulocytes and other cells after a danger signal by PAMPs or DAMPs. It appears to be mostly released through a non-Golgi associated pathway with an active non-classical secretion, which requires again PKC activation **(Vogl, *et. al.*, 2004)**. An additional more

recently discovered mechanism is the release of CLP bound to chromatin in neutrophil extracellular traps (NETs) **(McCormick, *et. al.*, 2010)**. The protein can also be passively secreted from necrotic cells after tissue insult has occurred **(Vogl, *et. al.*, 2004)**.

1.2.4. Extracellular Functions of Calprotectin

S100A8 and S100A9 bind their receptors with different affinity according to the protein conformation **(Bjork, *et. al.*, 2009)**. CLP heterodimer binds different cell-surface proteins like heparan sulphate proteoglycan, carboxylated N-glycan, and, directly or through carboxylated glycans, TLR4 and receptor for advanced glycated end products (RAGE) **(Leclerc, *et. al.*, 2009)**. TLR4 is the main CLP receptor34 and is preferentially bound by the heterodimer **(Vogl, *et. al.*, 2007)**. The signal transduction cascade is mediated by MyD88 and NF-kB, which translocate into the nucleus **(Ichikawa, *et. al.*, 2011)** and promote the expression of proinflammatory cytokines, such as TNF-a, interleukin (IL)-1b, IL-6, IL-8, IL-23, chemokine (CXC)-8, and other CXCs **(Sunahori, *et. al.*, 2006 ; Vogl, *et. al.*, 2007)**. receptor for advanced glycated end is also bound by CLP33 and activates inflammatory signaling pathways, including MAPK and NF-Kb **(Ichikawa, *et. al.*, 2011)**. Notably, the expression of S100A8 and S100A9 increases following NF-kB activation, thereby generating a positive feedback loop which amplifies inflammation via an autocrine and paracrine stimulation of the cells responsible for S100A8 and S100A9 production **(Vogl, *et. al.*, 2007)**. A major function of S100A8 and S100A9 is the regulation of leukocyte chemotaxis and tissue infiltration. Calprotectin induces the expression of integrin receptors on leukocytes, thus increasing their adhesion to fibrinogen, fibronectin, and endothelial cells **(Sunahori, *et. al.*, 2006)**. CLP causes a pro-inflammatory and thrombogenic response in the endothelium: it binds to endothelial cells through carboxylated glycans and TLR4 leading to cell activation. Following CLP activation, endothelial cells express CXCs, such

as IL-8 and MCP-1, and further S100A8 and S100A9. Endothelial cells also express VCAMs, ICAMs, and selectins on their surface which results in a chemotactic gradient, which then attracts PMNs and favors their binding to the endothelium **(Sunahori, *et. al.*, 2006)**. Calprotectin release leads to loss of cell–cell contacts and consequently alters the permeability of endothelium with leukocyte extravasation. It also triggers endothelial cell apoptosis and necrosis which are responsible for vascular and tissue damage **(Viemann, *et. al.* 2007).**

1.2.5. Putative Role in Adaptive Immunity

Along with its classical role as an endogenous activator of innate immune response, CLP might represent a connection between inflammation and the adaptive immune response. Calprotectin contributes to the induction of auto-reactive CD8 T cells during the activation process by antigen-presenting cells. This molecule is a co-stimulatory enhancer together with CD40/CD40 ligand signaling and leads to the loss of tolerance of T cells **(Loser, *et. al.*, 2010).** In a murine model of autoimmunity, the absence of S100A8 and S100A9 resulted in reduced IL-17 production by auto-reactive CD8 T cells and in lower autoantibody production **(Loser, *et. al.*, 2010)**. In addition to its pro-inflammatory activity, CLP exerts also a regulatory function in the adaptive immune system. Calprotectin overexpression in dendritic cells (DCs) is associated with an impaired T cell proliferation **(Averill, *et. al.*, 2011)**.

During inflammatory response, neutrophils and monocytes quickly arrive to the site of inflammation. The heterodimeric protein S100A8/A9, named as calprotectin which is an alarmin mainly derived by both cell types which play a critical role in inflammatory response, exerting its functions by binding to two patterns recognition receptors: Toll-like receptor and receptor of advanced glycation end products and activating pro-inflammatory signaling pathways leading to further recruitment and activation of immune cells **(Pruenster, *et.***

***al.*, 2016)**. Infection-induced inflammation is one of the main triggers that leads to calprotectin liberation **(Wang, *et. al.*, 2018)**.

1.3. Cystatin C

There are three distinct types of cystatins that share sequence and structure homology but differ in size, site of action, and disulfide bond topology **(Barrett, 1986)**. Human cystatin C (hCC) belongs to the type 2 cystatins that are generally secreted as extracellular polypeptides. Human cystatin C is broadly distributed and found in most body fluids **(Grubb, 2000)**.

1.3.1. Chemistry

Cystatin C (Cys C) contains four conserved cysteine residues that can form two disulfide bonds but, unlike other family members, was neither shown to be glycosylated (cystatin E/M and cystatin F) nor phosphorylated (chicken cystatin) **(Turk, *et. al.*, 2008)**. Cystatin C is a non-glycosylated protein with low molecular weight of 13 kDa consisting of 120 amino acids, belonging to cysteine-proteases family. It is produced at a constant rate by lysosomes of all nucleated cells in the organism and can be found in several biological fluids, such as serum, seminal liquid and cerebrospinal fluid **(Macissac, *et. al.*, 2011).** Because of its small size and positive charge, Cys C is freely filtered by glomerulus, where it is taken up and degraded by proximal tubular cells. It also does not provide tubular secretion and can therefore be used as a biomarker for renal function **(Dharnidharka, *et. al.*, 2002).**

1.3.2. Biological Roles

In terms of biological function, hCC is a target of proteolysis, and primarily functions as a protease inhibitor. It is degraded by cathepsin D and elastase **(Lenarcic, *et. al.*, 1991).** Uncontrolled proteolysis leads to an imbalance between active proteases and endogenous inhibitors, eventually leading to disease. The hCC concentration in blood correlates with the glomerular

filtration rate(GFR) **(Grubb, *et. al.*, 2014)**, an important marker of kidney health and the progression of diabetes, chronic kidney disease, etc. **(Roos, *et. al.*, 2007)**. Cystatin C is a cysteine protease inhibitor, also known as γ-trace basic protein or post-gamma globulin, which is encoded by the Cys C gene that can be continuously transcribed and expressed in all nucleated cells at a constant speed.. It is freely filtered in the glomeruli, completely reabsorbed by the renal tubular epithelial cells, and degraded within cells without returning to the blood. Renal tubular epithelial cells do not secrete serum Cys C (sCys C) into the lumen, therefore its serum concentration is mainly determined by glomerular filtration rate which is an important indicator of glomerular filtration **(Liu, 2012).** In addition, it has been reported that sCys C can be used to evaluate the survival rate of patients with sepsis **(Al-Beladi, 2015)**.

1.3.3. Cystatin C as Biomarker in Kidney Function Diagnosis

Several studies have shown that Cys C is an effective marker for diagnosing kidney damage, such as acute kidney injury **(Al-Tu'ma *et. al.*, 2017 ; Yong, *et. al.*, 2017)** and kidney failure, in patients with diabetes mellitus **(Yong, *et. al.*,2017)** . Kidney function and glomerular filtration rate (GFR) decline, the blood levels of cystatin C rise, previous studies have shown that serum levels of Cys C are more precise test of kidney function than serum creatinine levels **(Roos, *et. al.*,2007).** Cystatin C levels are less dependent on age, gender, ethnicity and muscle mass compared to creatinine **(Perrone, *et. al.*, 1992)**.

Cross-sectional studies (based on a single point in time) suggest that serum levels of cystatin C are a more precise test of kidney function (as represented by the glomerular filtration rate, GFR) than serum creatinine levels **(Roos, *et. al.*, 2007)**. It has been suggested that cystatin C might predict the risk of developing CKD, thereby signaling a state of 'preclinical' kidney dysfunction **(Shlipak, *et. al.*, 2006)**. Additionally, the age-related rise in serum cystatin C is

a powerful predictor of adverse age-related health outcomes, including all-cause mortality, death from cardiovascular disease, multimorbidity, and declining physical and cognitive function **(Justice, *et. al.*, 2018).**

However, inflammation does not cause an increase in the production of cystatin C, since elective surgical procedures, producing a strong inflammatory response in patients, do not change the plasma concentration of cystatin C. Levels seem to be increased in HIV infection, which might or might not reflect actual renal dysfunction **(Odden, *et. al.*, 2007).** The role of cystatin C to monitor GFR during pregnancy remains controversial **(Akbari, *et. al.*, 2005)**. Like creatinine, the elimination of cystatin C via routes other than the kidney increase with worsening GFR **(Sjostrom, *et. al.*, 2005).**

Cystatin C can be measured in a random sample of serum (the fluid in blood from which the red blood cells and clotting factors have been removed) using immunoassays such as nephelometry or particle-enhanced turbidimetry **(Croda, *et. al.*, 2007).**

The normal values decrease until the first year of life, remaining relatively stable before they increase again, especially beyond age 50 **(Kottgen, *et. al.*, 2008; Ognibene, *et. al.*, 2007).** Creatinine levels increase until puberty and differ according to gender from then on, making their interpretation problematic for pediatric patients **(Filler, *et. al.*, 2005).**

For optimal assessment, the serum/urinary levels of new biomarkers of AKI should increase rapidly at early stages of the injury, prior to the decrease in GFR **(Rossaint, *et. al.*, 2016),** and should not be affected by concomitant infections, diseases of the liver, and inflammatory conditions. Moreover, these markers should be specific and enable to differentiate the etiology of AKI **(Royakkers, *et. al.*, 2007).** Available evidence suggests that these criteria may be met by several proteins, including neutrophil gelatinase-associated lipocalin

(NGAL) (**Wheeler, *et. al.*, 2008),** kidney injury molecule-1 (KIM-1) **(Trof, *et. al.*, 2006)**, Cys C **(Mishra, *et. al.*, 2003)**, and some interleukins **(Bonventre** and Yang**, 2010).** Serum cystatin C is freely filtered through the glomerular membrane and completely reabsorbed and metabolized by the proximal tubular cells without secretion **(Dharnidharka, *et. al.*, 2002).** Acute Cys C changes may therefore provide more accurate AKI diagnosis; this would increase validity of novel biomarker and of AKI-outcome studies. Some studies have suggested that serum Cys C rise with AKI occurs before rise in serum creatinine, leading to evaluation of Cys C as an "early AKI biomarker" **(Parikh, *et. al.*, 2011)**. However, malignancy, corticosteroids and thyroid dysfunction may lead to Cys C variability **(Manetti, *et. al.*, 2005)**. Among the potential markers, sCys C performs a consistent accuracy in various conditions. For both the healthy individuals and CKD patients, serum Cys C has ever been proposed as a superior marker to Scr to evaluate GFR **(Filler, *et. al.*, 2005)**. Performed a meta-analysis to compare serum and/or urine cystatin C for diagnosis of AKI, and then they found Scys appeared to be a better biomarker in the prediction of AKI **(Zhang, *et. al.*, 2011)**.

1.4. Kidney Injury Molecule-1

Kidney Injury Molecule-1 (KIM-1) was identified by our group as the most up-regulated protein in the kidney proximal tubule after a wide variety of injurious influences including ischemia, nephrotoxicants, sepsis and immune related injury and its cleaved ectodomain is often used as a serum and urine marker for kidney injury **(Brooks, *et al.*, 2015).**

1.4.1. Chemistry and General Biological Roles

Kidney Injury Molecule-1, also identified as Hepatitis A Virus Cellular Receptor 1 (HAVCR1) in hepatocytes **(Kaplan, *et al.*, 1996)**, and T-cell immunoglobulin and mucin domain 1 (TIM-1), has been reported to be a

receptor for Ebola virus **(Kondratowicz, *et al.*, 2011)**, and dengue virus **(Meertens, *et al.*, 2012)**.

Kidney injury molecule-1 (KIM1, also known as TIM1, HAVCR1, or CD365) is primary expressed in kidney and drastically up-regulated in injured kidney proximal tubule in AKI or CKD, and plays crucial roles in inflammation infiltration and immune response **(Gardiner, *et. al.*, 2012).**

Based on previous studies, kidney injury molecule-1, a recently discovered transmembrane protein with extracellular mucin and immunoglobulin domains, is believed to play a role in tubule-interstitial damage **(Al-Tu'ma, *et. al.* , 2017 ; Ichimura, *et. al.*, 1998).** Kidney Injury Molecule-1 is not detectable in normal human or rodent kidneys but KIM-1 expression is increased more than that of any other protein in injured kidneys, and it is localized predominantly to the apical membrane of surviving proximal epithelial cells **(Ichimura, *et. al.*, 2008)**.

Normal kidney tissues rarely express KIM-1, except in the acute injury resulting from ischemia, hypoxia, toxicity, or some renal tubular interstitial, and polycystic kidney disease **(Wu, *et. al.*, 2018).** After injury to renal tubular cells, the extracellular section of KIM-1 is released into the renal tube cavity and is further shed into the urine, mediated by mitogen-activated protein kinase (MAPK) signaling pathways **(Zhang, *et. al.*, 2007)**.

Interestingly, KIM-1 has a phosphatidylserine receptor which enhances apoptotic bodies and necrotic debris phagocytosis. This molecule is, therefore, unique for giving epithelial cells a function characteristic of phagocytes; Figure (1-4) **(Visnagri, *et. al.*, 2015)**. It seems that KIM-1 down-regulates proximal tubular cell (PTC) cytokine secretion, modulates translational changes through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-_B) pathway and interaction with phosphatidylinositol3 PI3 kinase subunit p85 **(Brooks, *et. al.*, 2015)**. Experimentally, KIM-1 gene expression reflects

ongoing damage in various tubulointerstitial segments and in the renal cortex **(Chiusolo, *et. al.*, 2015)** . For these reasons, authors began considering KIM-1 a biomarker capable of identifying early AKI and may even hold a possible predictive role **(Tsigou, *et. al.*, 2013)**.

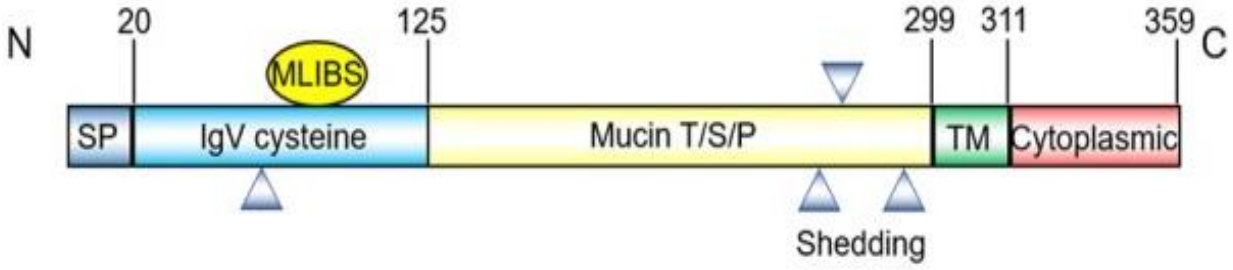


Figure (1-4): Structure of the Human KIM-1 protein. Schematic representation of KIM-1, showing the signal peptide, IgV, mucin, transmembrane, and cytoplasmic domains. The triangles are the predicted location of N-linked glycosylation sites. KIM-1, kidney injury molecule-1; MLIBS, metal ion-dependent ligand binding site; T/S/P, threonine /serine /proline ; TM, transmembrane(Visnagri, *et. al.*, 2015).

1.4.2. Physiological Functions of Kidney Injury Molecule-1

Kidney **I**njury **M**olecule-**1** functions as a phosphatidylserine receptor that recognizes and internalizes apoptotic cells **(Ichimura, *et. al.*, 2008)**. Kidney Injury Molecule-1 also functions as a scavenger receptor, mediating the uptake of modified low-density lipoprotein and necrotic-cell debris **(Ichimura, *et. al.*, 2008)**. Kidney Injury Molecule-1 expression transforms proximal tubular epithelial cells into semiprofessional phagocytes. In the immune system, KIM-1/ Tim-1 has been implicated in activation of T-helper 2 (Th 2), Th 1, and Th 17 differentiation **(Rennert, 2011)**. It has also been proposed to be an activating receptor in B cells, dendritic cells, and natural killer T cells **(Rennert, 2011)**. Many of the experiments that have led to these conclusions have relied on antibodies against KIM-1/ Tim-1, which have been presumed to be agonists or antagonists, or KIM-1 / Tim-1-Fc fusion proteins as key reagents **(Rennert, 2011).** In addition to its role in mediating phagocytosis, KIM-1 is

also involved in the repair process after injury to renal tubular epithelial cells. In vitro, transient KIM-1 overexpression can promote the migration and proliferation of renal tubular epithelial cells by activation of the ERK/MAPK signaling pathway.

1.4.3. Kidney Injury Molecule-1 as Biomarker

Recent studies explored the ability of KIM-1 to diagnose early and/or predict AKI development in special populations. KIM-1 also is a sensitive biomarker for chronic proximal tubular injury **(Gardiner, *et. al.*, 2012)**. In protein-overload nephropathy, increasing KIM-1 levels are associated with inflammation of the renal tubules **(van Timmeren, *et. al.*, 2006)**. Kidney Injury Molecule-1 is the only effective clinical biomarker for CKD associated with hypertension **(Hosohata, 2017)**. In more severe CKD, KIM-1 is an independent risk factor for progression to end-stage renal disease (ESRD) **(Alderson, *et. al.*, 2016)** . In decompensated liver cirrhosis patients, AKI may be early diagnosed by combining several biomarkers, such as KIM-1, NGAL, and sCys C **(Sun, *et. al.*, 2017)** . Elevated levels have also been identified in diabetic nephropathy **(Ornellas, *et. al.*, 2017),** while in transplanted patients it may help in early detection of allograft rejection associated AKI **(Szeto, *et. al.*, 2010)**. Notably, KIM-1 is overexpressed in renal cell carcinoma, therefore its extracellular domain can be detected in the urine of these patients **(Zhang, *et. al.*, 2014)**. Taking into consideration its specificity and sensitivity in early detection of renal injury, this biomarker may be used to detect renal dysfunction caused by nephrotoxic drugs **(Dieterle, *et. al.*, 2010)** .

Kidney Injury Molecule-1 is an established marker of ischemia and proximal tubular injury **(Zhang, *et. al.*, 2017)**. It was approved by the US Food and Drug Administration (FDA) as a biomarker of AKI in preclinical drug research **(Dieterle, *et. al.*, 2010).** This protein was proved to be particularly

useful in the diagnosis of AKI resulting from shock, surgery, or toxic damage and sepsis **(Liang, *et. al.*, 2010).**

1.5. Calprotectin, Cystatin C, and Kidney Injury Molecule-1 in Covid-19

1.5.1. Calprotectin in Covid-19

Calprotectin levels were significantly higher in Covid- 19 patients who required mechanical ventilation, similarly to CRP **(Shi, *et. al.*, 2020)**. A severe course of disease is characterized by a dysregulated immune response, suspected to be initiated by dysregulation of innate immune cells of the granulomonocytic lineage. The pro-inflammatory mediator calprotectin (S100A8/A9, MRP 8/14) is reported to be an early signal, mediating the cytokine storm associated with an increased severity of Covid-19 **(Silvin, *et. al.*, 2020)**. The expression of calprotectin is predominantly restricted to the intracellular compartment of neutrophil granulocytes, where it presents about half of the total cytosolic protein content. In contrast to routinely used inflammatory biomarkers such as CRP and PCT, it is released into the bloodstream without need for *de novo* protein biosynthesis. Thereby, circulating calprotectin possibly has a decisive kinetic advantage in that it might be one of the first responses of an organism to an inflammatory disease. Previous studies have reported significantly elevated levels of calprotectin in patients with severe Covid-19 and the possible ability of calprotectin to discriminate between mild and severe form of the disease **(Chen, L. *et. al.*, 2020).** In addition, elevated fecal calprotectin has been shown to associate with thromboembolic events in Covid-19 in the absence of gastrointestinal manifestations **(Giuffre, *et. al.*, 2020).**

1.5.2. Cystatin C in Covid-19

Cystatin C is a cysteine protease inhibitor secreted from human cells to blood circulation and directly interacts with human coronaviruses **(Zi and Xu,**

2018). Apart from the indication of renal function **(Fernando and Polkinghorne, 2020)**, serum cystatin C originated from human alveolar macrophages might also associate with lung inflammation and respiratory dysfunction **(Telo, *et. al.*, 2018)**. Furthermore, cystatin C was suggested as an independent predictive factor of high mortality among the elderly population **(Ingelfinger and Marsden, 2013).** As yet, little was known of the relationship between circulating cystatin C levels and disease severity or all-cause death in Covid-19. Several studies reported kidneys alterations, as reflected by increased serum creatinine, in Covid-19 patients. SARS-CoV-2 could directly infect kidney tubular cells, which express the ACE2 receptor on their cellular surface. Moreover, circulating mediators could interact with kidney- resident cells resulting in endothelial dysfunction, microcirculatory derangement, and tubular injury **(Joannidis, *et. al.*, 2020)**.

1.5.3. Kidney Injury Molecule-1 in Covid-19

Kidney Injury Molecule-1 has been identified as a new entry pathway for SARS-CoV-2 and its increase in correlation with disease severity as shown in study indicates that it may be used as a therapeutic target in the future **(Kerget, *et al.*, 2021)**. The interaction of KIM-1/TIM-1 with T cells plays an important role in immune response, allergy, asthma, autoimmune diseases, and response to viral infections **(Ichimura, *et. al.*, 2020)**. Initial evaluations of KIM1/TIM-1 in Covid-19 patients primarily focused on its relationship with acute kidney damage **(Luther, *et. al.*, 2020).** Like ACE-2 receptor, KIM-1/TIM-1 was found to facilitate the viral entry into cells via the IgV domain. Higher pro-inflammatory cytokine levels due to increase the presence of virus in blood may cause further progression of kidney damage **(Yang, X. *et. al.*, 2020)**. In studies with SARS-CoV and MERS-CoV, it was also determined that the IgV unit facilitates entry for other members of the family **(Rabaan, *et. al.*, 2020)**. In

another publication, it was suggested that KIM-1/TIM-1 receptors are abundant in the lungs and kidneys and that TW-37, a molecule that can inhibit anti-KIM-1/TIM-1 antibody and endocytosis, may be used as a therapeutic target **(Ichimura*, et. al.*, 2020)**.

1.6. Aim of the Study:

According to the previous background, the aims of the presented study include:

1. Measuring of each biomarker calprotectin, cystatin C and kidney injury molecule-1 in sera of Covid-19 to evaluatin severity among moderate and severe group of Covid-19 patients.
2. Measuring of the other biomarkers used in Covid-19 diagnosis.
3. Study the correlations between each of calprotectin, cystatin C and kidney injury molecule-1 with the other biomarkers in severe Covid-19 cases.

Chapter Two

Subjects , Materials and Methods

2. Subjects, Materials, and Methods

2.1. Subjects

The presented cross-sectional study was included a group of (91) patient samples with age ranged between (22 – 88) years and they are divided into: (45) of them were patients infected with moderate Covid-19 and (46) cases were patients infected with severe Covid-19. The patients with Covid -19 were selected from Al-Hayat unit, Al-Hussein Teaching Hospital, Al-Hussein Medical City / Kerbala Health Directorates / Kerbala - Iraq during Oct., 2020 to July, 2021. The sociodemographic aspects of the patients were collected through the self-reported technique (student questionnaire) including age, gender, and having any current chronic diseases (Hypertension, diabetes mellitus, ischemic heart disease, smokers or non-smokers) were taken from each patient.

The patients were considered to have severe / moderate Covid-19 depending upon fever, respiratory manifestations and radiological indicative of pneumonia. Patients were considered to have severe Covid-19 if any of the following changes was present:

(i) Respiratory distraction (≥ 30 / min)
(ii) Resting oxygen saturated $\leq 90\%$ or
(iii) Arterial oxygen (PaO_2) / fraction of inspired oxygen ≤ 300 mmHg .or
(iv) Respiratory failure requiring mechanical ventilation and require intensive care unit.

This investigation was approved by local medical ethics and all participants, information consent before the onset of study. The patients were registered and handed over a file for recording their data such as name, age, gender, weight, height, smoking states, current chronic diseases (diabetes mellitus, hypertension). Serum Calprotectin, Cystatin C, Kidney injury

molecule-1, C- reactive protein, Ferritin, Creatnine, Urea, Complete blood counts were investigated.

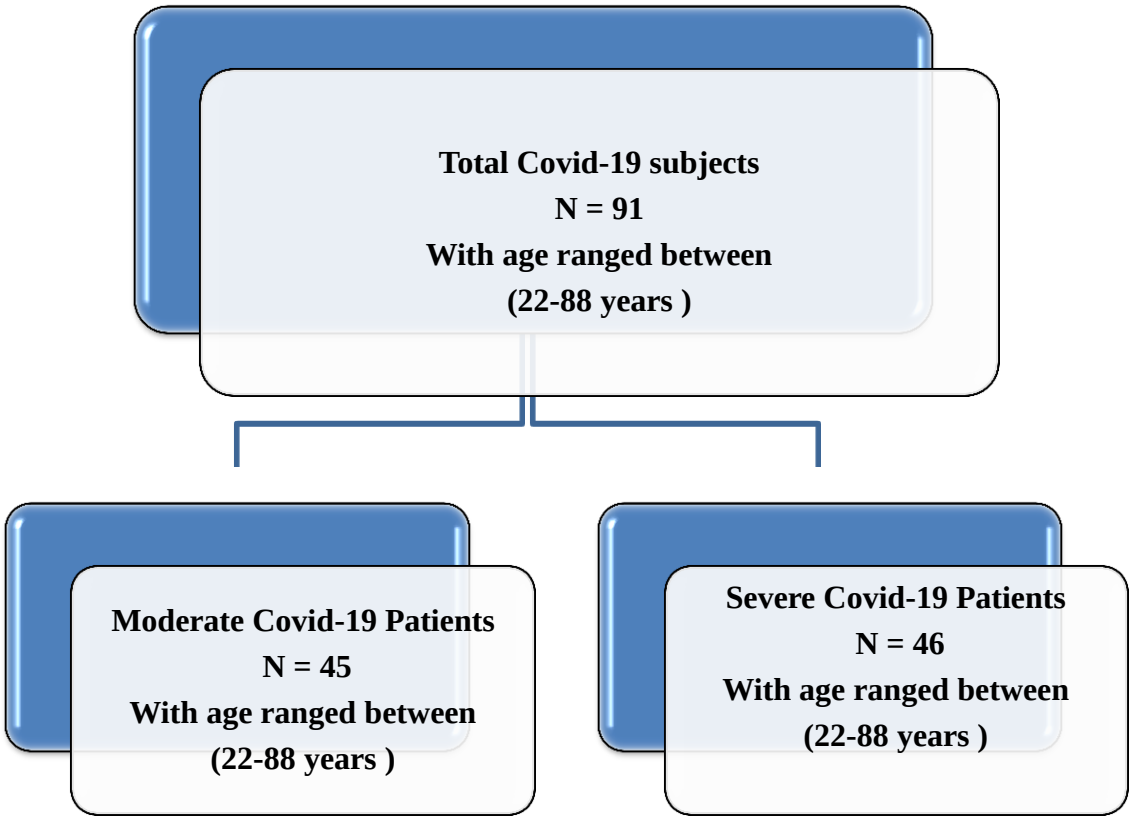


Fig. (2-1): Schematic representation of subjects groups

Exclusion criteria:

The study excluded patients with any chronic or immune diseases like receiving long term oral corticosteroid, anti-IL-6 or anti-TNF therapy and patients had history of vasculitis connective tissue disease. Patients suffering from cancer, systematic immune disease, and also thyroid gland diseases were also excluded.

Approval of the Ethical Committee

The protocol of the study was approved by Ethical Committee of College of Medicine, University of Kerbala, and committee of Al-hayat unit in Al-Hussein

Teaching Hospoital, Kerbala Medical City. Samples from serum were obtained after consent from patients or the patients' relatives.

A structured questionnaire was specifically design to obtained information which helps to select individuals according to the selection criteria of the study. Sociodemographic aspects of the patients were also collected through the self-reported technique (student questionnaire) which including: age, gender.

Blood Collection

Blood samples were collected from Al-hayat unit of Al Hussein Teaching Hospital. 5 ml of blood samples were drown by venipuncture using 5 ml disposable syringes. 3 ml of blood was left for (15 min) at room temperature in gel tube. Serums were separated by centrifuging for 10 minutes at approximately 300 x g. Serum samples were aliquot into two eppendrof tube and store at -20°C to avoiding multiple freezing-thawing cycles, and 2 ml of blood was collected by ethylene diamine tetraacetic acid (EDTA) anticoagulant tube. The samples were left on mixruller at room temperature to avoid formation of clot. The samples were used for determination of CBC.

2.2. Materials

2.2.1. Instruments

All the instruments and tools which are used in this study are shown below in the table 2-1.

Table (2-1): The instruments used in the study

Instrument	Suppliers
Centrifuge	HETTICH/ Germany
Deep Freeze	COOLTECH/ China
ELISA system	UNO/HUMAN/ Germany
XP-300™ automated hematology analyzer Sysmex	Japan
Cobas e 411	Germany
Cobas e 311	Germany
Roller Mixer	China
Various laboratory tools	Various suppliers

2.2.2. Chemicals and Kits

The chemicals and kits with their supplier which were used in this study are listed in table (2-2).

Table (2-2): Chemicals and kits which used in this study

Chemicals	Source
Human Calprotectin ELISA kit	Bioassay technology laboratory, China
Human Cystatin C Elisa kit	Bioassay technology laboratory, China
Human Kidney injury molecule-1ELISA kit	Bioassay technology laboratory, China
C-Reactive protein Kit	China
CBC Kit	Japan
Ferritin Kit	Maglumi, German
Creatinine Kit	Randox , U.K
Urea Kit	Mindray, China

2.3. Methods

2.3.1. Determination of Serum Calprotectin Levels

Enzyme Linked Immunosorbent Assay System (ELISA) was performed using sandwich method to determine the concentrations of serum calprotectin levels.

Principle:The plate was been pre-coated with human CLP antibody. CAL present in the sample was added and binds to antibodies coated on the wells. And then biotinylated human CLP Antibody is added and binds to CLP in the sample. Then Streptavidin-HRP was added and binds to the Biotinylated CLP antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and color develops in proportion to the amount of human CLP. The reaction was terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Provided: The following table indicate the reagents used for CLP determination (2-3).

Table (2-3): Reagents used for CAL determination

Components	Quantity
Standard Solution (640ng/ml)	0.5 ml x 1
Pre-coated ELISA Plate	12* 8 well strips x 1
Standard Diluent	3 ml x 1
Streptavidin-HRP	6 ml x 1
Stop Solution	6 ml x 1
Substrate Solution A	6 ml x 1
Substrate Solution B	6 ml x 1
Wash Buffer Concentrate (25x)	20 ml x 1
Biotinylated human CAL Antibody	1 ml x 1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation : All reagents should be brought to room temperature before use.

Standard: The 120µl of the standard Reconstitute the 120 µl (640 ng/ml) was reconstituted with 120µl of standard diluent to generate a 320 ng/ml standard stock solution. The standard was allowed to sit for (15 min) with gentle agitation prior to making dilutions. The duplicate standard points were prepared by serially diluting the standard stock solution (320 ng/ml) 1:2 with standard diluent to produce 160 ng/ml, 80 ng/ml, 40 ng/ml and 20 ng/ml solutions. Standard diluent was served as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month.

Table (2-4): Dilutions of standard solutions of

Standard Concentration	Standard No.	
320 ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
160 ng/ml	Standard No.4	120µl Standard No.5+ 120µl Standard Diluent
80 ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
40 ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
20 ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

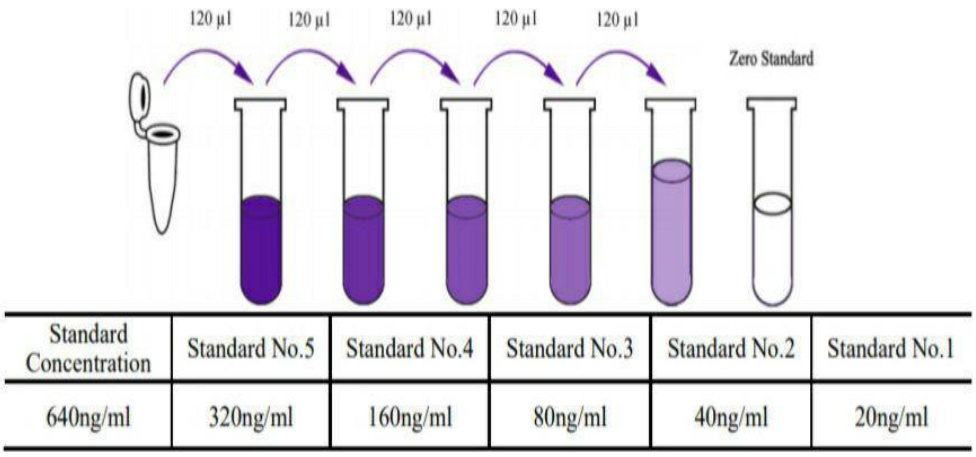


Figure (2-2): Dilutions of standard solutions of Calprotectin

Wash Buffer: 20 ml of wash buffer concentrate 25 X was diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer. When crystals had formed in the concentrate, the solution was mixed gently until the crystals had completely dissolved.

Assay procedure:

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use. The assay was performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. 50 µl standard was added to standard well. **Note:** Antibody did not added to standard well because the standard solution contained biotinylated antibody.
4. 40 µl sample was added to sample wells and then 10µl anti-CLP antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells (Not blank control well). They were mixed well. The plate was covered with a sealer and it was incubated 60 minutes at 37°C.
5. The sealer was removed and the plate was washed 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. 50 µl substrate solution A was added to each well and then 50µl substrate solution B was added to each well. Plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.
7. 50 µl Stop Solution was added to each well, the blue color changed into yellow immediately.

8. The optical density value of each well was determined by using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result:

Construct a standard curve by graphing the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and then drawing a best fit curve through the points on the graph. These calculations are best carried out using computer-based curve-fitting software, and the optimum fit line can be determined by regression analysis.

2.3.2. Measurement of Serum Cystatin C

Assay Principle:

This kit was an Enzyme-Linked Immunosorbent Assay (ELISA). The plate was been pre-coated with human Cys C antibody. Cystatin C present in the sample was added and binds to antibodies coated on the wells. And then biotinylated human Cys C Antibody was added and binds to Cys C in the sample. Then Streptavidin-HRP is added and binded to the Biotinylated Cys C antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and color develops in proportion to the amount of human Cys C. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm.

Table (2-5): Reagents used for Cys C determination

Components	Quantity
Standard Solution (4.8mg/dl)	0.5 ml x 1
Pre-coated ELISA Plate	12* 8 well strips x 1
Standard Diluent	3 ml x 1
Streptavidin-HRP	6 ml x 1
Stop Solution	6 ml x 1
Substrate Solution A	6 ml x 1
Substrate Solution B	6 ml x 1
Wash Buffer Concentrate (25x)	20 ml x 1
Biotinylated human Cys-C Antibody	1 ml x 1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pics

Reagent Preparation: All reagents should be brought to room temperature before use.

Standard: The 120 µl of the standard (4.8 mg/dl) was reconstituted with 120 µl of standard diluent to generate a 2.4 mg/dl standard stock solution. The standard was allowed for 15 min with gentle agitation prior to making dilutions. The duplicate standard points were prepared by serially diluting the standard stock solution (2.4 mg/dl) 1:2 with standard diluent to produce 1.2 mg/dl, 0.6 mg/dl, 0.3 mg/dl and 0.15 mg/dl solutions. Standard diluent was served as the zero standards (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month.

Table (2-6): Dilutions of standard solutions of Cystatin C

Standard Concentration	Standard No.	
0.15 mg/dl	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent
0.3 mg/dl	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
0.6 mg/dl	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
1.2 mg/dl	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
2.4 mg/dl	Standard No.5	120µl Original Standard + 120µl Standard Diluent

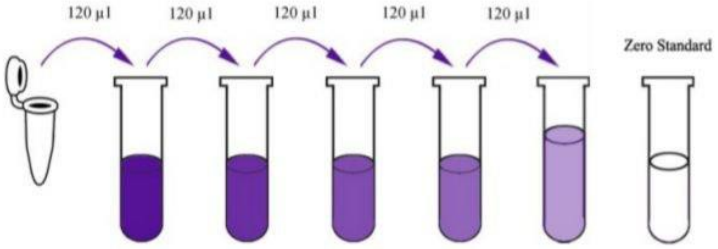


Table (2-3): Dilutions of standard solutions of Cystatin C

Wash Buffer: 20 ml of wash buffer concentrate 25 X was diluted into deionized or distilled water to yield 500 ml of 1x Wash Buffer. When crystals had formed in the concentrate, the solution was mixed gently until the crystals had completely dissolved.

Assay Procedure:

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use . The assay was performed at room temperature.
2. Determine the number of strips required for the assay. The strips were **inserted** in the frames for use. The unused strips should be stored at 2-8°C.
3. 50 µl standards were added to standard well. **Note:** Antibody did not added to standard well because the standard solution contained biotinylated antibody.

4. 40µl sample was added to sample wells and then 10µl anti-Cys C antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells (Not blank control well). They were mixed well. The plate was covered with a sealer and it was incubated 60 minutes at 37 °C
5. The sealer was removed and the plate was washed 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. 50 µl substrate solution A was added to each well and then 50 µl substrate solution B was added to each well. Plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark
7. 50 µl stop solution was added to each well, the blue color changed into yellow immediately.
8. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculation of Result:

Construct a standard curve by graphing the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and then drawing a best fit curve through the points on the graph. These calculations are best carried out using computer-based curve-fitting software, and the optimum fit line can be determined by regression analysis.

2.3.3. Measurement of Serum Kidney Injury Molecule-1

Assay Principle

This kit was an Enzyme-Linked Immunosorbent Assay (ELISA). The plate was been pre-coated with human KIM-1 antibody. Kidney Injury Molecule-1 present in the sample was added and binded to antibodies coated on the wells. And then biotinylated human KIM-1 Antibody was added and binds to KIM-1 in the sample. Then Streptavidin-HRP was added and binded to the Biotinylated KIM-1 antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and color developed in proportion to the amount of human KIM-1. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm.

Table (2-7): Reagents used for KIM-1 determination

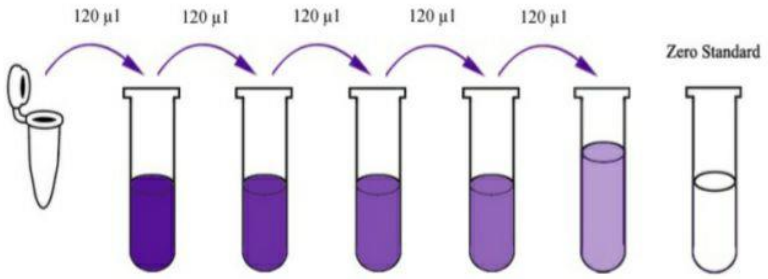
Components	Quality
Standard Solution (40 μg/ml)	0.5 ml x 1
Pre-coated ELISA Plate	12 x 8 well strips x 1
Standard Diluent	3 ml x 1
Streptavidin-HRP	6 ml x 1
Stop Solution	6 ml x 1
Substrate Solution A	6 ml x 1
Substrate Solution B	6 ml x 1
Wash Buffer Concentrate (25x)	20 ml x 1
Biotinylated human KIM-1Antibody	1 ml x 1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Reagent Preparation: All reagents were prepared freshly at room temperature before used.

1. The 120 µl of the standard (12.8 ng/ml) was reconstituted with 120µl of standard diluent to generate a 6.4ng/ml standard stock solution. The standard was allowed to sit for (15 mins) with gentle agitation prior to making dilutions. The duplicate standard points were prepared by serially diluting the standard stock solution (6.4 ng/ml) 1:2 with standard diluent to produce 3.2 ng/ml, 1.6ng/ml, 0.8ng/ml and 0.4ng/ml solutions. Standard diluent served as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month.
2. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25 X into deionized or distilled water to yield 500 ml of 1 X Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Table (2-8): Dilution of standard solutions of Kidney Injury Molecule-1

Standard Concentration	Standard No.	
0.15 ng/ml	Standard No.5	120µl Original Standard + 120µl Standard Diluent
0.3 ng/ml	Standard No.4	120µl Standard No.5+ 120µl Standard Diluent
0.6 ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
1.2 ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
2.4 ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
12.8ng/ml	6.4ng/ml	3.2ng/ml	1.6ng/ml	0.8ng/ml	0.4ng/ml

Table (2-4): Dilution of standard solutions of Kidney Injury Molecule-1

Assay Procedure:

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use. The assay was performed at room temperature.
2. Determine the volume of strips needed for the assay. For use, the strips were inserted into the frames. Unused strips should be kept at 2-8 °C.
3. 50 µl standards were added to standard well. **Note:** Antibody did not added to standard well because the standard solution contained biotinylated antibody
4. 40 µl sample was added to sample wells and then 10µl anti-KIM-1 antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells (Not blank control well). They were mixed well. The plate was covered with a sealer and it was incubated 60 minutes at 37°C.
5. After removing the sealer, the plate was washed five times with wash buffer. For each wash, soak wells in at least 0.35 mL wash buffer for 30 seconds to 1 minute. Aspirate all wells and wash with wash buffer five times, overfilling wells with wash buffer, for automatic washing. Using paper towels or some other absorbent material, blot the plate.

6. Each well added 50 of substrate solution A, followed by 50µl of substrate solution B. For 10 minutes at 37 °C in the dark, the plate was incubated and covered with a new sealer.
7. 50 µl Stop Solution was added to each well, the blue color changed into yellow immediately
8. Within 10 minutes of adding the stop solution, the optical density (OD value) of each well was measured using a microplate reader set to 450 nm.

Calculation of Result:

Construct a standard curve by graphing the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and then drawing a best fit curve through the points on the graph. These calculations are best carried out using computer-based curve-fitting software, and the optimum fit line can be determined by regression analysis.

2.3.4. Determination of C-Reactive Protein Level

Principle

The FinecareTM CRP Quantitative test is based on fluorescence immunoassay technology. The FinecareTM CRP Rapid Quantitative Test uses a sandwich immunodetection method. When the sample is added to the sample well of the Test Cartridge, the fluorescence- labled CRP antibody binds to CRP antigen in blood specimen. As the sample mixture migrates on the nitrocellulose matrix of test strip by capillary action, the complexes of detector antibody and CRP are captured to CRP antibody that has been immobilized on test strip. Thus, if more CRP antigen is in blood specimen, more complexes are accumulated on test strip. Signal intensity of fluorescence of detector antibody reflects the amount of CRP captured and FinecareTM FIA Meter shows CRP concentrations in blood specimen. The default results unit of FinecareTM CRP Rapid Quantitative test is displayed as x mg/L from Finecare TM FIA Meter.

The working range and the detection limit of the CRP Test system are 0.5 - 200 mg/L and 0.5 mg/L.

Procedure

1. One hundred µl of standard or sample was added to each well. Incubated for 90 minutes at 37 °C
2. Discarded the liquid, immediately added 100µL Biotinylated detection Ab working solution to each well. Incubated for 60 min at 37 °C.
3. Aspirated and washed for 3 times.
4. One hundred µl of HRP conjugate was added. Incubated for 30 minutes at 37 °C
5. Ninety µl of substrate reagent was added. Incubated for 15 minutes at 37 °C.
6. Fifty µl of stop solution was added. Determined the Abs value at 450 nm immediately.

Calculation

The concentration was calculated from the standard curve. The standard curve has demonstrated a direct relation between CRP concentration and the corresponding absorbance. The results have appeared automatically by the ELISA reader program from the calibration curve.

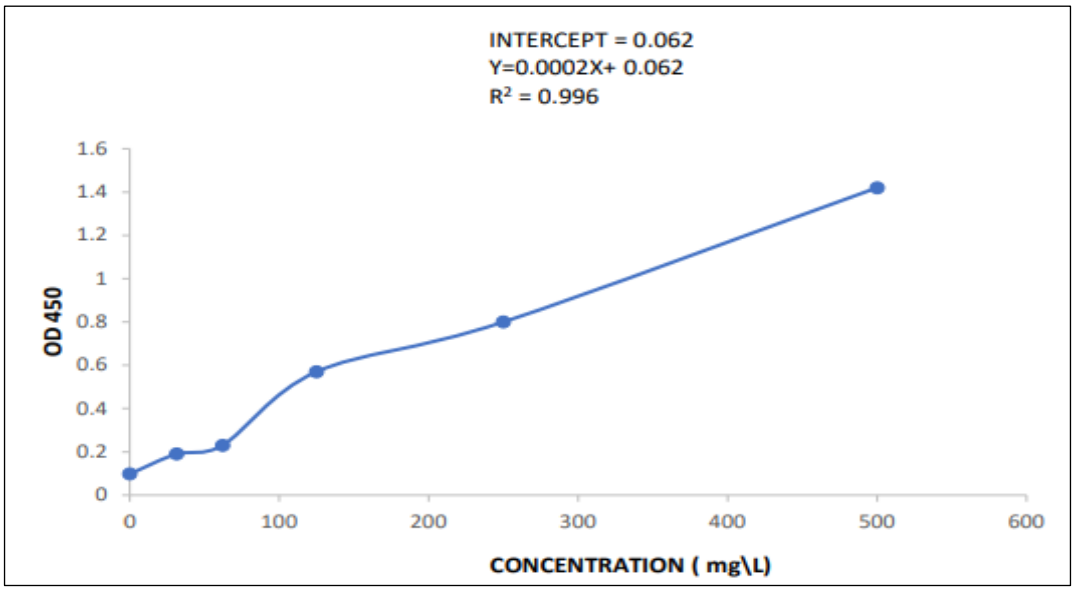


Figure (2-5): Standard curve of serum C-Reactive Protein

2.3.5. Determination of Serum Ferritin Level

Principle

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

Procedure

1. Allow Calibrators, QC and patient samples to reach ambient temperature.

2. Ensure that the amount of reagents, diluent, and wash solutions are adequate for the amount of samples to be run. You may place more than one bottle of

reagent at a time on the analyzer; however, avoid using more than one lot number of reagent for a single run.

3. Make sure the analyzer and/or tests required are not masked.

4. Check to see if calibration is required for the tests that will be run.

5. If running the same tests on all samples, go to the "Start" global button and set the "default profile".

6. Be sure to clear all previously programmed samples from the Data Review screen after backing up the data.

7. Perform the required maintenance on the e601 system.

8. The test result was read on the display of the Cobas test instrument.

Interpretation of test result

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

2.3.6. Determination of Serum Lactate Dehydrogenase Activity Level

Principle

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

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

Reagents- working solutions

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

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

Procedure:

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

Calculations

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

3.3.7. Determination of Complete Blood Count

The measures of CBC were done by XP-300™ Automated hematology analyzer Sysmex .

Principle:

- DC detection method for WBC .
- DC detection method for RBC/PLT .
- Non-cyanide haemoglobin analysis method for HGB .

Parameters: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYM%, MXD%, NEU%, LYM#, MXD#, NEU# RDW-SD, RDW-CV, PDW, and MPV.

Sample volume:

- Whole blood (WB) mode : Approximately 50µL .
- Pre-diluted (PD) mode : Approximately 20µL .

2.3.8. Measurment of Blood Urea

Reaction Principle:

$$\text{Urea} + 2H_2O \longleftrightarrow 2NH_4^+ + CO_3^{2-}$$

$$\alpha\text{-Oxoglutarate} + NH_4^+ + \text{NADH} \longleftrightarrow \text{L-Glutamate} + NAD^+ + H_2O$$

Urea was hydrolyzed by urease, and one of the products, ammonia, was helped to turn NADH to NAD^+ with the catalysis of GLDH. The absorbency decrease was directly proportional to the concentration of urea.

Table (2-9): Reagents Components and concentrations:

R1	Tris buffer ADP	120 mmol/l 750 mmol/l
	Urease GLDH	240 KU/l 0.4 KU/l
R2	NADH Oxoglutrate	1.2 mmol/l 25 mmol/l

Method: Urease-glutamate Dehydrogenase, UV method

Assay procedure:

Three sets of tubes (sample, standard and blank) were prepared as the following table (2-10).

Table (2-10) Assay procedure of urea determination

	Blank	**Sample**
Reagent 1	1000 µl	1000 µl
Dist. Water	15 µl	.....
Sample	.....	15 µl

The tubes were mixed and incubated for 2 min at 37C ., then reagent 2 was added :		
Reagent 2	250 µl	250 µl
The tubes were mixed thoroughly, incubated at 37C for 90 s and then the absorbance change was read value over a further 90 s.		
Change in Absorbance / min, ΔA_{min} = [ΔA sample]- [ΔA blank]		

Calculation: The analyzer was calculated the Urea concentration of each sample automatically after calibration .Conversion factor: mg/dl × 0.166 = mmol/l **;** Or: C sample = (ΔA sample/ΔA calibration) × C calibration

2.3.9. Measurement of Serum Creatinine Concentration

Principle: Creatinine was a colored complex formed when an alkaline solution reacted with picric acid. The amount of complex produced was proportional to the concentration of creatinine (**Henry, 1974**). The reagents used in this procedure are

1. Picric Acid 35 mmol/l
2. Sodium Hydroxide 0.32 mol/l

Assay procedure:

	Blank S0	**Standard S1**	**Sample**
Distilled	50 µl	--	--
Standard	--	50 µl	--
Sample	--	--	50 µl
Reagent	500 µl	500 µl	500 µl
The tubes were mixed and the absorbance was read after 30 seconds A1 of the standard and sample. The absorbance was read A2 of standard and sample at 2 minute later			

Calculation:

Concentration of creatinine in serum

$$\text{Conc. Creatinine} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \text{ X Standard concentration } \mu mol/l$$

$$\text{Conc. Creatinine} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \text{ X Standard concentration mg/dl}$$

Normal value = 0.6-1.1 mg/dl for men, 0.5-0.9 mg/dl for women

2.4. Statistical Analysis

Information from the questionnaire and all test results from patients samples were organized in several data sheets. The data analysis for this work was generated using the Real Statistics Resource Pack software for Mac(Release 7.2) of the resource pack for Excel 2016. Copy-right (2013 – 2020).

Descriptive statistics was performed on the participants' data of each group. Spearman correlation test was used to calculate the association coefficient (r) to assess the strength, direction and significance of association positive signed (r) indicated direct (positive association) and the negative signed indicated versed association, the value of (r) ranged from 0 (no association) and 1(perfect ssociation), the higher (r) value, the stronger the association . The distribution of the data was checked for normality using the Box plot test.

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

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Demographic Characteristics of Covid-19 Patients Groups

The clinical demographic characteristics and laboratory parameters of both patients groups of Covid 19 were summarized in Table (3-1).Table illustrated the mean age of participants which was within the age group of (22 – 88) years old that is higher in severe than in moderate. Older people are more likely to progress to severe disease than middle-aged adults or young people **(Liu, W. *et al*, 2020)**. Gender distribution among the studied groups was: 62% males, 38% females for patients group. These disparities in sex mainly relate to factors concerning social behaviour and human biology **(Gebhard, *et al*., 2020).** Zhao *et al*. found cell type-specific expression of the ACE2 receptor in type II alveolar epithelial cells is higher in males than in females **(Zhao, Y. *et al*., 2020)**.The diabetes mellitus and hypertension were higher in severe than moderate. Another report showed that the in patients with Covid-19 who developed most frequent comorbidities syndrome were hypertension (27%), the acute respiratory distress (6%) **(Wu, C. *et al*., 2020)**. diabetes (19%), and cardiovascular disease

There is growing evidence to support WHO's statements that smokers are at a higher risk of developing severe Covid-19 and consequent death **(Grundy, *et al*., 2020)**.

Table (3-1): Demographic characteristics of Covid-19 patients in severe and moderate groups

Characteristics		**Patient Group**		Normal Value
		Moderate	**Severe**	
Demographics	Age Mean(Median)	56.82(56)	63.04(62)	
	Gender (Male/Female)	(28/17)	(29/17)	
Medical History	Smoking(Yes/No)	(5/40)	(7/39)	
	DM(Yes/No)	(17/28)	(22/24)	
	HT(Yes/No)	(19/26)	(26/20)	
	Sat. O_2 Mean(Median)	91.87(92)	70.87(72)	95% or higher
Biochemical parameters Mean(Median)	CRP	85.58 (42)	63.65 (42.95)	0-6 mg/L
	LDH	409.31 (348)	524.31 (444)	240-480 U/L
	Ferritin	598.63 (490)	894.99 (630.4)	20-350 ng/mL
	S.Creatinine	2.67 (1.03)	1.11(0.79)	0.7-1.2 mg/dl
	S.Urea	57.708 (44.3)	54.45 (44.1)	12-45 mg/dl
	CLP	78.24 (64.98)	89.27 (65.14)	31.64-126.97 ng/ml
	Cys C	0.49 (0.48)	0.48 (0.47)	0.24-0.76 mg/dl
	KIM-1	1.701 (1.43)	1.506 (1.36)	0.76-3.26 ng/ml
Complete Blood Count Mean(Median)	RDW-CV	14.01 (13.80)	14.91(14.55)	11.5-14.5%
	WBC	14.47 (11.70)	14.99 (14.60)	4-11(10^9/L)
	NEU%	82.51(85.30)	84.77 (87.90)	39.3-73.7 %
	LYM%	10.84 (8.40)	8.77 (6.70)	18-45.3 %
	HGB	16.081(12.80)	12.62(12.55)	13.5-17.5 g/dL

3.2. Examination the distribution of data in the studied groups

In descriptive statistics, a box plot or boxplot (also known as box and whisker plot) is a type of chart often used in explanatory data analysis. Box plots visually show the distribution of numerical data and skewness through displaying the data quartiles (or percentiles) and averages.

Box plots show the five-number summary of a set of data: including the minimum score, first (lower) quartile, median, third (upper) quartile, and maximum score. The median is the average value from a set of data and is shown by the line that divides the box into two parts. In statistics, dispersion (also called variability, scatter, or spread) is the extent to which a distribution is stretched or squeezed. The smallest value and largest value are found at the end of the 'whiskers' and are useful for providing a visual indicator regarding the spread of measurements. On the other hand, figures also indicated that the interquartile ranges of the boxes regarding patients groups have more dispersion of a data set with indicated more variability.

3.2.1. Distribution (CRP, Ferritin and LDH)

Figure (3-1) to figure (3-3) demonstrate the distribution of CRP, Ferritin, and LDH in different stage of Covid-19 patients groups. The levels of CRP, Ferritin, and LDH were varied based on severity of disease. The median levels of CRP in moderate and severe groups were 42, 42.95 mg/l while the mean of ferritin in moderate and severe groups were 491.2, 630.1 ng/mL respectively and the mean of LDH in moderate and severe groups were 348, 444.23 U/L respectively, table (3-1).

A) C-reactive protein : Higher CRP levels are also linked to development of acute respiratory distress syndrome, which is observed in patients with severe Covid-19 **(Wu, J. *et. al.*,2020).** In COVID-19, the exact effect of CRP remains unclear, but it was reported that their level can be used for early diagnosis of pneumonia **(Chalmers, *et. al.*,2020),** and assessment of severe pulmonary infectious diseases. The elevated levels of CRP might be linked to the overproduction of inflammatory cytokines in patients with COVID-19. Cytokines fight against the microbes, Thus, CRP production is induced by inflammatory cytokines and by tissue destruction in patients with COVID-19. CRP levels in our studies increased in severe and moderate group, in others study CRP level could also be used in monitoring the progression and improvement of patients with Covid-19 **(Li, H. *et. al.*,2020)**. CRP was found at increased levels in the initial stage than those in the critical group **(Tan, *et. al.*,2020).**

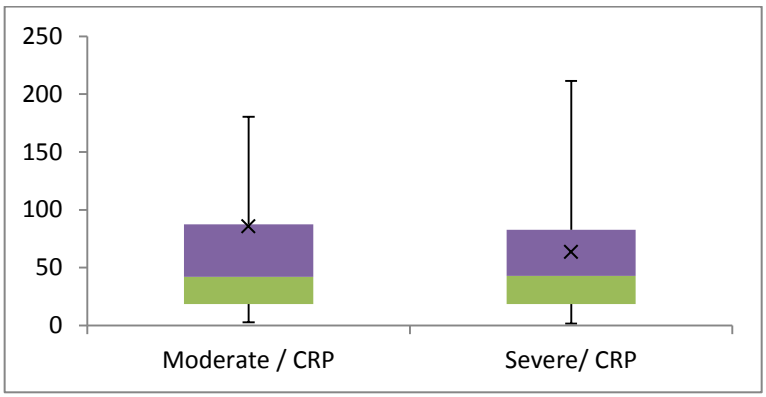


Figure (3-1): Boxplot of the Distribution of serum level of CRP mg/L in COVID-19 patients based on their disease severity.

B) Ferritin: Hyperferritinemia caused by the excessive inflammation due to the infection is associated with the admission to the intensive care unit and high mortality, and represents an indication to recognize high-risk patients to guide the therapeutic intervention to control inflammation **(Kernan and Carcillo, 2017).** Ferritin levels greater than

800 g/l were found in 100% of patients with severe disease and 30% of those with non-severe disease **(Ji, *et. al*., 2020).** Ferritin showed higher levels in non-survivors throughout the clinical course, and increased with disease deterioration **(Zhou, P. *et. al*., 2020).** Ferritin levels were also elevated in severe compared to moderate cases in a smaller retrospective study of 21 patients with Covid-19 conducted in China **(Chen**, Q. ***et. al*., 2020).** A study focusing on early hyperinflammation in Covid-19 patients evaluated the high levels of serum ferritin in the first seven days of hospitalization as a predictor for the cytokine storm syndrome, **(Henry, *et. al*., 2020)**,and a meta-analysis including 21 studies (3377 patients and 33 laboratory parameters) also demonstrated serum ferritin as a biomarker for potential progression to critical illness. **(Caricchio, *et. al*., 2020)**.

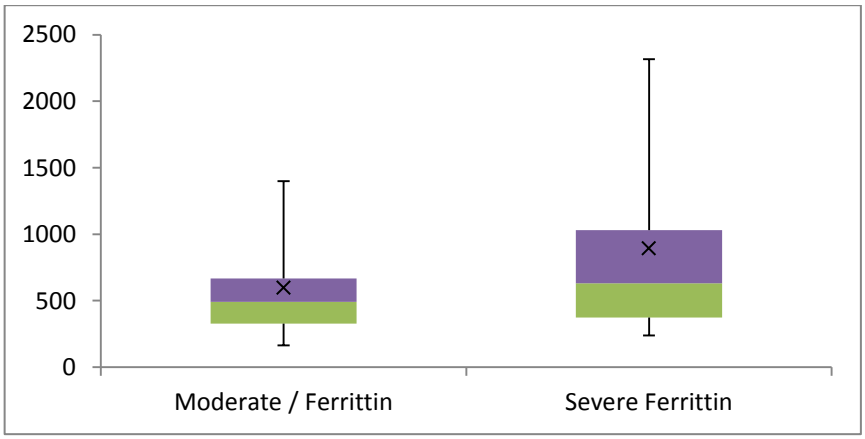


Figure (3-2): Boxplot of the Distribution of serum level of Ferritin ng/mL in COVID-19 patients based on their disease severity.

C) Lactate Dehydrogenase (LDH): To date, studies have shown that patients with severe Covid-19 have elevated serum LDH levels **(Zhang, B. *et al*., 2020)** , but no study has specifically evaluated its effect on the severity and mortality of COVID-19.

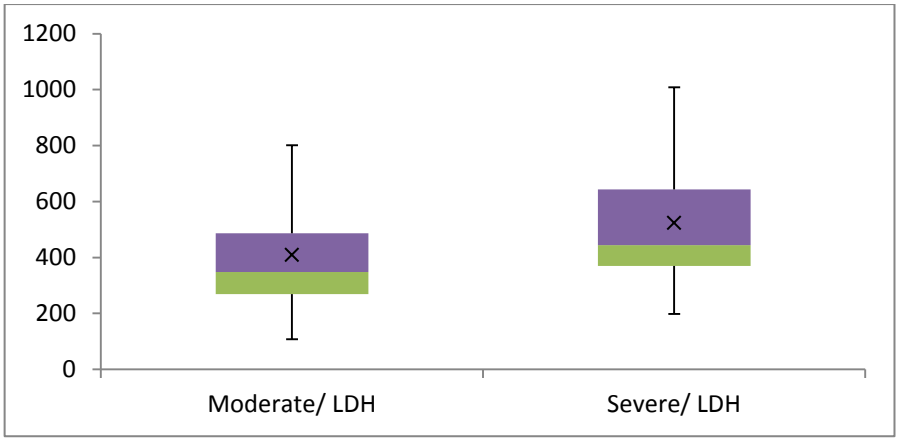


Figure (3-3): Boxplot of the Distribution of serum level of Lactate Dehydrogenase U/L in Covid-19 patients based on their disease severity.

3.2.2. Distribution of Calprotectin (CLP): Figure (3-4) demonstrate the distribution of CLP in different stage of Covid-19 patients groups. The levels of CLP were varied based on severity of disease. The median levels of CLP in moderate and severe groups were 64.98, 65.15 ng/ml.

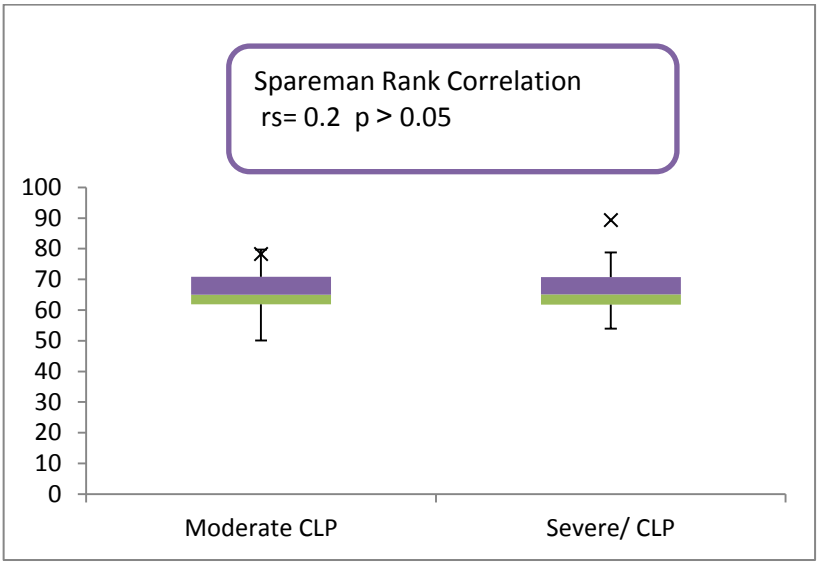


Figure (3-4): Boxplot of the Distribution of serum level of Calprotectin ng/ml in Covid-19 patients based on their disease severity

There was no significant correlation in CLP when compare it between moderate and severe group of Covid-19 patient group (rs= 0.2 p > 0.05), figure (3-4). Studies are ongoing, but there is not enough evidence at this time to support this finding and no scientific consensus whether or

not calprotectin can serve as a prediction of how serious the virus will be in some patients. Researchers will continue studying calprotectin in COVID-19 patients, but for now, calprotectin is still used primarily as a way for doctors to see if patients have inflammation in their intestines **(Shi, *et al*., 2020)**.

3.2.3. Distribution of Kidney Injury Markers (S,Creatinine, S.Urea, Cystatin C and Kidney Injury Molecule-1)

Figure (3-5) to figure (3-8) demonstrate the distribution of S.Cr, S.Urea, Cys C and KIM-1 in different stage of Covid-19 patients groups. The levels of theses biomarker were varied based on severity of disease. The median levels of S.Cr in moderate and severe groups were 1.03, 0.79 mg/dl while the median of serum urea in moderate and severe groups were 44.3, 44.1 mg/dl respectively, the median of Cys C in moderate and severe groups were 0.48, 0.47 mg/dl and the median of KIM-1 in moderate and severe groups were 1.43, 1.36 ng/mL respectively.

A) **Distribution S,Creatinine and S.urea:** To date, a number of studies have revealed that patients with Covid-19 may have renal function damage, and the clinical manifestations include rise in sCr, and AKI **(Henry, *et. al.*, 2020)** . Analyzed kidney function in 193 Covid-19 patients and found that 31% of patients had an elevated level of blood urea nitrogen (BUN) and 22% had increased serum Creatinine **(Li, Z. *et. al.*, 2020)**. Prospective cohort study of 701 Covid-19 patients, the incidences of elevated creatinine and BUN were 14.4 and 13.1%, respectively **(Cheng, *et al*., 2020).**

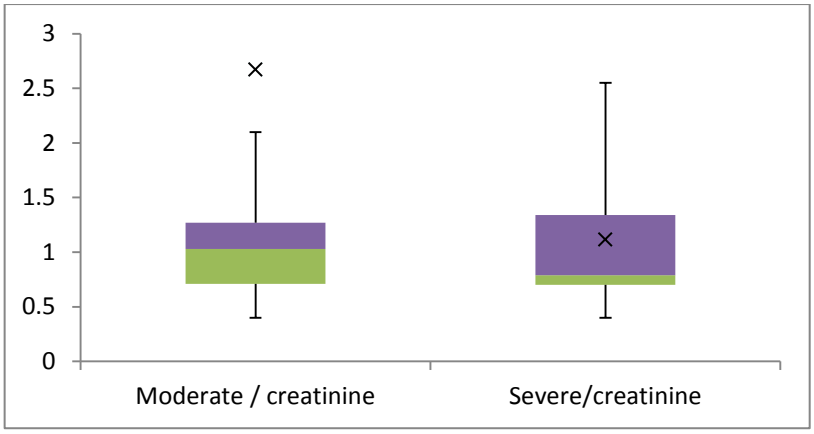


Figure (3-5): Boxplot of the Distribution of serum level of Creatinine mg/dl in Covid-19 patients based on their disease severity

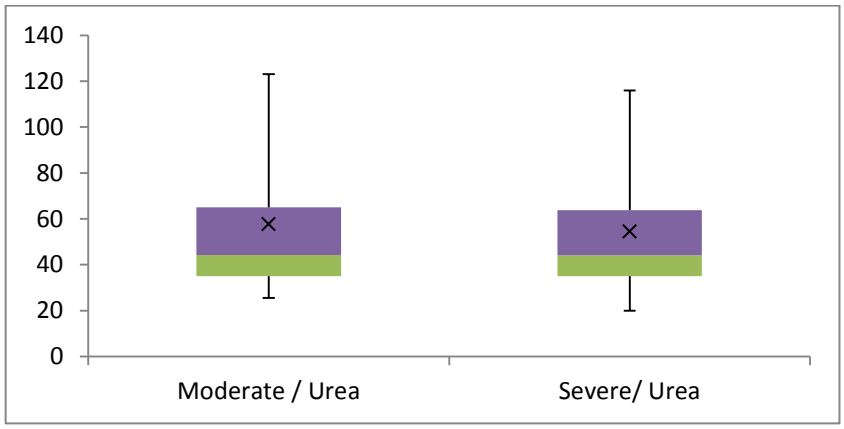


Figure (3-6): Boxplot of the Distribution of serum level of Urea mg/dl in Covid-19 patients based on their disease severity.

B) Distribution of Cystatin C: In support of While higher serum concentrations of cystatin C are likely to reflect the presence of kidney dysfunction, e.g., AKI, in patients with Covid-19, they might also be a marker of the excessive systemic inflammatory and pro-oxidant state that characterizes this group **(Fajgenbaum, *et al.*, 2010)**. In this study In Figure (3-4) there was no significant correlation in Cys C when compare it between moderate and severe group of Covid-19 patient group (rs= 0.3 p>0.05), figure (3-7).

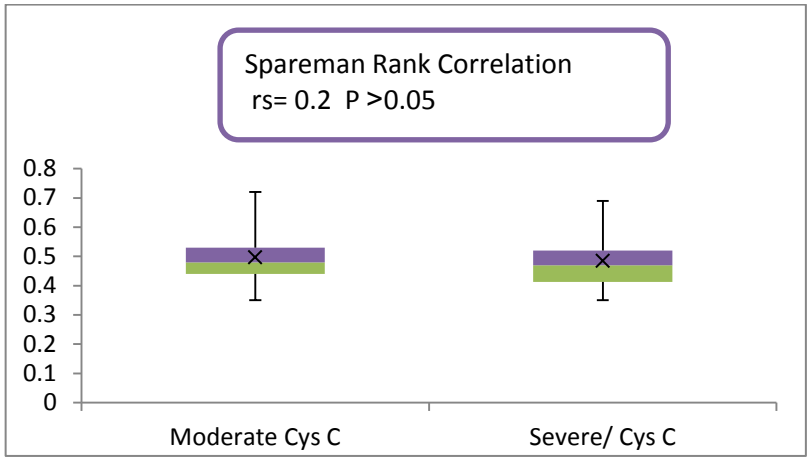


Figure (3-7): Boxplot of the Distribution of serum level of Cystatin C mg/dl in Covid-19 patients based on their disease severity.

Table (3-2) shows that correlation between Cys C, S.Cr and S.Urea in moderate and severe groups of Covid-19 patients. In severe group, there was a weakly significant positive-correlation between Cys C and S.Cr [rs=0.3, P value= 0.01].

Table (3-2): Correlation coefficients by Spearman rank test between Cystatin C and Creatinie and Urea based on severity of COVID-19 patients.

	Cyc C in Moderate patients		Cys C in Severe patients	
	rs	P value	rs	P value
S.Creatinine	0.14	0.3	0.3	0.01
S.Urea	0.16	0.2	0.04	0.7

Estimation of serum cystatin C can help early diagnosis of CKD, Compared with traditional renal function indexes, such as serum creatinine and urea nitrogen, Cys C can reflect glomerular filtration rate more accurately **(Iyare, *et al.*, 2019).** Serum creatinine is a more

sensitive index of kidney function compared to serum urea level. The mechanism of kidney injury following Covid-19 infection remains unclear, although the coexpression of ACE2 receptors and transmembrane serine proteases (TMPRSSs) is key for the entry of SARS-CoV-2 into host cells **(Zhang, B. *et al.*, 2020).** A single-cell transcriptome analysis showed that ACE2 and TMPRSS genes have relatively high coexpression in podocytes and proximal straight tubule cells **(Pan, X. *et al.*, 2020).** Therefore, kidney injury may be caused by direct viral infection. Moreover, cytokine storm syndrome, which is associated with sepsis following SARS-CoV-2 infection, might also cause kidney cell damage **(Mubarak, *et al.*, 2020).** Renal dysfunction may also result from volume depletion and multiple organ failure **(Selby, *et al.*, 2020).** A large retrospective study indicated that AKI occurred in approximately 90% of Covid-19 patients who required ventilation **(Hirsch, *et. al.*, 2020).**

C) Distribution of Kidney Injury Molecule-1: KIM-1 has been identified as a new entry pathway for SARS-CoV-2 and its increase in correlation with disease severity as shown in study indicates that it may be used as a therapeutic target in the future. KIM-1 has been associated with inflammation in renal tubular injury. Although the role of KIM-1 in AKI has been usually viewed as anti-inflammatory (as a receptor to phosphatidylserine, it increases the uptake of apoptotic and necrotic bodies) and involved in tubular cells' repair **(Song, *et al.*, 2019)**.

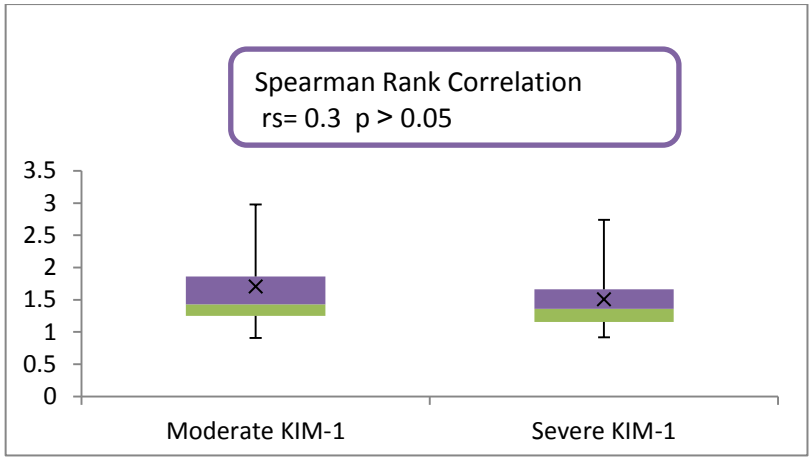


Figure (3-8): Boxplot of the Distribution of serum Kidney Injury Molecule-1 level of ng/ml in Covid-19 patients based on their disease severity.

Table (3-3) shows that correlation between KIM-1, CLP, Cys C, S.Cr and S.Urea in moderate and severe groups of Covid-19 patients. In severe group, there was a moderately significant positive-correlation between KIM-1 and CLP[rs=0.4, P value= 0.002].In modetate group there was weakly significant positive-correlation between KIM-1 and Cys C [rs=0.3, P value= 0.01] and in severe group there was strongly significant positive-correlation between KIM-1 and Cys C [rs=0.5, P value= 0.0001].

Table (3-3): Correlation coefficients by Spearman rank test between KIM-1 and CLP, Cys C, Creatinine and Urea based on severity of COVID-19 patients.

	KIM-1 in Moderate patients		KIM-1 in Severe patients	
	rs	P value	rs	P value
CLP	0.1	0.2	0.4	0.002
Cys C	0.3	0.01	0.5	0.0001
S.Creatinine	0.08	0.5	0.2	0.08
S.Urea	0.1	0.5	0.1	0.3

As a consequence of its role in enhancing the clearance of dead cells by the surviving tubular cells, KIM-1 may act to modulate immune response in AKI, and phagocytosis of apoptotic cells may downregulate the proinflammatory immune response **(Bonventre and Yang,2010).** Van Timmeren *et al*. reported that in various kidney diseases, KIM-1 expression in kidney bioptates was detected in areas of inflammation and fibrosis, while urinary KIM-1 increased in parallel to increased tissue expression and correlated with inflammation **(Van Timmeren, *et al*., 2007).** There was no significant correlation in KIM-1 when compare it between moderate and severe group of Covid-19 patient group (rs= 0.2 p>0.05), figure (3-8). There are not enough studies that illustrated the association between KIM1 and Covid-19 , but Notably, KIM1 and ACE2 co-expressed in kidney, colon, rectum, testis and gallbladder which are all among the target organs of SARS-CoV-2 **(Cha, *et. al*., 2020)** implicating a potential close correlation of KIM1 with Covid-19 manifestations.

Conclusion and Recommendation

Conclusions and Recommendations

1. Conclusion

- There is no significant correlation in CLP, Cys C and KIM-1 and cannot serve as indicator biomarkers when compare them among severe and moderate group.
- There is positive correlation between KIM-1 with Cys C in severe and moderate group, but only in severe group with CLP positive correlation.
- Significant positive correlation between Cys C and S.Cr in severe group of Covid-19 patients.

2. Recommendations

- ❖ Further research is warranted to better characterize and justify the routine clinical use of serial CLP assessments in Covid-19.
- ❖ Future studies using multivariate analyses are needed to identify independent predictors of severe disease and to define the best combination of markers to aid in the risk stratification and management of Covid-19 cases.
- ❖ Since most of the studies were retrospective in design, incomplete, and failed to report final outcomes due to the need for rapid publishing during the current pandemic, It might be a good idea to correlate the CLP, Cys C, and KIM-1 levels with pre/post Covid-19 cases in order to investigate their contributions through monitoring of disease or prediction of complications of disease.
- ❖ For make a superior information about the job of CLP, CysC, and KIM1 biomarkers in Covid-19, we recommend to creators to get different gatherings of Covid-19 patients (Mild and Critical) in the review and measure the GFR and contrast it and CysC and KIM1

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Appendix:

University of Kerbala – College of Medicine

Department of Biochemistry

M. Sc. Student Name: Hawraa Fadhil Abbas

Supervised by:

Prof. Dr. Fadhil Jawad Al-Tu'ma and Prof. Dr. Riyadh Mohe Al-Saiegh

((Experimental Data))

Sample No.:	O2:	Age:	Gender:
Chronic diseases: Hypertension T2DM Others		Smoking: Slightly Moderately Severe	
Inclusion criteria:			
Exclusion criteria:			

Biomarkers		
Biomarker	**Levels**	**Unit**
Ferritin		
Total LDH activity		
C-reactive protein		
CBC		
Serum Creatinine		
Blood Urea		
Calprotectin		
Cystatin C		
Kidney Injury Molecule-1		

الخلاصة

النبذة يسبب مرض فيروس كورونا التاجي المستجد متلازمة تنفسية حادة و وخيمة ، وانتشر الفيروس التاجي إلى العديد من البلدان وأصبح بمثابة جائحة عالمي. تحدث الوفاة نتيجة لمرض فايروس كورونا الشديد بسبب زيادة نقص الأكسجة في الدم ومتلازمة الضائقة التنفسية الحادة وفشل الأعضاء المتعددة.

تؤثر الجائحة على الكلى في ما يقرب من 3-9 ٪ من المرضى وتلف الكلى الحاد المتطور (AKI)وهو أعلى بكثير. يلعب قياس مستويات السيستاتين سي في الدم دورًا أكبر في تقدير وظائف الكلى .الكالبروتكتين هو عضو في عائلة بروتين S100 المرتبط بالكالسيوم وهو بروتين مغاير ، موجود في سيتوبلازم الخلايا الأحادية ، يلعب أدوارًا مهمة في الاستجابة الالتهابية في جسم الإنسان. استخدم كالبروتكتين مؤشرًا حيويًا مبكرًا في مرض فايروس كورونا و مع البكتيريا المصاحبة للعدوى والمرضى المعرضين لخطر الإصابات خطيرة. اما جزيء الجارحة للكلى هو بروتين سكري يتم تنظيمه في النبيبات القريبة بعد إصابة الكلى.

تهدف هذه الدراسة إلى تقييم الشدة في حالات كوفيد -19 عن طريق قياس مستويات كالبروتكتين وسيستاتين سي وارتباطهما بجزيء إصابة الكلى -1 وغيره من المؤشرات الحيوية.

طرق العمل و المواد: نوع الدراسة كان من الدراسات المستعرضة Cross-sectional study شملت هذه الدراسة على 91 عينة من مرضى كوفيد -19 19حالة شديدة و 45 مريضا منهم (56 ذكر ، 35 أنثى) مريض تراوحت اعمارهم بين (22-88) سنة في ردهة الحياة في مدينة الإمام الحسين الطبية في كربلاء. قسمت الحالات إلى ثلاث مجاميع إعتمادًا على شدة الإصابة (معتدلة و حادة).

لقد قيست مستويات المؤشرات الحيوية للكالبروتكتين, سيستاتين سي و الجزيئة الجارحة للكلى بوساطة تقنية المقايسة الإمتصاصية المناعية للإنزيم المرتبط ELISA اما مستويات عدد كريات الدم الكاملة في جهاز (XP-300™ Automated hematology analyzer Sysmex).

النتائج: وفقًا للنتائج المعروضة والتي شملت 46 حالة الشديدة 45 حالة متوسطة من مرضى كوفيد -19 ، هناك ارتباط إيجابي بين جزيء إصابة الكلى -1 وسيستاتين سي في الحالات الشديدة (p (p<0.01) في الحالات المتوسطة0.0>p) 5 (ومع كالبروتكتين (p <0.01 في

المجموعة الشديدة. كان هناك ارتباط ايجابي بين السيستاتين سي و مصل الكرياتين في الحالات الشديدة($p<0.05$)

الخلاصة: أوضحت النتائج التي تم الحصول عليها أن هناك علاقة ارتباط موجبة بين جزيء إصابة الكلى -1 وجزيء سيستاتين سي وكالبروتكتين. وارتباط إيجابي بين السيتاتين سي و مصل الكرياتينين.ولا توجد لهم علاقة بشدة المرض عند مقارنتهم مع حالات المرض الشديدة والمتوسطة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الطب
فرع الكيمياء والكيمياء الحياتية

مستويات الكالبروتكتين والسيستاتين سي في مرضى كوفيد -19 الحاد والمتوسط وعلاقاتهم بجزيء إصابة الكلى -1

رسالة ماجستير

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

من قبل

حوراء فاضل عباس

بكالوريوس تحليلات مرضية – جامعة كربلاء – 2018

اشراف

أ.د. فاضل جواد ال طعمة
فرع الكيمياء والكيمياء الحياتية

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فرع الطب الباطني

كلية الطب – جامعة كربلاء

2021 م **1443 هجـ**