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Correlation between serum level of (TNF-alpha, IL-10 and procalcitonin) and severity of COVID-19 disease

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ABSTRACT

COVID-19 is a contagious disease caused by the SARS-CoV-2 virus. Due to its similarity to SARS-CoV, the coronavirus disease (COVID-19) was initially referred to as SARS-CoV-2 Disease 2019. It has mortality rate appears to be approximately 3.8%. Acute lung injuries cause acute respiratory distress syndrome (ARDS) in most patients, which is the most prominent clinical feature of COVID-19. Adhesion molecules, immune cells, inflammatory cells, and cytokines significantly influence acute lung injury. This study aimed to assess the serum level of (TNF-alpha, IL-10, and procalcitonin) in COVID-19 patients compared to its level in healthy controls, to know the correlation between serum levels of these markers and clinical severity of COVID-19 disease, also to evaluate their role in the prognosis and to assess the using of TNF-alpha, IL-10, and procalcitonin as a predictable test for early detection of the patient who suspected to have COVID-19 disease. Serum level of TNF-alpha, IL-10, and procalcitonin was measured in 58 patients with COVID-19, ages ≥ 18 years, who attended the COVID-19 ward in Baghdad teaching hospital and outpatient clinic in Baghdad City, during the period from January 2021 to the end of May 2021, compared with 30 healthy controls matched in ages and sex to the patients, using Enzyme Linked Immuno-Sorbent Assay (ELISA) technique. TNF-alpha, IL-10, and procalcitonin serum levels were all substantially higher in patients than in healthy controls, with respective means and standard deviations of (14.62 5.32 compared 6.670 2.052) pg/ml, (13.053 7.822 versus 3.117 0.415) pg/ml, and (0.075 0.06 versus 0.035 0.011) ng/ml. There was a significant variation in serum level of TNF-alpha, IL-10, and procalcitonin between severe and non-severe patients with a higher mean ± SD for a severe group, (17.645±5.48 versus 11.147±2) pg/ml, (18.88±7.169 versus 6.396±1.34) pg/ml and (0.099±0.073 versus 0.047±0.0142) ng/ml, respectively. The higher levels were also observed in patients with poor outcomes with AUC in the ROC curve equal to (0.89, 0.92, and 0.83) for TNF-alpha, IL-10, and procalcitonin, respectively. Patients' elevated TNF-alpha, IL-10, and procalcitonin serum levels compared to the control group indicated the host's immune responses against the coronavirus infection. The serum concentration of TNF-alpha and IL-10 might be considered a reflective sign of the COVID-19 severity. These findings indicate different immuno-regulatory events during SARS-CoV-2 infection, which may be helpful in the early detection of severe COVID-19-infected patients.

The cytokine markers procalcitonin, IL-10, and TNF-alpha are used as a reliable tests for the early identification of those who may have severe COVID-19. This study aimed to measure the levels of (TNF-alpha, IL-10, and procalcitonin) in the serum of COVID-19 patients compared to their levels in healthy controls. Also, to know the correlation between serum levels of (TNF-alpha, IL-10 and procalcitonin) and clinical severity of COVID-19 disease.

Keywords: *serum , contagious , disease , COVID – 19 , virus*

1. Introduction

The SARS-CoV-2 virus causes the infectious condition known as coronavirus disease (COVID-19). The recently discovered coronavirus (COVID-19), known as SARS-CoV-2 disease 2019, is similar to SARS-CoV. (1). The pathogen of the outbreak recalled to our mind the horrible memory of the severe acute respiratory syndrome (SARS-2003, caused by another beta-coronavirus) that happened 17 years ago(2).

In January 2022, the cumulative number of confirmed cases and deaths globally was nearly 308 million and over 5.5 million, respectively(3). However, most patients had mild-to-moderate disease and recovered without requiring special treatment; about 10 to 20% progressed to severe or critical diseases, including pneumonia and acute respiratory failure. Serious illness is more likely to strike older persons and those with chronic diseases such as cardiovascular disease, diabetes, cancer, or chronic respiratory diseases. (4).

The primary pathological characteristic of COVID-19 is caused by lung damage, which in most severe cases results in acute respiratory distress syndrome (ARDS). Immune cells, cytokines, adhesion molecules, and inflammatory cells greatly influence acute lung damage. Previous research has demonstrated that some pro-inflammatory cytokines, such as those produced during acute pancreatitis and sepsis, play a significant role during acute lung damage. (5,6). They also demonstrated that infection with viral agents causes up regulation of cytokines such as Tumor Necrosis Factor-alpha (TNF-α), an essential mediator of inflammation (7,8). Gamma interferon (IFN-), IL-1, IL-8, IL-6, and TNF- are cytokine cytokines that increase inflammatory responses, whereas IL-4 and IL-10 suppress inflammation (9). A recent study suggests that hyper inflammation in SARS-CoV-2 patients is due to cytokines storm, especially in immunosuppressed patients, and as a result, the screening and managing of these cytokines could improve the mortality rate (8).

Several significant zoonotic viruses, including coronaviruses with significant genetic diversity, such as the Nipah virus, Hendra virus, and SARS-CoV, have been linked to bats as hosts (11,12). The emanation of SARS-CoV-2 was first observed when unexplained pneumonia cases were noted in the city of Wuhan, China (10). The causative virus of COVID-19 was rapidly isolated from patients and sequenced, with the results from China subsequently being shared and published in January 2020(14). The findings showed that it was a positive-stranded RNA virus belonging to the Coronaviridae family (a subgroup B betacoronavirus) and was new to humans(13). At first, analysis of the genomic sequence of the new virus (SARS-CoV-2) showed high homology with that of the coronavirus that caused SARS in 2002-2004, namely SARS-CoV (another subgroup B betacoronavirus) (15). As with the coronaviruses that cause SARS and MERS, human-to-human transmission of SARS-CoV- 2 was soon established (16), but the virus demonstrated much greater infectivity than these other two coronaviruses(17). The disease was officially named Coronavirus Disease-2019 (COVID-19, by WHO on February 11, 2020)(2).

In Early January 2022, the total number of worldwide cases was about 308 million, and the deaths were about 5.5 million; in Iraq, the total number of cases was about 2 million, and death was about 24,212 (3). A study of early transmission dynamics of COVID-19 revealed that the mean incubation period was 5-6 days, ranging from 1-14 days (18,56). It is thought that respiratory droplets are the primary means of transmission. Still, since the conjunctival epithelium is susceptible to infectious droplets and bodily fluids, it must avoid transmission through the ocular surface (19). Because SARS-CoV-2 nucleic acids were found in the stool

samples of COVID-19 patients with pneumonia and stomach symptoms, the fecal-oral pathway is thought to be responsible (20).

In addition, a 30-hour-old baby tested positive for SARS-CoV-2 infection has been described as a possible transmission pathway for vertical transmission between mothers and newborns (21). Similar to SARS-CoV, SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as its primary receptor, which is broadly expressed in vascular endothelium, respiratory epithelium, alveolar respiratory tissues(18,22) as demonstrated by successful live virus isolation from throat swabs and detection of viral subgenomic messenger RNA (sgRNA) in cells of the upper respiratory tract(22).

Coronavirus particles have been found by electron microscope analysis in the cytoplasm of epithelial cells from infected individuals' monocytes and macrophages (18). Direct or indirect exposure to the respiratory tract is the primary method of transmission. Remember that SARS-CoV-2 can actively reproduce in the upper, indicating that the bronchial and alveolar epithelial cells are the virus's main targets (1). When symptoms are mild and only affect the upper respiratory tract, the tropism of the upper respiratory tissue likely explains ongoing pharyngeal shedding of the virus and more active transmission of SARS-CoV-2 than SARS-CoV. Later in the disease's progression, COVID-19 is similar to SARS in that it replicates the virus in the lower respiratory tract and causes secondary viremia. This is followed by a severe attack on target organs such as the heart, kidney, and gastrointestinal tract and a sizable distal vasculature that expresses ACE2. This viral spreading process gets together with clinical deterioration, mainly occurring around the second week following disease onset. However, it has been recognized that disease exaggeration until the late stage is imputed to direct viral damage and a consequence of immune-mediated injury induced by SARS-CoV-2. It is undeniable that immune-mediated inflammation, just as it did in SARS, plays a significant part in the pathogenesis of COVID-19. A consistent drop in lymphocyte count and a notable increase in neutrophils were seen as COVID-19 progressed. In the meantime, inflammatory indicators were significantly increased (23).

The 17 kDa protein tumor necrosis factor (TNF), which has 157 amino acids and exists in solution as a homotrimer, is primarily controlled by soluble TNF-binding receptors. In humans, the gene maps to chromosome 6 (24). On activated macrophages, lymphocytes, and other cell types, TNF- is produced as a precursor from transmembrane TNF-, which is expressed as a cell surface type II polypeptide with 233 amino acid residues (26 kDa) (25,26). The soluble form of TNF-, which has 157 amino acid residues and weighs 17 kDa, is released after being broken down by metalloproteinases like TNF-converting enzyme (TACE). It mediates its biological activities through Type 1 and 2 TNF receptors (27,28). TNF- R1 and R2 are expressed in almost all nucleated cells(28). TNF-α is a cytokine produced by several cell types; however, the primary producers are monocytic lineage cells, such as macrophages(29). This cytokine is essential for the growth of tumors and other stationary or pathological situations such as infections, lesions, inflammation, and tumors (30). TNF-α induces inflammation, activates vascular endothelium, orchestrates the tissue recruitment of immune cells, and promotes tissue destruction(31). TNF- stimulates other immune cells after being released by macrophages, the body's first line of defense, and controls the production of additional proinflammatory cytokines during inflammatory responses (29).

TNF-α is the critical mediator of pulmonary edema in COVID-19 lung disease. The action of TNF alpha can explain the multi-organ failure found in severe COVID-19 disease (32). It is a vasoconstriction-causing cytokine that can cause ischemia in all organ systems (33).

TNF-α induces endothelial actin microfilament disruption and intercellular gap formation that determine transcellular permeability; it has been demonstrated to cause microtubule destabilization in human pulmonary artery endothelial cells (EC), TNF alpha at this moment-induced disassembly of the peripheral microtubule network. This is associated with TNFalpha–induced increases in the permeability of EC layers(34). The critical immunoregulatory cytokine interleukin 10 (IL-10) is mainly secreted by macrophages, although it is also produced by T-regulatory, (Th2) lymphocytes, dendritic cells, cytotoxic T cells, B lymphocytes, monocytes, and mast cells. There is a gene for IL-10 on chromosome 1. (35). Human IL-10 (hlL-10) has been shown to exist in solution predominantly as a homodimer, composed of two polypeptide chains of 160 amino acids (36). IL-10 was associated with the disease's severity; it principally contributed to the inhibition of the inflammatory response. The highly increased levels of IL-10 in severely infected patients might account for the negative feedback on systemic and local inflammation(37). In covid-19 disease, the increase in IL-10 reflects the exaggerated immune response, which triggers negative feedback via the IL-10 pathway on T cells. The elevation IL-2 in patients with poorer prognoses explains the trigger for such negative feedback on T cells through IL-10 synthesis. IL-10 directly inhibits T-cell activation and proliferation, reducing IL-2 (38).

Procalcitonin (PCT) is the 116-amino acid precursor of the hormone calcitonin(39). Its molecular weight is 13kDa, a prohormone of calcitonin produced by c-cells of the thyroid gland and intracellularly cleaved by the proteolytic enzyme into the active form (40). Procalcitonin is a proinflammatory marker that could benefit the diagnosis of infection. In the past, procalcitonin levels have been estimated to diagnose sepsis or guide antibiotic therapy, but it was not determined if it would differentiate between sepsis and other causes of inflammation. Procalcitonin was a useful biomarker indicating bacterial infection(41).

Around 15% of patients require inpatient care for moderate to severe pneumonia, which affects around 80% of SARS-CoV-2 infections and is typically treatable with outpatient care (46). Asymptomatic people are essential reservoirs of infection throughout the incubation period, which makes epidemic prevention and disease control challenging (54,55).

Fever (83%–98%), cough (50%–82%), exhaustion (25%–44%), shortness of breath (19%–55%), and muscle discomfort (11%–44%) are the most typical symptoms of SARS-CoV-2 infection(42,43). A few days before the onset of fever, some individuals may experience sputum production, rhinorrhea, chest tightness, sore throat, nausea, vomiting, diarrhea, headache, and anosmia, demonstrating that fever is an important but not the only early sign of infection (42). Patients with dyspnea and hypoxemia sometime might quickly progress into acute respiratory distress syndrome (ARDS), septic shock, blood clotting dysfunction, and even multiple organ failure in about one week(48,50), overall mortality rate appears to be approximately 3.8% (42,44,47,48,49). Almost all critically sick patients have additional medical issues, such as diabetes, hypertension, kidney disease, and cardiovascular disease. Furthermore, patients with these comorbidities who have COVID-19 have relatively high mortality rates (51). The severity of COVID-19 patients is also related to age and death among those aged ≥40. Studies have shown

that the morbidity rate is lower in children and infants than adults(52,53). Although many COVID-19 patients suffer from respiratory symptoms, SARS-CoV-2 can also lead to several extra pulmonary manifestations, including thromboembolic complications, cardiac injury and arrhythmia, acute coronary syndromes, acute renal injury, gastrointestinal (GI) symptoms, liver function impairment, hyperglycemia and diabetic ketosis, neurologic deficits, and dermatologic complications(57).

The method used most frequently during the current pandemic is direct nucleic acid detection or detection via nucleic acid amplification, both known as "molecular tests." Another method is immunological testing, which determines the effects of a virus infection on the host. While some specialist laboratories may also be able to define the cellular immune response, this is typically focused on identifying virus-specific antibodies (58). Current lab diagnostic tests for coronavirus include reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR (rRT-PCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (59,60). Laboratory specimens, including nasopharyngeal and oropharyngeal swab tests, have become a standard assessment for diagnosis of COVID-19 infection(61). The RT-PCR results usually become positive after several days (2-8 days)(62). Although real-time reverse transcription polymerase chain reaction (RT- qPCR) is the current recommended laboratory method to diagnose SARS-CoV-2 acute infection, several factors, such as the requirement of special equipment and skilled staff, limit these time-consuming molecular techniques(63). When diagnosing COVID-19, around 10 days or more after the beginning of symptoms, antibody testing can play a primarily complementary role to rtPCR assays in determining the present infection (64,65). There are four major tests of antibody detection for SARS-CoV-2; IgA, IgM, or IgG antibodies produced by B cells:

1. Rapid diagnostic tests (RDT)
2. Enzyme-linked immunosorbent assays (ELISA)
3. Neutralization assays
4. Chemiluminescent immunoassays

The antibody tests for SARS-CoV-2 have a low specificity within the first week of exposure and increase in the second and third weeks(66). Current clinical reports show that antibodies against SARS-CoV-2 viral particles develop around a week after infection, with peak IgM antibody levels at 10 to 13 days, and persist for 32 to 40 days, and IgA which appear between day 8 to 10 and peaks around two weeks to 20 days. In contrast, the IgG antibodies peak around 17 days and persist for 7 weeks (67,68). Recently, several easy-to-perform rapid antigen detection tests were developed and recommended in some countries as the first line of diagnosis(63). Several non-specific blood counts and biomarkers are used in predicting the severity of COVID-19 infection, such as IL-6, CRP, ESR, ferritin, LDH, WBC, D-dimer, platelet count, and lymphocyte count(69). For patients suffering from fever, sore throat, fatigue, coughing, or dyspnea coupled with recent exposure, the diagnosis of COVID-19 infection can be established with typical chest computerized tomography (CT) characteristics despite negative RT-PCR results. Typical CT findings included bilateral pulmonary parenchymal ground-glass and consolidative pulmonary opacities, sometimes with a rounded morphology and peripheral lung distribution(70).

The primary methods currently used are symptomatic and supportive care, which includes monitoring vital signs, maintaining blood pressure and oxygen saturation levels, and treating consequences, including secondary infections or organ failure (70). Some protease inhibitors,

including lopinavir, darunavir, and atazanavir, may be used to treat COVID-19(71); in some nations, individuals with COVID-19 are receiving emergency therapy with the lopinavir-ritonavir combination (72,73), Lopinavir-ritonavir alone or in combination with interferon (INF)-β – an inflammation regulator molecule – has been listed by WHO as options for "solidarity" clinical trial for COVID-19(74). lopinavir-ritonavir treatment was associated with a better outcome but did not significantly accelerate the clinical improvement of severe COVID-19 infection(73).

Another strategy to combat SARS-CoV-2 infection involves targeting the reverse transcription step by blocking RdRp and therefore preventing viral replication(71); Remdesivir is a NtRTI drug that is worthy of a "solidarity" clinical trial for COVID-19, according to WHO(74). Adult patients admitted to the health centre with Covid-19 and signs of a lower respiratory tract infection recovered faster (78). combination Interferon beta-1a to remdesivir was not better than remdesivir alone in hospitalized patients with COVID-19 pneumonia. Patients who required high-flow oxygen at baseline had worse outcomes after treatment with interferon beta-1a(79). Another drug that affects viral replication is Ribavirin, which hinders the RNA capping from destabilizing the viral RNA and finally obstructs replication(71). Ribavirin is one of the recommended drugs administered in combination with either IFN alpha or lopinavir-ritonavir(75).

The clinical outcomes of coronavirus and similar outbreaks do not support using corticosteroids. There is no unique reason to expect that patients with COVID-19 infection will benefit from corticosteroids, and such a treatment may be harmful (80,81). Since corticosteroids suppress lung inflammation (47), corticosteroids could be prescribed at the right time for suitable patients.

Chloroquine is a widely-used antimalarial and autoimmune disease drug reported to be a potential broad-spectrum antiviral drug (76,77). Chloroquine is well known for its ability to prevent virus infection by raising the endosomal pH necessary for virus/cell fusion and interfering with the glycosylation of SARS-CoV cellular receptors (82). Chloroquine was previously included in the recommendations for the prevention and treatment of COVID-19 pneumonia (83,84). Hydroxychloroquine is a chloroquine analog with fewer concerns about drug-drug interactions (85). Both chloroquine and hydroxychloroquine have immunomodulatory effects and can suppress the immune response (86,87). Recently, discontinuing hydroxychloroquine from at least four large studies effectively marks the end of efforts at repurposing chloroquine or hydroxychloroquine for COVID-19 infection(88).

According to WHO guidelines to prevent COVID-19 transmission, the following instructions was recommended(45):

a. maintaining good hygiene and wearing personal protective equipment (wearing a face mask, washing hands more frequently with soap and water, using hand sanitizer more frequently, cleaning the house, and covering the nose and mouth when coughing or sneezing).
b. Avoiding social gatherings, traveling to the hospital or other healthcare facilities, congested areas, and traveling less, particularly to areas affected by COVID-19.
c. Get vaccinated when it is your turn.

The mechanisms through which various vaccinations provide protection vary. However, the body is left with a supply of "memory" T-lymphocytes and B-lymphocytes after receiving any form of the vaccine, which will continue to remember how to combat that virus in the future. There are now three main COVID-19 vaccine kinds that have been approved or permitted for use in emergencies (89):

mRNA vaccines contain components of the COVID-19-causing virus that urge our cells to produce a protein that is safe and specific to the virus. The genetic material from the vaccine is destroyed once our cells produce copies of the protein.

Protein subunit vaccines include harmless pieces (proteins) of the virus that causes COVID-19 instead of the entire germ.

Vector vaccines include a modified strain of a virus other than the COVID-19 virus. There is COVID-19-causing virus material inside the modified virus's outer shell. It is referred to as a "viral vector." The genetic material inside the viral vector instructs cells to produce a protein specific to the virus that causes COVID-19 once it has entered our cells. These instructions tell our cells to duplicate the protein.

Research shows that COVID-19 vaccines effectively prevent COVID-19, but certain COVID-19 vaccines require more than one injection to complete the primary series.

2. Subjects, Materials, and Methods

2.1 Study design

This prospective case-control study was conducted at Baghdad teaching hospital - Medical City, at the covid-19 ward, from January 2021 to the end of May 2021.

After explaining the nature of the study to them, ethical approval and informed consent were obtained from each participant.

2.2 Subjects

2.2.1 Patients group:

A total of 58 patients aged between (18-70) years, 25 males and 33 females diagnosed with COVID-19 by PCR and were on medical treatment.

All patients had their comprehensive medical histories collected, and a questionnaire was used for this purpose, as in (Appendix 1).

Investigations like WBC count, hemoglobin, serum LDH, serum ferritin, D-dimer, serum Interleukin-10, serum TNF-alpha and serum procalcitonin were done on the patients.

Classification of COVID-19 severity non-severe (mild/moderate) versus severe (severe/critical) was done according to WHO Living guidance for clinical management of COVID-19 (90).

The acute disease includes patients meeting one or more of the following:

1. **Acute respiratory distress syndrome (ARDS):**

- Onset: within one week after the onset of new or worsened respiratory symptoms or a recognized clinical insult (such as pneumonia).
- Chest imaging: (radiograph, CT scan, or lung ultrasound): bilateral opacities not fully explained by volume overload, lobar or lung collapse, or nodules.
- Origin of pulmonary infiltrates: respiratory failure not fully explained by cardiac failure or fluid overload. Need objective assessment (e.g.echocardiography) to exclude hydrostatic cause of infiltrates/edema if no risk factor present
- Oxygenation impairment in adults :

• Mild ARDS: 200 mmHg < PaO2/FiO2a ≤ 300 mmHg (with PEEP or CPAP ≥ 5 cmH2O).
• Moderate ARDS: 100 mmHg < PaO2/FiO2 ≤ 200 mmHg (with PEEP≥5 cmH2O).
• Severe ARDS: PaO2/FiO2 ≤ 100 mmHg (with PEEP ≥ 5 cmH2O).

2. **Sepsis**: in Adults: acute organ failure that poses a risk of death brought on by a dysregulated host response to a suspected or confirmed infection.

Signs of organ dysfunction include altered mental status(delirium), challenging or fast breathing, low oxygen saturation, reduced urine output, fast heart rate, weak pulse, cold extremities or low blood pressure, skin mottling, laboratory evidence of coagulopathy, thrombocytopenia, acidosis, high lactate, or hyperbilirubinemia.

3. Septic shock: Adults: persistent hypotension despite volume resuscitation, requiring vasopressors to maintain MAP ≥ 65 mmHg and serum lactate level > 2 mmol/L

4. Acute thrombosis: Acute venous thromboembolism (i.e., pulmonary embolism), acute coronary syndrome, acute stroke.

Severe disease :

Adolescent or adult with one of the following conditions in addition to clinical indications of pneumonia (fever, cough, or dyspnea):

- respiratory rate > 30 breaths/min
- severe respiratory distress
- SpO2< 90% on room air.

While the diagnosis can be made on clinical grounds, chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications.

Moderate disease: Adolescent or adult with clinical signs of pneumonia (fever, cough, dyspnoea, fast breathing) but no signs of severe pneumonia, including SpO2 ≥ 90% on room air.

Mild disease: Symptom of COVID-19 without evidence of viral pneumonia or hypoxia.

2.2.2 Healthy control group:

It included 30 healthy subjects with no signs and symptoms suggesting viral infection. They were 7 males and 23 females, and their ages and sex were matched with patient groups. Also, a detailed history was taken from them (Appendix 2).

Exclusion criteria were

1- pregnant or lactating women.
2- Patients with malignancy.
3- Patients on autoimmune therapy.

2.3 Materials

2.3.1 Equipment and Instruments

Table 2-1. Equipment and Instruments

no	Equipment and materials
1.	Absorbent filter paper/ China
2.	Adhesive strips/ China
3.	Automated microplate washer / China
4.	Calibrated variable precision micropipettes and disposable plastic pipette tips. / China
5.	Centrifuge/ China
6.	Deep freezer (-20)/ Germany

no	Equipment and materials
7.	Disposable glove/U.A.E
8.	ELISA microplate reader (wavelength: 450 nm) and printer/ China
9.	Plate cover/ China
10	Semilogarithmic graph paper/ China
11.	Serum separator tube (SST) for blood drainage/ U.A.E
12.	Sterile disposable syringes /U.A.E
13.	Timer/ China
14.	Tourniquet/ China
15.	Vortex mixer/ China
16.	1.5 ml Eppendorf tubes/U.A.E
17.	37°C incubator/ China

No.: number, ELISA: enzyme-linked immune-sorbent assay

2.3.2 Kit

In this study, we used 2 ELISA kits were used from / CUSABIO/ China:

1. Human Tumor Necrosis Factor α (TNF-α) Enzyme-linked Immune-Sorbent Assay Kit For the quantitative determination of human tumor necrosis factor α (TNF-α) concentrations (pg/ml) in serum, plasma, cell culture supernates, tissue homogenates, cell lysates.

CSB-E04740h / CUSABIO/ China, principle: the sandwich.

The kit provided one 96-well microplate pre-coated with an antibody specific for TNF-α.

2. Human Interleukin 10 (IL-10) Enzyme-linked Immune-Sorbent Assay KIT for the quantitative determination of human interleukin 10 (IL-10) concentrations(pg/ml) in serum, urine, cell culture supernates, ascitic fluid, cerebrospinal fluid (CSF), saliva.

CSB-E04593h/ CUSABIO/ China, principle: sandwich

The kit provided one 96-well microplate pre-coated with an antibody specific for IL-10.

Procalcitonin and ferritin are measured by the fully automated Cobas E411 analyzer manufactured in Germany by Roche, which employs their proprietary ElectroChemiLuminescence (ECL) technology for immunoassay analysis. CBC is measured by a fully automated hematology analyzer Sysmex Made in Japan. Automated biochemistry analyzer C311 Cobas measured LDH, D.diamer, and CRP from Roche made in Germany.

2.3.3 Reagents and Chemicals:

Table 2-2: List of chemicals and Reagents for TNF-α

No	Chemicals and Reagents	Quantity
1.	Assay plate (12 x 8 coated Microwells)	1(96 wells)
2.	Standard (Freeze-dried)	2
3.	Biotin-antibody (100 x concentrate)	1 x 120 µl
4.	HRP-avidin (100 x concentrate)	1 x 120 µl
5.	Biotin-antibody Diluent	1 x 15 ml
6.	HRP-avidin Diluent	1 x 15 ml
7.	Sample Diluent	1 x 50ml
8.	Wash Buffer (25 x concentrate)	1 x 20 ml

No	Chemicals and Reagents	Quantity
9.	TMB Substrate	1 x 10 ml
10.	Stop Solution	1 x 10 ml
11.	Adhesive Strip (For 96 wells)	4
12.	Instruction manual	1

Table 2-3: List of chemicals and Reagents for IL-10

No	Chemicals and Reagents	Quantity
1	Assay plate (12 x 8 coated Microwells)	1(96 wells)
2	Standard (Freeze-dried)	2
3	Biotin-antibody (100 x concentrate)	1 x 120 µl
4	HRP-avidin (100 x concentrate)	1 x 120 µl
5	Biotin-antibody Diluent	1 x 15ml
6	HRP-avidin Diluent	1 x 15 ml
7	Sample Diluent	1 x 50 ml
8	Wash Buffer (25 x concentrate)	1 x 20 ml
9	TMB Substrate	1 x 10 ml
10	Stop Solution	1 x 10 ml
11	Adhesive Strip (For 96 wells)	4
12	Instruction manual	1

2.4 Method

2.4.1 Blood sample collection and preparation

Five ml of whole blood was obtained from each subject included in this study at the time between days 9 to 14 after the onset of symptoms, under an aseptic technique by using a sterile 5ml syringe and disinfection of the skin with 70% ethanol before venipuncture, then placed in a sterile serum tube (gel tube) which is labeled with patient name, age and date of collection.

2.4.2 Serum preparation and storage

Blood samples were allowed to clot in the gel tubes for two hours in an upright position at room temperature (23°C) and then centrifuged for 15 minutes at 1000 rpm; then, the supernatant was carefully transferred using the pipette to Eppendorf tubes and immediately frozen in aliquots at -20°C till use.

2.4.3 Detection of TNF-alpha by ELISA technique

2.4.3.1. Reagents preparation

1. Standard

First, the standard vial was centrifuged at 6000 rpm for 30s. Then we reconstituted the standard with 1.0 ml of sample diluent.

After the standard had been thoroughly reconstituted, it was mixed once again, and before dilutions were made, it was allowed to sit for at least 15 minutes with light agitation.

Seven eppendorf tubes numbered from (0-6) were prepared, then we pipetted 250 µl of sample diluent into each tube. A series of 2-fold dilutions were created using the stock solution. Before the subsequent transfer, each tube was blended correctly. The high standard (500 pg/ml) is the standard undiluted. The zero standards is Sample Diluent (0 pg/ml).

Tube	S7	S6	S5	S4	S3	S2	S1	S0
Pg/ml	500	250	125	62.5	31.2	15.6	7.8	0

2. Biotin-antibody

We centrifuged the vial before opening it.

Biotin-antibody requires a 100-fold dilution, so we added 10 µl of Biotin-antibody + 990 µl of Biotin-antibody Diluent.

3. HRP-avidin Diluent

The vial was centrifuged before opening. HRP-avidin requires a 100-fold dilution, so we suggested that a 100-fold dilution equals 10 µl of HRP-avidin + 990 µl of HRP-avidin Diluent.

4. Sample diluent

One vial of sample diluent contained 50 ml of the solution and was ready to be used.

5. Wash Buffer

20 ml of Wash Buffer Concentrate was diluted into 500 ml distilled water.

6. TMB Substrate

One vial of substrate contained 10 ml of Tetramethylbenzidine and was ready to be used.

7. Stop Solution

One vial contained 10 ml of acidic solution.

2.4.3.2.Assay procedure

1. All reagents and samples were allowed to reach room temperature before use.
2. Samples were centrifuged again after thawing before the assay.
3. One hundred µl of standard and sample were added per well.
4. the plate was then sealed with the adhesive strip provided and incubated for 2 hours at 37°C.
5. the liquid was removed from each well, but without washing.
6. Then, each well received 100 l of Biotin-antibody, and a fresh adhesive strip was used to cover the plate.
7. Then, it was incubated for 1 hour at 37°C.
8. After incubation, each well was aspirated and washed; this process was repeated two times for a total of three washes. The washing process was done by filling each well with wash buffer (200µl) using the multi-channel pipette, and it was allowed to stand for 2 minutes.
9. Then, the liquid was completely removed at each step.
10. After the last wash, any remaining wash buffer was removed by decanting through inverting and blotting the plate against clean paper towels.
11. One hundred µl of HRP-avidin was added to each well, and the microtiter plate was covered with a new adhesive strip and then incubated for 1 hour at 37°C.
12. The aspiration/wash process was repeated five times, as in step 8.
13. 90 µl of TMB Substrate was added to each well, then incubated for 15-30 minutes at 37°C and protected from light.
14. Then, to thoroughly mix the 50 µl of Stop Solution that was poured into each well, the plate was lightly pounded.

15. lastly, the optical density of each well was determined within 5 minutes using a microplate reader set to 450 nm.

2.4.4. Detection of IL-10 by ELISA technique

2.4.4.1. Reagents preparation

1. Standard

First, the standard vial was centrifuged at 6000 rpm for 30s. Then the standard was reconstituted with 1.0 ml of Sample Diluent

The standard was reconstituted correctly and allowed to sit for at least 15 minutes with gentle agitation before dilutions.

We used the stock solution to produce a 2-folds dilution series. Seven Eppendorf tubes numbered from (0-6) were prepared, then we pipetted 250 µl of Sample Diluent into each tube. Before the subsequent transfer, each tube was adequately blended. The high standard (800 pg/ml) is the undiluted standard. The zero standard is the Sample Diluent (0 pg/ml).

Tube	S7	S6	S5	S4	S3	S2	S1	S0
Pg/ml	800	400	200	100	50	25	12.5	0

2. Biotin-antibody

The vial was centrifuged before opening. Biotin-antibody requires a 100-fold dilution, so we added 10 µl of Biotin-antibody + 990 µl of Biotin-antibody Diluent.

3. HRP-avidin Diluent

The vial was centrifuged before opening. HRP-avidin requires a 100-fold dilution, so we suggested that a 100-fold dilution equals ten µl of HRP-avidin + 990 µl of HRP-avidin Diluent.

4. Sample diluent

One vial of sample diluent contained 50 ml of the solution, ready to be used.

5. Wash Buffer

Twenty ml of Wash Buffer Concentrate was diluted into 500 ml distilled water.

6. TMB Substrate

One vial of substrate contained 10 ml of Tetramethylbenzidine, and it was ready to be used.

7. Stop Solution

One vial contained 10 ml of acidic solution

2.4.4.2. Assay procedure

1. all reagents and samples were allowed to reach room temperature before use.
2. The samples were centrifuged again after thawing before the assay.
3. One hundred µl of standard and sample were added per well.
4. Then, the plate was sealed with the adhesive strip provided and incubated for 2 hours at 37°C.
5. the liquid was removed from each well, but without washing.
6. Then, each well received 100 µl of Biotin-antibody, and a fresh adhesive strip was used to seal the plate.
7. Then, it was incubated for 1 hour at 37°C.

8. After incubation, each well was aspirated and washed; this process was repeated twice for three washes. The washing process was done by filling each well with Wash Buffer (200µl) using the multi-channel pipette, and it was allowed to stand for 2 minutes.
9. Then, the liquid was removed at each step.
10. After the last wash, any remaining wash Buffer was removed by decanting through inverting and blotting the plate against clean paper towels.
11. One hundred µl of HRP-avidin was added to each well, and the microtiter plate was covered with a new adhesive strip and then incubated for 1 hour at 37°C.
12. The aspiration/wash process was repeated five times, as in step 8.
13. ninety µl of TMB Substrate was added to each well, then incubated for 15-30 minutes at 37°C.
14. Then, 50 µl of Stop Solution was added to each well to ensure thorough mixing, and the plate was softly tapped.
15. Lastly, the optical density OD of each well was determined within 5 minutes using a microplate reader set to 450 nm.

2.4.5 Interpretation of the results

The standard curve is obtained by plotting the mean OD-values of the standards 0 – 7 on the y-axis against their respective known concentrations on the x-axis, then the unknown samples are read directly in pg/ml from their measured OD values as shown in figure(2.1),(2.2).

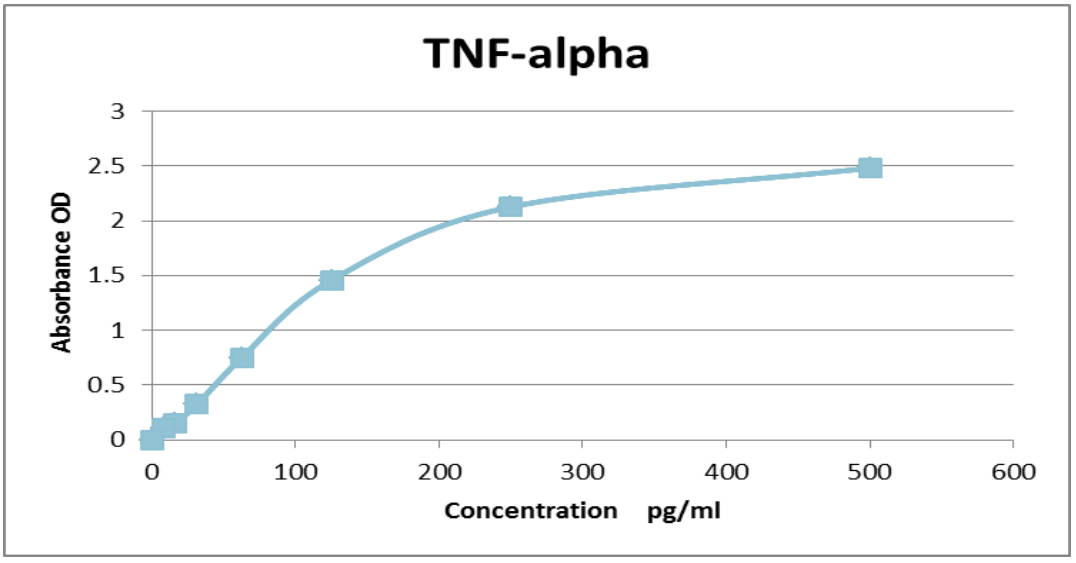

Figure 2.1: the standard curve of TNF-alpha concentrations

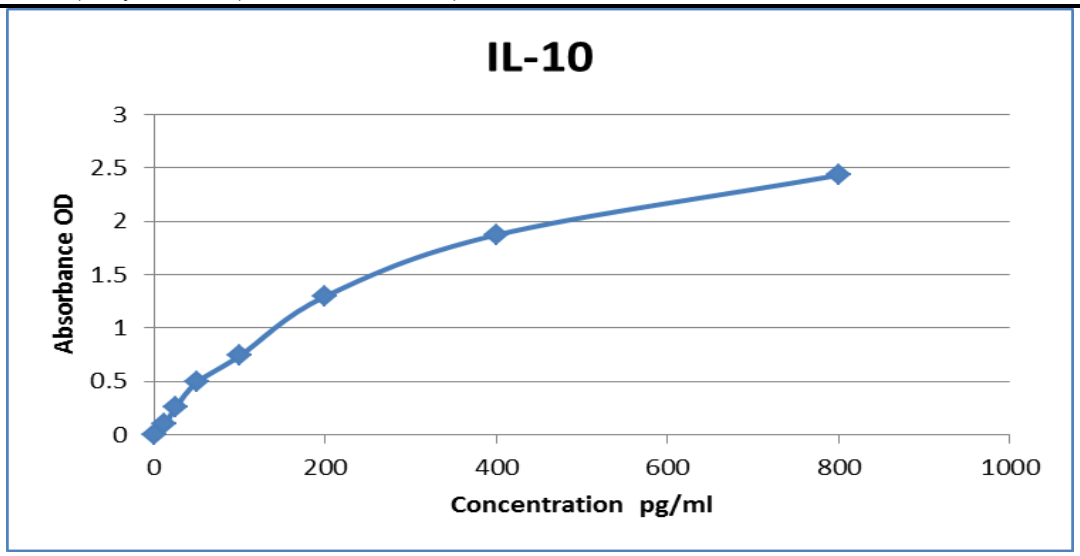

Figure 2.2: the standard curve of IL-10 concentrations

2.4.6.statistical analysis

The gathered information was entered into a 2016 version of Microsoft Excel before being imported into the statistical application SPSS V24. To present descriptive statistics, tables, and graphs were employed. To decide whether there was a genuinely massive distinction between the related method for mathematical information, a t-test with a free example was used. The significance of the connection between relevant categorical variables was determined using the Chi-Square test. ROC Curve was used to determine the parameters' predictability to see each parameter's sensitivity and specificity after deciding the area under the curve and cut-off point and determining a specific cut-off. P-value ≤ 0.05 was considered as a discrimination point for significance.

3. Results

3.1. Demographic and clinical characteristics

The results of this study 88 subjects were included, 58 patients and 30 control. Table 3-1 presents the distribution of patients and controls groups according to demographic and clinical variables; the mean age of cases was (51.2±16.1) which was not differ significantly from the mean age of the control group (45.7 ± 10.5) years, P-value =0.059. Also, no significant association was found between patients and control groups regarding sex, education, and occupation, with p-value= 0.068, 0.842, 0.158, respectively. About 55 (94.80%) of patients stated that they had family contact with infected persons; a highly significant association was noticed between family contact and getting COVID-19, compared to the control group P-value=0.001. It was found that 13 (22.40%) of patients in this study had DM, and a significant association was found between being diabetic and getting covid-19 compered to controls P-value=0.005. Regarding hypertension, 17 out of 58 (29.30)% of the patients had hypertension; a highly significant association was noticed between being hypertensive and getting covid-19 compared to controls, P-value=0.001. Having a history of congestive heart failure had no significant association with getting covid-19 infection compared to controls, P-value=0.141.

Table 3-1: Association between COVID-19 and studied variables

Studied variables				COVID-19 Patients		Healthy Controls		p-value
		No	%	No	%	No	%	
Age		88	49.3±14.6	58	51.2±16.1	30	45.7±10.5	0.059
Sex	Male	32	36.36	25	43.10%	7	23.30%	0.068
	Female	56	63.64	33	56.90%	23	76.70%	
Education	Primary and less	36	40.91	25	43.10%	11	36.70%	0.842
	Intermediate or secondary	27	30.68	17	29.30%	10	33.30%	
	University	25	28.41	16	27.60%	9	30.00%	
Occupation	Employed	26	29.55	20	34.50%	6	20.00%	0.158
	Not employed	62	70.45	38	65.50%	24	80.00%	
family contact	Family contact	55	62.50	55	94.80%	0	0.00%	0.001
	No family contact	33	37.50	3	5.20%	30	100.00%	
DM	Yes	13	14.77	13	22.40%	0	0.00%	0.005
	No	75	85.23	45	77.60%	30	100.00%	
Hypertension	Yes	17	19.32	17	29.30%	0	0.00%	0.001
	No	71	80.68	41	70.70%	30	100.00%	
CHD	Yes	4	4.55	4	6.90%	0	0.00%	0.141
	No	84	95.45	54	93.10%	30	100.00%	

No: number, DM: diabetes mellitus CHD: congestive heart failure
P –value ≤ 0.05

3.2 Percentage of COVID-19 symptoms among studied cases

Clinical characteristics of patients with different disease severity are shown in (Table 3-2) which shows that body ache was the leading complaint of the patients which account (94.8%), followed by fever and fatigue, which accounted for (91.4%) of patients, headache, and loss of appetite were (86.2%) of patients, patients complaining of nausea, cough, SOB, and sore throat (84.5%), (77.6%), (62.1%), and (41.4%) respectively.

The percent of patients who were complaining of vomiting, sputum, diarrhea, and chills were (27.6%), (20.7%), (13.8%), and (12.1%) respectively, which were less frequent.

Table 3-2: The percentage of each symptom in the COVID-19 patients group*

Symptoms	Count No.	Percent
Body ache	55	94.8%
Fever	53	91.4%
Fatigue	53	91.4%
Headache	50	86.2%
Loss of Appetite	50	86.2%
Nausea	49	84.5%
Cough	45	77.6%
SOB**	36	62.1%
Sore throat	24	41.4%
Vomiting	16	27.6%

Symptoms	Count No.	Percent
Sputum	12	20.7%
Diarrhea	8	13.8%
Chills	7	12.1%

*patient group included 58 patients infected with covid-19
**SOB: shortness of breath No.: number

3.3 The measured parameters in comparison between COVID-19 patients and healthy controls groups

(Table 3-3) shows the comparison of blood and immune-inflammatory parameters between the patients and control groups. There were many differences in the mean values between the patients and control groups. The patients' group had a higher mean WBC count (7.234 ± 3.120×10^9/L) versus (5. 483 ±0.816 ×10^9/L), higher neutrophil percentage (71.17±9.9%) versus (60.63±5.3%), higher serum ferritin (460.95±264.3) versus (92.93±33.18 ng/ml), higher lactate dehydrogenase (LDH) serum levels (596.862 ±158.5U/L) versus (293.5±34 U/L), a higher serum levels of D-dimer among covid-19 case (0.92± 0.69 mg/l) versus (0.272±0.141 mg/l), a significantly higher C-reactive protein (CRP) serum levels (22.128±13.21 mg/l) versus (2.893±0.99 mg/l), the P-value was 0.001 for all.
However, the patients' group had a lower lymphocyte percentage than the controls group (22.379± 9.64%) versus (32.367±2.83 %), P-value 0.001.
The procalcitonin serum levels were higher in the patients' group than in the controls group (0.075±0.06 ng/ml)versus (0.035±0.011 ng/ml), P-value= 0.001.
The mean concentration of TNF-alpha among covid-19 patients (14.621±5.32) pg/ml was significantly higher than that of the control group (6.67±2.052) pg/ml, P-value=0.001.
The mean concentration of IL-10 among covid-19 patients (13.053±7.82) pg/ml was significantly higher than that of the control group (3.117±0.415) pg/ml, P-value=0.001.

Table 3-3: The comparison of measured parameters between COVID-19 patients and healthy Controls groups

Measured parameters	Group	N	Mean	Std. Deviation	p.value
WBC1x10^9/L N.R(3.5-9.5) x10^9/L	patient	58	7.234	3.120	0.001
	Control	30	5.483	0.816	
LYM(%) N.R(20-50)%	patient	58	22.379	9.460	0.001
	Control	30	32.367	2.834	
Neutrophil(%) N.R(40-75)%	patient	58	71.179	9.906	0.001
	Control	30	60.633	5.372	
Ferritin	patient	58	460.955	264.364	0.001

Measured parameters	Group	N	Mean	Std. Deviation	p.value
N.R(20-200)ng/ml	Control	30	92.930	33.182	
LDH N.R(135-350)U/L	patient	58	596.862	158.506	0.001
	Control	30	293.500	34.030	
D.Dimer N.R(<0.5)mg/l	Patient	58	0.921	0.692	0.001
	Control	30	0.272	0.141	
CRP N.R(0.0-1)mg/l	patient	58	22.128	13.217	0.001
	Control	30	2.893	0.999	
TNF-alpha N.R(0.0-8.1)Pg/ml	Patient	58	14.621	5.3267	0.001
	Control	30	6.670	2.052	
IL10 N.R(0.0-9.1)Pg/ml	Patient	58	13.053	7.822	0.001
	Control	30	3.117	0.415	
Procalcitonin N.R(0.02-0.05)ng/ml	Patient	58	0.075	0.060	0.001
	Control	30	0.035	0.011	

WBC: white blood cell, LYM: Lymphocyte, LDH: lactate dehydrogenase, CRP: C-reactive protein, TNF-alpha: tumor necrosis factor-alpha, IL-10: interleukin 10, ng: Nano gram, pg: pictogram, ml: milliliter, mg: milligram. N.R: normal range.
p-value ≤ 0.05

3.4 Evaluating the performance of the measured parameters in the early detection of COVID-19

Figures (3.1), (3.2), and Table (3-4) show the (AUC), significant cut-off point, sensitivity, and specificity of the measured parameters (TNF-alpha, IL-10, procalcitonin, LDH, ferritin, D-dimer, CRP and neutrophil in addition to Lymphocyte in (figure 3.2). The biomarkers derived in this study successfully detected true positives (AUC).

When looking at Figures (3.1) and (3.2) and Table (3.4) the (AUC) for the neutrophil was (0.839 [95% CI =0.756-0.922]), for Lymphocyte was (0.863[95% CI=0.783-0.942]), for ferritin was (0.934[95% CI =0.878-0.991]), for LDH was (0.973[95% CI =0.938-1.0]), for D-Dimer was (0.931[95% CI=0.877-0.985]), for CRP was (0.961[95% CI=0.923-1.0]), for TNF- alpha was (0.976[95% CI=0.939-1.0]), for IL-10 was(0.96.4[95% CI=0.918-1.0), and for Procalcitonin was (0.845[95% CI=0.766-0.923),(all P- values < 0.001).

All of the markers had high sensitivity in detecting the infected patients (81,79.3,94.4, 94.8, 91.4, 91.4, 96.6, 96.6, and 74.1) for neutrophil, Lymphocyte, serum ferritin, LDH, D-dimer, CRP, TNF-alpha, IL-10 and procalcitonin respectively.

The specificity for neutrophil, Lymphocyte, ferritin, LDH, D-dimer, CRP, TNF-alpha, IL-10, and procalcitonin was (73.3, 90, 86.7, 83.3, 80, 90, 93.3, 93.3 and 80) respectively.

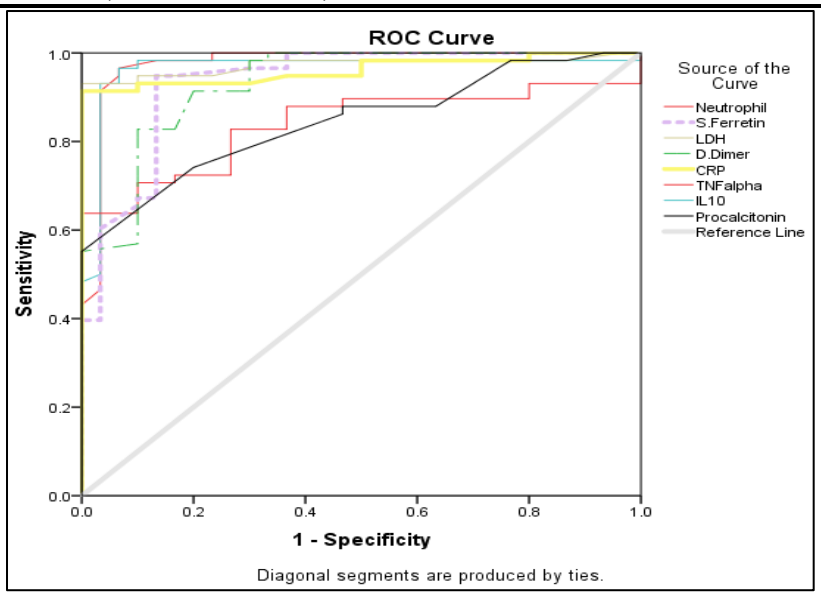

Figure 3.1: ROC curves of measured parameters according to the detection of covid-19.

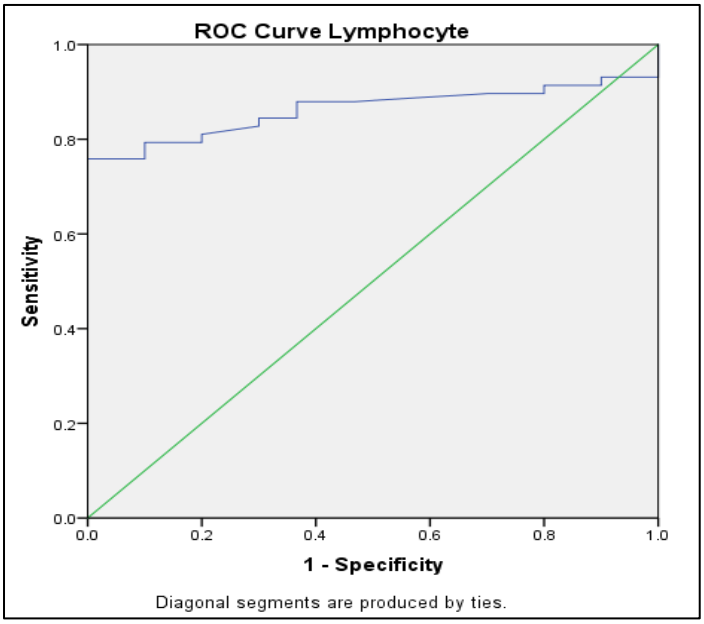

Figure 3.2: ROC curves of the Lymphocyte according to the detection of covid 19

Table 3-4: The values of measured markers in the detection of covid-19 infected persons

Test Result Variable(s)	Area under curve	SE	PV	95% CI				
				LB	UB	Cut-off point	Sensitivity	Specificity
Neutrophil%	.839	.042	0.001	.756	.922	63.85	81	73.3

Lymphocyte% *	.863	.041	0.001	.783	.942	28.9	79.3	90
Ferretin ng/ml	.934	.029	0.001	.878	.991	145.5	94.4	86.7
LDH U/l	.973	.018	0.001	.938	1.0	326.0	94.8	83.3
D.Dimer mg/l	.931	.028	0.001	.877	.985	.425	91.4	80
CRP mg/l	.961	.020	0.001	.923	1.0	4.35	91.4	90
TNF-alpha Pg/ml	.976	.019	0.001	.939	1.0	8.65	96.6	93.3
IL-10 Pg/ml	.964	.024	0.001	.918	1.0	5.05	96.6	93.3
Procalcitonin ng/ml	.845	.040	0.001	.766	.923	.045	74.1	80

*only in the case of Lymphocytes, smaller test result variable(s) values indicate more substantial evidence for an actual positive state.
The actual positive state is Sever.

AUC: Area under the curve, SE: standard error, PV: present value, CI: confidence interval, LB: lower bound, UB: upper bound, LDH: lactate dehydrogenase, CRP: C-reactive protein, TNF-alpha: tumor necrosis factor-alpha, IL-10: interleukin 10, ng: Nano gram, pg: pictogram, ml: milliliter, mg: milligram

.

3.5 Association of studied parameters with disease severity in COVID-19 patients

Figure (3.3) shows that 31 (53%) of COVID-19 cases in this study were found to be severe (severe and critical), and 27(47%) were non-severe (mild to moderate) according to WHO Living guidance for clinical management of COVID-19 (90).

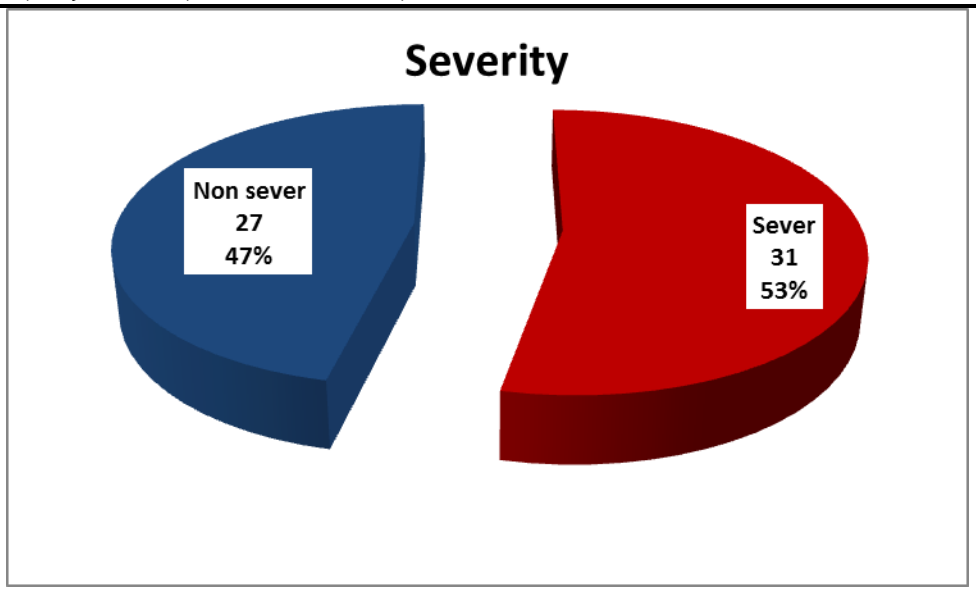

Figure3.3 distribution of studied COVID-19 cases according to the severity

Table 3-5 shows that the mean of measured parameters among severe (31 cases) and non-severe (27 cases) was significantly higher in the severe than in the non-severe group, mean of WBC count was (8.794± 3.288 $\times 10^9$/L) versus (5.444 ± 1.625$\times 10^9$/L), the mean neutrophil percentage was (77.058% ±5.6060%) versus (64.430%±9.5046%), the mean of ferritin concentration was (625.032±264.4197)ng/ml versus (265.237±92.366)ng/ml, the mean of LDH concentration was(674.194± 155.4137)U/l versus (508.074±109.0603)U/l, the mean of D-dimer was (1.2926 ±0.77312)mg/l versus (0.4948±0.09044)mg/l, the mean of CRP was (31.448±10.0924)mg/l versus (11.426±6.5416)mg/l, the mean of TNF-alpha concentration was (17.645±5.4884)pg/ml versus (11.148± 2.0045)pg/ml, the mean of IL-10 cocentration was (18.881 ±7.1693)pg/ml versus (6.396±1.3406) pg/ml, the mean of procalcitonin was(0.0991±0.07326)ng/ml versus (0.0470± 0.01429)ng/ml, all P-value= 0.001
The mean lymphocyte percentage was lower in severe than non-severe (16.848± 6.5257%) versus (28.730± 8.2899%), P-value 0.001
The difference in hemoglobin value between the severe and non-severe groups was nonsignificant.

Table 3-5: Differences between means of measured parameters and COVID-19 severity in patient group.

Measured parameters	Severity*	N	Mean	S. Deviation	P value
WBC x10^9/L	Severe	31	8.794	3.2886	0.001
	Non severe	27	5.444	1.6254	
Lymphocyte%	Severe	31	16.848	6.5257	0.001
	Non severe	27	28.730	8.2899	
Neutrophil%	Severe	31	77.058	5.6060	0.001

	Non severe	27	64.430	9.5046	
Hemoglobin g/dl	Severe	31	13.648	1.7299	0.17
	Non severe	27	12.689	2.2363	
Ferritin ng/ml	Severe	31	625.03	264.4197	0.001
	Non severe	27	265.23	92.3669	
LDH U/l	Severe	31	674.19	155.4137	0.001
	Non severe	27	508.07	109.0603	
D.Dimer mg/l	Severe	31	1.2926	.77312	0.001
	Non severe	27	.4948	.09044	
CRP mg/l	Severe	31	31.448	10.0924	0.001
	Non severe	27	11.426	6.5416	
TNFalpha pg/ml	Severe	31	17.645	5.4884	0.001
	Non severe	27	11.148	2.0045	
IL10 pg/ml	Severe	31	18.881	7.1693	0.001
	Non severe	27	6.396	1.3406	
Procalcitonin ng/ml	Severe	31	.0991	.07326	0.001
	Non severe	27	.0470	.01429	

SD: standard deviation, WBC: white blood cells, LDH: lactate dehydrogenase, CRP: C-reactive protein, TNF-alpha: tumor necrosis factor-alpha, IL_10: interleukin 10, WBC: white blood cell, LDH: lactate dehydrogenase, CRP: C-reactive protein, TNF-alpha: tumor necrosis factor-alpha, IL-10: interleukin 10, ng: Nanogram, pg: pictogram, ml: milliliter, mg: milligram. P-value ≤0.05

3. Discussion

Cytokine-mediated inflammatory responses have been associated with pulmonary inflammation and acute lung injury in SARS (91, 92), MERS-CoV (93), and in recent SARS-CoV-2 (47, 94) infections.

In the current study, the age of patients with Covid-19 disease ranged between 18-70 years (mean=51.2±16.1), which agrees with the study by Chuan Qin (94), but our study disagreed with the previous study by sex distribution as there was a female predominance in our study, this difference may be due to small sample size.

Regarding clinical characteristics and comorbidities, most infected patients presented with fever, fatigue, respiratory symptoms, and gastrointestinal symptoms, which correlate with the study by Zang X (8) and another study by Chuan Qin(80); also, there were correlations between having hypertension and diabetes, and COVID-19 infection which agrees with same studies, but in our study there was no relation between COVID-19 and cardiac disease which disagreed with studies as mentioned above.

TNF-α has been shown to have a significant role in the pathophysiology of Acute Lung Injury (ALI) (95), and serum levels of TNF-α were found to be higher in patient groups, which is comparable to Merza MY et al.'s findings in Erbil city (1). According to reports, some individuals with coronavirus infections of the respiratory system had higher blood concentrations of TNF-α (96,98). The sever cases had higher levels than non-severe, which agree with Feldmann M et,

and Gong J et, (96,98) respectively, and disagree with Merza MY et, which reported an insignificant difference between severe and non-sever group (1).

The serum concentrations of IL-10 were elevated in the patient group with significantly higher in severe than non-sever groups, similar to Chaolin Huang's study(27) and Merza MY et, (1). In acute lung injury, it has been demonstrated that IL-10 inhibits TNF-α and neutrophil activation (97).

In a meta-analysis study published in(the Polish Archives of internal medicine journal), they report that the significantly higher serum levels of tumor necrosis factor-alpha (TNFα) and IL -10 in patients with a more severe clinical course of COVID-19 and emphasize a predictive role of IL -10 cytokine for development of acute disease requiring intensive care support(99).

The levels of PCT were elevated in the patient group, with higher in sever groups in comparison with non-sever, which agrees with several studies which reported that elevated PCT is positively associated with the severity of COVID-19(100,101). In normal physiological conditions, thyroid parafollicular C cells synthesize and release the PCT. However, it can also be synthesized in many extrathyroid tissues during bacterial infection, which is mediated by increased concentrations of tumor necrosis factor-alpha (TNF-α)(102). PCT may indicate the severity of COVID-19 infection in patients (39).

The levels of LDH, CRP, D-dimer, and ferritin were significantly higher in severe than non-sever cases, which is in close corroboration with the studies performed by Keddie, S. et al., who have demonstrated increased levels of these biomarkers concerning the severity of COVID-19 (103). Regarding The CBC parameters, lymphocyte count was significantly lower in the severe group than in the non-severe group(106,107). White blood cell count, and neutrophil count, were higher in patients with severe forms (103,105).

Our results indicated that the measured parameters, including TNF-alpha and IL-10, were of significant diagnostic value as analyzed by the ROC curve with the sensitivity of (96.6% and 96.6%) respectively, which agree with the study by Han H et (110). The study performed by Ying Chi et; in china; demonstrated that IL-10 might help identify asymptomatic infections among suspected cases and close contacts and was of significant diagnostic value (104).

The PCT serum levels were sensitive in predicting COVID-19 in our study (74.1%), which disagrees with the study by Liu Zm et (109).

The patient's outcome also correlates with the serum levels of measured immune biomarkers, as patients with higher levels of TNF-alpha and IL-10 were associated with severe illness and poor prognosis, as in a study by Emir Y. et(108).

The procalcitonin level is directly associated with poor outcomes; patients with elevated PCT levels had a higher risk of overall mortality than those with normal PCT levels. COVID-19 patients with elevated PCT levels were more likely to develop critically severe disease (109).

5.1.Conclusions

1. Patients' elevated serum levels of TNF-alpha, IL-10, and procalcitonin compared to the control group indicated the host's immune responses against the coronavirus infection.
2. The serum concentration of TNF-alpha, IL-10, and procalcitonin might be considered a parameter for the COVID-19 severity.

3. These findings indicate different immunoregulatory events during SARS-CoV-2 infection, which may be helpful in the early detection of severe COVID-19-infected patients.

5.2.Recommendations

In light of the previous conclusion, we recommend the following points:

1. Further studies on a larger sample of COVID-19 patients in a multicenter will be required to support our findings.
2. In future studies, we will conduct follow-up studies in patients with COVID-19 and determine the levels of measured markers after recovery.
3. We recommend testing the immune markers used in the study to detect many other infectious diseases early.
4. We also recommend including the effect of medical treatment on the serum level of measured markers by obtaining before and after-treatment serum levels.

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Conflict of Interest: The authors certify that he has NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical statements: Treat those with whom we work and those we serve with civility and consideration. Actively we strive to merit the respect, trust, and confidence of colleagues, customers, and the public.

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Appendices

Appendix 1

Patient ID:

Name : Age: Gender:
Residency: Occupation:
Education :
History of family contact:
Signs and symptoms:
Fever : Fatigue : Dry Cough:
Headache : chills:
Loss of appetite: Body aches: Shortness of breath:
Mucus or phlegm: sore throat : nausea:
Diarreah:
Vomiting :
Duration of symptom:
Past medical hx :
DM
HT
CHD
RS COPD. Asthma
Renal
Malignancy
Past surgical
Radiological investigation finding (if present):
Lab test result :
IL-10
TNF alpha
CBC:
Serum ferritin
Serum LDH
D-dimer
CRP

Appendix 2

Patient ID:
Name : Age: Gender:
Residency: Occupation:
Education :
History of family contact:

Past medical hx :
DM
HT
CHD
RS COPD. Asthma
Renal
Malignancy
Past surgical
Radiological investigation finding (if present):
Lab test result :
IL-10
TNF alpha
CBC:
Serum ferritin
Serum LDH
D-dimer
CRP