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Study the Serum Level of Perforin among COVID-19 Patients: Forensic Study

A Research paper

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By

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بسم الله الرحمن الرحيم

{ ويسألونك عن الروح قل الروح من أمر ربي وما أوتيتم من العلم إلا قليلا }

صدق الله العلي العظيم

سورة الاسراء (الآية ٨٥)

Certification

I certify that this research paper was prepared under my supervision at the department of Biology, College of science, University of Babylon, as a partial requirement for the Degree of Higher Diploma in Forensic Evidence.

Signature:

Name : **Professor. Dr. Hussein Oliewi Muttaleb**

Date:

In view of available recommendation, I forward this research paper for debate by the Examination Committee.

Signature:

Name: **Assist. Prof. Dr. Adi Jassim Abd AL-Rezzaq**

Title: Chairman of Biology

Date:

Dedication

I dedicate this work for my mother, you will always be in my heart and mind.

For soul of my father, who made me the man I am.

For my lovely family my wife and my children

For my dear brothers and sisters .

Adnan

Acknowledgments

After the help of ALLAH for me in completing this study I am thankful and grateful to his almighty. I like to thank all those who helped me in completing this study in the best way. I'll start with my Supervisor Professor. Dr. Hussein Oliewi Muttaleb who was the best help for me by providing advice, guidance and evaluation along the preparation period of this research.

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Also my gratitude goes to all the patients for their cooperation in achieving this research.

Adnan

Summary :

Human coronaviruses (hCoVs), termed hCoV-OC43 (1st. hCoV), hCoV-229E (2nd. hCoV), SARS-CoV (3rd. hCoV), hCoV-NL63 (4th. hCoV), hCoV-HKU1 (5th. hCoV), MERS-Cov (6th. hCoV) and SARS-CoV2 (7th. hCoV) were seven human coronaviruses push clinical alarm of outbreak from animal to human.

COVID-19 caused by by SARS-CoV2 virus was the ling lasting pandemics among those seven outbreaks and announce high attention and global efforts to contain it with less victims. The current study aim was to evaluate cytotoxic T-cells (CTLs)-medicated immunity and renal failure among COVID-19 patients. During a period of 30 days from 1st. June to 30 July 2021 60 COVID-19 patients (36 female and 24 male) were included in study and recorded all data. Blood urea, serum creatinin and perforin were measured.

The results revealed that most engaged age groups were (39-58 years, female 13 (21.6%), male 11(18.34%); (59-78 years, female 16 (26.66%), male 9 (15%). C-reactive protein (CRP) positive and complete blood count CBC show lymphocytopenia as screening diagnostic test for suspicion of COVID-19. The results showed that there is significant differences (Sig. value 0.0001) between COVID-19 patients with chronic diseases and non- chronic in lung damage and low Spo2.

Concern renal function test (urea and creatinin) serum levels among COVID-19 patients the results revealed significant increase in urea level for both male (sig. value 0.0059*) and female (sig. value 0.0032*) when compared with control. Non-significant difference was observed for creatinin between male and female COVID-19 patients compared with control. The Perforin serum level of patients was same those of healthy control. The study concludes, elderly female were more prevalent than other in COVID-19, COVID-19 patients with chronic diseases history have high lung damage involvement along with low Spo2 than those without chronic diseases. Additionally kidney function may be retarded due to COVID-19 and

monitored by urea and creatinin level. Moreover Perforin serum level were same level when compared with healthy control due to, they elderly and may be convalescent.

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1.Introduction and literatures review

1.1. Introduction:

The coronavirus disease 2019 (COVID-19) outbreak is a worldwide emergency, as its rapid spread and high mortality rate has caused severe disruptions. The number of people infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, is rapidly increasing worldwide. Patients with COVID-19 can develop pneumoni (Zhu et al., 2020 ,Huang et al., 2020). a newly emerged respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has recently become pandemic.

Most patients with COVID-19 exhibit mild to moderate symptoms, but approximately 15% progress to severe pneumonia and about 5% eventually develop acute respiratory distress syndrome (ARDS), septic shock and/or multiple organ failure(Huang et al., 2020, Xu, Z et al., 2020).

SARS- CoV- 2 apparently succeeded in making its transition from animals to humans on the Huanan seafood market in Wuhan, China. However, endeavours to identify potential intermediate hosts seem to have been neglected in Wuhan and the exact route of transmission urgently needs to be clarified.The initial clinical sign of the SARS- CoV- 2- related disease COVID- 19 which allowed case detection was pneumonia.

More recent reports also describe gastrointestinal symptoms and asymptomatic infections, especially among young children (Chan JF et al., 2020). Like other viruses, SARS- CoV- 2 infects lung alveolar epithelial cells using receptor- mediated endocytosis via the angiotensin- converting enzyme II (ACE2) as an entry receptor (Zhou P et al., 2020).

Those who did not survive their illness compared with survivors had higher D-dimer levels, fibrin(ogen) degradation products (FDP) and longer PT and APTT values. Abnormal coagulation parameters were evident early after hospitalization and in some patients, fibrinogen concentrations and antithrombin activity decreased over time (Tang et al., 2020). Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causing Covid-19 is one of the most important devastating emerging infectious diseases, nowadays. Covid-19 was classified as a public health emergency of international concern (PHEIC) on 30 January 2020 (A.J. Rodriguez-Morales et al., 2020).

Severity of Covid-19 was different among patients Thus, the main aim of this study was to determine some of immunological parameter relatedness to severty of disease by the flowing objectives :

1- Collection of blood samples from Covid-19 patients
2- Dimorphic and clinical history of patients .
3- Measurement of urea, serum creatinin perforin levels in patients serum.

1-2 : Literatures Review

1-2-1:History of Pneumonia

Pneumonia the most deadly communicable disease with risk factors include children less than five , adults older than 75 years of age, colder climates and hospitalization (Blasi et al.,2007). In 2008, pneumonia occurred in approximately 156 million children (151 million in the developing world and 5 million in the developed world) because malnutrition, overcrowding, and the lack of proper housing are prevalent risk factors (McAllister et al.,2019). The WHO estimates that one in three newborn infant deaths are due to pneumonia (Ujunwa and Ezeonu , 2014).

About 200 million cases of viral community-acquired pneumonia occur every year−100 million in children and 100 million in adults , Viral pneumonia is first described by Hobart Reimann in 1938. had established the practice of routinely typing the pneumococcal organism, and distinction between viral and bacterial strains (John and Hodges ,1988). The SARS epidemic began quietly at the turn of the 21st century. Middle East respiratory syndrome (MERS) is first reported in September 2012 in Saudi Arabia, following isolation of MERS-CoV from a male patient who died from severe pneumonia and multiple organ failure. On December 2019 Atypical unknown pneumonia is first recorded in Wuhan city, Hubei province. Patients have showed high fever (more than 38 C°), dry cough,malaise, and breath difficulties (Schwartz and Graham 2020). the most frequent viral agents include influenza virus followed by respiratory syncytial virus, parainfluenza virus, adenoviruses and Coronavirus (Jain et al,2015) .In 2002, coronavirus infections (SARS-CoVs) spread in Guangdong, south China, causing high fever, breathlessness and pneumonia, and rapidly spread to various regions around the world . The infection has spread in 26 countries, resulting about 8096 cases and 774 deaths. Whereas MERS-CoV is first detected in Saudi Arabia in 2012. An outbreak of pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that started in Wuhan- China, has become a global pandemic infection, 2494 cases are infected by the virus, of which 858 died in more than 25 countries. In March 2020, the disease affected more than 150 countries and territories around the world (Helmy, et al. 2020). In Iraq, the first confirmed case of COVID-19 has been reported in Najaf province for the Iranian student came from Iran on 24 February 2020, followed by 4 cases from one family in Kirkuk province on 25 February, they have also a travel history to Iran .An additional case is

recorded on 27 February in Baghdad, for a patient who recently visited Iran. 74 confirmed cases and 8 fatalities have been reported across Iraq as of 12 March 2020 (Flaih et al.,2020) . The confirmed cases jumped to 1415 on 16 April 2020, with 78 fatalities are recorded by 24 May 2020, the confirmed cases of COVID-19 reached 4469 and reported 160 deaths, while 2738 patients recovered from the infection (Sarhan et al.,2020).

1.2.2: Pathophysiology

Pathogens spread to the lungs through inhalation of droplet , aspiration of fluid, or hematogenous spread , Pneumonia results if host defense mechanisms are unable to keep the respiratory network free from infectious agents (Jain et al., 2015) .

1. viral pneumonia: starts when viral particles enter the body, usually through the nose or mouth. The virus travels to the lungs where they infect the pneumocytes or other cells in the alveoli and airways , there is an interstitial inflammation with infiltrate in the alveolar, causing damage to ciliated epithelium surfaces .The lungs become congested, hemorrhagic and intracellular viral inclusions may form. Local host defenses, such as mucociliary clearance, or secretion of specific secretory IgA antibodies can remove some of the virus particles. However, if mucociliary clearance is impaired or secretory IgA antibodies are absent, infection continues to spread. Respiratory epithelial cells are invaded, and viral replication occurs. Newer viruses then infect larger numbers of epithelial cells, shut off the synthesis of critical proteins, and ultimately lead to host cell death (Lee *et al*., 2016).

Viruses use specialized proteins found on their surface to attach to other proteins on the surface of cells in the lungs. For example, SARS-CoV-2,

uses a Spike protein on its surface to attach to the protein angiotensin converting enzyme 2 (ACE2) on cells in the lung. After attaches it uses the machinery of the cell to make new copies of the virus. Pneumocytes infected by a virus can become damaged and die.The body responds to this injury by replacing the thin type 1 pneumocytes with the thicker, stronger type 2 pneumocytes. Injured pneumocytes also release signals that attract lymphocytes to lungs. As in bacterial pneumonia, fluid fills the air spaces which makes difficult to breathe hyaline membranes (Figure 1-1) describe thick pinks bands of tissue on the inner surface of the alveoli (Katherina et al.,2020). These hyaline membranes are often seen in combination with type 2 pneumocytes and fluid filling the air spaces and alveolar walls. Difficulty in breathing because the thickened alveolar walls make it more difficult for oxygen to exchange between the lungs and the blood. The changes associated with viral pneumonia on x-ray or CT scan will also appear more grey or white because the alveoli have less air than normal in these area (Caswell ,2019).

Bacterial pneumonia starts when bacteria enter the lungs and alveoli from the nose or mouth. Bacteria can also travel to the lungs from other parts of the body through the blood .Bacteria in the alveoli will grow within th air space until they are detected by the body’ s immune system, which will attempt to remove the bacteria from the body. Small capillaries surrounding the alveoli will open to increase the amount of blood flowing to the lungs lead to inflammatory cells including neutrophils to reach the lungs this known as acute inflammation (Katherina et al.,2020). The green or yellow sputum produced when a person with pneumonia coughs is made up of millions of neutrophils and debris from damaged tissue and dead bacteria. It is a sign that active inflammation is taking place in the lungs. Although neutrophils are required to kill and remove the bacteria

in the lungs, they can also damage the pneumocytes lining the alveoli (Katherina et al.,2020).

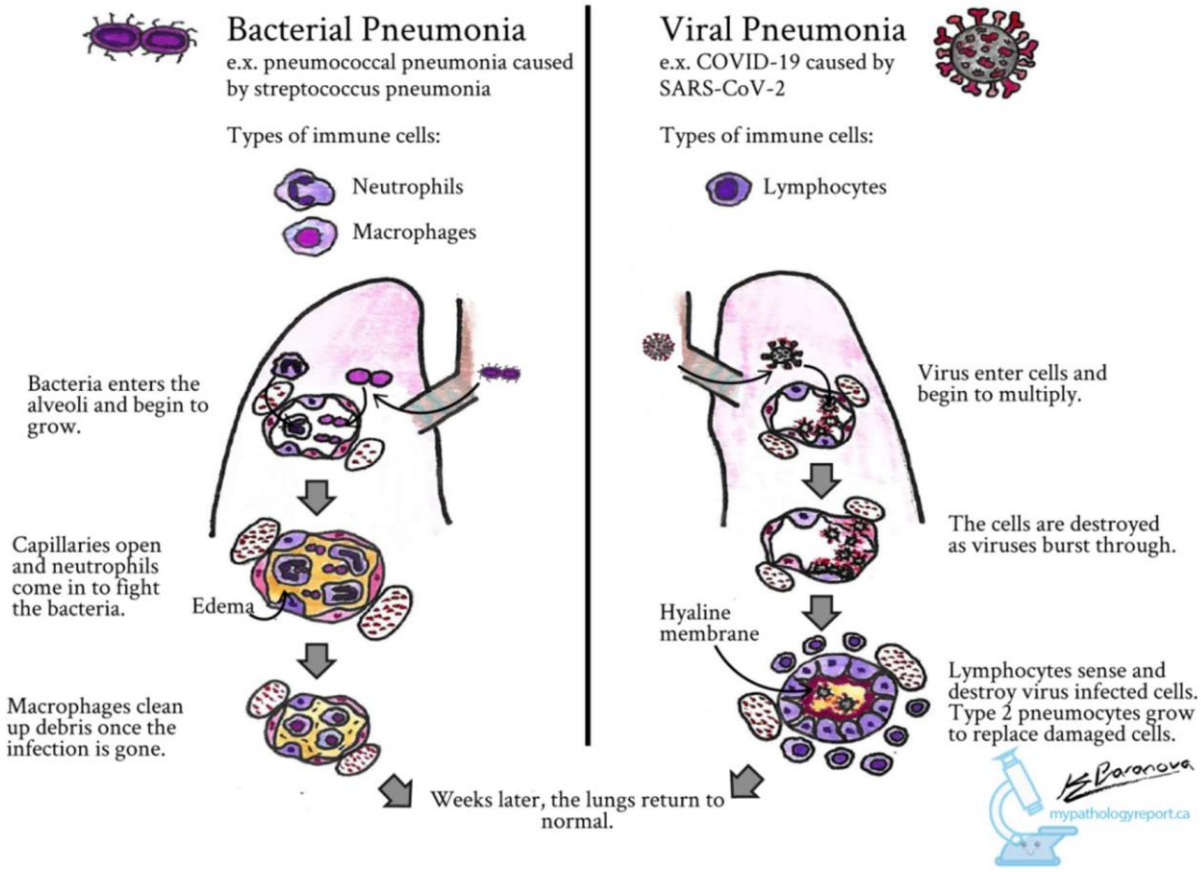

Figure (1-1): the pathophysiology of pneumonia in viral or bacterial causes (Katherina et al.,2020).

The combination of damaged pneumocytes with extra blood flow can cause fluid to fill the air spaces. This process is called edema. Because the fluid prevents air from getting into the alveoli, a person with pneumonia can have difficulty breathing. So , areas of the lung with infection and edema are described as showing consolidation (Çalık et al,2018).

1.2.3: Etiology

There are various causes of pneumonia including bacteria, viruses, and fungi, The populations most at risk for pneumonia are children under five

years, people aged 65 or over, and people with pre-existing health problems. this study focusing on viral pneumonia by coronaviruses .

1.2.4: Coronaviruses

Coronaviruses are important human and animal pathogens that belong to the family Coronaviridae, suborder Cornidovirineae. It named for their large spikes projecting from the envelope giving the virus a crown-like shape of approximately 100 nm (Bleibtreu et al.,2018). The envelope consists of a lipid bilayer derived from the cell membrane of the host and four structural proteins, spike (S), envelope (E), membrane (M), and nucleoprotein (N), as well as a variable number of nonstructural proteins(figure 1-1). SARS-CoV-2 recognizes angiotensin-converting enzyme 2 (ACE2) to attach to cells, particularly respiratory epithelial cells of the host. This process is dependent on the host serine proteaseTMPRSS2, which cleaves viral spike protein at the S1/S2. S2 subunit allows for fusion of viral and cellular membranes (Li et al.,2020). At the end of 2019, a novel coronavirus, was identified as the cause of a cluster of pneumonia cases in Wuhan- China. It rapidly spread, resulting in an epidemic throughout China, with sporadic cases reported globally. From December 2019 till now, the disease, which resulted in several millions cases with many deaths, was caused by a novel type of coronavirus, termed COVID-19 (Ralph , et al., 2020). Patients with COVID19 usually developed a high fever followed by clinical symptoms of cough and shortness of breath. SARS-CoV-2 belonged to species Severe acute respiratory syndrome-related coronavirus with two other zoonotic coronaviruses, SARS-CoV and MERS-CoV, introduced to humans in the 21st century (Gorbalenya et al. 2020).

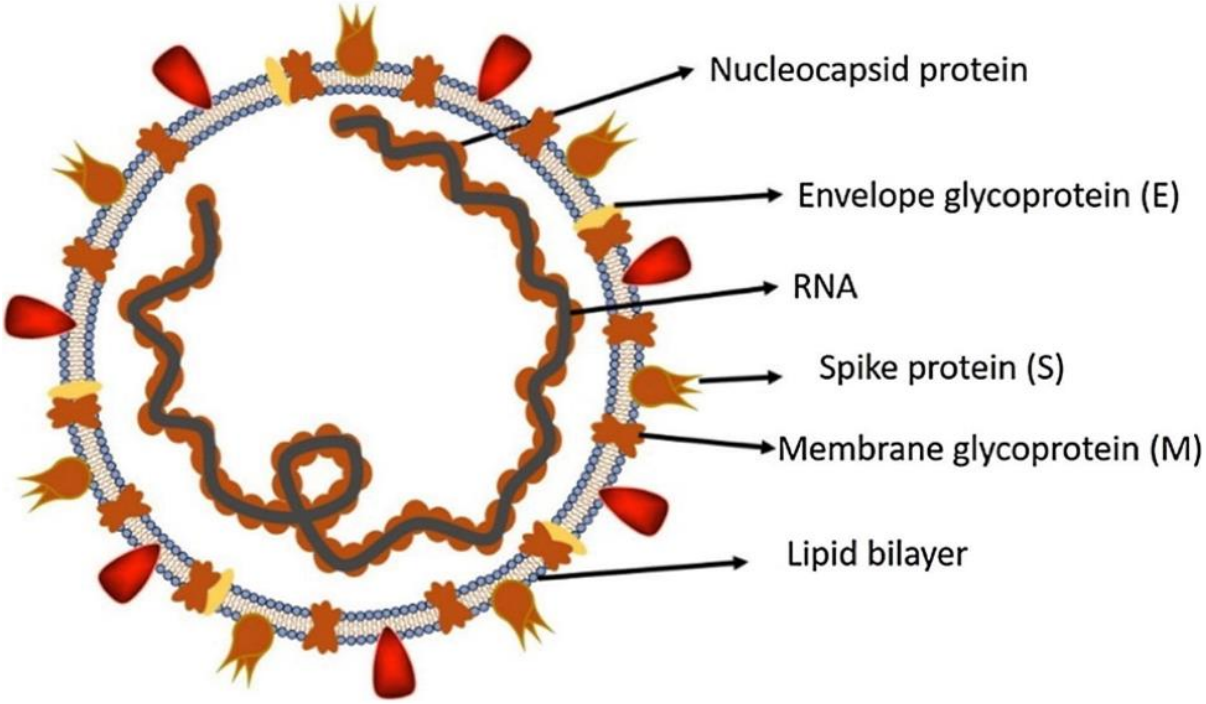

Figure 1-1. A structure of Respiratory Syndrome (SARS) coronavirus((Singhal,2020)

1.2.5: Immune response to SARS-CoV Infection

1.2.5.1: Innate immune response

Lungs are the vital organs designed not only for the gaseous exchange but also serve as a major immune organ to protect the host from diseases caused by the pathogen inhalation during respiration along with allergens and xenobiotics (allergic asthma, pneumonia). Innate Immune Response to SARS-CoV Infection appear in human alveolar type II cells are capable of initiating robust innate immune responses that could contribute to the cytokines and chemokines measured in the lungs and sera of patients with SARS. In addition, chemo-attractants released by epithelial cells to recruit inflammatory cells and to initiate the innate immune response (Qian, et al., 2013). Immune host defense plays an important role in the outcome of bacterial pneumonia, and modulation the inflammatory response as an adjunctive treatment strategy. Normally, bacteria are prevented from reaching the alveoli by several

defense mechanisms located along the upper airway but when bacteria reach the alveoli are usually phagocytosed and killed by alveolar macrophages. When these normal protective mechanisms are overwhelmed, several complex defense systems are triggered. The invasion of pathogens produces a vigorous inflammatory response, including the recruitment of neutrophils. Neutrophils exert microbicidal effects involving several oxidative and enzymatic processes (Weiss ., 1989).

In addition, complement products can promote the killing of bacteria by neutrophils and macrophages (Zisman et al. ,1997) Also , a crucial role of a complex network of cytokines in the initiation and maintenance of inflammation during bacterial infection . Although excessive proinflammatory cytokine production during severe infection may have deleterious effects, but the beneficial role of pro-inflammatory cytokines in local host defense were suggested, (Bachert and Hopken 2001) . Three major cell types line the airway: the ciliated cell, the mucous secreting goblet cell, and the secretory Clara cell. In addition, in the upper airways, there are submucosal glands that contribute to airway secretions. It remains controversial at present if the lung below the glottis is sterile or if there is a lung microbiome. Many recent studies have focused on the ability of respiratory epithelium to respond to pathogens through PRRs such as Toll like receptors (TLRs). Most TLRs (TLR1‑6, 9) are found on respiratory epithelium and the function of TLRs in response to several pathogens resulting in lower respiratory infection has been well characterized. A compartment of lymphocytes residing in the respiratory tract epithelium over the epithelial membrane and between the epithelial cells known as bronchus associated lymphoid tissue (BALT) that comprised from B cells a major immune cell population to generate IgA , T cells , DCs. BALTs also have high endothelial venule (HEV), which serves to transport lymphocytes and antigens to and from the circulation. The IgA produced may bind to the lymphocytes to increase their Ab-dependent cytotoxic action. The secreted IgA also protects against viral and bacterial infections along with the allergy. These pulmonary innate immune cells

serve as antigen-presenting cells (APCs) and secrete several cytokines and chemokines to regulate both the pulmonary innate and adaptive immunity . The pulmonary microbiota helps in the pulmonary immune system development, tolerance induction, and its homeostasis. The pulmonary innate immune response during pneumonia initiates with the activation of residential innate immune cells (AECs, AMs, etc.) inducing the neutrophil infiltration into the lungs (Invernizzi, 2020).

1.2.5.2: Adaptive immune response

Adaptive immunity plays a critical role in pulmonary immunity to many pathogens and is the increasing focus of vaccine induced immunity. Immune response of T cells MERS‐CoV and SARS‐CoV are β‐coronaviruses that can cause fatal lower respiratory tract infections and extra pulmonary manifestations. T cells, CD4+ T cells, and CD8+ T cells particularly play a significant antiviral role by balancing the combat against pathogens and the risk of developing autoimmunity or overwhelming inflammation. CD4+ T cells promote the production of virus‐specific antibodies by activating T‐dependent B cells. However, CD8+ T cells are cytotoxic and can kill viral infected cells. CD8+ T cells account for about 80% of total infiltrative inflammatory cells in the pulmonary interstitium in SARS‐CoV infected patients and play a vital role in clearing CoVs in infected cells and inducing immune injury. (Li, et al., 2020). Depletion of CD4+ T cells is associated with reduced pulmonary recruitment of lymphocytes and neutralizing antibody and cytokine production, resulting in a strong immune‐mediated interstitial pneumonitis and delayed clearance of SARS‐CoV from lungs(Fan et al., 2020).

Dendritic cells (DCs) play a key role in innate immune and adaptive immune responses. As the strongest antigen‐presenting cells in the organism, they effectively stimulate the activation of T‐lymphocytes and B lymphocytes, thus

combining innate and adaptive immunity. Immature DCs have strong migration ability, and mature DCs can effectively activate T cells in the central link of start‐up, regulation, and maintenance of immune responses (Li,and Fan 2020).

Cytotoxic CD8+ T cells (CTLs) secrete molecules such as perphorine, granzymes, and INF-γ to eradicate the virus from host cells. and CD4+ helper T lymphocytes (Th) assist cytotoxic T cells and B cells by producing inflammatory cytokines . However, persistent SARS-CoV stimulation can deplete T lymphocytes in such a way as to reduce their function and prevent cytokine production .This may be induced by the inhibitory cytokine IL-10, which has been detected in the peripheral blood of patients with COVID-19 and, in addition to preventing the proliferation of T lymphocytes, may also promote their depletion. IL-6 and TNF-α also seem to be specifically involved in CD4+ and CD8+ T-cell depletion (which leads to the lymphopenia that is frequently observed in COVID19 patients treated in intensive care units) because their concentrations seem to be inversely proportional to the number of T lymphocytes(Crisafulli, et al., 2020).

In a healthy condition, angiotensin-converting enzyme 2 (ACE2) converts angiotensin II to angiotensin 1‐7 which stimulates endothelial cells to produce nitric oxide (NO). NO helps the vessels to vasodilate and suppresses platelet aggregation. In COVID-19, SARS-CoV-2 occupies ACE2 and the angiotensin II level increases, which result in vasoconstriction and decreased blood flow. Von Willebrand factor (VWF) stored in Weibel Palade body is released into the circulation, promoting clot formation. Decreased ADAMTS13 levels could contribute to thrombus formation within the vasculature. Thrombus formation in disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome. (Iba, et al., 2020).

1.2.5.3: cytokines

Cytokines are soluble protein molecules that facilitate communication between different compartment of the immune System (Kumar et al.,2020). That regulate a number of physiological and pathological functions including innate immunity, acquired immunity and a plethora of inflammatory responses (Dinarello 2000). There are presently over 30 cytokines with the name interleukin (IL) , other cytokines have retained their original biological description, such as tumor necrosis factor (TNF) (Uceyler et al.,2010). Some cytokines clearly promote inflammation and are called suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines by virtue of their ability to suppress genes for pro-inflammatory cytokines such as IL-1, TNF- α and the chemokines .

Another class of genes that are pro-inflammatory are chemokines, which are small peptides that facilitate the passage of leukocytes from the circulation into the tissues. The cells regulated by cytokines must express a receptor for the factor. Thus, cells are regulated by the quantity and type of cytokine to which they are exposed and by the expression of up regulation and down regulation of cytokine receptor (Dinarello 2000).

Perforin is a potent pore-forming protein and permits cytotoxic proteases, such as granzyme B, to enter the cytoplasm of virally infected target cells. Upon recognition of a target cell by cytotoxic cells, an immune synapse is formed and perforin and granzymes are secreted into the synaptic cleft. Perforin then forms pores in the target cell membrane, which allows granzyme proteases to enter the target cell cytosol, leading to cell death. Supporting the pivotal role of this protein in immune responses to viral infections, perforin knockout mice cannot protect themselves against viruses.(Voskoboinik,et al.,2015)

Several factors affect variance in perforin expression (Fig 1) including age. There appears to be easier ‘perforin exhaustion’ in the elderly, which was shown in a series . Compared with those from younger donors, peripheral natural killer

(NK) cells from elderly subjects consumed up to 12 times more perforin following culture with target cells (K562 leukemic tumor cells), and synthesized significantly less perforin in response to a stimulus (PHA)(Mariani,et al.,1996)

It is reasonable to think that perforin bearing the A91V change could be related to suboptimal activation and effector capacities of CD8 and/or natural killer (NK) cells. In the context of a viral infection, the correct function of these cells is required to contain the viral replication, clear the virus and overcome the infection. Ineffective killing of SARS-CoV-2 infected cells might lead to a sustained activation of lymphocytes and macrophages contributing to the cytokine storm and hyper inflammation that characterizes sCOVID-19.(Mancebo ,et al.,2006)

2- Materials and Methods:

2.1: Materials:

2.1.1: Instrument and Equipments

The equipment used in the current study were listed in the table (2-1) below.

Table (2-1) : Instruments and Equipment in this study

Table (2-1): Materials and Kits

Instruments and Equipment	Origin	Company
Disposables	China	Homecare
Tubes	China	Citoglass
Centrifuge	Germany	Hittech
Freezer	Lebanon	Concord
Chemistry analyzer	Japan	Fujifilm
ELISA reader+Washer	USA	Bioteck
Incubator	Germany	Memmert
Autoclave	Japan	Hirayama

2.1.2: ELISA Kit and Haematological kits test

Table (2-2): ELISA Kit and Haematological kits test

Kit	**Origin**	**Company**
Urea , creatinine kit	Japan	Fujifilm
Perforin ELISA kit	China	Elabscience

2.2: Methods:

2.2.1: Patients and Control Group

This case- control study involves 60 (36 female and 24 male) patient COVID 19 whose admitted to COVID 19 Wards in Merjan medical city during June to July 2021 in Babylon Province. All patients were diagnosed based on previous clinical report and clinical examination. These cases are compared with twenty-eight healthy control subjects, All subject with ages ranged from (15- >55) years, all of them is asked to fill a questionnaire and all had no family history of any disease. All patients suffering from Covid-19 pneumonia were included and excluded other type of respiratory disease.

2.2.2: Ethical considerations

The approvals were obtained from all the participants (patients and healthy) and after obtaining the fundamental approvals form the official authoritis, the following information is recorder (patient name ,age ,sex, date infection, chronic disease).As well as record the percentage of oxygen Sop2, C.T.scan. and whether the treatment is given (plasma, Rimidisver, Altamira).

2.2.3: Collection of samples

Fife milliliters of venous blood sample was taken from all subjects. A tourniquet is applied directly on the skin around the arm. The skin over the vein is sterilized with 70% ethyl alcohol from the subjects before blood collection. then the blood samples are transferred into Gel tube for serum separation, the blood left for about 30 minutes in room temperature for clotting and then centrifuged at 3000 rpm for 2 minutes. Then the serum is collected in sterile Eppendorf tube in four repeaters and kept frozen at -20 C˚.

2.2.4: Immunological study

2.2.4.1: Estimation of serum Human perforin

ELISA kit applied to the in vitro quantitative determination of Human Perforin concentrations in serum .

A. Test principle

ELISA kit uses Sandwich-ELISA as a method. The 96 micro titer plate provided in this kit has been adsorbed with an antibody specific to Human perforin . Samples or Standards were added to the appropriate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for perforin were added to well and allowed to bind. Avidin-Horseradish Peroxidase (HRP) conjugate were added to each well and incubated . Free components are washed away after that chromatogenic substrates are added to all wells and allowed to incubated . Only those wells that contain perforin , biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value was proportional to the concentration of perforin .The concentration perforin in the samples by comparing the OD of the samples to the standard curve.

B. Reagent preparation

1-All samples and kit components are brought from refrigerator before use.

2-Preparation of Standard Solution : Reconstitute the lyophilized recombinant protein to make 50 pg/ml of perforin solution by 1ml standard and sample diluents buffer was added to a tube of lyophilized protein. The tube has been shacked gently with vortex, taking care not to foam and then kept at room temperature for 10 min. and mixed thoroughly.

Dilution method : Take 7 EP tubes, 500 µl of Reference Standard &Sample Diluent was added to each tube. 500 µl from 50 pg/ml perforin Working solution was pipetting to the first tube and mix up to produce 25,500 pg/ml working solution . Pipette 500 µl of the solution from the former tube in to the latter one according to this step . Then 500 µl is moved

from each tube to another to prepared series dilute. Each tube has been thoroughly before next transfer .

3. Wash Buffer – 30 ml of Concentrated Wash Buffer added to 750 ml of deionized H2O to prepared Wash Buffer

4. Biotinylated Detection Ab Working Solution : The stock tube was centrifuged before use, then diluted by Biotinylated Detection Ab Diluent(1:100). The required amount was calculated before experiment (100µL/well).

5. Concentrated HRP Conjugate – Concentrated HRP Conjugate were diluted by adding concentrated HRP Conjugate Diluent(1:100).

6. Substrate Reagent- The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again because it is sensitive to light and contaminants.

C . Assay procedure

1. Sample: 100µL of standard, blank, or sample per well is added. The blank well is added with reference standard and sample diluent. The plate covered with plate sealer, then incubated for 90 min at 37℃.

2. Biotinylated Detection Ab: The liquid of each well was removed, without washed. Immediately 100µL of biotinylated detection Ab working solution was added to each well . The plate covered with plate sealer. Gently tap the plate to ensure full mixing, then incubated for 1 h at 37°C.

3. Wash: Each well aspirated and washed, repeating the process three times. Washing done by filling each well with wash buffer. Complete removal of liquid at each step is essential. After the latter wash, the remaining wash buffer was removed by aspirating or decanting. The plate was upturned and putted it against thick clean absorbent paper.

4. HRP Conjugate: 100µL of HRP conjugate working solution is added to each well. The plate covered with the plate sealer, then incubated for 30

min at 37°C.

5. Wash: The washing process is repeated for five times as conducted in step 3.

6. Substrate: 90µL of substrate solution was added to each well, the plate covered with a new plate sealer, then incubated for about 15 min at 37°C. The plate was protected from light, the reaction time can be shortened or extended according to the actual color change, but not more than 15 min.

7. Stop: 50µLof stop solution is added to each well, then the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.

8. Optical density (OD): The optical density of each well was determined at once, using a micro-plate reader set to 450 nm. IL-17 and ACE -2 concentration of unknown samples and control groups are calculated from the standard curve.

2.2.4.2: detection Urea and Creatinin

Dry chemistry test for Urea and Creatinin by Fujifilm (Dri-Chem NX500i)

1- opining the foil and withdraw the test chip.

2 -putting the chip in chamber inside the analyzer.

3 -placing the 1ml tube containing serum sample in sample holder along with tip.

4-starting the analyzer automatically and the Results will appear 5 minutes later.

2.2.5: Statistical Analysis

Graph Pad prism version 7.05 . Numerical data were tested for normal distribution using the T test. data were presented as the mean ± standard deviation of different parameters.

2.2.6: Biosafety and Hazard Material Disposing

Biosafety aspects followed during the work include disposing of all contaminated supplies by autoclaving and then incineration. All benches cleaned with alcohol before and after the work.

Results and Discussion :

The current study aim was to evaluate cytotoxic T-cells (CTLs)-medicated immunity and renal failure among COVID-19 patients. The study include 60 patients (36 female with age Mean±SD 57.86±15.85 years) and (24 male with age Mean±SD 54.5±14.89) admitted to COVID-19 wards in Merjan medical city, Hilla, Iraq during one month's 1st June 2021 to 30th. June 2021. The results showed that the most engaged age groups were (39-58 years, female 13 (21.6%), male 11(18.34%); (59-78 years, female 16 (26.66%), male 9 (15%) (Table 1).

Table (1): Distribution of COVID-19 patients among age group

Gender	Mean±SD	Age group	No. (%)
Female	57.86±15.85	19-38	4 (6.66)
		39-58	13 (21.66)
		59-78	16 (26.66)
		79-98	3 (5)
Male	54.5±14.89	19-38	3 (5)
		39-58	11(18.34)
		59-78	9 (15)
		79-98	1(1.68)

The elderly may represent a specific cluster of high-risk patients for developing COVID-19 with rapidly progressive clinical deterioration (Perrotta et al., 2020). Svartengren et al (2005), showed, in individuals aged 19–81 years, an age-related decline in the clearance of inhaled particles in the small airway region, suggesting this finding as one factor responsible for the high prevalence of respiratory symptoms among the elderly. In elderly persons the gradual decrease in the number of cilia and ciliated cells in the airway, decreasing of upper airway size may give an explanation of the different prevalence of COVID-19 in elderly. Nevertheless, a progressive and relatively linear increase in nasal cavity volume with increasing age coupled

with an age-dependent decrease of nasal resistance might represent determinants for a higher prevalence of COVID in the elderly population (Xu et al., 2019).

Regarding to immune changes in the elderly, a disruption of the innate and adaptive immune system was observed, resulting in an extensive production of cytokines and inflammatory mediators the so-called inflamming process as well as a more profound depletion of CD4+ cells that consequently lead to a disproportionate cytokine storm and a reduced virus clearance (Aw et al., 2007; Napoli et al., 2020).

The patients were selected randomly and the results revealed that all patients were positive for C-reactive protein (CRP) and complete blood count CBC show lymphocytopenia as screening diagnostic test for suspicion of COVID-19 (Table 2). The results showed that there is significant differences (Sig. value 0.0001) between COVID-19 patients with chronic diseases and non in lung damage and low Spo2 (Table 3).

Table (2): Primary diagnostic assays of COVID-19 patients

Gender	no. of patients	CRP	CBC remark
Female	36	Positive	lymphocytopenia
Male	24	Positive	lymphocytopenia

Table (3): Clinical investigations of COVID-19 patients

Gender	Status	no. of patients	CT(%) Mean±SD	Spo2 Mean±SD
Female	chronic diseases	28	42±0.18	85±0.15
	non-chronic diseases	8	21±0.12	93±0.24
Significance			0.0001*	0.0001*
Male	chronic diseases	17	42±0.37	89±0.18
	non-chronic diseases	7	24±0.23	93±0.43
Significance			0.0001*	0.0001*

During infectious or inflammatory disease states, CRP levels can activate the classical complement cascade of the immune system and modulates the activity of phagocytic cells, supporting the role of CRP in the opsonization of infectious agents and dead or dying cells. CRP level on admission was a sensitive and early indicator for COVID-19 severity . Moreover, CRP level was positively correlated with lung lesion at tomographic scans (Chalmers et al., 2019; Chen et al., 2020; Wang et al., 2020)

Regarding our results, higher lymphopenia level was found in the severe cases, but the assessment of the prognosis value of the biological parameters in correlation with the severity of the disease demonstrated that CRP level was more relevant (Ahnach et al., 2020). Lymphopenia was detected in severe COVID-19 patients (85%) and suggested as a severity predictor. They also reported that low lymphocyte count and poor prognosis were related to aging. Lymphopenia could have occurred in COVID-19 patients via four

mechanisms: (a) viral attachment to the cell surface receptor ACE2 infect lymphocytes that lead to lymphocyte death; (b) the possible role of coronavirus in the destruction of lymphoid organs; (c) induction of lymphocyte apoptosis by the production of tumor necrosis factor-α and interleukin-6, and (d) inhibition of lymphocyte production during metabolic acidosis. Although the pathogenesis of COVID-19 remains unclear, lymphopenia was observed in most of the patients. Aging and chronic illness lead to endothelial dysfunction that dismounts cell-cell adhesions, promotes endothelial cell death, extravasation that resulted in lymphopenia (Liao et al., 2002; Bermejo-Martin et al., 2018; Elhassadi et al., 2020; Tan et al., 2020; Xu et al., 2020).

Concern renal function test (urea and creatinin) serum levels among COVID-19 patients the results revealed significant increase in urea level for both male (sig. value 0.0059*) and female (sig. value 0.0032*) when compared with control. Non-significant difference was observed for creatinin between male and female COVID-19 patients compared with control. The Perforin serum level of patients was same those of healthy control (table 4,5,6)

Table (4): difference of Urea, creatinin and Perforin levels among COVID19 Male patients and control

Test		COVID-19 Male (n=24)	Control (n=28)	Sig. Value
Urea	Mean	**47.14**	**31**	0.0059*
	S.D.	**28.27**	**8.60**	
Creatinin	Mean	**0.90**	**0.94**	0.8189
	S.D.	**0.90**	**0.18**	
Perforin	Mean	**25.06**	**27.45**	0.4110
	S.D.	**9.17**	**11.28**	

Table (5): difference of Urea, creatinin and Perforin levels among COVID19 female patients and control

Test		COVID-19 female (n=36)	Control (n=28)	Sig. Value
Urea	Mean	**55.72**	**31**	0.0032*
	S.D.	**41.87**	**8.60**	
Creatinin	Mean	**1.02**	**0.94**	0.6574
	S.D.	**0.93**	**0.18**	
Perforin	Mean	**25.30**	**27.45**	0.4391
	S.D.	**10.70**	**11.28**	

Table (6): difference of Urea, creatinin and Perforin levels among COVID19 male and female patients

Test		COVID-19 Male (n=24)	COVID-19 female (n=36)	Sig. Value
Urea	Mean	**47.14**	**55.72**	0.3835
	S.D.	**28.27**	**41.87**	
Creatinin	Mean	**0.90**	**1.02**	0.6148
	S.D.	**0.90**	**0.93**	
Perforin	Mean	**25.06**	**25.30**	0.9286
	S.D.	**9.17**	**10.70**	

The results of this study was in agreement with those of Guzik et al., (2020) whose found that 27% of the 59 patients with COVID-19 (including 28 severe patients) had elevated urea nitrogen levels and the serum creatinine level was elevated in 19% of the patients. A report on 99 patients showed that 6% elevated serum urea nitrogen, 3% of the patients displayed elevated serum creatinine (Yang et al., 2020). Ye et al., (2021) found that an increase in blood urea at 24 h of hospitalization was associated with a composite of clinical outcomes and in-hospital mortality in patients with COVID-19. The

importance of dynamically monitoring blood urea may therefore be justified in the treatment of COVID-19. Mahmoudi et al., (2020) found that increased level of urea occurred at duration of 2–4 days after the onset of viral infection.

Perforin is a potent pore-forming protein and permits cytotoxic proteases, such as granzyme B, to enter the cytoplasm of virally infected target cells. Upon recognition of a target cell by cytotoxic cells, an immune synapse is formed and perforin and granzymes are secreted into the synaptic cleft. Perforin then forms pores in the target cell membrane, which allows granzyme proteases to enter the target cell cytosol, leading to cell death (Voskoboinik et al., 2015)..

In addition, perforin is known to be an important component of the human immune response to usual respiratory viral infections such as those that produce common cold symptoms (known etiological pathogens to include coronaviruses) (Schmidt and Varga, 2018).

Once reaching the SARS-Cov2 infected cells, CD4+ T helper (Th) cells interact with CD8+ T cells, which drive the cytotoxic response that kills cells infected with the virus. The CD8+ T cells directly recognize viral peptides presented at the surfaces of infected cells, causing apoptosis (a form of programmed cell death) and preventing the virus from spreading further. Alveolar macrophages recognize the neutralized viruses and the apoptotic cells (killed by the CD8+ T cells) and clear them by phagocytosis. This then results in recovery from the viral infection (Chen and Wherry, 2020).

Cytotoxic T lymphocytes (CTLs), CD8+, are a specialized population of immune cells that is able to selectively kill infected cells and consequently eliminate viruses. Usually, CD8 T lymphocytes mediate adaptive cytotoxic

T cell responses (Malyshkina et al., 2017). Cytotoxic cells can contribute to virus control by eliminating infected cells. T cells responding to viral antigens expand and differentiate from cells with a naive phenotype into subpopulations of terminally differentiated (Wiesel et al., 2009; Schmidt and Varga, 2018). SARS-CoV-2 infection is associated with a reduction in CD8+ (Chen et al., 2020; Nie et al., 2029).

Our results in agreement with Westmeier et al., (2020) whose found that in young patients, granzyme A or B and perforin levels were increased in mild and moderate cases. Conversely, in elderly COVID-19 patients, there was a reduced expression of granzyme and perforin (Westmeier et al., 2020). Kang et al., (2020) and Zenarruzabeitia et al., (2021), found that higher levels of perforin and granzyme B were associated with the severity of the disease, suggesting that NK cells from patients with moderate and, even more with severe disease, have the potential to eliminate more efficiently target cells. The apparent increase in the levels of perforin and granzyme B in NK cells from COVID-19 patients that correlated with the disease severity.

Singh et al., (2021) found that the percentage of CD8+ T cells was decreased in moderate and convalescent patients compared to healthy control . so the characterized the cytotoxic potential of CD8+ T cells based on granzyme A and perforin levels and found that there was a tendency of decreased granzyme A expression in mild and moderate patients compared with healthy control. In COVID-19 patients, increased production of inflammatory cytokines inversely correlated with perforin-expressing NK and T cells, with these potentially cytotoxic cells greatly decreased in ICU versus non-ICU patients (Bordoni et al., 2020). Another study suggested that decreased perforin and granzyme levels in CD8+ T cell and NK cells is

associated with severely afflicted COVID-19 patients (Mazzoni et al., 2020).

Conclusion

Conclusions:

1-Eldrly female were more prevalent than other in COVID-19.

2-COVID-19 patients with chronic diseases history have high lung damage involvement along with low Spo2 than those without chronic diseases.

3- Kidney function may be retarded due to COVID-19 and monitored by urea and creatinin level

4- Perforin serum level were same level when compared with healthy control due to, they elderly and may be convalescent.

Recommendation

The current study highly recommended that following:

1- Studying the correlation between the serum level and gene expression of CD8 T cell, Perforin and Granzyme-A, -B in ICU-young COVID-19 patients and non-ICU.

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الخلاصة

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم
قسم علوم الحياة

دراسة مستوى المصل من ال Perforin في مرضى COVID-19: دراسة عدلية

بحث

مقدم الى كلية العلوم / جامعة بابل

كجزء من متطلبات نيل شهادة الدبلوم العالي في الأدلة الجنائية

من قبل

عدنان دوهان عبد الواحد راضي

بكالوريوس علوم حياة

جامعة بابل ٢٠٠٥

باشراف

أ. د. حسين عليوي مطلب فضيل

١٤٤٣ هـ

م ٢٠٢١

الخلاصة:

فيروسات كورونا البشرية يطلق عليها hCoVs- hCoV -hCoV-OC43 (1st. hCoV), 229E (2nd. hCoV) SARS CoV (3rd. hCoV), hCoV-NL63 (4th. hCoV), hCoV-HKU1 (5th. hCoV), MERS-Cov (6th. hCoV) and SARS-CoV2 (7th. hCoV)

وكانت سبعة فيروسات . وكورونا بشرية تدفع الانذار السريري لتفشي المرض من حيوان الى انسان وكان COVID -19الناجم عن فيروس SARS- COV2 و الأوبئة الدائمة بين هؤلاء السبعة في تفشي المرض والاعلان عن اهتمام كبير وجهود عالمية لاحتوائه مع عدد اقل من الضحايا وكان الهدف من الدراسة الحالية هو تقيم الخلايا التائية السامة للخلايا CTL المناعية العلاجية والفشل الكلوي بين مرضى COVID -19 خلال فترة ٣٠ يوما"(الاول من يونيو الى ٣٠ يوليو من عام ٢٠٢١ تم تضمين ٦٠ مريضا" COVID-19

وكانت(٣٦ انثى و٢٤ ذكر) وسجلوا جميع البيانات وتم قياس اليوريا في الدم والكرياتنين والبيرفوررين

اظهرت النتائج ان معظم الفئات العمرية المشاركة كانت (٣٩- ٥٨ سنة)اناث١٣(٢١,٦%) والذكور ١١(١٨,٣٤%) . واعمار (٥٩-٧٨ سنة) كانت اناث١٦(٢٦,٦٦%) وذكور ٩(١٥%).

البروتين التفاعلي CRPايجابي وتعداد العام لكريات الدم CBCيظهر قلة اللمفاويات كاختبار فحص تشخيصي للاشتباه في COVID-19

واظهرت النتائج وجود فروق ذات دلالة احصائية ذات قيمة(0.0001)بين مرضى COVID-19

المصابين بأمراض مزمنة وغير المصابين في تلف الرئة. وانخفاض في SPO2واختبار وظائف الكلى (اليوريا والكرياتنبن)في مستويات مصل الدم بين مرضى19- COVID.

واظهرت النتائج زيادة معنوية في مستوى اليوريا لكل من الذكور ذات قيمة (0.0059) والاناث (0.0032) عند المقارنة مع الكونترول (السيطرة) لوحظ وجود فرق غير معنوي في الكرياتنين بين مرضى كوفيد ١٩ من الذكور والاناث مقارنة مع مجموعة السيطرة وكان مستوى مصل بيرفورين لدى المرضى نفس مستوى السيطرة .

وخلصت الدراسة الى ان الاناث المسنات كانت اكثر انتشارا" من غيرها في كوفيد ١٩.

ان مرضى كوفيد -١٩ والمصابين بإمراض مزمنة لها تاريخ في تلف الرئة جانبا" الى جنب في انخفاض مستوى SPO2 بالمقارنة مع الغير مصابين بإمراض مزمنة .

بالإضافة الى ذلك قد تتأخر وظائف الكلى بسبب كوفيد -١٩ ويتم مراقبتها عن طريق مستويات اليوريا والكرياتنين علاوة على ذلك كان مستوى مصل البيرفورين ننفس المستوى عند مقارنتة بمستوى السيطرة بسبب تقدم في السن وقد يكونون في فترة نقاهة.

.

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Chapter Three: Results & Discussion

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List of Abbreviations

Symbol	Description
CD4+	Cluster of differentiation 4
CBC	Complete blood count
CRP	C- reactive protein
APCs	antigen-presenting cells
ELISA	Enzyme linked immunosorbent assay
IgA	Immunoglobulin A
IL-6	Interleukin-6
IL-8	Interleukin-8
NK	Natural Killer
OD	Optical density
SARS-CoV-	severe acute respiratory syndrome coronavirus 2
WHO	World health organization
TNF-α	Tumor necrosis factor- alpha
ACE2	Angiotensin converting enzyme- 2
MERS	Middle East respiratory syndrome
CD8	Cluster of differentiation 8

IL-1	Interleukin-1
IL-12	Interleukin-12
BALT	bronchus-associated lymphoid tissue
MERS	Middle East respiratory syndrome
TLRs	Toll- like receptor
HEV	high endothelial venule
C0VID-19	Corona virus disease 2019
	Conclusions
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Abstract:

COVID-19 is most important pandemics during 21st century. It caused by new coronavirus called SARS-Cov2. High morbidity and mortality rates were recorded and the world move rapidly for suitable medication and vaccination to lowering the prevalence and improve recovery. The aim of the current study was to evaluate the cytotoxic T-cells killing efficiency of SARS-Cov2 infected cells via measuring the level of perforin and Granzyme B. during a period of one month, June 2021, 88 serum samples were collected from (36 female with COVID-19, 24 male with covid-19 and 28 healthy male), to measure CRP, CBC, CT%, Spo2, Perforin and Granzyme B. the results revealed that, all patients have positive CRP results along with reduction of lymphocyte in complete blood count (CBC) of all patients. The age group with high prevalence was >55 years old (female 21 (34.86), Male 12 (19.92). The chronic diseases associated with age progression includes diabetes mellitus, cardiac diseases and hypertension may be related with score of lung damage and oxygen consumption as showed by results (Sig. value 0.0001) .

The results of Perforin serum level show non-significant differences between COVID-19 male patients with control (Sig. value 0.4110). Also non-significant differences between COVID-19 female patients with control (Sig. value 0.4391). Non-significant differences was also observed between female and male COVID-19 patients (Sig. value 0.9286). Concern Granzyme B serum level the results documented only significant decreasing between male COVID-19 patients and control (Sig. value 0.0158*).

The study conclude that, the most of the inward COVID-19 patients were from elderly. Female prevalence of COVID-19 patients was the highest. Moreover all COVID-19 patients with complications or chronic diseases have highest score of lung involvement and low Spo2 when compared with those with no complications. Additionally Both of Perforin and Granzyme B serum level were decreased when compared with healthy control due to, they elderly and may be convalescent.

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IgA	Immunoglobulin A
IL-6	Interleukin-6
IL-8	Interleukin-8
NK	Natural Killer
OD	Optical density
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
WHO	World health organization
TNF-α	Tumor necrosis factor- alpha
ACE2	Angiotensin converting enzyme- 2
MERS	Middle East respiratory syndrome
CD8	Cluster of differentiation 8
IL-1	Interleukin-1
IL-12	Interleukin-12
BALT	bronchus-associated lymphoid tissue
MERS	Middle East respiratory syndrome
TLRs	Toll- like receptor
HEV	high endothelial venule
C0VID-19	Corona virus disease 2019
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