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Granzyme B Serum Level As Forensic Marker with Severity of COVID-19 patients

A Research paper
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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

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Dedication

I dedicate this work for soul of 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 .

Mohammad

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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 Assist. Prof. Dr. Noor Salman Kadhim 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.

Mohammad

الخلاصة :

يعد مرض كوفيد -١٩ من اهم الاوبئة خلال القرن الحادي والعشرين سببها فايروس كورونا جديد يسمى SARS-Cov2 حيث تم تسجيل معدلات عالية من الامراضية والوفيات . ويتحرك العالم بسرعة للحصول على الدواء المناسب والتطعيم لتقليل الانتشار وتحسين الشفاء.

كان الهدف من الدراسة الحالية هو تقييم كفاءة قتل الخلايا التائية السامة للخلايا المصابة ب SARS-Cov2 عن طريق قياس مستوى البيرفورين وگرانزايم بي خلال فترة شهر واحد / يونيو ٢٠٢١ تم جمع ٨٨ عينة توزعت بين (٣٦ انثى مصابة بكوفيد -١٩ و ٢٤ ذكر مصاب بكوفيد-١٩و ٢٨ اصحاء) لقياس CRP ,CBC ,CT%,SPO2 وبيرفورين وگرانزايم بي .

اظهرت النتائج ان جميع المرضى لديهم نتائج CRP ايجابية الى جانب قلة الخلايا اللمفاوية في تعداد الدم الكامل CBC. وكانت الفئة العمرية ذات الانتشار المرتفع اكبر من ٥٥ عاما" .في الاناث بسبة %٢١ (٣٤,٨٦) والذكور بنسبة %١٢(١٩،٩٢).

قد ترتبط الامراض الزمنة المرتبطة بتقدم العمر مثل داء السكر وامراض القلب وارتفاع ضغط الدم بدرجة تلف الرئة واستهلاك الاوكسجين كما هو موضح في النتائج ذات القيمة (٠،٠٠٠١)

اظهرت نتائج مستوى مصل البيرفورين اختلافا" غير معنوي بين مرضى كوفيد -١٩ الذكور و الاصحاء ذات قيمة (٠،٤١١٠) .كما تتوجد ايضا" فروق غير معنوية بين الاناث المصابات بكوفيد-١٩ و الاصحاء ذات دلالة احصائية (٠،٤٣٩١). كما لوحظت فروق غير معنوية بين الاناث والذكور من مرضى كوفيد-١٩ ذات قيمة (٠،٩٢٨٦).

فيما يتعلق بمستوى مصل گرانزايم بي فقد وثقت النتائج انخفاضا" كبيرا" فقط بين مرضى كوفيد-١٩ الذكور والاصحاء ذات القيمة (٠،٠١٥٨).

خلصت الدراسة ان معظم مرضى كوفيد-١٩ في الردهات كانوا من كبار السن .و كان معدل انتشار مرض كوفيد-١٩ في الاناث هو الاعلى . علاوة على ذلك فان جميع مرضى كوفيد -١٩ الذين يعانون من مضاعفات او امراض مزمنة لديهم اعلى درجة من تأثير الرئة وانخفاض SPO2 عند مقارنتهم مع اولئك الذين ليس لديهم اي مضاعفات.

بالإضافة الى ذلك انخفاض مستوى كل من بيرفورين وگرانزايم بي في المصل عند مقارنتهم بالأصحاء نظرا" لكونهما شديدي التعافي وقد يكونان في حالة نقاهة.

Summary

COVID-19 is the most important pandemics during 21st century. It is caused by new coronavirus called SARS-Cov2. High morbidity and mortality rates were recorded and the world moves rapidly for suitable medication and vaccination to lowering the prevalence and improve recovery. The aim of the current study is 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), and 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($P_=$ 0.0001) .

The results of Perforin serum level show non-significant differences between COVID-19 male patients and control (p. = 0.4110). Also non-significant differences between COVID-19 female patients with control (Sig. value 0.4391). Non-significant differences were 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 had 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 in patients when comparing with a healthy control, and to, they elderly might be during convalescent period.

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Units of Measurement

NO.	Item	Unite
1	CRP	mg/mI
2	Perforin	Pg/mL
3	Granzyme B	Pg/mL
4	SPO2	**%**
5	C.T Scan	**%**

List of Abbreviations

Symbol	Description
A91V	Perforin variant gene
ACE2	Angiotensin converting enzyme- 2
ADAMTS13	ADAM Metallopeptidase With Thrombospondin Type 13
AECs,	alveolar epithelial cells
AMs	alveolar macrophages
APCs	antigen-presenting cells
BALT	bronchus-associated lymphoid tissue
C1q.	Complement ,component 1q
CBC	Complete blood count
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
COVID-19	Corona virus disease 2019
CRP	C- reactive protein
CTLS	Cytotoxic CD8+ T cells
CXCL10	C-X C motif chemokine ligand-10
DCs	Dendritic cells
E	envelope
ECM	enhanced extracellular matrix
ELISA	Enzyme linked immunosorbent assay
GzmB	Granzyme B
HE	hemagglutinin esterase
HEV	high endothelial venule
HR1	heptad repeat regions 1
HR2	heptad repeat regions 2
HRP	Horseradish Peroxidase
ICU	Intensive care unit
IgA	Immunoglobulin A
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-8	Interleukin-8

IL-12	Interleukin-12
K562	Human cell line(from human blood)
M	membrane
MCP-1	Monocyte chemoatractant proein-1
MERS	Middle East respiratory syndrome
N	nucleoprotein
NK	Natural Killer
NO	nitric oxide
OD	Optical density
ORFs	open reading frameworks
PHA	Phyto hem agglutinin
PICs	Pro inflammatory cytokines
PRR	pattern recognition receptor
S	spike
S1/S2	Antigen of SARS-cov-2
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
S.D	Standard deviation
Th	T helper
TLR1	Toll- like receptor 1
TLR6	Toll- like receptor 6
TMPRSS2	Trans membrane protease serine 2
TNF-α	Tumor necrosis factor- alpha
VWF	Von Willebrand factor
WHO	World health organization
α- CoV	Alphacoronavirus
β- CoV	Betacoronavirus
γ- CoV	Gammacoronavirus
δ- CoV	Deltacoronavirus
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1.Introduction and literatures review

1.1. Introduction

An outbreak of pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), named coronavirus disease -2019 (COVID-19) that started in Wuhan- China, has become a global pandemic.SARS-CoV-2 enter host cells via the angiotensin-converting enzyme 2(ACE2) receptor, which is expressed in various human organs after binding to spike a surface glycoprotein on virus (Synowiec, *et al*., 2021). The virus-induced inflammatory responses, including the excessive expression of proinflammatory cytokines (PICs) and chemokines as IL-1, IL-6, IL-12, IL-8 were found in COVID-19 patients especially in severe cases (Huang *et al*., 2020). The host immune system is essential for the resolution of infection, but on the other hand can serve as a crucial player in the pathogenesis of the major clinical complications of the disease. Several studies have shown various changes in selected immune parameters during the pneumonia infection by comparing infected and healthy, severe and non -severe patientes.

In Covid-19 pandemic the death reach to 1,754,754 globally and the total number of the infected had reached 79,231,893, According to the weekly epidemiological update released by WHO on 29 December 2020 (Karako, *et al*.,2021).

Severty 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- Demographic and clinical history of patients .

3- Determinant of CRP, perforin and Granzyme B levels in patients serum.

1-2 : Literatures Review

1-2-1: Historical review

The SARS-CoV epidemic, which began in Southern China in the autumn of 2002 and spread to 29 countries or regions, resulting in 8096 cases and 774 deaths, marked the beginning of the twenty-first century (WHO 2002 – 2003, Hon *et al*.; 2003, WHO 2003).

SARS' etiologic agent (an unidentified coronavirus; SARS-CoV) was isolated and its genome sequenced in record time (WHO 2003, Marra *et al*.; 2003, Drosten *et al*.; 2003, Rota *et al*.; 2003, Ksiazek *et al*.; 2003, Homes *et al*.; 2003). The mini pandemic was brought under control within 7 months of its onset, thanks to an unprecedented global public health campaign (WHO. 2003).

The first case of Middle East respiratory syndrome (MERS) was discovered in Jeddah, Saudi Arabia, in June 2012, and the majority of cases have occurred in the Arabian Peninsula (Zumla *et al*.; 2015). The World Health Organization (WHO) has received reports of over 2,400 cases worldwide, with over 850 deaths (WHO. 2019). MERS-CoV is a zoonotic virus that has a reservoir host in dromedary camels (Reusken *et al*.; 2016, Wernery *et al*.; 2017, Paden *et al*.; 2018). Bats are a probable original reservoir; coronaviruses similar to MERS-CoV have been found in bats, although there is no epidemiologic evidence of their function in transmission (Corman *et al*.; 2014).

An unknown disease emerged at the end of 2019 and became the subject of attention. Pneumonia-like symptoms and lung fibrosis is caused by the disease (Zhou F. *et al*.; 2020). It all started in Wuhan, Hubei Province, China, a city of 11 million population (Adhikari *et al*.; 2020, Callaway *et al*.; 2020, Chen *et al*.; 2020, Fisher and Wilder-Smith 2020, Jingchun *et al*.; 2020). On December 31, 2019, China was the first country to announce this pneumonia with an unknown cause to the WHO country office (WHO 2020). Since then, it has registered thousands of new COV-19 cases.

In China, the disease increased in late January and early February (Callaway *et al*.; 2020). Covid-19 had expanded to 19 other nations by January 31,2020, infecting 11,791 people and causing 213 deaths. On January 30,2020, the WHO declared the COVID-19 epidemic a Public Health Emergency of International Concern (WHO 2020, Adhikari *et al*.; 2020). It has also spread globally, affecting vast populations in Iran, South Korea, and Italy, as well as resulting in an increase in cases in over 150 countries around the world (Callaway *et al*.; 2020).

On December 29,2019, the World Health Organization designated the novel coronavirus as coronavirus disease 2019 (COVID-19) (WHO, 2020). The virus is also known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (WHO, 2020). According to reports, several people infected with pneumonia of unknown origin were related to a local seafood market in Wuhan, China, in December 2019 (WHO, 2020).

The coronavirus outbreak has been linked to the Wuhan seafood market, according to the WHO (Sun K. *et al*.; 2020). Scientists began researching the source of the current coronavirus almost immediately. On January 10,2020, Prof. Yong Zhang's research group was the first to publish the COVID-19 genome (Adhikari *et al*.; 2020).

SARS-CoV-2 is not country-specific virus. It was extremely infectious, spreading to over 100 countries in the last two to three months and affecting over 300000 persons on the world. As of March 24, 2020, the following populations are impacted: China, Republic of Korea, Australia, Malaysia, Japan, Singapore, New Zealand, and others in the Western Pacific Region. A total of 195,511 positive cases were reported in the European Region (Italy, Spain, Germany, the United Kingdom, Norway, and so on), with 24,087 of them occurring in just one day. In a single day, there were 10,189 recorded cases and 1447 deaths (WHO, 2020).

Across Southeast Asia, there have been 1990 confirmed cases with 65 deaths. This outbreak affected a total of 27,215 people in the Eastern Mediterranean Region, with 1877 people dying as a result of it. In the Americas, there have been 49,444 reported cases and 565 fatalities, with an average of 12,428 new cases and 100 deaths per day. In the end, 1305 confirmed cases and 26 deaths have been recorded in the African Region (WHO, 2020).

Coronavirus disease has quickly spread throughout Europe, North America, Asia, and the Middle East, with the first cases recorded in Latin America and Africa. According to the WHO, the number of countries, states, or territories affected by the coronavirus had risen significantly outside of China by March 16, 2020, with 143 countries. A pandemic has been announced for Cov-19 by the Director-General of the World Health Organization due to the rising levels of infections and severity (Trevor *et al*.; 2020). Tedros Adhanom Ghebreyesus, Director-General of the World Health Organization, said on March 13, 2020 the Europe has been the pandemic's epicenter (Trevor *et al*.; 2020).

1.2.2: Classification of Coronavirus

Coronaviruses are members of Coronaviridae family, and Orthocoronavirinae subfamily of the Nidovirales order. Among RNA viruses, CoVs have the largest genomes, with genome sizes ranging 26 - 32 kb. Depending on genetic and antigenic criteria, coronaviruses are classified into four genera: alphacoronavirus (α-CoV), betacoronavirus (β-CoV), gammacoronavirus (γ-CoV), and deltacoronavirus (δ-CoV) (Woo *et al*.; 2012, Lu R. *et al*.; 2020). Bats and mice act as reservoirs for alpha and beta coronaviruses, while birds serve as reservoirs for gamma and delta coronaviruses (Li Q. *et al*.; 2020). SARS-CoV-2 belongs to the subgenus Sarbecovirus of the genus Betacoronavirus, according to phylogenetic analysis as shown in Figure (1-1) (Lorusso *et al*.; 2020).

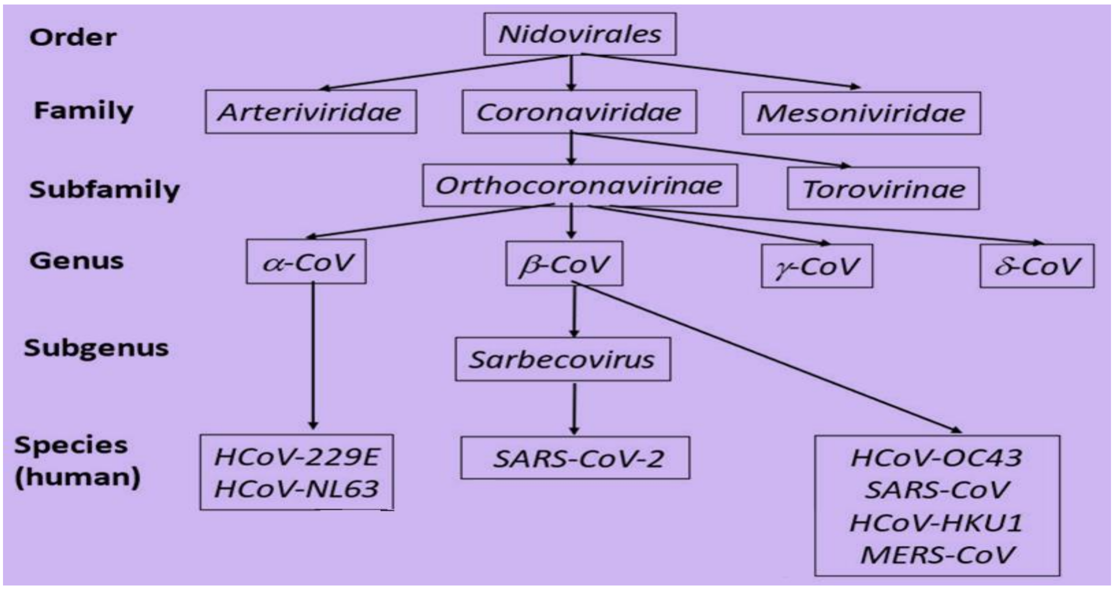

Figure (1-1): Classification of Coronaviruses (Woo *et al*.; 2012, Lu *et al*.; 2020).

1.2.3: Structure of coronavirus

Coronaviruses are large particles viruses that are spherical in shape and have spikes that form a surface projection (Singer *et al*.; 2014). The particles are about 125 nanometers in diameter. The envelope diameter is 85 nm, and the spikes are 20 nm long; however, SARS-spikes CoV-2's are larger, increasing its pathogenicity.

The viral envelope, like any other membrane, was made up of a lipid bilayer and a variety of proteins with structural functions, including membrane (M), envelope (E), and spike (S) (Singer *et al*., 2014) in a ratio of E:S:M 1: 20:300 (Susan 2020). The particle's total number of spikes is about 74 (Yousif *et al*., 2013). However, as shown in figure (1-2), SARS-CoV-2 has another short projection of a proteinous structure known as hemagglutinin esterase (HE) (McIntosh .1974).

The spikes are folded into homotrimers and are divided into two sections, S1 is the spike's head structure, with receptor-binding domains (RBD) that contain the signal peptide, and S2 is the spike's stem, with heptad repeat regions (HR1 and HR2) and a putative fusion peptide (F). The transmembrane domain and endo-domain are also present. All of these subunits aid in the activation of these subunits to facilitate fusion, which is essential for pathogenesis and maintaining envelope integrity (Cui *et al*.; 2019). Finally, the nucleocapsid is made up of nucleic acid (positive-sense single-stranded RNA genome) folded on several copies of proteins (nucleocapsid, N). The organization is organized in a continuous bead-on-a-string pattern (Cui *et al*.; 2019). All of these structures are essential for the virus's defense when it is outside of host cells (Decaro *et al*.; 2011).

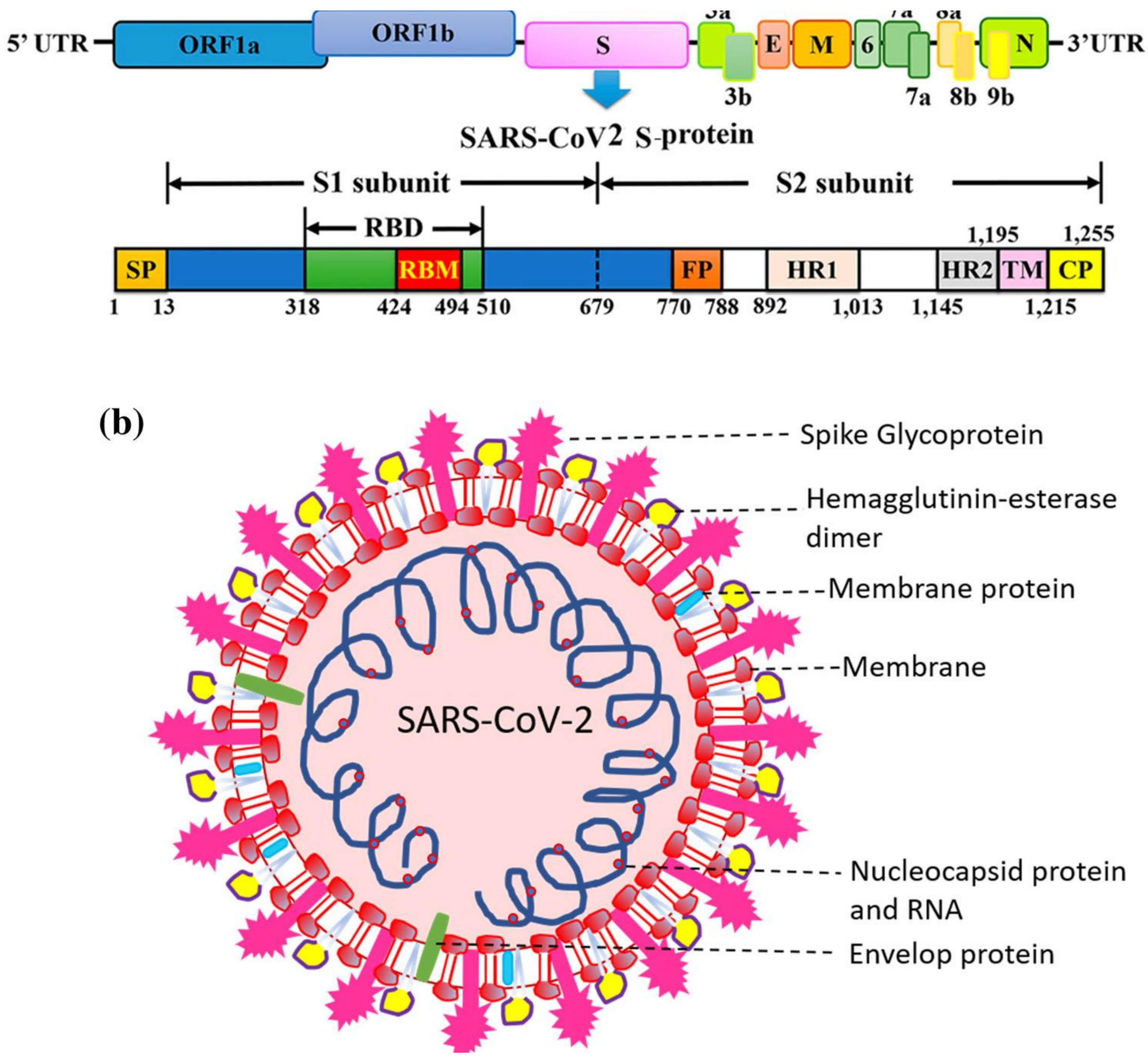

Figure (1-2): Schematic representation of the genome organization

1.2.4: Human receptors of coronavirus

Coronavirus infection (SARS-CoV-2) can be transmitted by bats and humans since it is a member of the Nidovirus order. The Angiotensin-converting enzyme 2 (ACE2) receptors, which can be found in a variety of organs, including the heart, lungs, kidneys, and digestive system, are complementary in form to the spike shape, allowing effective attachment and making it easier for the virus to reach the target cells (Rabi *et al*.; 2020).

This binding occurs in the S protein domain of SARS-CoV-2 receptors, which is closely linked to ACE2 of human and bat (Qianqian *et al*.; 2021). Following the entrance and attachment routes, the membrane of viral and the cell of host fuse (Qianqian *et al*.; 2021).

Next fusion, the type II membrane serine protease (TMPRSS2) on the cell surface of host removes ACE2 and activates the spike-like S proteins to be attached to the receptor (Rabi *et al*.; 2020). When S proteins are activated, they cause configurable changes that enable the virus to invade cells (Ge *et al*.; 2013).

The key determinants of entry for this virus are both proteins (TMPRSS2 and ACE2). Nasal epithelial cells, exactly cup/secretory cells and ciliary cells, have the maximum expression of ACE2 in the respiratory system, according to Fox 2020.

Furthermore, the implanted SARS-CoV-2 later the genomic material will be released into the cytoplasm, and it will become nucleoplasmically localized (Qianqian et al.; 2021). The genetic material of this virus would be mRNA, which is prepared for translation into a protein (Qianqian *et al*.; 2021).

This virus's genomic material has been supplemented by around 14 open reading frameworks (ORFs), every one encodes different set of structural and non-structural proteins that aid in the virus's survival and virulence. By contributing to the ribosome frame shifting event, the genetic parts that encode non-structural proteins first convert to ORF1a and ORF1b to create two great superimposed proteins, pp1a and 19 pp1ab, during the transformation stage (Marquardt *et al*.; 2020).

The structural and accessory proteins are then generated from the sub-genomic proteins like M, S, and E, after that they are separated in the endoplasmic reticulum and moved to the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). In this time being, an earlier transcribed genomic material program will enter N protein in nucleocapsid form and progress to ERGIC. Nucleocapsid can encounter some other structural proteins in this compartment and create small portfolio vesicles for exocytosis outside the cell Figure (1-3) (Masters *et al*.; 2006, Fehr *et al*.; 2015).

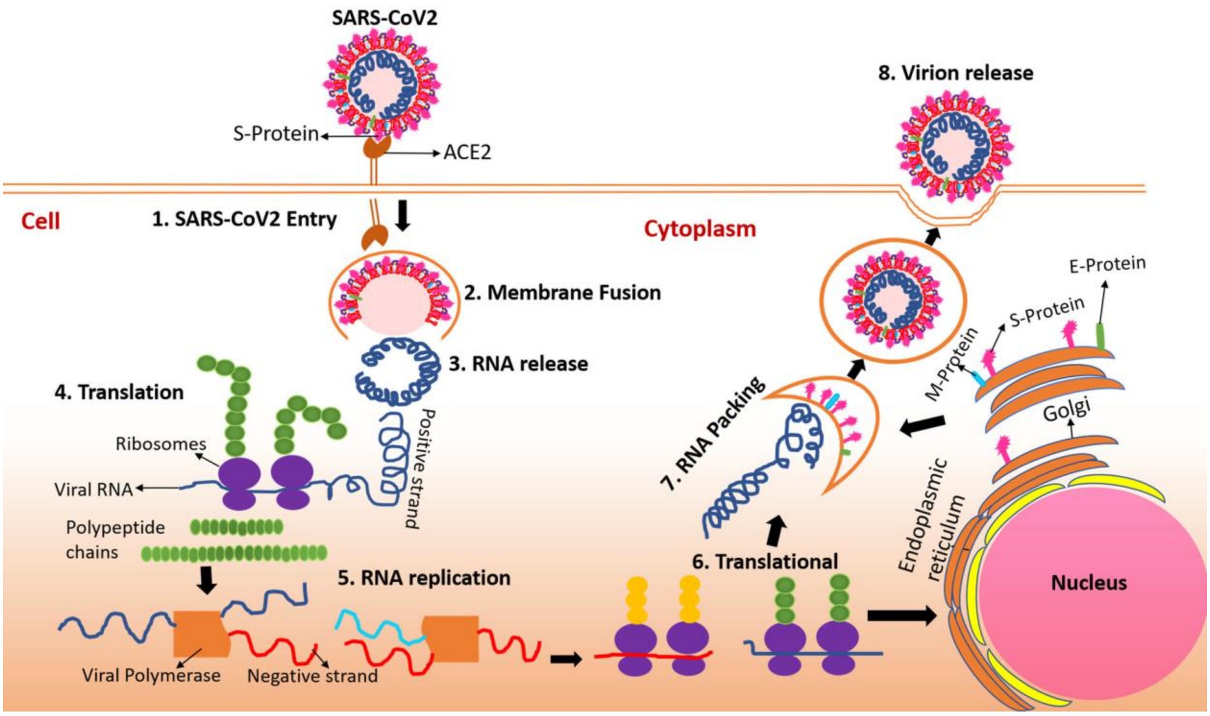

Figure (1-3): The schematic diagram of the mechanism of COVID-19 entry and viral replication and viral RNA packing in the human cell.

1.2.5: Immune response to SARS-CoV2 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 Perforin , granzymes B, 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 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)

Granzyme B (GzmB) is a serine protease traditionally identified as being involved in lymphocyte-mediated apoptosis. Secreted in combination with the pore-forming protein, perforin, which facilitates entry into the target cell, GzmB initiates apoptosis.(Masson *et al.*,1987).

However, during effector–target cell engagement, approximately one-third of GzmB disperses away from the immunological synapse and into the extracellular space.(Isaaz *et al*., 1995).

Further, in a more recent shift in paradigm, other cell types can also express and secrete GzmB, including B cells, basophils, mast cells, neutrophils, dendritic cells, and keratinocytes. (Turner *et al*.,2019) GzmB is expressed at low levels in healthy tissue, but is dramatically upregulated in aged skin and diabetic ulcer.(Parkinson *et al*.,2015) .

Multiple validated substrates of GzmB have been described in skin including vitronectin10, decorin11, and fibronectin.(Boivin *et al*.,2012) .

Perforin-independent GzmB-mediated cleavage of these and other proteins has been linked to impaired collagen remodeling12, enhanced extracellular matrix (ECM) degradation. (Buzza *et al*.,2005) .

GzmB progressively accumulates in conditions associated with age and chronic inflammation, suggesting a role in inflammaging (chronic, low-grade inflammation associated with aging.(McElhaney *et al*.,2012).

The role of GzmB in disease appears to be context-dependent, and varies according to the cell source and where GzmB accumulates in the skin. (Turner *et al*.,2019)

C-reactive protein (CRP) is an annular (ring-shaped) pentameric protein found in blood plasma, whose circulating concentrations rise in response to inflammation. It is an acute-phase protein of hepatic origin that increases following interleukin-6 secretion by macrophages and T cells. Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via C1q. CRP is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes). It is a member of the pentraxin family of proteins. It is not related to C-peptide (insulin) or protein C (blood coagulation). C-reactive protein was the first pattern recognition receptor (PRR) to be identified. CRP binds to the phosphocholine expressed on the surface of dead or dying cells and some bacteria. This activates the complement system, promoting phagocytosis by macrophages, which clears necrotic and apoptotic cells and bacteria (Pepys *et al*., 2003).

2- Materials and Methods

2.1: Materials

2.1.1: Instrument and Equipment

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

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

2.1.2: ELISA Kit and Serological kits test

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

Kit	Origin	Company
CRP kit	Spain	Spainreact
Perforin, Granzyme B 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 admitted to the COVID 19 Wards in Merjan medical city during June 2021 in Babylon Province. All patients were diagnosed based on previous clinical report and clinical examination. These cases were compared with twenty-eight healthy control subjects, All subject with ages ranged from (15- >55) years, all of them were 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 approval from the official authorities. The following information is recorded (patient name ,age ,sex, date infection,and chronic disease).As well as recorded the percentage of oxygen SpO2, C.T.scan, and whether the treatment is given (plasma, Rimidisver, Altamira).

2.2.3: Collection of samples

Five milliliters of venous blood sample were taken from all subjects. A tourniquet is applied directly on the skin around the arm. The skin over the vein was sterilized with 70% ethyl alcohol from the subjects before blood collection. then the blood samples were 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 was collected in sterile Eppendorf tube in four repeaters and was kept frozen at -20 C°.

2.2.4: Immunological study

2.2.4.1: Estimation of serum Human perforin and Granzyme B

ELISA kit was applied to the in vitro quantitative determination of Human Perforin and Granzyme B concentrations in serum .

A. Test principle

ELISA kit for Perforin and Granzyme B uses Sandwich-ELISA a same method. The 96 micro titer plateprovided in this kit has been adsorbed with an antibody specific to Humanperforin . 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 were washed away after that chromatogenic.substrates are added to all wells and allowed to incubated .Only those wells that contain Perforin, Granzyme B, biotinylated detection antibody and Avidin HRP conjugate will appear blue in color. The enzyme-substrate reaction isterminated 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 tothe concentration of Perforin. The concentration of Perforin , Granzyme B in the samples was measured by comparing The OD of the samples to the standard curve.

B. Reagent preparation

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

2-Preparation of Standard Solution : Reconstitute the lyophilized

recombinant protein to make 50, 1000 pg/ml of Perforin, granzyme B respectively solution by 1ml standard and sample diluents buffer was added to a tube of lyophilized protein The tube shacked gently with.
vortex, taking care to avoid foam formation 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, 1000 pg/ml perforin ,granzyme B respectively Working solution was pipetted to the first tube and mixed up to produce 25,500 pg/ml working solution . Pipetted 500 µl of the solution from the former tube in to the latter one according to this step . Then 500 µl was 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°C.

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 was repeated for five times as conducted in step3 .

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 no 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.

2.2.4.2: detection of CRP

CRP was detection by using latex agglutination

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.

3: Results and discussion

The requirements to get rid COVID-19 patients include sufficient immune response controlling the infection along with suitable treatment. The current study aims to evaluate the cytotoxic T-cells killing efficiency of SARS-Cov2 infected cells via measuring the level of perforin and Granzyme B. The results revealed that all patients had 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) (Table 1). 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) (table 2).

Table (3-1): Distribution of COVID-19 patients among age group

Age group	Female (n=36 (%)	Male (n=24 (%)
15-24 Years	2 (3.32)	0 (0)
25-34 Yers	0 (0)	3 (4.98)
35-44 Years	5 (8.3)	2 (3.32)
45-54 Years	8 (13.28)	7 (11.62)
>55 years	21 (34.86)	12 (19.92)
Age Mean±SD	57.86±15.85	54.5±14.89

Table (3-2): Clinical investigations of COVID-19 patients

Gender	Chronic diseases	no. of patients	lung damage (CT%) Mean	Spo2 (Mean)	CRP (mg/l)
Female	chronic	28	42	88	>10
	non-chronic	8	18	94	>10
Male	chronic	17	40	91	>10
	non-chronic	7	24	93	>10
	significant Value		0.0001*	0.0001*	

* significant differences

It seems that the elderly more susceptible to COVID-19 and this hypothesis can be interpreted due to the reduction in number and size of ciliated cells of airway and decreased nasal resistance in elderly). Additionally the elderly was significantly associated with more extensive lung involvement, lower oxygen saturation levels, and higher need for oxygen supplementation. Consequently, these patients had more severe COVID-19 and required mechanical ventilation and ICU admission more often than younger patients (Guo *et al*., 2020; Lian *et al*., 2020). Elderly people are possibly less compliant than younger subjects to follow authorities' recommendations such as wearing a face mask or social distancing (Daoust, 2020), the risk of developing a severe form increases with the number of comorbidities (Fu *et al*., 2020).

Regardin to immune changes in the elderly, a disruption of the innate and adaptive immune system was observed, (Xu *et al*., 2019)resulting in an extensive production of cytokines and inflammatory mediators the so called inflammaging 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).

In COVID-19, the exact effect of CRP remains unclear, but it was reported that their level can be used for early diagnosis of pneumonia , and assessment of severe pulmonary infectious diseases (Warusevitane *et al*., 2016). 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)

The results of Perforin serum level showed 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*) (Table 3,4,5).

Table (3-3): difference of Granzyme B and Perforin levels among COVID19 Male patients and control

Test		COVID-19 Male (n=24)	Control (n=28)	Sig. Value
Granzyme B	Mean	**178.96**	**319.97**	0.0158*
	S.D.	**21.91**	**275.21**	
Perforin	Mean	**25.06**	**27.45**	0.4110
	S.D.	**9.17**	**11.28**	

* significant differences.

Table (3-4): difference of Granzyme B and Perforin levels among COVID19 female patients and control

Test		COVID-19 female (n=36)	Control (n=28)	Sig. Value
Granzyme B	Mean	**293.15**	**319.97**	0.7413
	S.D.	**352.27**	**275.21**	
Perforin	Mean	**25.30**	**27.45**	0.4391
	S.D.	**10.70**	**11.28**	

Table (3-5): difference of Granzyme B and Perforin levels among COVID19 male and female patients

Test		COVID-19 Male (n=24)	COVID-19 female (n=36)	Sig. Value
Granzyme B	Mean	**178.96**	**293.15**	0.1192
	S.D.	**21.91**	**352.27**	
Perforin	Mean	**25.06**	**25.30**	0.9286
	S.D.	**9.17**	**10.70**	

When the SARS-CoV-2 virus infects epithelial cells, such as those found in the airways, it replicates inside the cells, using the host cell's biochemical machinery. This causes the host cell to undergo programmed cell death, releasing molecules called damage-associated molecular patterns (e.g. nucleic acids and oligomers) leading to producing IL-6, CXCL10 and MCP-1 which recruit the T cell to the site of infection (Tay *et al*., 2020).

One prominent cause of lymphopenia may be an enhanced migration of T cells into infected compartments. Despite the lymphopenia, expanded virus-specific CD8+ T cells can be detected in COVID-19 patients. The CD8+ T cells are specific toward several proteins of SARS-CoV-2(Anft *et al*., 2020; Grifoni *et al*., 2020; Thieme *et al*., 2020; Weiskopf *et al*., 2020). During the early phase of the immune response, CD8+ T cells reacted against the spike, membrane, and nucleocapsid proteins. The T lymphocytes of convalescent patients responded to structural proteins or nonstructural proteins, which provides evidence of the development of memory to different viral proteins after infection (Le Bert *et al*., 2020).

A key mechanism of functional CTLs is the elimination of virus-infected cells through the induction of apoptosis of target cells after cell-to-cell contact with effector CD8 T cells. To perform cytotoxic functions, CTLs produce and accumulate effector molecules like the serine proteases granzymes (Gzms) and the pore-forming protein perforin in cytotoxic granules. Additionally, the release of Gzms from activated T cells contributes to the development of inflammation in infected organs. Gzms also change the intracellular matrix and support the migration of lymphocytes, while perforin is necessary for the entry of Gzms into target cells. After the formation of an immunological synapse and the degranulation of cytotoxic granules, Gzms enter target cells, where they initiate multiple pathways leading to the cell death of the infected cell, terminating intracellular virus replication through the loss of the host cell (Voskoboinik *et al*., 2015; David *et al*., 2020).

Our results are agreed 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). 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).

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 cytokineswere 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).

The role of age in the current pandemic has received great attention. The elderly even when mostly healthy, exhibit greater numbers of exhausted T and NK cells, and chronic low-grade sterile inflammation, characterized by high serum concentrations of C reactive protein, IL-6 and IL-8 (Akbar and Gilroy, 2020). Additionally, senescent cells, which potentiate the pro-inflammatory milieu, increase in the elderly, and are associated with impaired lung function (Campisi, 2016). Similarly, it has been shown that perforin expression by NK cells declines significantly after the age of 70 years and that children have higher levels than adults (Cunningham *et al*., 2020).

Conclusion

The current study conclude the following:

1-Most of the inward COVID-19 patients were from elderly.

2-Female prevalence of COVID-19 patients was the highest.

3-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.

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

Recommendation

The current study is highly recommended that following:

1-Studing the serum level and gene expression of Perforin and Granzyme B in relation to IL-6, CD4 and CD8 markers.

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Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Conclusions and Recommendations

References

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