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CORRELATION OF IMMUNOLOGICAL MARKERS LEVELS (IL-2Rα, CD107a & FasL) WITH THE SEVERITY IN COVID-19 INFECTION

A Thesis

Submitted to the College of Medicine and the Committee of Post Graduate Studies \ University of Karbala in Partial Fulfillment of the Requirements of Master Degree of Science in Medical Microbiology

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بسم الله الرحمن الرحيم

يرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ

صدق الله العلي العظيم

Certification

We certify this thesis was prepared under our supervision at the College of Medicine, University of Karbala, as a partial fulfillment of the requirement for the Degree of Master of Science in Medical Microbiology.

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DEDICATION

To our time Imam, the guardian of the matter, and the commander of the nation, ***Al-Hujjah Ibn Al- Hassan*** *"God accelerated his appearance", who has always been my patron to almighty gracious Allah in the completion of my project and passing the difficult stages of it.*

To the soul of my father and my mother ….Who I hope they are happy for my graduation.

To my faithful husband Dr. Dhia M. AlDeffaei …..Who was always standing next to me in every moment of this journey? His limitless support and encouragement made this work come into existence.

I present this humble work…….

FATEMA

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SUMMRY:

Those coronavirus infection 2019 (COVID-19) pandemic may be the A large portion separate emergency influencing around the world, it affects both human lives and global economy, according to WHO there are 156,496,592 confirmed cases including 3,264,143 deaths up to 6 May 2021, The causative agent of COVID-19 is Coronavirus, lymphocyte count has been a marker of interest since the first COVID-19 publication. Lymphopenia related to the disease severity, many immunological markers such as IL2Rα, CD107a and FasL can affect lymphopenia.

To evaluate immunological markers (IL2Rα, CD107a and FasL) in mild and sever COVID-19 patients, and correlation with disease severity and lymphopenia.

The patients enrolled in this study divided into two groups: mild and sever according to patients signs and symptoms. Total 88 proofed COVID-19 patients their lymphocytes count and immunological markers (IL2Rα, CD107a and FasL) were measured by serological techniques sandwich ELISA and statistically correlated.

In group of mild disease, the mean age 43.09 years ± 14.3 (18-75 years), 22 males and 22 females, male to female ratio was 1:1, their WBC was 6.44 ± 2.2, ranging from 3.4 to 12.4, the lymphocytes were 2.8 ± 0.71 ranging from 1.6-4.2. In sever group patients: the mean age 57.7 years ± 12.7 (26-80 years), 25 males and 19 females, male to female ratio was 1.3:1 their WBC was 12.1 ± 4.6, ranging from 4.1 to 26.6, Lymphocytes were 1.29 ± 0.69 ranging from 0.3-3.4. There was a significant difference between mild and severe cases for age, WBC and lymphocytes ($P<0.05$). Gender distribution was not significantly related to disease severity ($P>0.05$). Lymphopenia was not found in mild cases but 59.1% of severe cases had lymphopenia (**$P<0.05$**). There was a significant difference between mild and severe cases for IL2-Ra and CD107 ($P<0.005$) no significant relation between FasL and disease severity in our specimen.

Measuring serum levels of IL-2R, CD107a and FasL in COVID-19 patients will serve a sensitive marker for monitoring patients conditions and provide a suitable tool to verify which patient will develop sever lymphopenia and may need care in intensive care unit

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List of abbreviations

Symbols	Descriptions
3CLpro	coronavirus main protease
ACE2	angiotensin-converting enzyme 2
ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation-induced cell death
ARDS	Acute respiratory disease syndrome
BSTI	British Society of Thoracic Imaging
CBC	Complete Blood Count
CCL2	Chemokine (C-C motif) ligand 2 (CCL2) is also referred to as monocyte chemoattractant protein 1 (MCP1)
CDC	Centers for Disease Control
cDNA	DNA copy
CK	Creatine kinase
COVID-19	The coronavirus disease 2019
CoVs	Corona Viruses
CRP	C-reactive protein.
CT	Computed tomography
CXCL10	C-X-C motif chemokine ligand 10 (IP-10)
CXR	Chest X-ray
DHL	lactic dehydrogenase
DW	Distilled water
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
Fas	First apoptosis signal
FasL	First apoptosis signal ligand
GGO	Ground glass opacities
GISAID	Global Initiative on Sharing All Influenza Data
HRP	Horseradish peroxidase
ICTV	international Committee for the Taxonomy of viruses
IFNγ	Interferon gamma
IL-1,2,6, etc.	Inter-Lukin-1,2,6, etc.

IL-2R	Inter-Lukin-2 receptor
LAMP-1	lysosomal-associated membrane protein-1
MERS	Middle East respiratory syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	Massinger Ribonucleic acid
NK	Natural killer cells
OD	Optical density
ORF1	first open reading frame.
PAMPs	Pathogene-associated Mollecular Pattern
PCR	Poly chain reaction
PLpro	papain-like protease
PRR	Pattern Recognition Receptor
RBD	Receptor –Binding Domain
RdRP	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
SARS	severe acute respiratory syndrome
SARS-CoV	acute respiratory syndrome coronavirus
SGPT	glutamic-pyruvic transaminase
ssRNA	single-stranded ribonucleic acid
TCR	T cell receptor
TLR	Toll-like Receptor
TMB	Tetra-methyl benzidine
TMPRSS2	TM protease serine 2
TNF-α	Tumor Necrotic Factor-alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
WHO	World Health Organization.

Chapter One

Introduction

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Literature Review

Chapter One

1.1-Introduction:

The coronavirus disease 2019 (COVID-19) pandemic is the most sever crisis affecting worldwide, it affects both human lives and global economy, this newly diagnosed infection spreads rapidly between peoples and countries, according to WHO it affect more than 70 million person and more than 1.5million death, researches indicate that this disease was recently discovered in the Chinese city of Wuhan, and then turned into a pandemic that affected all countries of the world, many medical protocols have been tried in management, only the social distancing and quarantine have proven to be the most effective strategy to mitigate the disease spread (Sprung CL *et al*., 2020).

The causative agent of COVID-19 is Coronavirus; microbiologist already know about the human coronavirus since the 1960s. But it rarely causing a significant attention, in 2003, there was an outbreak of severe acute respiratory syndrome (SARS) in mainland China and Hong Kong caused by the acute respiratory syndrome coronavirus (SARS-CoV), in 2012, another outbreak of Middle East respiratory syndrome (MERS) in Saudi Arabia, the United Arab Emirates and the Republic of Korea caused by the Middle East respiratory syndrome coronavirus (MERS-CoV), fortunately in both circumstances the outbreaks were contained (Fehr AR. and Perlman S., 2015).

Patients with COVID 19 primarily complain from symptoms related to the upper respiratory system involvement, Lower respiratory tract infection related symptoms including dry cough, fever, and dyspnea were reported (Huang C. *et al*., 2020). In addition, headache, dizziness,

generalized weakness, vomiting and diarrhea were observed (Shi H., *et a*l., 2020).

Laboratory biomarkers are essential to predict the severity of COVID-19, lymphocyte count has been a marker of interest since the first COVID-19 publication. Lymphopenia related to the disease severity (Liu W. *et al*., 2020, Liu Y. *et al*., 2020 and Wang D, *et al*.2020).

Risk factors for disease severity are the elevated level of C-reactive protein (CRP), serum ferritin, lactic dehydrogenase (LDH), D-dimer, procalcitonin, prothrombin time, Creatine kinase (CK), glutamic-pyruvic transaminase (SGPT), urea, and creatinine (Kermali M., *et al.*, 2020).

The immunological markers that related to disease severity and the bad prognosis are: reduced level of CD4+ T and CD8+T lymphocytes, NK cells and elevated levels of IL6, TNF-α, IL-8, IL-10, IFN-γ, IL-2R, GM-CSF, and IL-1 β. (Chen G., *et al*., 2020)

1.2- Aim of study:

The study to evaluate immunological biomarkers (IL2Rα, CD107a and FasL) in mild and sever COVID-19 patients, and correlation with disease severity and lymphopenia.

1.3- Literatures review:

1.3.1 Coronavirus family

The international Committee for the Taxonomy of viruses (ICTV) has approved the naming of more than 40 coronaviruses. Most of which infect animals. The SARS-CoV-2 virus is the seventh known coronaviruses that infect humans. Of these only four consider as community acquired and are contagious for human for a very long time, the rest three – SARS-CoV, MERS-CoV and SARS-CoV-2 appear to have infect human population more recently. Unfortunately, these three result in a high mortality rate. (Decaro N. and Lorusso A. 2020)

Coronavirus gain its name from the crown-like spikes on the viral surface, coronaviruses infest many animals, where they can cause gasterointestinal tract, respiratory tract or even central nervous system diseases in different severities. Coronaviruses initially classified into three distinct groups 1, 2, and 3 coronaviruses (Brian DA. and Baric RS., 2005) depending on the genotype and serology, the Coronavirus Study Group of the International Committee for the Taxonomy of Viruses (ICTV) classified coronaviruses into three genera, *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*, (Fehr AR. and Perlman S. 2015). Both alphacoronaviruses and betacoronaviruses were found in mammals, while gammacoronaviruses were found in birds and recently proved to infect mammals (Jonassen CM, *et al*, 2005, Mihindukulasuriya KA *et al*, 2008). Another new genera of CoVs called *Deltacoronavirus* had also been identified in pigs and birds (Woo PC, *et al*, 2009). The coronaviruses depend on a special replication mechanism lead to a high rate of new recombination and a high mutation rates, so adapt to new hosts and ecological niches (Lai MM, 1997 and Herrewegh AA., *et al*, 1998).

1.3.2 Coronavirus structure

Coronavirus (CoV) is a single-stranded ribonucleic acid (ssRNA). This type of virus may be a concealed entrapping non-segmented, positive-sense and its genome measure something like 26 to 32 kb., It may be the biggest known RNA virus. SARS-CoV-2 3' end encodes structural proteins, including spike (S) glycoproteins1,2, film (M) glycoproteins, and also envelope (E) Also nucleocapsid (N) proteins (Fig. 1. 1). Furthermore of the genes encoding structural proteins, there are particular genomic locales encoding for viral proteins needed to replication, furthermore will other non-structural proteins, for example, such that those papain-like protease (PLpro) What's more coronavirus primary protease (3CLpro). (Jie Cui *et al*. 2018).

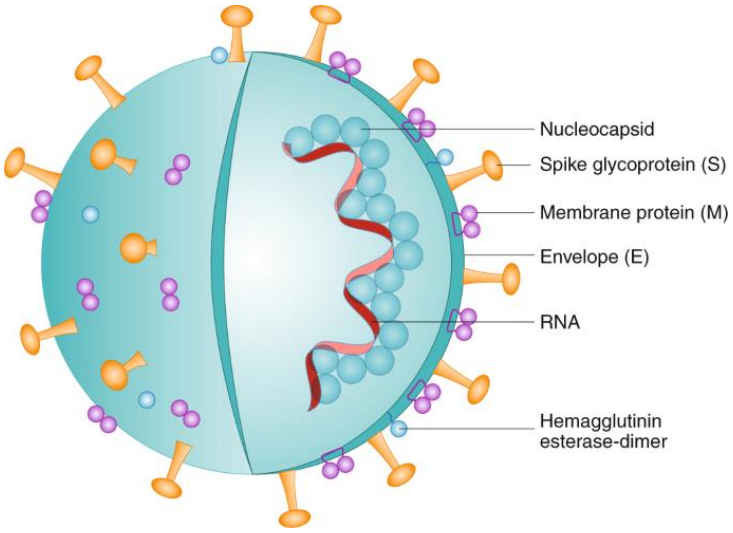

Fig-1.1: Schematic representation of SARS-CoV-2 structure. (Ziebuhr, J. *et al*. 2000)

There are many glycosylated S proteins units cover the surface of SARS-CoV-2, it binding to the host cell receptor angiotensin-converting enzyme 2 (ACE2), initiating cell invasion by the virus. In the ACE2 receptor on the host cell membrane there is type 2 TM serine protease called TM protease serine 2 (TMPRSS2), it promotes virus entry into the cell by activating the viral S protein. At whatever point the infection enters

the cell its RNA is delivered, polyproteins are interpreted from the RNA genome, and replication and record of the viral RNA genome happen by protein cleavage and gathering of the replicase–transcriptase complex. Viral RNA is repeated, and primary proteins are blended, gathered, and bundled in the host cell, after which viral particles are delivered. These proteins are fundamental for the viral life cycle (Huang CL *et al*., 2020).

Structure of the S protein

It consists of an extracellular N-terminus, a transmembrane (TM) domain anchored in the viral membrane, and an intracellular C-terminal segment. It measures 180–200 kDa size, it exists in a metastable state normally, but when it interacts with host cell receptor a perfusion conformation occurs, there will be rearrangement of the structure, this allows the virus to fuse with the host cell membrane. The glycoprotein spikes are coated with polysaccharide molecules to camouflage them to get rid of the host immune system during entry [Watanabe Y *et al.*, 2020). S protein length is 1273 aa, it consists of a signal peptide (amino acids 1–13) located at the N-terminus, the S1 subunit (14–685 residues), and the S2 subunit (686–1273 residues); these S1 subunit is responsible for receptor binding while the S2 region responsible for membrane fusion. In the S1 subunit, there is an N-terminal domain (14–305 residues) and a receptor-binding domain (RBD, 319–541 residues); the fusion peptide (FP) (788–806 residues), heptapeptide repeat sequence 1 (HR1) (912–984 residues), HR2 (1163–1213 residues), TM domain (1213–1237 residues), and cytoplasm domain (1237–1273 residues) comprise the S2 subunit. (Xia S, *et al*., 2020).

S protein form a crown-like projection surrounding the viral particle seen as a characteristic bulbous, (Fig. 1. 2). Based on that structure, the S1

subunit form the bulbous head while the S2 subunit form the stalk region. The design of the SARS-CoV-2 trimeric S protein has been controlled by cryo-electron microscopy at the nuclear level. (Wrapp D, *et al*.,2020)

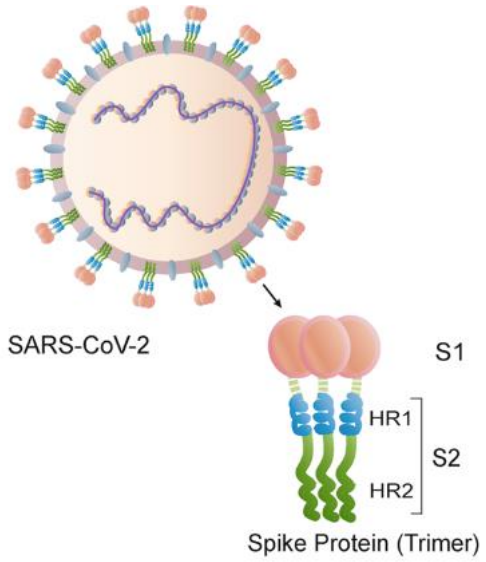

Fig. 1-2: Structure of the SARS-CoV-2 S protein. (Fehr AR, *et al* 2015)

S protein exists as an inactive precursor in the native state, while it activated by the host cell proteases in viral infection state, by cleaving it into S1 and S2 subunits, this is important for activating the membrane fusion domain after viral entry into host cells.(Hoffmann M, *et al*., 2020). The cleavage site of SARS-CoV-2 S protein still not reported in counter to other coronaviruses.(Du L, *et al*., 2007)

1.3.3-Mechanism of infection:

Cellular infection happens by pathogenic virus might induce humoral and cellular immunities which is essential to wipe out the viral disease. In any case, an uncontrolled or deficient immune response might prompt immunopathology and cause extreme harm to patients (Kruse, *et al*., 2020). The known mechanism of cellular infection of SARS-CoV-2 is mediated by the cell surface receptor ACE2 (Fig. 2). SARS-CoV-2 seems to be able to infect any cell that were genetically modified to express solely the ACE2 receptor (Zhou, P. *et al*, 2020). SARS-CoV-2 mostly infect the respiratory and gastrointestinal tracts, because the ACE2 receptors are expressed by epithelial cell their (Malik Y. S *et al*, 2020, Chen, W. H *et al* 2020). Other researches indicates that the expression of ACE2 can be done by brain cells, cardiomyocytes, seminal vesicle cells, renal proximal tubules and gallbladder epithelium (Hikmet, , *et al*, 2020).

The symptoms of COVID-19 patient enhanced these theories as hyposomia, nausea, vomiting, headache and cerebral damage (Baig, *et al*, 2020). Another less potentially SARS-CoV-2 receptor is the L-SIGN (CD209L), which is type II transmembrane glycoprotein of the C-type lectin family, this receptor expressed mainly in human lung alveolar epithelial type II cells and endothelial cells. It is responsible for SARS-CoV entry into host cells but to lesser degree than ACE2 receptor (Kliger, Y., *et al*, 2005). Therefore, SARS-CoV-2 cat target CD209L as well.

The first viral component that attack host cells and interact with it is the surface-exposed S glycoprotein, being encoded by the most variable region of the SARS-CoV genome, the S gene (Zhou, *et al*, 2020). The S protein is a major component accounting for viral binding, fusion and further entry into target cells. It composed of two main functional units.

The SARS-CoV-2 S1 subunit contains the receptor-binding domain (RBD), which affinity for binding to host receptor varies among different CoVs, so it controls the infection process and so it controls the severity of the disease (Walls, A. C. *et al*, 2020). The SARS-CoV-2 S2 subunit is involved in the virus fusion with the host-cell membrane (Cui, J. *et al*, 2019).

SARS-CoV, MERS-CoV and SARS-CoV-2 share the same mechanisms of binding and/or invading host cell through a several steps due to the RBD genomic similarity (Ahmed, *et al*, 2020). The virus entry is a complicated process that begin with the detection of the RBD 394 glutamine residue by the lysine 31 residue on the human ACE2, then adopting specific conformations that will permit a fast dissociation of the S1 and S2 subunits (Walls, *et al*, 2020). (Fig. 1.3). This proteolytic priming of the S protein by host proteases is also mandatory to release the fusion peptide, that enable fusion of virus and host cell membrane, and subsequent release of SARS-CoV-2 RNA into the cytoplasm (Letko,. *et al*, 2020).

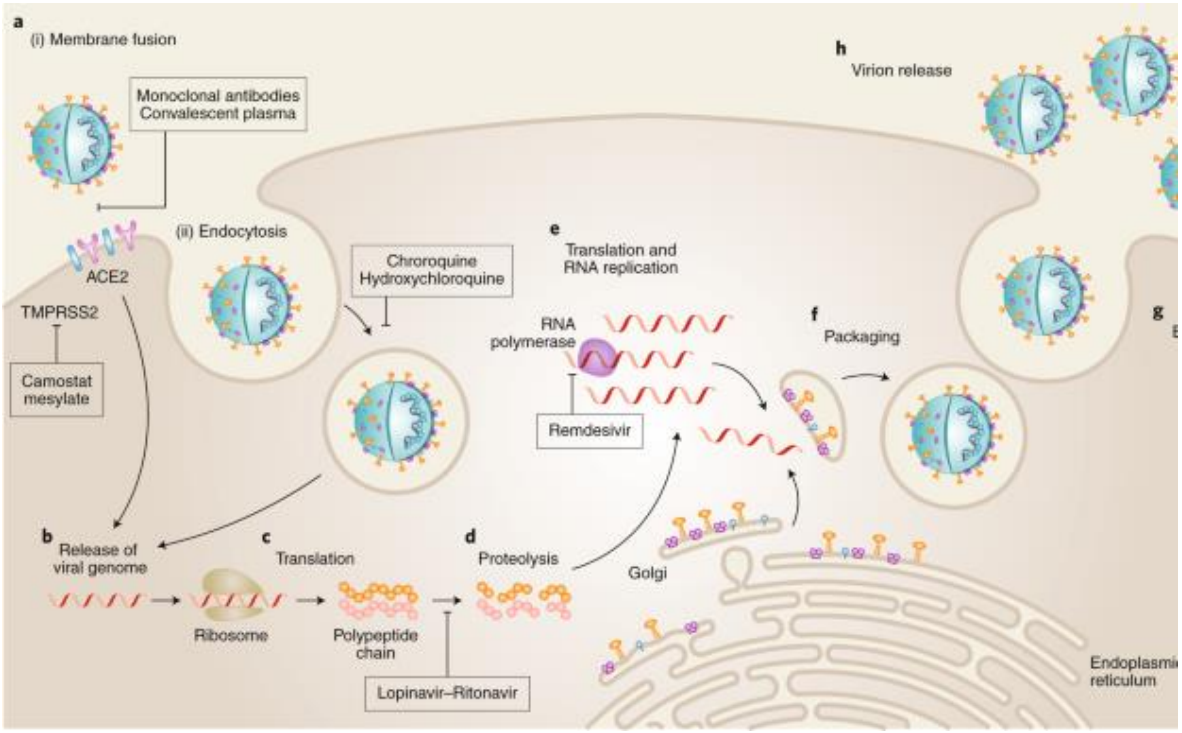

Fig. 1.3 SARS-CoV-2 is internalized by the cell via (i) membrane fusion or (ii) endocytosis. The SARS-CoV-2 spike binds to the angiotensin-converting enzyme 2 (ACE2) via its receptor-binding domain (RBD) and further releases its RNA (b), which will be translated into viral proteins (c,d). e–h, These proteins will form a replication complex to create additional RNA (e) that will further assemble with the viral proteins into a new virus (f), which will be released (g,h). The transmembrane protease serine 2 (TMPRSS2) is a protease shown to affect virus entry, even though its knockout does not inhibit cell infection by SARS-CoV-2. TMPRSS2, transmembrane protease serine 2; ACE2, angiotensin-converting enzyme 2. (Walls, A. C. et al., 2020)

When the virus enters into host cells, RNA genome serves as mRNA for the first open reading frame (ORF1), then it translated into viral replicase polyproteins, which divided into small products by viral proteinases. These products accumulated on double-membrane vesicles that become sites for RNA viral synthesis through two stages. The first stage constitutes the genome replication, the second stage includes the subgenomic RNA transcription and further translation into structural and accessory proteins via additional ORF (Kliger, Y. *et al.*, 2020). These structural proteins are essential for RNA synthesis by RNA-dependent

RNA polymerases (RdRP) in order to replicate the genomic RNA which subsequently released when fusion with plasma membrane occur (Li, X. *et al.*, 2020). The double-membranes help the virus to escape from host immune responses by lacking the pattern recognition receptors (PRR), the activation of which is mandatory for triggering host innate immunity against these viral invading pathogens (Li, G. *et al*, 2020).

1.3.4-Immunity against viral infection:

There are three types of immunity in human body, innate immunity (which is the rapid response against infections), adaptive immunity (which is the slow response), and the passive immunity which has two elements either: natural immunity that received from the maternal origin, and active immunity which received via medicine like vaccination. SARS-CoV2 transmitted through respiratory droplets via coughing and sneezing, it enters respiratory system through nasal cavity and starts replicating. The main viral receptor is ACE2 to which the S protein spike is pinched inside the host cell, then furin enzyme which is found in the host cell, plays an essential role for the viral entry, here the viral propagation start, where it can be detected by nasal swabs, in respiratory tract the virus faces a more active innate immune response. Meanwhile the disease manifested clinically and an innate response cytokine may be predictive of the subsequent clinical course (N.L. Tang, *et al*., 2005)

A-INNATE IMMUNITY

Innate immunity involves many proteins in activation and development one of these is the Toll-like receptors (TLR), it is a family of 11 transmembrane receptor proteins, act by recognition of pathogen-associated molecular patterns (PAMPs). Coronavirus trigger release of many significant body cytokine such as IL-6, IL-1, TNF (P Conti *et al*.,

2020). Innate immunity is the first mechanism of body defense against viral infections where the virus is recognized by specific TLR. Both SARS-CoV and MERS viruses related to TLR3, which has a specific role in the development of an immune response against coronaviruses (Totura AL, *et al*., 2015). Dendritic cells, placental cells, and pancreatic cells are the most common body cells that highly expressed TLR3, its activation through TRIF (TIR-domain-containing adapter-inducing interferon-β) pathway, determines the activation of IRF3 (interferon regulatory factor 3) and NF-kB (Uematsu S and Akira S. 2007)

Activation of TRIF pathway result in increased production of type I interferons (IFN alpha and beta) and other key cytokines include those involved in adaptive immunity (e.g., IL-2 and IL-4), proinflammatory cytokines and interleukins (ILs) (e.g., interferon (IFN)-I, -II, and -III; IL-1, IL-6, and IL-17; and TNF-α); and anti-inflammatory cytokines (e.g., IL-10). (Totura AL, *et al*., 2015). Many research certified an abnormal high level of the following cytokines and chemokines in COVID-19 patients: IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, M-CSF, G-CSF, GM-CSF, IP-10, IFN-γ, MCP-1, MIP 1-α, hepatocyte growth factor (HGF), TNF-α, and vascular endothelial growth factor (VEGF) (Huang C, *et al*., 2020, Liu K., *et al*., 2020, Chen C., *et al*., 2020, Costela-Ruiz VJ, *et al*., 2020).

B- Adaptive immunity

The adaptive immune system defense against invasive microbes by the antigen-specific pathway, it consists of three major lymphocyte types: B cells (antibody producing cells), CD4+ T cells (helper T cells), and CD8+ T cells (cytotoxic, or killer, T cells), these elements can be important in protection against viral infections. Most of existed human

vaccines work on the basis of protective antibody responses, with neutralizing antibodies being the most common mechanism of action, expression of type I IFN (IFN-I) and other pro-inflammatory cytokines are the primary host reaction against virus entry, which are suppressed by SARS-CoV and MERS-CoV (Prompetchara, E., et al., 2020), also the inhibition of cellular components of host immunity has also been reported for SARS-CoV-2 and MERS-CoV infections. The latter led to the downregulation of gene expression related to antigen presentation (Menachery, et al, 2018) while increased levels of exhausted CD8+ T cells and loss of CD4+ T cell function were found in the peripheral blood of patients infected with SARS-CoV-2 (Zheng H. Y. et al., 2020)

So, to control and eliminate any infection and particularly the SARS-CoV-2, the induction of a balanced host immune response is crucial, employing adaptive and innate immune responses, as well as events mediated by the complement system. From one side, an uncontrolled immunity may result in damaging of lung parenchyma, functional impairment and reduced lung capacity (Kruse R. L., 2020). On the other side, immune insufficiency or misdirection may increase viral replication and cause tissue damage (Li G. *et al.*, 2020).

1.3.5-CYTOKINE STORM

As a response to infection cytokines release resulting in different symptoms ranging from mild to severe manifestations, its mild response manifestation includes mild flu-like symptoms as increased body temperature, myalgia, arthralgia, nausea, rash, and depression. At the same time of immune activation, human body activate compensatory-repair processes to restore deformed organ function. In 1993 Ferrara JL et al described for the first time the term “cytokine storm” as a graft- vs.-host

disease (Ferrara JL *et al*.,1993), since that time it extended to describe the similar sudden cytokine releases associated with autoimmune, sepsis, cancers, acute immunotherapy responses, and infectious diseases (Chousterman BG *et al*.,2017, Shimabukuro-Vornhagen A *et al*.,2018). A very high levels of cytokines (IFN, IL, chemokines, CSF, TNF, etc.) release occur as result of immune system over-activation in response to infection, drug, and/or some other stimuli, these high level causing severe inflammatory responses start locally and spread systemically via circulation, causing collateral damage in tissues. (Gupta KK *et al*., 2020).

A cytokine storm is nearly always pathogenic due to its harmful effects, however the local and systemic cytokine responses to infection are essential parts of the host's response to infection, when natural killer (NK) cells and macrophages produce these cytokines along with activation of T cells and humoral responses this can help resolve infection, accompanied by effector mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC). (Borrok MJ *et al*., 2015).

These immune responses are triggered to control the pathogen. Such as local cytokines, IFN-α/β and IL-1β, which secreted by epithelial cells can protect nearby cells by stimulating IFN-stimulated gene expression then leading to activating NK cells. This increment in NK cell's lytic potential and then secretion of IFN-γ.(Chatenoud L, *et a*l., 1989). In addition to that, activation of myeloid resident cells (macrophages) by IFN-γ amplifies subsequent TLR-mediated stimulation, involving release of high levels of TNF, IL-12, and IL-6, which, in turn, can further modulate NK cells.(Channappanavar R, *et a*l., 2017) With disease progression, an additional cytokine responses occur by T cell and antibody responses, resulting in massive or sustained antigen release and added TLR ligands from viral-induced cytotoxicity.(Li T, *et al*., 2006). During that the

Cytokine release syndrome are under the control of host-related factors of pathogen related factors such as anti-inflammatory responses or genetics factors or even reduced pathogen load, if this allowed to develop, it could result in tissue damage and organ failure. For example, IL-10 and IL-4 controls a negative feedback mechanism for cytokine release, dysregulation of this mechanism can result in cytokine release syndrome or cytokine storm, another evidence of that is using anti IL-6 (tocilizumab) in treatment of advanced cases can disrupt the formation of cytokine storm.(Tisoncik JR, *et al*., 2012, Cifaldi L, *et al*., 2015).

In severe-to-critical COVID-19 disease, a lethal cytokine storm can result in sharp lymphocytes and NK cell counts reduction and elevations in levels of D-dimer, C-reactive protein (CRP), ferritin, and procalcitonin.(Chen N, *et al*., 2020), diffuse alveolar damage characterized by hyaline membrane formation and infiltration of interstitial lymphocytes in pulmonary tissue, collateral tissue damage and organ failure.(Zhang Y, *et al*., 2004, Chien JY, *et al*., 2006,).

1.3.6- Symptoms of COVID-19 disease:

COVID-19 patients presented by one or more of the following systemic symptoms as fever, fatigue, myalgia, rigor, arthralgia, or as upper respiratory tract symptoms as sore throat, rhinorrhea, nasal congestion, hyposmia, hypogeusia, otalgia, or lower respiratory tract symptoms involve any cough weather productive or dry cough, dyspnea, chest pain, hemoptysis, wheeze, patients may have presented with gastrointestinal symptoms as diarrhea, nausea, vomiting, abdominal pain, central nervous system symptoms could be headache, ataxia, confusion. (Grant MC. *et al*., 2020)

COVID-19 Patients may experience a range of symptoms, from no symptoms to critical illness, in general, patients with SARS-CoV-2 infection can be classified into five groups according to the severity of illness, However, the criteria for each group may overlap or vary across clinical guidelines and clinical trials, and a patient's clinical status may change over time.

- Asymptomatic patients: Individuals who test positive RT-PCR but they have no symptoms.
- Mild Illness: Individuals who have saturation of oxygen (SpO2) ≥94% on room air and have any of the following symptoms (e.g., sore throat, fever, insomnia, cough, myalagia, headache, muscle ache, nausea, vomiting, diarrhea) but they do not have dyspnea, or abnormal chest imaging.
- Moderate Illness: Individuals complaining from lower respiratory disease by clinical assessment or imaging and who have saturation of oxygen (SpO2) ≥94% on room air.
- Severe Illness: Individuals with reduce oxygen saturation (SpO2 <94%) on room air, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) <300 mmHg, respiratory rate >30 breaths per minute, or lung infiltrates >50% by CT scan.
- Critical Illness: Individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction.

The risk factors for progression to severe COVID-19 are the older age (older than 65years), cardiovascular disease, diabetes, chronic obstructive lung disease, obesity, cancer, chronic kidney disease (Verity R, *et al*., 2020).

1.3.7- Complications of Coronavirus infection:

Many complications had been reported post-COVID-19 infection, according to Zhu J. et al, 2020 the most common are:

- Acute Respiratory Distress Syndrome (ARDS)
- Acute Respiratory Failure
- Pneumonia
- Secondary bacterial Infection
- Acute Liver Injury
- Acute Cardiac Injury
- Acute renal Injury
- Septic Shock
- Disseminated Intravascular Coagulation
- Venous clots
- Multisystem Inflammatory Syndrome in Children
- Chronic Fatigue
- Rhabdomyolysis

1.3.8-Lymphopenia:

Major causes of lymphopenia in COVID-19 patients are:

1- SARS-COV-2-infect lymphocytes and cause apoptosis, where it was clear that both MERS-CoV and SARS-CoV directly infect T cells and induce cell apoptosis (Chu H. *et al*, 2016)

2- The serum level of pro-inflammatory cytokines, such as TNF-α and IL-6, have been closely correlated with lymphopenia, the resultant

inflammatory cytokine storm is likely a key factor behind the observed lymphopenia (Mazzoni A. *et al*, 2020).

3- SARS-COV-2-mediated bone marrow impairment, where there were high expression of CXCL10 (IP-10) and CCL2 (MCP-1) which can bone marrow impairment (Cheung CY. *et al*, 2005).
4- Exhaustion of T cells in COVID-19 infection may result in lymphopenia. Increased cell surface expression of programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin domain 3 (Tim-3) in both CD4+ and CD8+ T cells may explain the cell exhaustion (Diao, *et al*, 2020).

Overall, lymphopenia and increased levels of certain pro-inflammatory cytokines such as IL-1, IL-2R, IL-6, IL-12, IFN-γ, and TNF-α have been closely associated with the disease severity (Tavakolpour S. et al, 2020), soluble FasL has been proved to be correlated with lymphopenia in many disease such as infection with malaria (Kern P. *et al*, 2000), another biomarker of CD8 T cell exhaustion is decrease in expression of some T cell activating immune marker such as CD107a and IF-Ɣ (Diao, *et al*, 2020).

1.3.9- IL-2 receptor (IL-2R)

The survival and immune function of T-cell are depending on the availability of essential cytokines. One of these is Interleukin-2 (IL-2) which is α-helical protein measure 15.5 kDa, is able to culture T cells for long term in vitro, it poses significant effects on T lymphocytes proliferation, survival and ability to activation-induced cell death (AICD), also on their differentiation, production of cytokine, and immune tolerance

(Malek, T. R., *et al.*, 2008, Dooms, H. and Abbas, A. K., 2006 and Refaeli, Y., *et al.*, 1998).

IL-2 receptor (IL-2R) has three subunits (Fig 1.4), CD25 is α-subunit, CD122 is β-subunit and CD132 which is the γ-subunit. cytoplasmic tails of CD122 and CD132 are responsible exclusively about IL-2 signaling, while CD25 is not involved because it has a short cytoplasmic tail. CD25 has important role due to its highest affinity for IL-2 among the individual subunits and it play as an affinity converter. IL-2 signaling may occur in the absence of CD25 at high concentrations by the effects of CD122 and CD132, which form the intermediate-affinity IL-2R. However, all three subunits are required to respond to low concentrations of IL-2 this occur by forming the high-affinity IL-2 receptor (Malek, T. R., *et al.*, 2008).

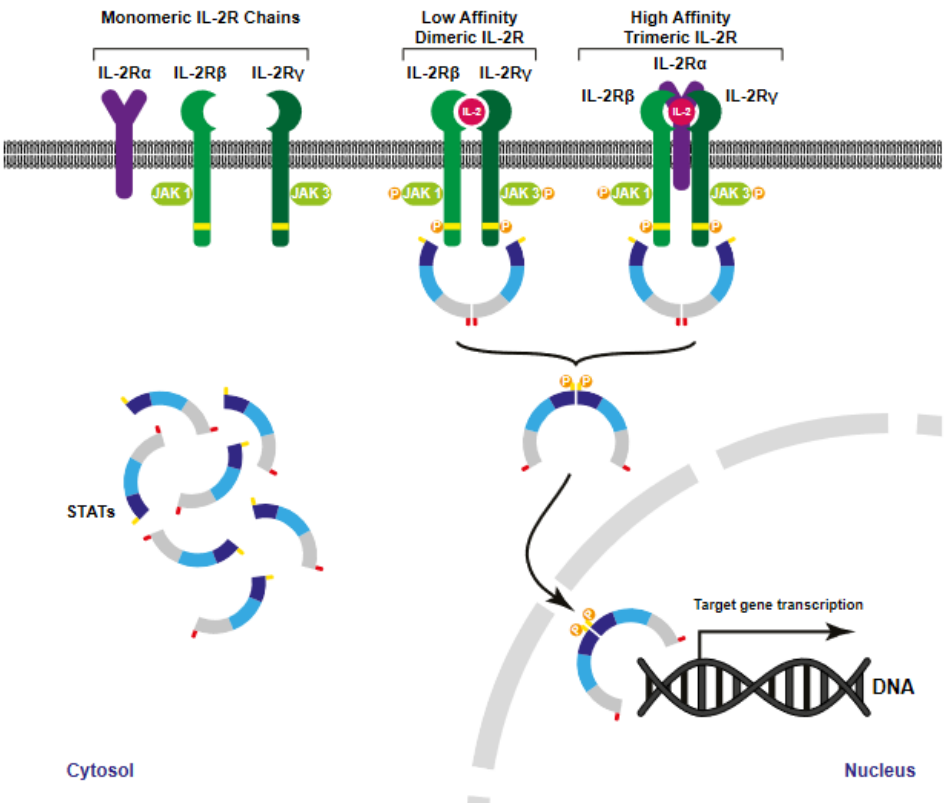

Fig 1.4 Schematic representation of the IL-2 receptor chains (IL-2Rα, IL-2Rβ, and IL-2Rγ) (Malek, T. R., *et al*., 2008)..

Many important cytokine serum levels increased in COVID-19, one of these is the soluble IL-2R, its increment seem to be correlated with the severity of the disease. Interleukin-2 exerts both stimulatory and regulatory immune functions via secretion by activated T-helper lymphocytes, its activity mediated by binding to IL-2R. A few immune cells are expressing the IL-2R at their surface as activated T lymphocytes, natural killer cells, activated B cells and monocytes. The soluble form of the IL-2R seems to be produced by proteolytic cleavage of IL-2Rα, and the release of soluble IL-2R into the circulation is proportional to its membrane expression, so the high blood levels of soluble IL-2R can reflect the immune cell activation in different inflammatory and malignant diseases. The expression of IL-2R on the surface of pulmonary endothelial cell and its binding with IL-2 has been also demonstrated thus induce a pulmonary-edema. Thus, blood level IL-2R may increase due to induction by the expression of endothelial cells, IL-2R and IL-2 response may be a part of COVID-19 pathophysiology through the direct action on the endothelium. (Lodigiani C. *et al*, 2020 and Maeda M. *et al,* 2020)

1.3.10- CD107a :

Natural killer (NK) cells are a subgroup of granular lymphocytes that has no T-cell receptor (CD3) but express CD56 surface receptor (Cooper M.A., *et al*., 2001). It serves important role in eliminating a number of viral infections and in clearance of some tumor cell (Yokoyama, W.M., Scalzo, A.A., 2002). These cells are essential part of the innate immune response because they are able to lyse tumor cells and virally infected cells without prior antigen sensitization. They act as important connection between the

innate and adaptive immune response as they secrete large amounts of cytokines and chemokines that can determine the adaptive immune response. Generally, these cells present in peripheral circulation, attracted to tissue parenchyma by some sort of chemokines secreted by the infected or malignant cells (Moretta. *et al.,* 2002). These cells contain vesicles which has high concentrations of cytolytic granules in their cytoplasm, (Cooper., *et al.,* 2001). Fig 1.5 exhibiting principle of CD107a degranulation. The vesicles contain many cytolytic proteins such as perforin and granzyme that are uniquely designed to induce target cells apoptosis when release (Burkhardt *et al.,* 1989; Tschopp, J., Nabholz, M., 1990). As a result of NK cell activation, they follow the integration of complex signals from both activating and inhibitory receptors on the surface of these cells, they rapidly release these granules at the immunological synapse inducing target cell apoptosis (Cooper M.A., *et al*., 2001; Moretta A. *et al.*, 2002; Cerwenka, A., and Lanier, L.L., 2001). The membrane of these released cytolytic granules is lined by a lysosomal-associated membrane protein-1 (LAMP-1 or CD107a or CD107a antigen, or LGP120, or LAMPA) (Winchester, B.G., 2001; Peters P.J., *et al*., 1991).

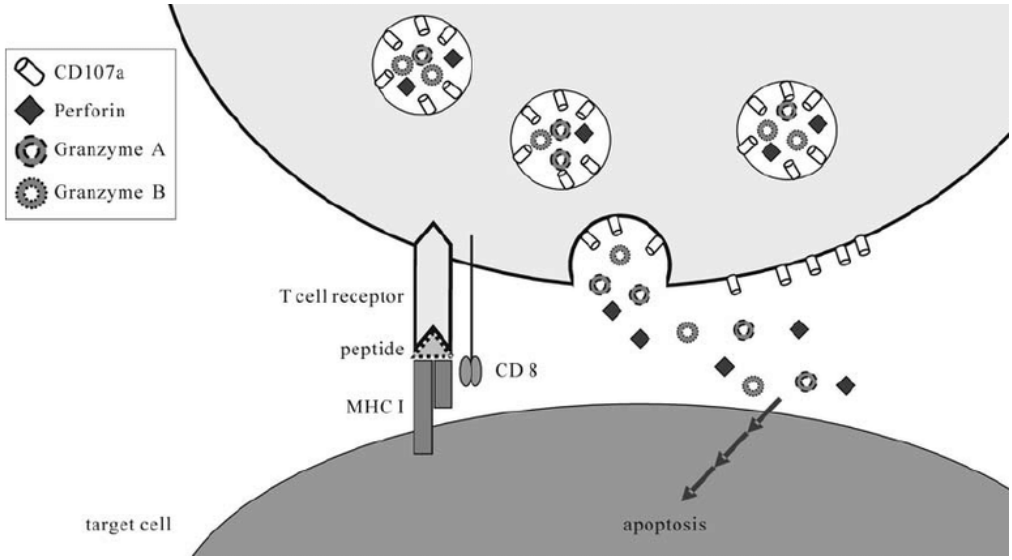

Fig 1.5 The principle of CD107a degranulation assay (Moretta A. *et al*., 2002).

CD107a is a highly glycosylated single pass type 1 membrane protein belonging to the lysosome- associated membrane protein family, it represents approximately 50% of the proteins in the lysosomal membrane (Fukuda, M., 1991). LAMP family members have short cytosolic tails, interact with trans Golgi mediators that are involved in sorting and targeting proteins to the lysosomal pathway (Winchester, B.G., 2001). On the luminal side, the highly glycosylated portion of the vesicle's molecule protect the cellular membrane from the effects of the lytic enzymes contained in the granules, subsequently, they protect the extracellular membrane of the effector cell following degranulation (Fukuda M, 1991). CD107a expression on the cell surface has been described as a marker of cytotoxic CD8+ T-cell degranulation (Betts, M.R., *et al.*, 2003). COVID-19 patients showed decreased percentages of CD107a compared with healthy persons, suggesting the functional exhaustion of CD8+ T-cell in COVID-19 patients (Zheng M, *et al*., 2020)

1.3.11- FasL:

First apoptosis signal Ligand (FasL) is a 40 kDa type II membrane protein, also known as CD178, CD95L, or TNFSF6, a member of the tumor necrosis factor (TNF)-receptor. its primary function is the induction of apoptosis triggering by binding to FAS, the ligation of Fas with FasL results in the activation of a caspase cascade that initiates cell death (Ashkenazi A, and Dixit VM. 1999).

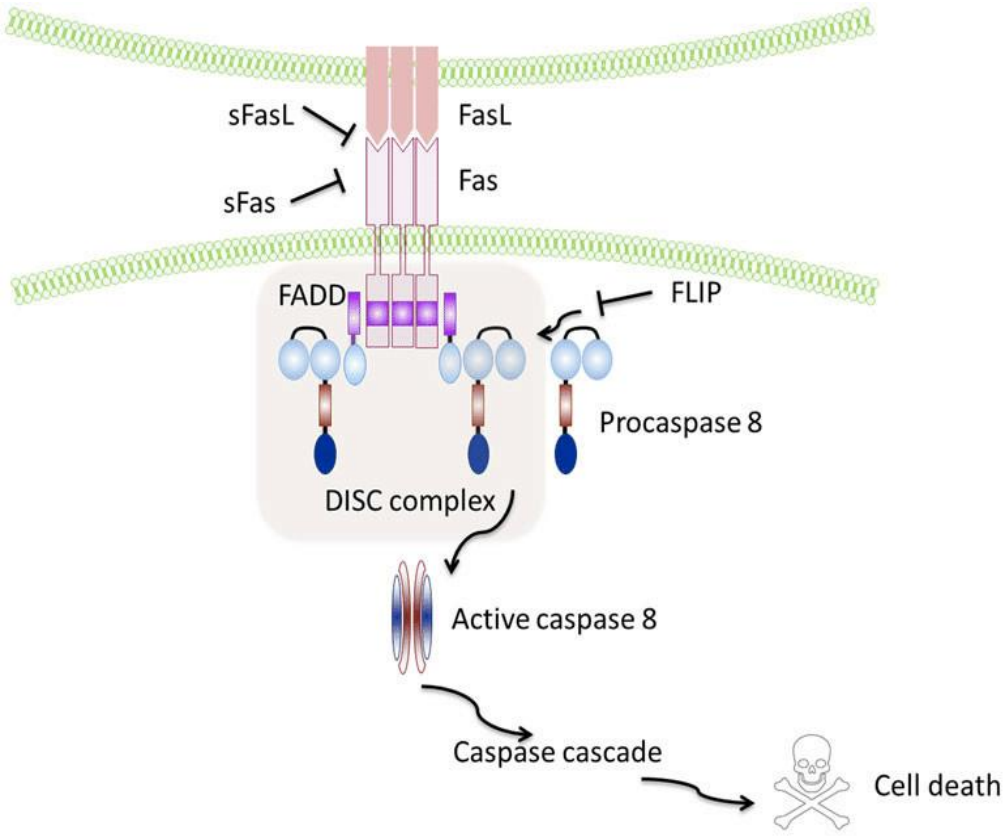

Fig 1.6 Schematic representation of the Fas-FasL pathway (Chervonsky AV., 1999).

The Fas/FasL signaling pathway is essential for apoptosis to maintain immune system homeostasis as in Fig 1.6. Along the usual physiological immune response, apoptosis has an essential role to delete potentially pathogenic autoreactive lymphocytes from the peripheral circulation and tissue parenchyma, minimizing tissue damage that inevitably caused by immune responses (Chervonsky AV., 1999). The re-stimulation of T cell receptor (TCR) that is previously activated and expanded in the absence of appropriate co-stimulation will induces activation-induced cell death (AICD) (Mercep M., 1989), serves as a mechanism of eliminating the over-activated T cells, such as autoreactive T cells in autoimmune diseases. Fas/FasL-mediated apoptosis can control the unlimited expansion of CD8 and CD4 cells (Shi YF. *et al.*, 1989).

Throughout COVID-19 disease progression, FasL causes severe immune system dysregulation. This results in two major pathological problems – reduced lymphocyte counts (lymphopenia) and inflammatory apoptosis, resulting in pneumonia and acute respiratory distress syndrome (ARDS), respectively (Girija A. S. S. *et al*., 2020)

1.3.12- Diagnosis of COVID-19 disease:

Final diagnosis can be achieved by clinical assessment in correlation with laboratory tests, these tests may be direct to detect the causative agent or indirect by measuring systemic response of the body to the infection, direct measure by detecting genetic material of SARS-CoV-2, The most common used procedure for that genetic material identification of SARS-CoV-2 is real-time polymerase chain reaction (RT-PCR), Currently there are two types of COVID-19 tests used in all over the world. The first type use molecular assays for diagnose SARS-CoV-2 viral RNA by using a polymerase chain reaction (PCR)-based techniques or nucleic acid hybridization-related strategies. The other type of assay involve both serological and immunological assays, which rely on detecting antibodies produced by the patient as a result for the viral infection or on detection of antigenic proteins in infected patients. Both types of tests sharing different purposes in management of the COVID-19 pandemic. Both types for SARS-CoV-2 viral RNA identifies viral infected patients during the acute phase of the disease, also the serological testing subsequently identifies patients who have produced antibodies to the virus and could be potential plasma donors. It also serves the ability to follow contacts persons and monitor the immune status of peoples over time. (Jones H., 2020).

Molecular assays for detecting viral nucleic acids

SARS-CoV-2 is a single-stranded, positive-sense RNA virus, its entire genetic sequence was uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) platform on January 10, 2020, since that, many diagnostic kits were available (Tan R., 2020)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is considered to be the gold standard for identification of SARS-CoV-2 virus, it depends on the ability of amplification of a scanty amount of viral genetic material in a sample, by using upper respiratory system samples from suspected patients using oral and nasal swabs. Other studies use samples from serum, stool, or ocular secretions. (Xia J. *et al*., 2020: Kujawski S. A. *et al*., 2020).

RT-PCR test was done by either one (single step) or two (multiple steps) procedure. The one (single) step real-time RT-PCR utilize a single tube containing the necessary specimen to produce the full RT-PCR reaction while the other type real-time RT-PCR use multiple tubes to run the amplification and reverse transcription reactions, this type has a higher sensitivity and greater flexibility than the first process. It needs less material and allows for the ability to stock cDNA for quantification of multiple targets. (Wong M. L.; Medrano J. F. 2005). Because of faster time requirement, limited sample handling, reduced bench time, and cross-contamination between the RT and real-time PCR steps, the single -step procedure is preferred approach, up to the time of writing these wards most of molecular diagnostic assay have used the real-time RT-PCR procedure targeting different SARS-CoV-2 genom, including the spike (S) protein, ORF1b or ORF8 regions, and the nucleocapsid (N), RNA-dependent RNA

polymerase (RdRP), or envelope (E) genes. (Coronavirus Test Tracker, 2020) RT-PCR. Among symptomatic persons, sensitivity and specificity were 72.1% and 98.7%, respectively.(Brihn A, *et al* 2021)

Supportive tests

Other tests weather laboratory or imaging tests could demonstrate the manifestations of COVID-19 disease, its complications, and may detect the risk factors for complications.

Laboratory tests

Starting with Complete Blood Count (CBC) where there usually there is reduces lymphocyte count (lymphopenia)(less than $1.3x10^9$/L), reduced eosinophilic count (eosinopenia), and the ratio of neutrophil/lymphocyte count ≥ 3.13, these results related to disease severity and bad prognosis. Reduced platelets count (thrombocytopenia) is related to cardiovascular complications and also bad prognosis (Vabret N., *et al*., 2020). and biochemical tests, CRP, LDH, serum Ferritin and D dimers and others. Lymphopenia caused by the multifactorial mechanism that includes the cytopathic effect of the virus resulting in apoptosis, and bone marrow suppression by inflammatory cytokines (Azkur A.K., *et al*,. 2020).

Imaging tests

Chest X-Ray and computed tomography scan are of great value in detecting diseased lung, but they cannot infer the causative agent, infection with influenza or mycoplasma infections can yields same image, lung interstitial disease is another radiological differential diagnosis (Foust Am, *et al*., 2020). The CT scan is more sensitive than chest X-ray, bilateral consolidations accompanied by ground glass opacities are the most common findings in COVID19 patients (Sultan OM, *et al.*, 2020).

CT scan findings:

CT scans considered a diagnostic tool when RT-PCR tests are not available and when there is clinical suspicion with negative RT-PCR test, recent international radiological guidelines do not recommend CT scan for all patients and consider the RT-PCR test is specific for disease diagnosis these including the British Society of Thoracic Imaging (BSTI), the Centers for Disease Control (CDC) and the American College of Radiology (ACR) (Nair A. *et al*., 2020, Centers for Disease Control and Prevention, 2019 and Recommendations for the use of chest radiography and computed tomography (CT) for suspected COVID-19 infection. 2020), the sensitivity of CT in COVID patient reaching to 98% (Ai, *et al*, 2019), while CXR sensitivity was increases from 55% at ≤ 2 days to 79% at > 11 days after symptom onset.(Jacobi A, *et al*, 2020).

Ground glass opacities (GGO) definition is an areas of hazy appearance by radiological term mean increase in lung density, mostly due to partial filling of airspaces with fluid (pus), bronchial and vascular markings do not obscure but still seen in controversy to consolidation where it is pulmonary opacity that obscure underlying vessels and bronchial walls (Z. Ye, *et al*., 2020). Many previous studies identified GGO and consolidations as the earliest and predominant CT findings in COVID-19 patients (R. Lu, *et al*., 2019). Early in the course of the disease the GGO was predominantly unilateral, multifocal, and subpleural in distribution, then the opacities diffuse bilaterally and became more confluent with the end of first week after the onset of symptoms, some GGO changes to consolidation opacities within 1-3 weeks (H. Shi, *et al.*, 2020). Consolidation is the second predominant feature in COVID-19 patients, its presence may indicate disease severity (Z. Ye, *et al*., 2020). Another common manifestation is the crazy-paving pattern where a large

GGO contain septal and interlobular thickening, adjacent foci of high and low attenuation, usually its presence associated with reticular fibrotic changes (S. L. Aquino, *et al.*, 1998 and S. E. Rossi, *et al.*, 2003). Other CT findings are tree in bud opacities, pleural effusions, pleural thickening and lymphadenopathy (Z. Cheng *et al*., 2020), Figure 1.7 and 1.8 exhibiting the most common CT findings.

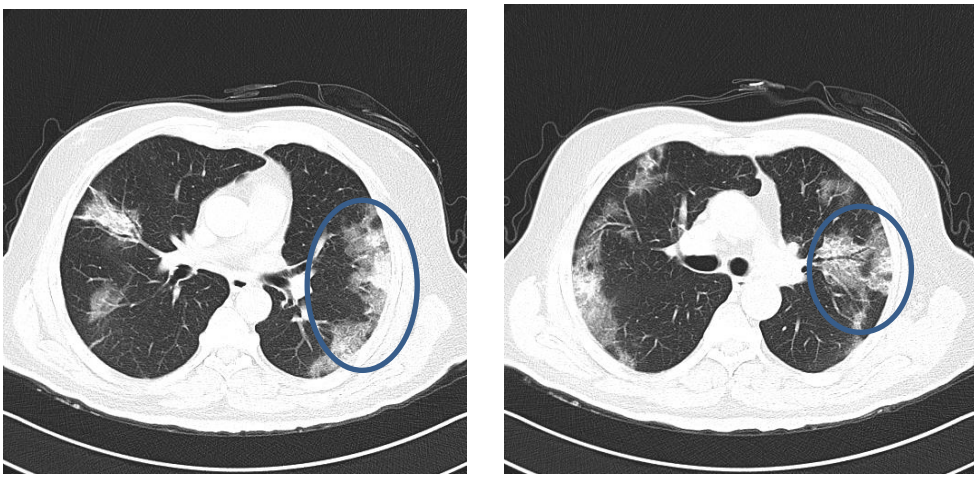

Fig-1.7: CT scan images of 55-year-old male with positive RT-PCR. Axial thin-section CT scan shows bilateral subpleural patchy ground-glass pulmonary opacity. (Z. Cheng et al., 2020)

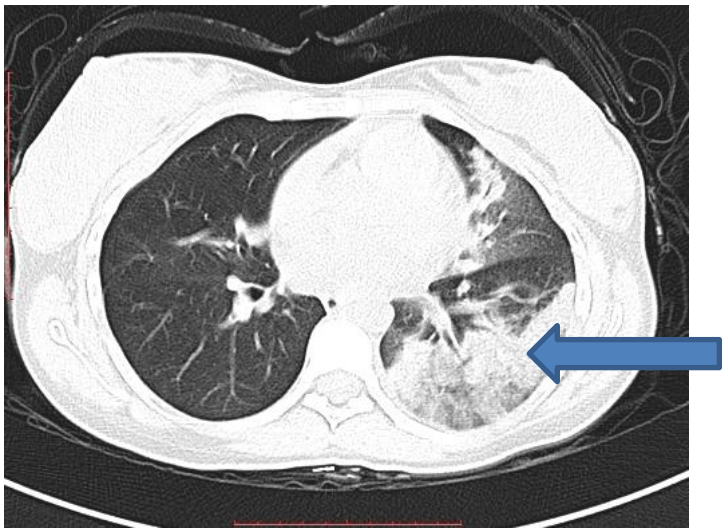

Fig-1.8: CT scan images of 38-year-old female with positive RT-PCR. Axial thin-section unenhanced CT scan shows left lower lobe large patchy pulmonary consolidation opacity. (Z. Cheng et al., 2020)

1.3.13- Treatment:

Yet there is no proven effective cure for COVID-19 infection, the most common treatment for COVID-19 patients is supportive treatment. Although multiple anti-viral drugs, including remdesivir and lopinavir plus ritonavir, were used in clinical trials (Chen N. *et al*., 2020). Both efficacy and even safety of these drugs still under clinical evaluation and are unclear yet. All immune-mediated lung injury and acute respiratory distress syndrome (ARDS) are associated with adverse outcomes in patients with COVID-19 (Xu Z. *et al*., 2020). Some centers used antimalarial drug in treatment of COVID in early period of the pandemic (Singh B. *et al* 2020)

1.3.14- Prevention:

For controlling any contagious diseases like COVID-19, multiple essential steps should be stated starting with education of people, isolation of infected persons, prevention of disease spread, controlling the transmission, vaccinations and finally treatment of affected persons, each step is critical as stated by the WHO (WHO Director-General's opening remarks at the media briefing on COVID-19, 2020). Minimizing the transmission of COVID19 infection may be possible by following recommendations:

Home isolate, by staying away from any immediate contact with any healthy or diseased individual, in light of the fact that even healthy individual could be an asymptomatic patient, keeping social removing rules by staying away from crowded public places and keeping up with somewhere around two meters of distance between every individual, avoiding shaking hands and kissing when greeting others; maintaining the personal hygiene by frequent hands washing for at least 20 seconds using

soap and water or any hand sanitizer with at least 70% alcohol, especially after touching common surface areas, using the bathroom, or shaking hands, avoiding touching eyes, nose, and mouth with unwashed hands; and disinfecting surfaces using household sprays or wipes. Using a medical mask (especially N95) or a respirator (especially FFP3) can effectively minimize disease spread also used masks should be discarded with special care. (Organization WH. - 11 March 2020).

For those health workers, the use of medical face shields and wearing protective suits are recommended. The idea of wearing gloves is not recommended for general public and is not an adequate protection against COVID-19 comparing with frequent hand washing, because gloves can easily be contaminated, but it is highly recommended for medical workers (Desai A, *et al*., 2020).

In terms of vaccines, there are a large number of vaccination strategies against SARS-CoV, MERS-CoV being tested in animals, including a live-attenuated virus, viral vectors, inactivated virus, subunit vaccines, recombinant DNA, and proteins vaccines. Although, until now, there has not been any approved vaccine against SARS-CoV-2, several clinical trials have been launched for testing the effects of various vaccines against SARS-CoV-2. (Desai A, *et al*., 2020).

Chapter Two:

Methodology

Chapter Two: Methodology

2.1. The study design:

This is cross sectional study a retrospective study. Consecutive patients who are already or newly diagnosed to have COVID-19 infection matched for age and gender.

2.2. Study group selection:

A random selection of all enrolled candidates (mild and sever COVID-19 patient's groups) was carried out for the period from August 14, 2020 to December 23, 2020 in Al-Imam Al-Hussein-medical city in Kerbala, Iraq.the center dealing with COVID-19 patients as out-patients clinics and inpatients involving the intensive care and respiratory care units.

2.2.1. Patients sampling:

A total of 88 patients who were attending respiratory care unite in Al-Imam Al-Hussein-medical city, as in- and out-patients were enrolled in the study. were classified as :

Severe cases: (1) symptoms of lower respiratory infection with reduce oxygen saturation (SpO2 <94%) (2) patient with cytokine release syndrome (one or more of the following: a- S.ferritin >300ng/mL with doubling within 24hr, b- S.ferritin >600ng/mL at presentation, c-LDH >250U/L, d-elevated D-Dimer>500ng/mL, e-S.IL6 ≥ three times upper normal limit.

Mild cases are those who have upper respiratory symptoms without any risk factors, no sign of pneumonia in imaging. who have saturation of oxygen (SpO2) ≥94% on room air

The age groups ranging between 18 to 75 years. The patient's genders were 41 females and 47 males.

2.2.2. Clinical data:

Information including age,gender, severity of disease (mild and sever cases), WBC counts, CRP, LDH, serum Ferritin and D dimers and more others collected directly from patients or their family members via a questionnaire sheets.

2.2.3. **Inclusion criteria:**

All patients were included had clinical manifestations were highly suggestive for COVID-19. Patients diagnosed to have COVID-19 by Positive PCR test and physician and supported by other blood and biochemical tests.

2.2.4. **Exclusion criteria**:

1. All patient with negative RT-PCR.
2. Patients with a known oncological disease.
3. Patients with a known immunological disorder (autoimmune disease, immune deficiency).
4. Patients admistration steroid or immune-modulating treatment.

2.3. Ethical issues: Ethically, the selection of the study groups and data collection were accomplished after we have been taken the approval from University of Karbala/ College of Medicine, Department of Medical Microbiology. and approval from Karbala Health Directorate to work in its hospitals and a practical supervisor has been set in hospital to follow the workflow. In addition, the permission and agreement of laboratory department of each hospital to work in the corresponding immunity unit of lab also have been taken. Regarding blood samples, we obtained the permission from patients themselves or their relatives or use the collected blood from patients to perform serological or chemical tests, either as part of their follow-up regimen (admitted patient) or to screen or confirm

diagnosis of COVID-19 the permission taken only to use the blood samples of both candidates (mild and sever) were informed that these tests are for research and are not diagnostic, and interestingly, all participants were cooperative and good respondents. Permission of patients to publish the study.

2.4. Blood samples processing: The 5ml freshly aspirated venous blood samples using disposable syringes were collected sequentially from patients, at the aspiration unit in the lab of the hospital were the patients attended. The blood will be withdrawn from each patient will be divided in to two parts: **1st part** in a gel tubes and submitted to a barcode to be labeled and sent to biochemistry unit in the lab, where they centrifuged at 3000 revolutions per minute (rpm) for 10 minutes to separate serum. Later, from each sample, we took 1/2 to 1 ml of serum in a plane tube, labeled, and kept in the deep freeze (for immunological markers IL2R, CD107a, Fasl), while the original tubes kept (Cobas8000,ROCH) auto-analysis to pass in the daily run of (CRP, LDH, S. ferritin, d-dimer) that done by biochemistry unit staff of the mentioned labs.**2nd part** :in EDTA tubes for hematological test(CBC,PT,PTT) that done by hematology analyzer (Sysmex XN 350,Japan).

2.5. Laboratory instruments used in the study: Down, a list of all laboratory instruments and equipment used during the study:

Table (2.1): Laboratory instruments and equipment

Instrument	Manufacturer origin
Disposable syringe (5ml)	Medi, China
Gloves	Malaysia
Gel and plane test tubes	Jordan
Cylinder: : 1000ml MBL, Boro	Germany

100ml Hlalab	
Multi-channel 100 M pipette	Slamed,Germany
Micropipettes 100 M, 1000M	Slamed, Germany
Plastic rack	China
Centrifuge	Kokusan, Japan
Distillator Nuve,	Turkey
Cold incubator	Memmert, Germany
Deep freezer	ARCTIKO, Poland
ELISA reader and printer	BioTek, USA
ELISA automated washer	BioTek, USA
Vortex	England .

2.6-The laboratory technique: The biochemical and hematological tests which currently used as a second step in the diagnosis and follow-up for Covid-19 patient, and the study marker (CD107a, IL2Ra, FasL), all are measured by sandwich ELISA (Enzyme-Linked Immunosorbent Assay) technique.

2.7- Materials (kits) used in the study: Three different kits had been used in this study.

2.7.1- ILR2α ELISA kit: this ELISA kit from MYBIOSOURCE (MBS2703776,USA) was used for serum IL2Rα level measurement. The kits contain the basic components required for the development of sandwich ELISAs to measure natural and recombinant human interleukin receptor (IL2Rα) this test are used for in vitro quantitative measurement of ILR2α in serum, cell culture supernatants, plasma samples and other biological fluids. (MYOBIOSOURCE ELISA). All the kit contents are illustrated in the table (2.2) below.

Table (2.2): The reagents of IL2Rα ELISA kit ((MYOBIOSOURCE ELISA) Reagents

CONTAINT	**AMOUNT**
Assay plate ready to use ,	96-well strip plate
Human IL2Ra Standard	2 vials
Detection Reagent A	1×120µL
Detection Reagent B	1×120µL
TMB Substrate(Tetra-methyl benzidine)	1×9mL
Wash Buffer (30×concentrate)	1×20mL
Plate Sealer for 96 wells	4
Standard Diluent	1×20mL
Assay Diluent A	1×12mL
Assay Diluent B	1×12mL
Stop solution	1×6mL
Instruction Manual	1

2.7.2- Human CD107a(LAMP1) ELISA KIT: this ELISA kit from MYBIOSOURCE (MBS76060776,USA) was used for serum CD107a level measurement. The kits contain the basic components required for the development of sandwich ELISAs to measure natural and recombinant human CD107a(LAMP1) this test are used for in vitro quantitative measurement of CD107a in serum, cell culture supernatants, plasma samples and other biological fluids. (MYOBIOSOURCE ELISA). All the kit contents are illustrated in the table (2.3) below.

Table (2.3): The reagents of CD107a ELISA kit ((MYOBIOSOURCE ELISA) Reagents

CONTENT	AMOUNT	**storage**
Quantity Assay plate	8*12 (96-well strip plate)	2-8℃/-20℃
Lyophilized standard	2 vials	2-8℃/-20℃
Dilution for sample andstandard	20ml	2-8℃/-20℃
Antibody labeled with Biotin	120ul	2-8℃/-20℃
Dilution Buffer for Antibody	10ml	2-8℃/-20℃
conjugate HRP-Sreptavidin	120ml	2-8℃/-20℃
dilution Buffer for Conjugate	10ml	2-8℃/-20℃
Substrate	10ml	2-8℃/-20℃
Stop Solution	10ml	2-8℃/-20℃
Wash Buffer(25x)	30ml	2-8℃/-20℃
Sealer Plate	5	

2.7.3 Human Factor-Related Apoptosis ligand (FASL): This ELISA kit from COSABIO (Lot:Y08225091,CHINA) This test for the quantitative determination of human factor-related Apoptosis ligand (FASL) Concentration in serum, plasma, cell culture supernates. The kits contain the basic components required for the development of sandwich ELISAs to measure natural and recombinant human FASL. All the kit contents are illustrated in the table (2.4) below.

Table (2.4): The reagents of FASL ELISA kit ((COSABIO ELISA) Reagents.

Reagents	**Quantity**
Assay plate	1(96 wells)
Standard(freeze dried)	2 vial
Biotin-labeled Antibody(100x concentrate)	120ul
Biotin-labeled Antibody Dilution Buffer	15ml
HRP-avidin (100x concentrate)	120ul
HRP-avidin Dilution Buffer	15ml
Sample Dilution	50ml
TMB Substrate	10ml
Stop Solution	10ml
Wash Buffer(25x)	20ml
Plate Sealer	5pieces
Instruction manual	1 copy

detection range of each of the markers is demonstrated in the table (2.5) below.

Table (2.5): The detection ranges of serum CD107a, FASL and IL2Ra levels

The marker	Minimum detectable dose	Detection range
ILR2α	Less than 5.8pg/ml	15.6-1,000pg/ml
CD107a	Less than 0.156ng/ml	0.156-10ng/ml
FASL	7.81pg/ml	31.25-2000pg/ml

2.8-The method (procedure): The serum CD107a, FASL and IL2Ra levels measurements of the total patient's sera carried out concomitantly in two separated runs, were tested at the Immunity unit of Al-Imam Al-Hussain medical city lab. The first run in 23/12/2020, while the second run accomplished at the same lab in 24/12/2020.The same instructions of an individual marker were referred for both runs.

2.8.1-The procedure for the immunological markers measurement:

1. All kits should be stored at 2-8C and to be used within the expiration date of each kit.
2. It is important to brought all reagents to room temperature (18-25C) before use for 30 minutes.
3. The steps and reagents of each ELISA assay may vary, and it is better to used the test specific information for the assay being worked with.
4. Distilled water (DW) from Distillates in the biochemistry unit of the lab, putted in a graduated glass container (cylinder/1000ml), to be used for preparation of reagents.
5. By used PH indicator, we measured the PH of the DW and was acidic (5.7).
6. Be sure that pipettes used ware calibrated.
7. Used graduated glass containers to prepare the reagents.
8. patients and their samples passed in the run were listed.
9. The required information from both kits admitted into the ELISA reader, to facilitate the OD measurement of each individual marker.
10. Early in the morning of the work day, all samples to be tested were brought out the deep freeze and allowed to thaw, also the kit brought out the refrigerator to reach room temperature before use.

11. Labeling of test tubes and containers of each marker separately was carried out.

2.8.1.1- Serum FasL level measurement: We strictly refer to the Assay Layout Sheet (User Manual; COSABIO) instruction:

1-Principle: This assay was sandwich enzyme immunoassay quantitative technique. The microplat has been pre-coated with Antibody specific for FasL.

Pipetted the Standards and samples into the wells and the immobilized antibody will been bound by present FasL. any unbound substances will be removed, a specific conjugated with labeled biotin-antibody specific for FasL is added to the wells. Then the well washed.after that conjugated labeled with avidin-horseradish peroxidase (HRP) is added to the wells. After a wash any unbound avidin-enzyme reagent will be remove, and then color develops , when substrate solution was added to the wells and stopped The color development and the intensity of the color is measured. color in proportion to the amount of FasL bound in the initial step.

2-Reagents preparation for FasL COSABIO kit: According to instructions of COSABIO User Manual, we prepared the reagents:

Biotin-antibody (1×): before opening Centrifuge the vial. It need 100-fold dilution. A suggested 100-fold dilution was added 10 µl of Biotin-antibody to 990 µl of Biotin-antibody Diluent.

HRP-avidin (1×): before opening Centrifuge the vial. a 100- fold dilution It requires. A suggested 100-fold dilution is 10µl of HRP-avidin was added to 990 µl of diluent of HRP- avidin.

Buffer for wash (1×): Mix gently to dissolve any crystals if present. Diluted 20 ml of Wash Buffer concentrate (25×) into 480ml of DW to prepare 500 ml of Wash Buffer (1×).

Again, PH indicator used and the Wash Buffer was neutral (PH=7.2) and kept in the refrigerator.

Standard: the standard vial centrifuge at 6000-10000pm for 30s. the standard was Reconstituted with 1.0 ml of ready to use Sample Diluent (SD) (don't use other diluent). in a plane tube, to get a stock solution of 2000pg/ml(S7) which serves as the high standard and allow it to sit for a minimum of 15 minutes with gentle agitation prior to making dilution.

3-Assay procedure: a-We preparing standards by taking seven plane tubes labeled sequentially (S7-S0), as shown in the table (2-6) below.

Table (2-6) FASL standards arrangement in the plate

Tube	S7	S6	S5	S4	S3	S2	S1	S0
Pg/ml	2000	1000	500	250	125	62.5	31.25	0

a- 250µl of SD were pipetted in the tubes from S6 to S0, and the stock Solution (S7)

used to produce a 2-fold dilution series by pipetting a 250µl of S7 into S6, then a 250µl of S6 into S5 and repeated sequentially to S1. The S0 contain only Sample Diluent SD and serve as Standard zero (0 pg/ml).

b- The patient's samples sequentially arranged in a plastic rack according to their name's list.

c-The plastic micro titer plate (96 wells) was unsealed from the pouch, placed on the working bench to start addition using a multi-channel pipette graduated to 100µl.

d- A100µl of standard started from S0 to S7 in wells, and 100µl of undiluted samples were added to the remaining 88wells, covered with adhesive strip, and incubated for 2 hours at 37℃.

*Note: Changing of pipette tips between additions of each standard and samples

* During incubation period, all other reagents of the kit prepared as mentioned above. *After incubation, only removing of the liquid of each

well was carried out without washing. **e-**A100µl of Biotin-antibody (1x) added to each well, covered with a new adhesive strip, and incubated for 1 hour at 37℃.

* During incubation period, priming of the ELISA automated washer to FASL wash buffer done, and a three-wash program chosen.

*Aspiration of liquid and washing for 3-times carried out, filling each well with 200µl of wash buffer per wash cycle.

*Complete removal of any remaining wash buffer ensured by inverting the plate and blotting against clean paper towels.

f-A 100µl of HRP-avidin (1x) was added to each well, covered with a new adhesive strip, and incubated for 1 hour at 37℃.

Aspiration of liquid and washing for 5-times carried out, and complete removal of any remaining wash buffer ensured by inverting the plate and blotting against clean paper towels. **g-**Addition of 90Ml of TMB Substrate to each well, incubated for 15-30 minutes at 37C, and protected from light, with observation once every 10 minutes for change of color.

h- 50µl of Stop Solution was added to each well and gentle tapping to ensure thorough mixing done.

i-Determination of the optical density (OD) of each well within 5 minutes, using a reader of microplate set to 450 nm.

j- Results Calculation: the duplicate readings for each standard and sample Average will be taken and subtract the zero standard OD average.Astandard curve will be plotted by the mean absorbance of each standard on the x-axis and the concentration on the y-axis and draw a best fit curve on the graph through the points. The samples used were undiluted, so no need to multiply the concentration from the standard curve by dilution factor.

2.8.1.2- Serum IL2Rα level measurement: The IL2Rα ELISA kit (MYOBIOSOURCE ELIS) this kit has provided with pre-coated microplate with an antibody specific to IL2Rα.Then, added Avidin conjugated to Horseradish Peroxidase (HRP) to each microplate well and incubated. Next, added TMP Substrate (Tetra-methyl benzidine) solution only those well contain IL2Rα, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color .The reaction(enzyme-substrate) was terminated by addition of sulphuric acid solution and measured spectrophotometrically at wavelength of 450±10nm.the IL2Rα concentration was determined by comparing the O.D(optical density)of the samples to the standared curve.

2. Reagents preparation for IL2Rα: Reagents preparation was accomplished according to instructions of (MYOBIOSOURCE) User Manual:

Standard: The Standard reconstituted with 1.0ml of standard diluent**,** kept at room temperature for 10 minutes, gently shake (not to foam).the concentration of the standard in the stock solution is 1000pg/ml.and then 7 tubes will be prepare which containing 0.5ml of standard diluent and according the table (2-7) below ,produce double dilution series .

Table (2-7) IL2Rα standards arrangement in the plate.

Tube	1	2	3	4	5	6	7	8
Pg/ml	1000	500	250	125	62.5	31.2	15.6	0

Before the next transfer mix each tube thoroughly and the last tubes with standard diluent is the blank as 0pg/ml.

Detection Reagent A and Detection Reagent B Centrifuge the stock detection A and B before use dilute them to the working concentration 100-fold with Assay diluent A and B respectively .

-Wash Buffer (1×): Mixed gently to dissolved any crystals if present. to prepare 600 ml of Wash Buffer (1×). Added 20 ml of Wash Buffer concentrate (30×) into 580ml of DW (distilled water). the Wash Buffer was neutral (7.2) and PH indicator used and kept in the refrigerator.

-TMP substrate: aspirate the needed dosage of the solution with sterilized tip and do no dump the residual solution into the vial again.

3-Assay procedure : The procedure of the kit:

a- wells for diluted standard, blank and sample could be determine. prepared 7 wells, 1well for blank. added 100µl each 0f dilution standards (read reagent preparation), blank and sample into the appropriate wells. covered with the plate sealer. incubated for1 hour at 37℃.

b-Removed the liquid of each well, don't wash.

c- 100µl of **Detection Reagent A** working solution was added to each well, cover with plate sealer and incubated for 1hour at 37℃.

d- Aspirated the solution and wells were washed with 350µl of wash solution ,using auto-washer ,and let it sit for 1-2 minute .totally wash 3 times. After the last wash, remove any remaining wash buffer by aspirating or decanting. invert the plate and blot it against absorbent paper.

e- 100µl of **Detection Reagent B** working solution was added to each well, coverd with plate sealer and incubated for 30 minute at 37℃.

f- Aspiration\wash was repeated as mentioned above in **step d**-.for total 5 times.

g- Later on 90µl of **substrate** solution was added to each well and the microplate, covered with plate sealer and incubated away of direct light for10- 20 minutes at 37℃.

h-A 50µl of stop solution was added to each well. and then determination of OD of each well carried out immediately using a reader for microplate set to 450 nm±10

Results calculation: For each standard and sample average the duplicate readings and subtract the average zero standard OD. the a standard curve Construct by plotting the absorbance mean of each standard on the x-axis against the y-axis for concentration and draw on the graph a best fit curve through the points. The samples used were undiluted, so no need to multiply the concentration from the standard curve by dilution factor.

2.8.1.3. Reagents preparation for CD107a (LAMP1): Reagents preparation was accomplished according to instructions of (MYOBIOSOURCE) User Manual:

-Standard: Reconstitute the Standard with 1.0ml of standard diluent**,** kept for 10 minutes at room temperature, shake gently (not to foam).the concentration of the standard in the stock solution is 10ng/ml. and then prepare 7 tubes containing 0.3 ml of standard diluent and produce double dilution series according the table (2-8) below.

Table (2-8) CD107a standards arrangement in the plate

tube	1	2	3	4	5	6	7	8
ng/ml	0	0.156	0.312	0.625	1.25	2.5	5	10

Added 0.3ml from above standard solution into 1st tube and mixed them thoroughly. 0.3ml from 1st tub transfer to 2nd tube mixed them thoroughly.

0.3ml from 2nd tube transfer to 3rd tube mixed them thoroughly, and so on. for the blank control Sample dilution buffer was used only.

Wash Buffer (1×): Mixed gently to dissolve any crystals if present. to prepare 780 ml of Wash Buffer (1×) must be Dilute 30 ml of Wash Buffer concentrate (25×) into750ml of DW (distilled water). Again, the Wash Buffer was neutral (7.2) PH indicator used and kept in the refrigerator.

Biotin-antibody (1×): Before opening, Centrifuge the vial. It needs a 100-fold dilution. 10 µl of Biotin-antibody was added 990 µl of Biotin-antibody Dliuent

HRP- Sreptavidin conjugate (1×): Before opening, Centrifuge the vial. It a 100- fold dilution requires. We take 10µl of HRP-avidin and added 990 µl of HRP- Streptavidin Dliuent.

Sample dilution: the sample was diluted with the provided dilution buffer and well mix before testing .we need to be diluted at least two fold with sample dilution buffer .

3-Assay procedure:

a-The plate was washed 2 times before adding standard,sample and control(blank) wells.

b-A 100µl each 0f dilution standards (read reagent preparation), blank and diluted sample were added into the appropriate wells, covered with the plate sealer. incubated for 90 minute at 37℃.

c- Aspirated the solution and washed with 350µl of wash solution to each well using auto-washer , and let it sit for 1-2 minute .totally wash 2 times.

d- A 100µl of diluted **Biotin-antibody** were added to each well, and the plate covered with new adhesive strip, and incubated for 1 hour at 37℃.

e- Aspiration\wash was repeated 3 times

f- A 100µl of the working dilution of **Streptavidin-HRP** were added to each well, the plate covered, and incubated for30 minutes at 37℃ away of direct light.

g- Aspirated the solution and washed with 350µl of wash solution to each well using auto-washer, and let it sit for 1-2 minutes, totally wash 5 times .

h-Later on 100µl of substrate was added to each well , and the plate covered with new adhesive strip ,and the microplate incubated away of direct light for 10-20 minutes at 37℃.

i-To stop reaction, a 50µl of stop solution was added to each well, and determination of OD of each well carried out immediately using a reader for microplate setted at 490 nm.

4- Results calculation: the average of duplicate readings for each sample and standard then subtract the zero standard OD average. Draw a standard curve by plotting on the x-axis the mean absorbance of each standard against the y-axis contain concentration and draw on the graph a best fit curve through the points. The samples used were diluted, so need to multiply the concentration by dilution factor.

2.9- Waste disposal: Remnants of procedure which is involve syringe, gloves, tubes, biological materials, chemicals and microplate consider as a biohazardous material dispose according to protocol followed by Al-Hussein medical city lab-disposal approach.

Table (2.9) Reference range serum of biochemical tests:

The test	Reference range
CRP	0-6 mg/L
Ferritin	13-350 ng/ml
D-dimer	0-500 ng/ml
LDH	100-250 U/L

2.10- Statistical Analysis

Statistical analyses were performed using SPSS statistical package for social Sciences (version 20.0 for windows, SPSS, Chicago, IL, USA).

Data normality distribution was examined using Shapiro-Wilk test. CRP, LDH, s. ferritin, D. dimer, IL2a, FasL and CD107 were found to be not normally distributed. Quantitative data are represented as mean, standard deviation and range for normally distributed data or median, IQR (Interquartile range) for non-normally distributed data. Qualitative data are represented as count and percentage.

Correlation test was done to test the relation of immunological markers with other parameters. Student's t-test or Mann-Whitney U test was used to test differences in severity status according to data distribution.

ROC analysis (Receiver Operating Characteristic) was used as an indicator of disease activity. P value of <0.05 was considered statistically significant.

Chapter Three:

Results

Chapter Three: Results

3-Results:

3.1- Demographic characteristics of patients with COVID-19 (age, gender):

The study included 88 patients, have been proved to be COVID-19 by RT-PCR, the patients visit the respiratory clinic in Al-Imam Al-Hussein medical city in Karbala (the only medical center dealing with COVID-19 patients), 44 patients were complaining from mild symptoms, treated as outpatients while another 44 patients were admitted to the hospital complaining from severe symptoms, we analyzed each group separately as demonstrated in table 3-1 and figure 3-1, the 1st group was the mild symptoms patients, their mean age 43.09 years ± 14.3 (18-75 years), 22 males and 22 females, male to female ratio was 1:1. Their WBC was 6.44 ± 2.2, ranging from 3.4 to 12.4, the Lymphocytes was 2.8 ± 0.71 ranging from 1.6-4.2. the 2nd group was the severe symptoms patients, which have mean age of 57.7 years ± 12.7 (26-80 years), 25 males and 19 females, male to female ratio was 1.3:1. Their WBC was 12.1 ± 4.6, ranging from 4.1 to 26.6, while the Lymphocytes was 1.29 ± 0.69 ranging from 0.3-3.4.

Table 3-1: Demographic distribution, WBC and lymphopenia in the studied cases and relation to disease activity

	Disease activity							
	Mild (44 cases)				Severe (44 cases)			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Age P=**0.005***	43.09	14.40	18.00	75.00	57.77	12.76	26.00	80.00
WBC P=**0.005***	6.44	2.21	3.40	12.40	12.10	4.67	4.10	26.60
Lymphocyte P=**0.005***	2.88	0.71	1.60	4.20	1.29	0.69	0.30	3.40
Gender P=0.669	Count		%		Count		%	
Male	22		50.0		25		56.8	
Female	22		50.0		19		43.2	
Lymphopenia P=**0.005***								
Present (<1.3 $x10^9$/L)	0		0.0		26		59.1	
Absent (>=1.3 $x10^9$/L)	44		100.0		18		40.9	

There was a significant difference between mild and severe cases for age, WBC and lymphocytes (P<0.05). Gender distribution was not significantly related to disease activity (P>0.05). Lymphopenia was not

found in mild cases but 59.1% of severe cases had lymphopenia (**P<0.05**). done by student's t-test.

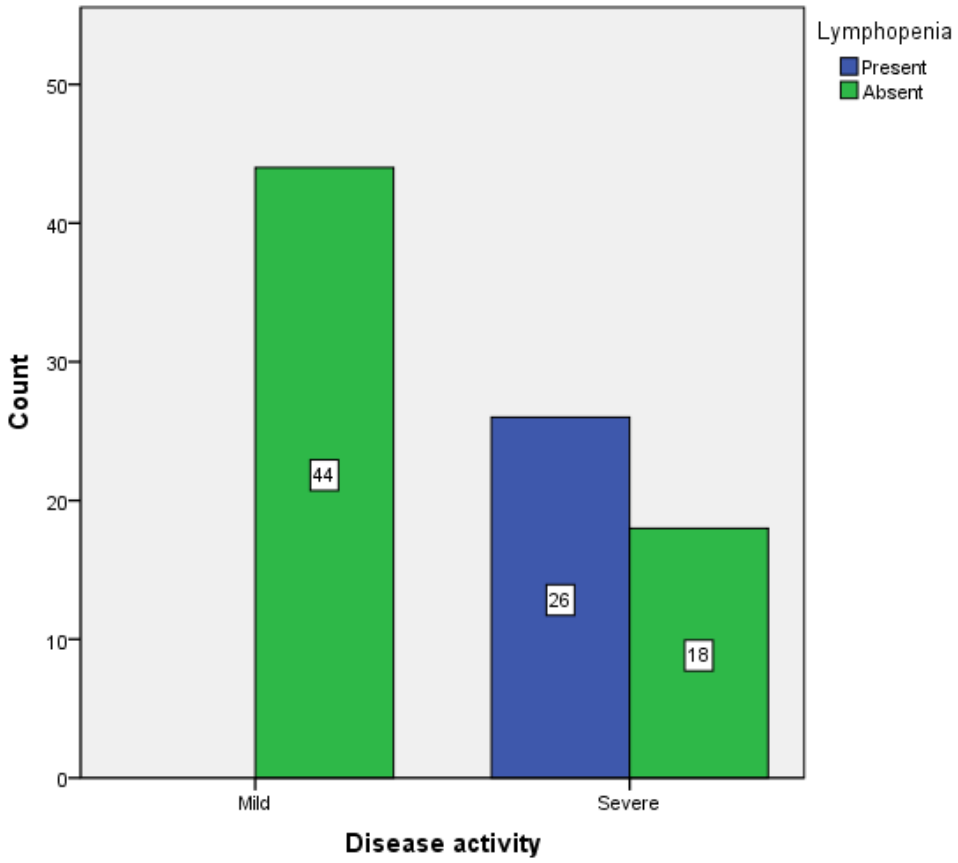

Fig 3-1: Lymphopenia in studied cases according to disease severity

3.2- Biochemical markers characteristics of COVID-19 patients (CRP and D.dimer):

There was a significant difference between mild and severe cases for CRP and D. dimer ($P \leq 0.05$) (According to Mann-Whitney U test). No LDH and S. Ferritin tests were done for mild cases.

There was elevated CRP in both mild and sever cases, median value in mild cases was 7 mg/L comparing with 35.9 mg/L in severe cases, the relationship was significant $P \leq 0.005$ as demonstrated in table (3- 2):

D.dimer values were elevated in both groups, ranging from 114-633ng/ml in mild cases, while in severe cases ranging from 256-4428ng/ml, there was a significant relationship $P \leq 0.005$ as demonstrated in table (3- 2).

3.3- Immunological markers characteristics of COVID-19 patients (IL2-Ra, FasL and CD107):

There was a significant difference between mild and severe cases for IL2-Ra and CD107 ($P < 0.005$). No significant relation between FasL and disease severity in our specimen, this according to Mann-Whitney U test,

The IL2-Ra values in mild cases ranging between 4.1-35.7 (median 16.2), while in severe cases ranging from 11-83.9 (median 24.5), Low levels of CD107 were seen in severe cases (0.2-0.7) comparing with mild cases values (0.5-5.4). This demonstrated in table (3- 2):

Table 3-2: Studied parameters and relation to disease activity in the studied sample

	Disease activity							
	Mild (44 cases)				Severe (44 cases)			
	Mean	IQR	Min	Max	Mean	IQR	Min	Max
CRP P=**0.005***	10.85	10.45	1.80	74.00	46.1	57.3	5.2	95.1
LDH	Not done				533.8	236.0	280.3	1080.0
S.ferritin	Not done				963.6	369.0	228.7	3989.0
D.Dimer P=**0.005***	310.8	204.0	114.0	633.0	883.1	349.0	256.0	4428.2
IL2Ra P=**0.005***	16.3	10.25	4.10	35.70	33.2	23.4	11.0	83.9
FasL P=0.217	15.5	4.75	7.80	34.20	6.66	16.0	4.0	34.4
CD107 P=**0.005***	1.80	0.95	0.50	5.40	0.50	0.18	0.2	0.7

3.4- The correlation between lymphopenia and both biomarkers and immunological markers of severe COVID-19 patients:

There was a significant difference between presence and absence of lymphopenia in severe cases for FasL($P<0.05$). There was no significant difference in CRP, LDH, S. Ferritin, D.dimer ($P=0.384$), IL2Ra and CD107 between the presence and absence of lymphopenia ($P>0.05$). Table (3-3) summarized by Mann-Whitney U test this relationship.

Table 3-3: Relation of CRP, LDH, S. Ferritin, D. dimer, IL2Ra, FasL and CD107 to the presence or absence of lymphopenia in severe cases.

	Lymphopenia							
	Present				Absent			
	Mean	IQR	Min	Max	Mean	IQR	Min	Max
CRP P=0.364	50.44	60.2	6.1	95.1	39.9	44.9	5.2	91.9
LDH P=0.095	487.4	270.4	280.3	885.10	600.8	341.3	323.1	1080.0
S.Ferrtin P=0.242	1124.2	532.2	228.7	3989.00	731.7	369.9	321.4	1058.0
D.dimer P=0.384	832.7	360.9	256.0	3393.00	956.1	343.7	357.0	4428.2
IL2Ra P=0.981	32.6	25.8	11.0	75.50	34.2	12.8	14.5	83.9
FasL P=**0.005**	7.1	20.0	7.3	34.40	76.1	2.4	4.0	16.4
CD107 P=0.114	0.47	0.17	0.21	0.70	0.54	0.18	.30	0.7

3.5- The correlation between lymphocytes and other parameters of sever COVID-19 patients:

To test the relation of lymphocytes to immunological parameters and other studied parameters, correlation test was used in severe cases, this demonstrated in details in Table (3- 4),

Lymphocytes: There was a significant negative correlation with FasL (r=-0.687, P=0.005) figure (3- 2), while positive correlation with CD107 (r=0.360, P=0.016) figure (3- 3). There was also a significant positive correlation with LDH (r=0.340, P=0.024) figure (3-4).

No significant correlation was found between lymphocytes and IL2Ra, CRP, S.ferrtin and D.dimer.

IL2Ra: There was a significant positive correlation with LDH (r=0.304, P=0.045) figure (3- 5), and a negative correlation with S. Ferritin (r=-0.328, P=0.030) figure (3- 6).

FasL: There was a significant negative correlation with CD107 (-0.469, P=0.001) figure (3- 7).

CD107: No significant correlation was found between CD107 and IL2Ra, CRP, S.ferrtin LDH and D.dimer.

Table 3-4: Correlation between lymphocytes to other studied parameters in severe cases

Correlations (severe cases N=44)

		Lymphocyte	IL2Ra	FasL	CD107
IL2Ra	Pearson Correlation	.204			
	Sig. (2-tailed)	.185			
FasL	Pearson Correlation	**-.687****	-.016		
	Sig. (2-tailed)	**.000**	.917		
CD107	Pearson Correlation	**.360***	-.024	**-.469****	
	Sig. (2-tailed)	**.016**	.875	**.001**	
CRP	Pearson Correlation	-.141	-.124	.154	-.050
	Sig. (2-tailed)	.362	.424	.319	.746
LDH	Pearson Correlation	**.340***	**.304***	-.231	.003
	Sig. (2-tailed)	**.024**	**.045**	.132	.984
S.ferrtin	Pearson Correlation	-.135	**-.328***	.047	-.006
	Sig. (2-tailed)	.383	**.030**	.760	.970
D.dimer	Pearson Correlation	.259	-.119	-.200	.075
	Sig. (2-tailed)	.090	.441	.193	.630

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

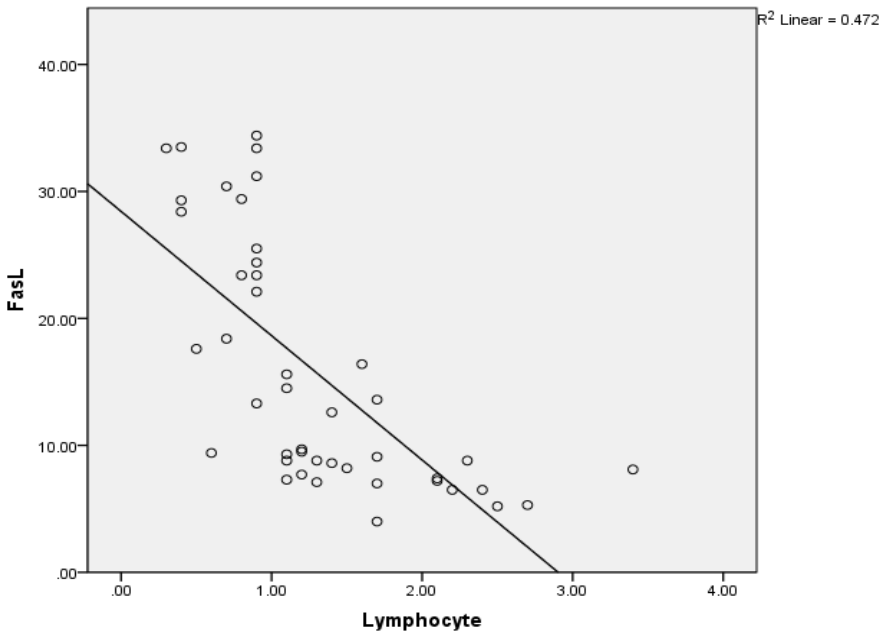

Fig 3-2: Correlation of lymphocytes with FasL in severe cases

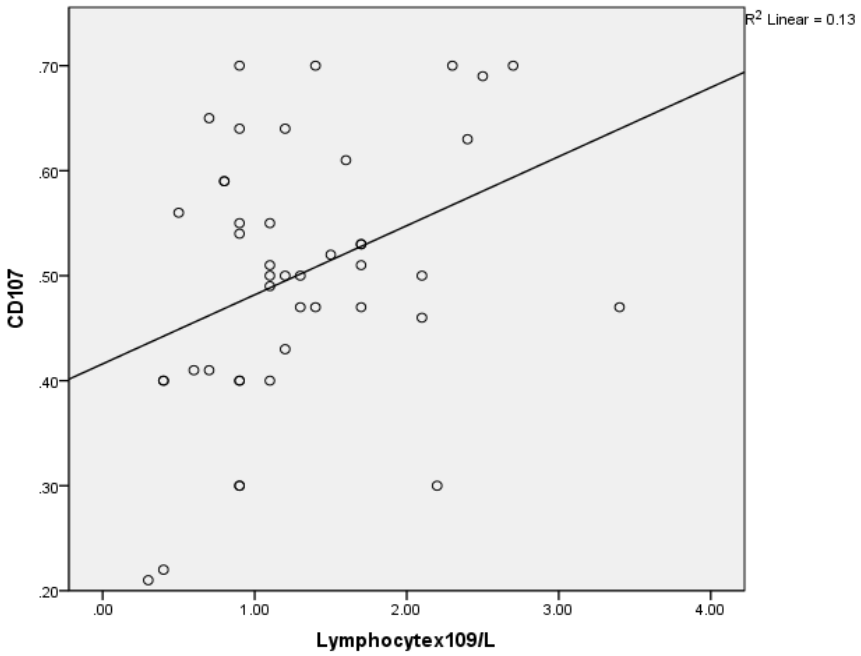

Fig 3-3: Correlation of lymphocytes with CD107 in severe cases

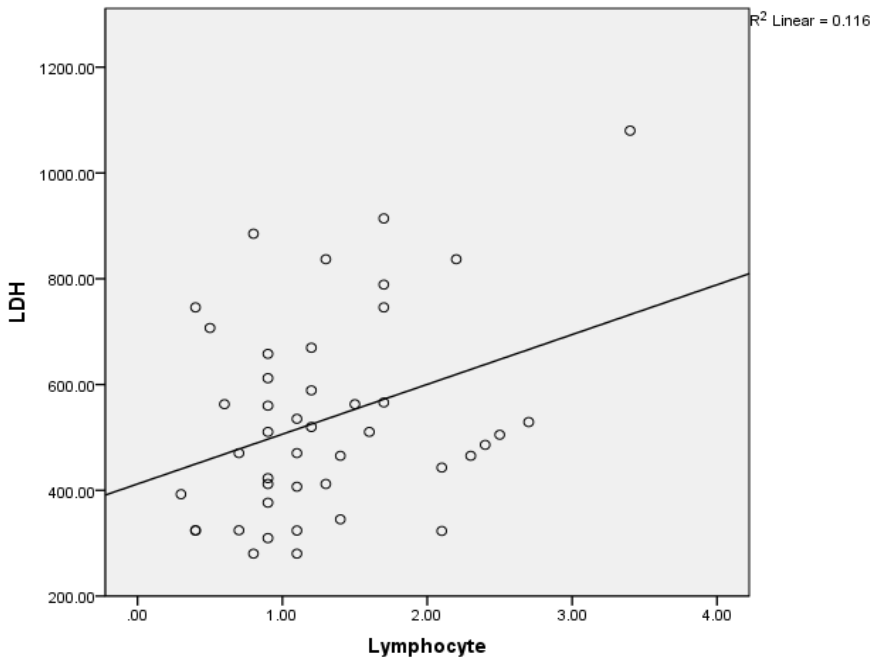

Fig 3-4: Correlation of lymphocytes with LDH in severe cases

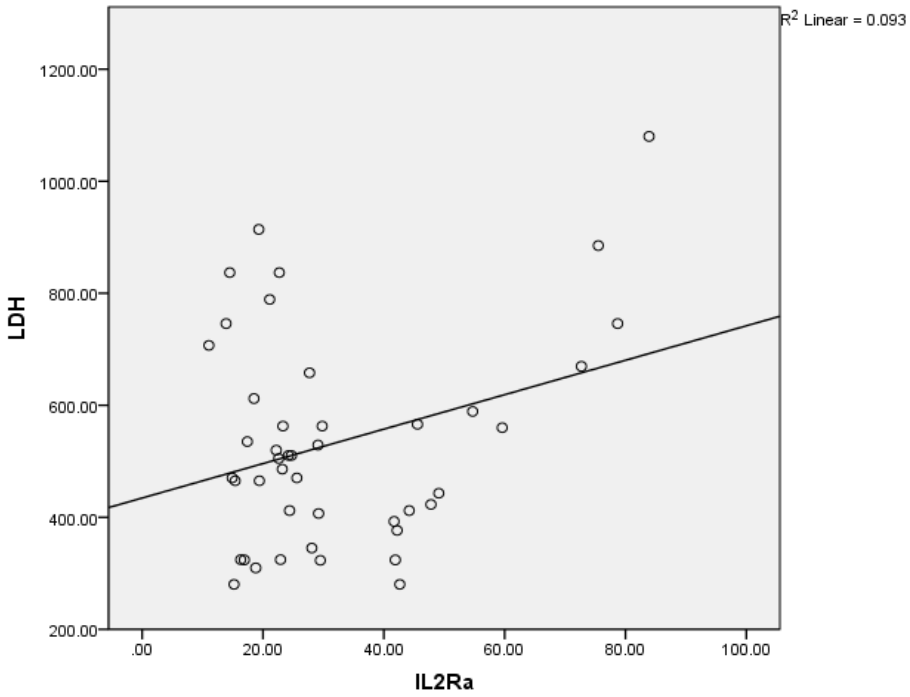

Fig 3-5: Correlation of IL2Ra with LDH in severe cases

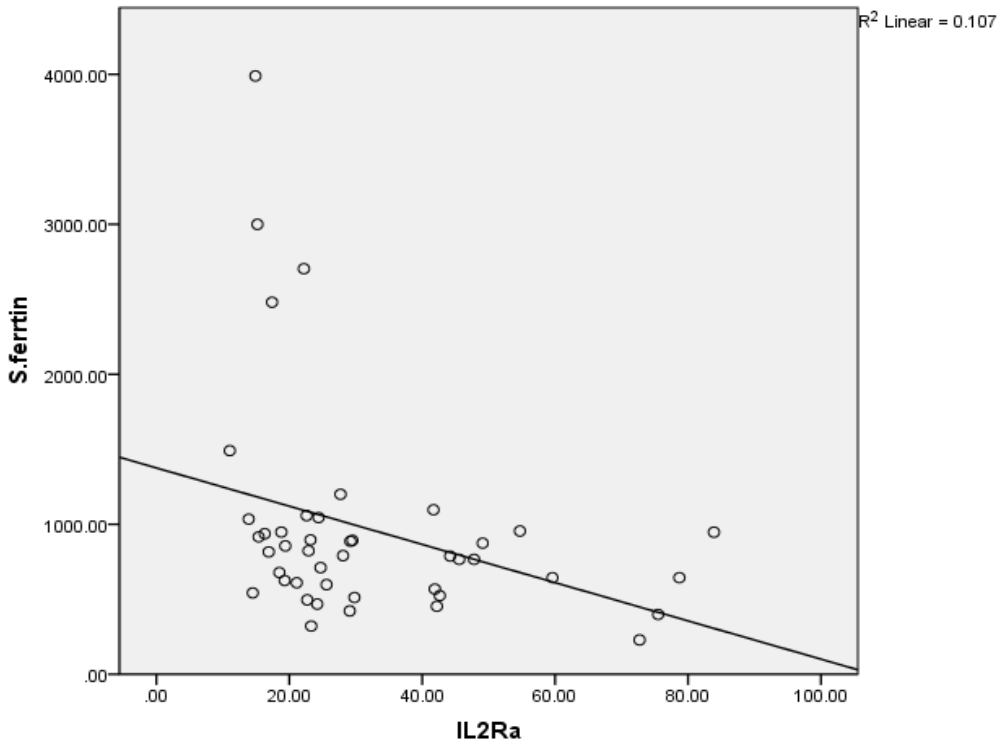

Fig 3-6: Correlation of IL2Ra with S. ferritin in severe cases

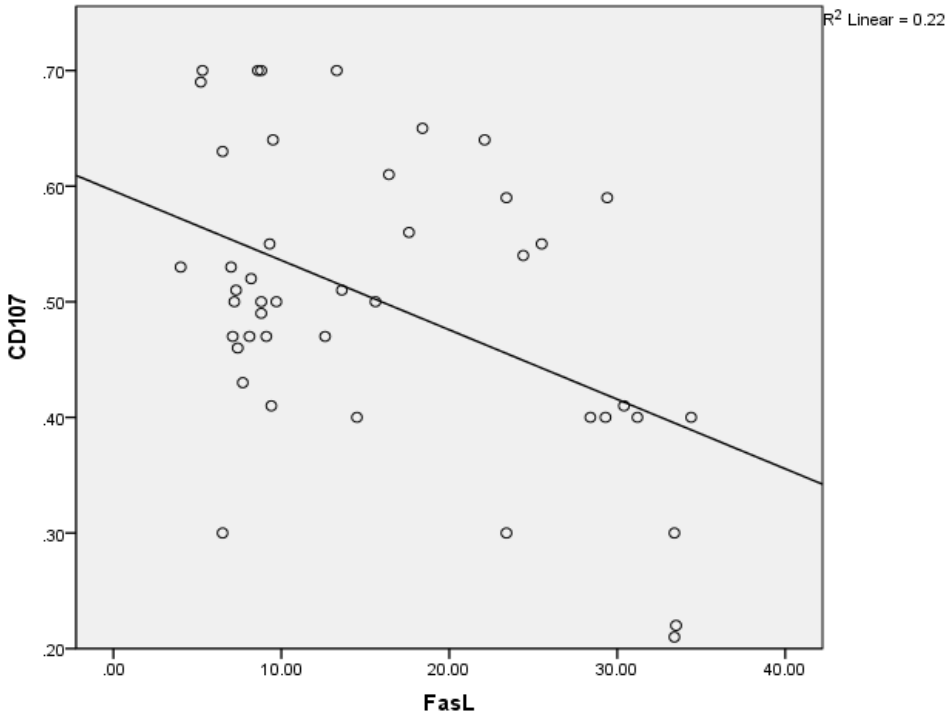

Fig 3-7: Correlation of FasL with CD107 in severe cases

3.6- The correlation between lymphopenia, biomarkers and immunological markers of mild COVID-19 patients:

To test the relation of lymphocytes to immunological parameters and other studied parameters, correlation test was used in **mild cases**.Table (3-5)

Lymphocytes: There was a significant negative correlation with FasL (r=-0.446, P=**0.002**),

Table 3-5: Correlation between lymphocytes to other studied parameters in mild cases. **Correlations mild cases N=44**

		Lymphocyte	IL2Ra	FasL	CD107
IL2Ra	Pearson Correlation	.141			
	Sig. (2-tailed)	.362			
FasL	Pearson Correlation	**-.446****	.077		
	Sig. (2-tailed)	**.002**	.621		
CD107	Pearson Correlation	-.019	-.078	-.111	
	Sig. (2-tailed)	.902	.614	.472	
Age	Pearson Correlation	.008	-.011	-.065	-.096
	Sig. (2-tailed)	.959	.944	.673	.535
WBC	Pearson Correlation	-.033	.038	.090	-.065
	Sig. (2-tailed)	.831	.806	.562	.676
CRP	Pearson Correlation	.200	-.171	.033	-.222
	Sig. (2-tailed)	.194	.268	.829	.148
D.dimer	Pearson Correlation	-.067	-.123	-.280	.185
	Sig. (2-tailed)	.664	.426	.065	.230

**. Correlation is significant at the 0.01 level (2-tailed).

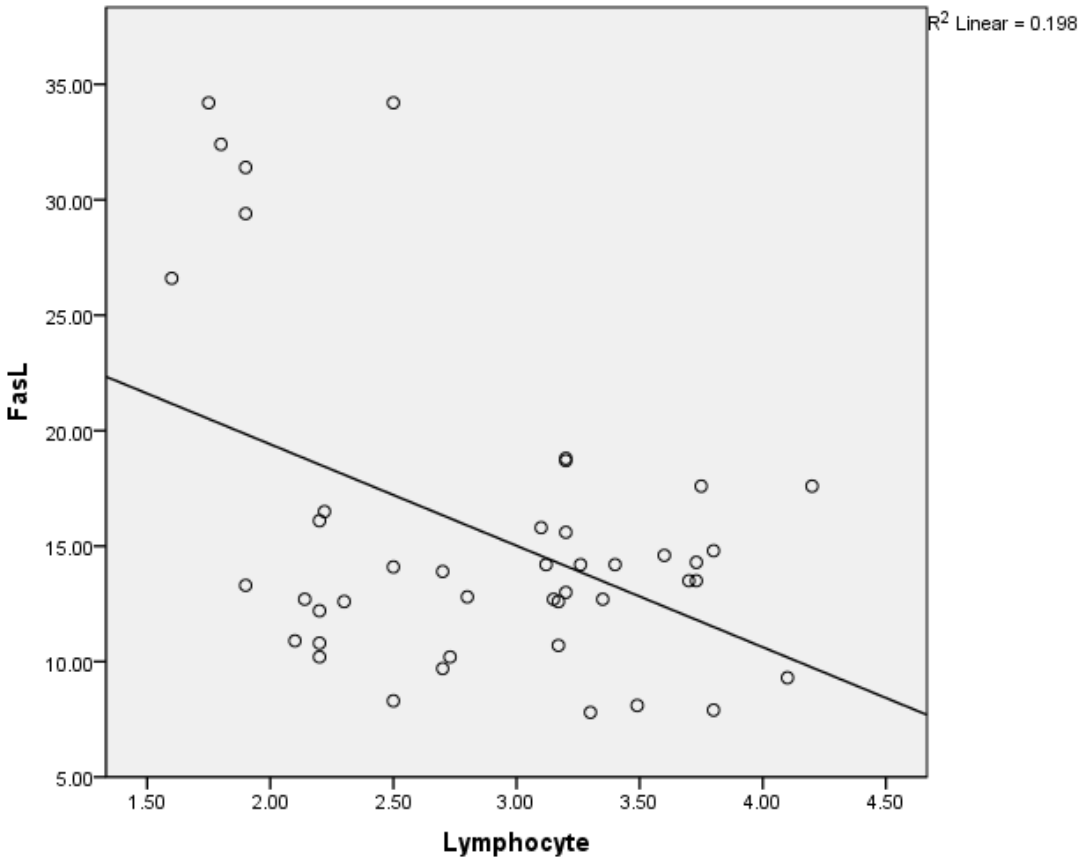

Fig 3-8: Correlation of lymphocytes with FasL in mild cases

3-7 ROC results

Studying D. dimer ROC results showed a significant area under the curve of 92.8%, **P<0.005** (Table 3- 6) for indicating severity of disease activity.

Table 3-6: ROC D.dimer results

Area Under the Curve

Test Result Variable(s):D.dimer

Area	Std. Error[a]	P value	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
92.8%	0.026	**0.005***	0.877	0.980

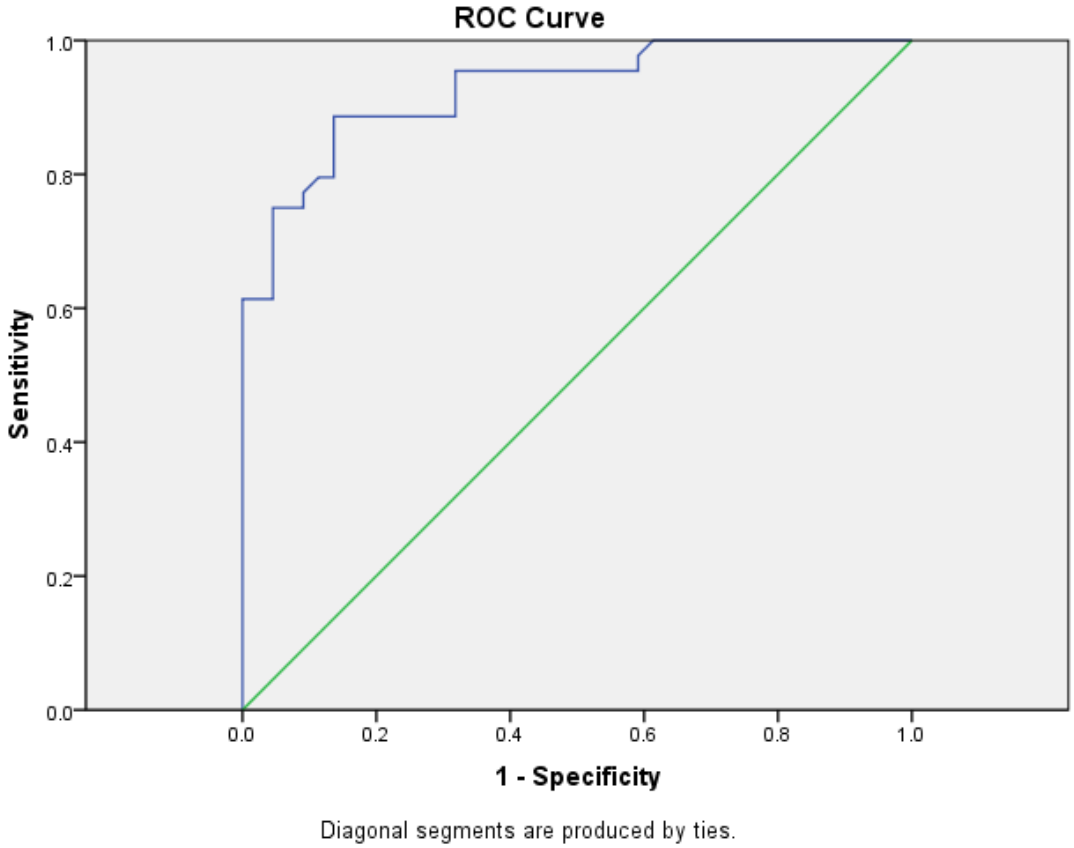

Fig 3-9: D. dimer ROC

At a D. dimer value of 432.50, the sensitivity was 88.6% and the specificity was 86.4% for indicating a severe outcome for corona virus infection.

Chapter four:

Discussion

Discussion

COVID-19 established its prevalence from December 2019, it quickly spread all over the world, and then it became complex public health challenge negatively affecting humanity and business. In this study, we report 88 patients with RT-PCR-confirmed SARS-CoV-2 infection, all of which presented with upper or lower respiratory tract symptoms and fever, mild cases suffer from upper respiratory tract infection, while those with sever condition complaining from lower respiratory tract disease (pneumonia), the severity of infection was influenced by amount of viral load, age and co-morbid conditions (Xu Z. *et al*. 2020, Huang C. *et al*, 2020 and Wang D. *et al*, 2020).

4-1 Demographic characteristics of patients with COVID-19

4-1-1 Age and gender

As in similar recently published data there was significant correlation between age and disease severity, elderly patient more prone to sever disease (Jin J-M. *et al*, 2020 and Hou H. *et al*, 2020), even mortality was higher in older age group as mention by Wang D.W. *et al*, 2020, the explanation for that is the depressed immunity due to comorbidity in elderly patients, resulting in more sever disease and wider complications, the mean age of our patients with mild disease was 43 years versus 57.7 years for sever disease patients. There was no significant difference in age distribution between female and male patients, this agreed by Jie Qian. *et al*, 2020 and Maleki Dana, P. *et al*, 2020. Wang C. *et al*, 2020, but not accepted by Gebhard C. *et al*, 2020 and Jin J.M. *et al*, 2020, men are biologically older than women (Blagosklonny M.V.2010), this finding goes with what we observe that males were more than females in sever cases, gender differences in the presentation time to health care may give

an explanation for increase the rate of men with COVID-19 which need hospitalization. in spite of no correlation between gender and disease severity in this specimen, still male gender represents a risk factor for disease severity as found by a meta-analysis done by Peckham H. *et al*, 2020 involving more than three million reported global cases, males were three times more than females that requiring intensive care unit this may be due to the effect of estrogen in female which have immune enhancing effects but testosterone secreted by testes have immune suppressive effect (A jay and Per-Erik, 2020), but the overall incidence for infection was equal, this agreed by Global Health 5050, Hou H. *et al*, 2020 and Jin J. M. *et al*, 2020.

4-2 Lymphopenia in studied cases according to disease activity:

WBC-count was significantly correlated in mild and sever cases, as it well known COVID-19 patients presented with leukopenia (Huang CL. *et al*, 2020, Wang DW. *et al*, 2020 and Liu J-Y. *et al*, 2020), then in late stages of the disease some sever and critical patients shows leukocytosis, mostly due to secondary bacterial infections (neutrophilia) as agreed by Huang G, *et al* 2020.

During SARS and MERS previous infections lymphopenia was well known reported (De Wit E. *et al*, 2016). Recent studies found similar findings in COVID-19 patients, particularly in ICU patients (Chen N. *et al*, 2020). The main cause of lymphopenia in COVID-19 infection still unclear but some authors relay on inhibition of the cellular immunity and apoptosis following overactive immune system, as reported in SARS infection (Chan PK, Chen GG 2008). In our patients lymphopenia also correlated well in mild and sever diseases **(P=0.005)**, this also was well-known in recent published data (Guan WJ. *et al*, 2020, Holshue ML. *et al*, 2019, Chen XY.

et al, 2020 and Wu CM. *et al*, 2020), in mild cases lymphopenia was not evident the explanations are:

1. Small number of patients in a relatively short time for collecting data.
2. Our mild cases were presented early within 1st five days of symptoms and as we know lymphopenia develop gradually in disease course.

4-3 Biochemical markers characteristics of COVID-19 patients (CRP and D.dimer):

CRP and D. dimer were closely correlated with disease severity as it seen by many articles (Wang D. *et al*, 2020 , Zhu J. *et al*, 2020 and Valerio L. *et al*, 2021), both biomarkers were used as a marker of disease severity assessment and pulmonary embolism as sited by Becher Y. *et al*, 2020., higher levels of D. dimer were reported in both mild and sever disease patients, the sensitivity of this test was 88.6% after 432.5 ng/ml level.

D. Dimer was very important marker of severity this finding was seen by a Chinese group reported that D-dimer levels of ≥2.0 μg/mL on admission were associated with a 51.5 times increased mortality relative to D-dimer levels of <2.0 μg/mL in a cohort of 343 COVID-19 patients, this finding of D-dimer levels as a negative prognostic marker was also noted in other studies conducted in world wild (Zhang L, *et al*, 2020).

The level of LDH were elevated in both groups (normal range 140-280U/L) (Holmes RS *et al*, 2009), this representing cell death (apoptosis), these values correlated well with lymphopenia and IL-2Ra, the results were concomitant with the recent published data by Zhang Z meta-analysis (Zhang Z. *et al*. 2020).

In sever covid-19 patients there was elevated levels of IL-2Ra and FasL while decrease level of CD107a that correlate significantly with the level of lymphocytes count, these markers can be used as marker for disease severity and prognostic markers.

4-4 Relation of biological markers with lymphopenia

In severe cases D-Dimer, LDH, S. Ferrtin and CRP was in their higher values, however with disease progress, this elevation was not correlated positively with lymphopenia, one of important causes could be the rise of WBC due to secondary infections, second point of view is need for dynamic study for these markers, a single read can be misleading due to certain patient condition (presence of other disease, certain drug use etc..) some researchers use neutrophil-lymphocyte ratio as a marker of systemic inflammation for following these biomarkers as done by jin YH. *et al*, 2020. And it was a sensitive measure more than lymphocyte count alone, D.Dimer level of 432.5ng/ml (=0.9mg/L) was found to be a sensitive predictor for ICU admission in patients with COVID-19 infection.

4-5 Immunological markers characteristics of COVID-19 patients:

4-5-1 CD107a:

It is well known that CD107a protects cytotoxic lymphocytes from damage during degranulation, while any interference with CD107a expression can cause the death of cytotoxic lymphocytes during degranulation that is the matter in COVID19 sever patients, the study and others articles (De Biasi S, *et al*,2020 and Cossarizza, A. *et al*, 2020) find

a strong correlation between degranulation marker CD107a and disease severity, the CD107a was positively correlate with lymphocytes count, low levels were recorded in sever lymphopenia this due to CD107a-deficient NK cells are more susceptible to apoptosis after target cell encounter this agreed by Cohnen A. *et al* 2013, finding also noted by Zheng M. *et al*, 2020, the peripheral NK cell count and function are significantly affected in SARS–CoV-2 infection as it usual in other viral infections as in CMV, NK cells in patients with severe COVID-19 have been found to have decreased functional markers as CD107a and cytokine expression (e.g., TNF). Only few mature NK cells have been found in critical COVID-19 patients, furthermore their ability to communicate with other WBC and exert their cytolytic functions is reduced (Salome B. *et al*, 2020)

Low level of CD107a due to defective induction of surface expression was associated with certain subtypes of hyper-inflammatory immunodeficiency syndromes (Marcenaro S. *et al*, 2006 and Bryceson YT. *et al*, 2007), SLE (Ng WL. *et al*, 2006) and also in psychological stress (Duggal N. A. *et al*, 2006), the high sensitivity of CD107a in lymphopenia faced low specificity toward certain pathologies make this immunological marker of low value for assessing lymphopenia in COVID-19 patients but still it is good assessment factor for disease severity.

4-5-2 IL-2Ra

IL-2 is the cornerstone in the proliferation of T cells and in the generation of effector and memory T cells as found by Rosenberg S.A. 2014 and Ross, S. H. & Cantrell, D. A. 2018). It also involved in adaptive immunity and increases glucose metabolism to promote the proliferation and activation of T cells, B cells and NK cells as mentioned by Turner

M.D., *et al*. 2014. We found elevated levels of IL-2Ra in both mild and sever patients, higher levels seen in sever patients, a significant correlation was noted between level of IL-2Ra and disease severity, this also seen by Huang C. *et al*, 2019, Liu K. *et al*, 2020. and Mehta P. *et al*, 2020, there were higher levels of IL-2Ra in sever cases this was goes with Hou H. *et al*. 2020 findings, but this was not significant correlation in sever patients, this may due to differences in patient medical hospital (presence of secondary infection or types of drug used), IL-2R has been shown to be elevated in various immunological disorders, starting with hemophagocytic syndromes (M. Lin et al, 2017), granulomatous disease like tuberculosis and sarcoidosis (S. Takahashi,*et al*. 1991 and J.C. Grutters, *et al*. 2003), elevated levels have been reported in T-cell mediated diseases, like MS (L.M. Maier. *et al*. 2009), type 1 DM (C. Giordano. *et al*,1988), and rheumatoid arthritis, G. Semenzato. *et al*,1988), In general, increased levels of IL-2R in patients with autoimmune diseases are considered to reflect T-cell activity. Hence, it is not surprising that it increasing in COVID-19 and the physicians can use it to assess disease severity but it should carry in mind that it is of low specificity.

4-5-3 FasL

Our data on increased level of FasL expression are in line with data by Zheng H.Y *et al*,2020 showing an exhausted T-cell phenotype in patients with severe COVID-19 infection, this increment in level of FasL in COVID-19 may suggest an increase in lymphocytes apoptosis. This hypothesis can be supported by our findings of an inverse correlation between FasL expression and lymphocyte population. Moreover, level of FasL inversely correlated with the level of CD107a in sever disease, both mild and sever cases exhibiting high levels but statistically speaking no significant difference were seen between both groups (disease severity), an

early sampling of COVID-19 patients (1st week of symptoms) may explain these results, this was unaccepted by Bellesi S *et al*, 2020 and Wang D. *et al*, 2020, who decline a significant correlation with disease severity.

As we reported its level correlated well with lymphopenia, and since lymphopenia is the whole mark of COVID-19, FasL can be used as a sensitive marker for disease severity. FasL plays an essential role in various conditions including aging process and pulmonary pathologies, we emphasis on its immunological role in COVID-19, many articles show that FasL increase with age in the blood serum of healthy individuals (Pinti M. *et al*, 2003, Goto M. 2008, Jiang S. *et al*, 2008), This may explain why elderly patients are more prone to severe COVID-19 complications, not just that but FasL increases also in patients complain from Werner syndrome which is a typical accelerated aging disease (Goto M. 2008), in addition high serum level of FasL was seen in certain pulmonary diseases including interstitial lung diseases (ILDs) such as hypersensitivity pneumonitis, asbestosis and interstitial pneumonia (Kuwano K. *et al*, 2002), furthermore it elevated in patients with chronic obstructive pulmonary disease (COPD) where its level directly related to the disease symptoms (Kosacka M, *et al*, 2016), all the previous data add an important information to the physician about the outcome of COVID-19 in elderly patients especially those with previous unhealthy lungs where it is a challenging issue to isolate the patients of high risk from developing cytokine storm and other COVID-19 complications.

From the forty-four sever patients eight patients were admitted to the ICU in a critical condition, they had a significant high levels of the immune markers, this in line with Zhang Y, *et al*, 2020 and Chi Y, *et al* 2020.

Conclusion & Recommendation

Conclusion:

1-Following the serum level of IL-2R and CD107a in COVID-19 patients will serve as a marker for monitoring patients conditions and provide a suitable tool to verify which patient may need care of the intensive care unit.

2-CRP and D. dimer were positively correlated with disease severity, D.dimer could be used as marker for disease severity and prognostic markers.

3-FasL level was correlated with disease severity but not with lymphopenia.

Recommendations

1. Serum level of IL-2R, CD107a and FasL should be done for large size sample of COVID-19 patients early in disease course and then another follow up tests done if the patient acquires sever disease.
2. Studying the expression of the CD107 on NK cells by flow cytometry.
3. Study about IL2R, Fas and FasL gene polymorphism in a COVID-19 patients as a genetic risk factor for development of sever disease.
4. Study IL2R, CD107 and FasL in bronchial wash and comparing with serum level in same patients.

Chapter Five: Reference

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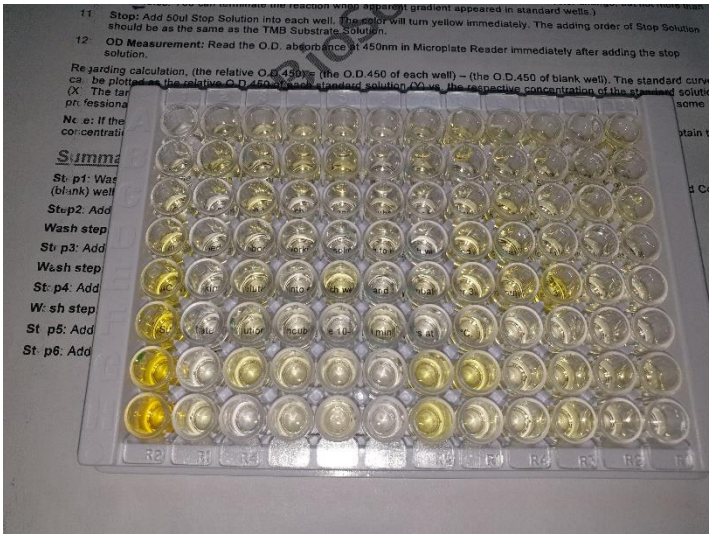
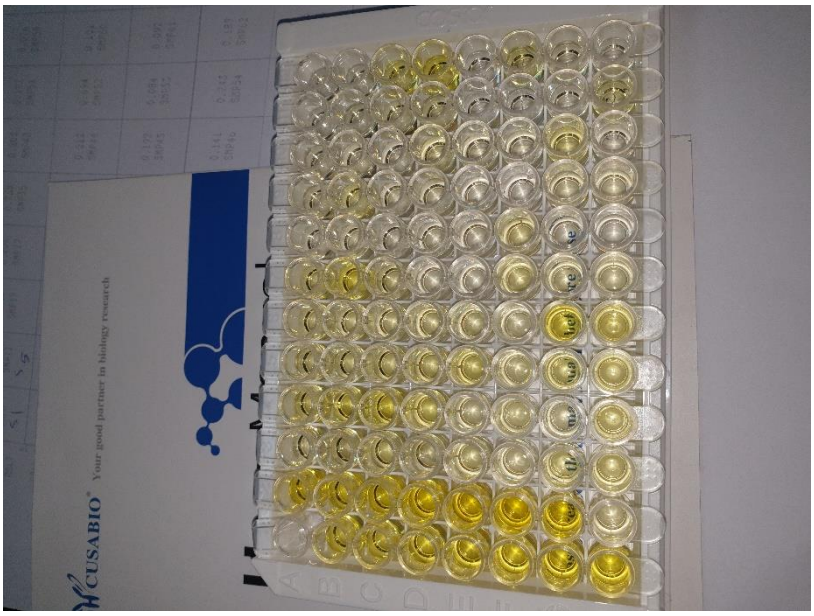
Appendix

lAppendix 1

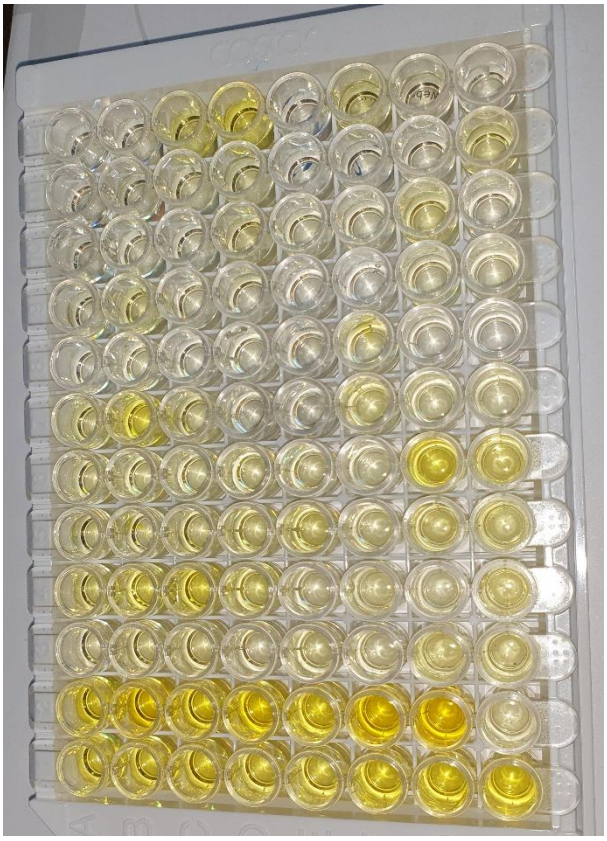
ELISA plate for FasL



ELISA plate for IL2-R



ELISA plate for CD107



ELISA reader

Patient quasionare

Patient name Patient number	
age	
gender	
Presentation day	
symptoms	
D-Dimer	
S. Ferretin	
LDH	
CRP	
CBC	

الخلاصة

تعد جائحة فيروس كورونا 2019 من أشد الأزمات التي أثرت بشكل كبير على العالم ، فهي تؤثر على كل من حياة الإنسان والاقتصاد العالمي ، وفقًا لمنظمة الصحة العالمية ، هناك 156،496،592 حالة مؤكدة بما في ذلك 3264،143 حالة وفاة حتى السادس من آيار 2021، ان العامل المسبب لـمرض COVID-19 هو فيروس كورونا ، وقد كان تعداد الخلايا الليمفاوية موضع اهتمام منذ أول انتشار للمرض حيث أن نقصها مرتبط بشدة المرض ، ان العديد من الواسمات المناعية مثل IL2Rα و CD107a و FasL لها تأثير على تعداد الخلايا الليمفاوية.

هدفت الدراسة الى تقييم العلامات المناعية (IL2Rα و CD107a و FasL) في مرضى COVID-19 الخفيف والشديد، والعلاقة مع شدة المرض و تعداد الخلايا الليمفاوية. تم تقسيم المرضى المسجلين في هذه الدراسة إلى مجموعتين: اصابات خفيفة وشديدة اعتمادا على علامات وأعراض المرضى. تمت دراسة 88 مريضًا تم إثبات إصابتهم بفيروس كوفيد تسعة عشر في مدينة الامام الحسين عليه السلام الطبية التعليمية في كربلاء بين الفتره اب 2020- كانون الاول 2020,واجريت الاختبارات المعمليه من خلال تقنيات مصليه ساندويتش (ELISA) وتم قياس الواسمات المناعية (IL2Rα و CD107a و FasL) للمرضى وربطها إحصائيًا. في مجموعة الأمراض الخفيفة كان متوسط العمر 43.09 سنة ± 14.3 (18-75 سنة) ، 25 ذكر و 19 أنثى ، نسبة الذكور إلى الإناث كانت 1: 1 ، WBC لديهم 6.44 ± 2.2 ، تتراوح من 3.4 إلى 12.4 ، الخلايا الليمفاوية كانت 2.8 ± 0.71 تتراوح من 1.6-4.2. في مجموعة المرضى الشديدة: متوسط العمر 57.7 سنة ± 12.7 (26-80 سنة) ، 22 ذكر و 22 أنثى ، كانت نسبة الذكور إلى الإناث 1.3: 1 كانت WBC لديهم 12.1 ± 4.6 ، تتراوح من 4.1 إلى 26.6 ، الخلايا الليمفاوية كانت 1.29 ± 0.69 يتراوح بين 0.3-3.4. كان هناك فرق كبير بين الحالات الخفيفة والشديدة للعمر ، WBC والخلايا الليمفاوية (P <0.05). لم يكن التوزيع بين الجنسين مرتبطًا بشكل كبير بشدة المرض (P> 0.05). لم يتم العثور على انخفاض في عدد الخلايا اللمفاوية في الحالات الخفيفة ولكن 59.1 ٪ من الحالات الشديدة كانت تعاني من انخفاض في عدد الخلايا اللمفاوية (P <0.05). كان هناك فرق كبير بين معدل الواسمات والحالات الشديدة لـ (IL2-Ra و CD107 p<0.005). لا توجد علاقة ذات دلالة إحصائية بين FasL وشدة المرض في عينتنا.

قياس مستويات المصل من IL-2R و CD107a و FasL في مرضى COVID-19 سيخدم علامة حساسة لمراقبة حالات المرضى ويوفر أداة مناسبة للتحقق من المرضى الذين يعانون من نقص حاد في الخلايا اللمفاويه والذين سيصابون بمرض شديد وقد يحتاج إلى رعاية في وحدة العناية المركزة.

وزارة التعليم العالي والبحث العلمي
كلية الطب
جامعة كربلاء
قسم الأحياء المجهرية الطبية

دراسة العلاقة بين مستويات الواسمات المناعية (FasL, CD107a, IL-2Rα) مع شدة المرض في مرضى كوفيد-19

أطروحة

مقدمة الى كلية الطب ولجنة الدراسات العليا / جامعة كربلاء كجزء من استيفاء متطلبات درجة الماجستير في علم الأحياء المجهرية

الباحث

فاطمة عبد الله منخي

بأشراف

أ. الدكتورة ألاء سعد العتابي
دكتوراه في علم المناعة

أ. الدكتورة ضمياء مكي حمزة
دكتوراه أحياء مجهرية

2021م **1442 هـ**