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ASSOCIATION OF IL-6 AND TNF ALPHA LEVELS WITH HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN TREATED COVID-19 PATIENTS

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ABSTRACT

This study aimed to assess the role of IL-6 and TNF-alpha levels with other hematological and biochemical parameters in disease status on response to treatment of SARS-COV-19 patients. Characterized Coronavirus disease 2019 (COVID-19) by a cytokine storm manifested by elevation in the levels of many inflammatory and biochemical markers and changes in the blood indices. The most of the biochemical markers used to detect COVID-19, namely; D- dimer, serum ferritin, Lactate dehydrogenase, C-reactive protein, Alanine aminotransferase, Aspartate aminotransferase were shown to be positively correlated with severity of the COVID-19 symptoms.

KEYWORDS: COVID-19, IL-6, TNF, Hematological.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It causes serious respiratory illness such as pneumonia and lung failure and it was first reported in Wuhan, the capital of Hubei, China.[1] In March, 2020, the World Health Organization (WHO) declared that the outbreak of coronavirus disease 2019 (COVID-19) has become a global pandemic. Currently, the virus is spreading in almost all continents.[2]

This virus is belongs to the subfamily Coronavirinae in the family Coronaviridae and the order Nidovirales.[3] Coronaviruses are enveloped viruses with a single-strand, positive-sense RNA genome approximately 26–32 kb in size, which is the largest known genome among all RNA viruses. The term ‘coronavirus’ refers to the appearance of coronavirus virions considering the protruding spike proteins on their surface that look like a crown under electron microscopy (“*corona*” means crown).[4]

SARS-CoV-2 is spread primarily via respiratory droplets during close face-to-face contact. Infection can be spread by asymptomatic, pre symptomatic, and symptomatic carriers.[5] Entry of SARS-CoV-2 depends on the binding of S proteins covering the surface of the virion to the cellular ACE2 receptor.[6] After entering respiratory epithelial cells, SARS-CoV-2 provokes an immune response with inflammatory cytokine production accompanied by a weak interferon (IFN) response. This is followed by the infiltration of macrophages and neutrophils into the lung tissue, which results in a cytokine storm.[7]

Particularly, SARS-CoV-2 can rapidly activate pathogenic Th1 cells to secrete pro-inflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6). GM-CSF further activates $CD14^{+}CD16^{+}$ inflammatory monocytes to produce large quantities of IL-6, tumor necrosis factor-α (TNF-α), and other cytokines**.** These inflammatory mediators further damage the epithelial cells lining and reach into the blood circulation where it causes damage to other organs.[8]

Interleukin -6 (IL – 6) is an important member of the cytokine network and plays a central inflammation.[9] IL-6 can be produced by almost all stromal cells and immune system cells, including B-lymphocytes, T-lymphocytes, macrophages, monocytes, dendritic cells, mast cells, and other non-lymphocytic cells such as fibroblasts, endothelial cells, keratinocytes, glomerular mesangial cells and tumor cells.[10]

More evidence suggest that critically ill patients with severe respiratory failure and SARS-CoV-2 have either immune dysregulation or macrophage-activation syndrome, both of which are characterized by pro-inflammatory cytokines. The immune dysregulation, in particular, is driven by the Interleukin-6 (IL-6) and not by Interleukin-1beta (IL-1beta).[11]

Two key features of this immune dysregulation are: over-production of pro-inflammatory cytokines by monocytes and lymphocyte dysregulation with CD4 lymphopenia.[11] Tumor necrosis factor (TNF)-α, an important member of the TNF superfamily of ligands, is a pleiotropic pro-inflammatory cytokine.[12]

TNF-α in some diseases like influenza and COVID-19 are associated with lung injuries. Studies have demonstrated that high levels of TNF- α are observed in plasma and alveolar fluid lavage of patients with ARDS. Elevated levels of cytokines lead to increased endothelial permeability and decreased alveolar fluid clearance caused by down-regulation of sodium channels in the epithelium.[13]

2.1 COVID-19 disease

In December 2019, Coronavirus Disease 2019 (COVID-19), a fatal zoonotic disease, occurred in Wuhan, Hubei Province, China. The disease which was caused by 2019 novel coronavirus (2019-nCoV) has rapidly spread. The pathogen could cause severe respiratory syndrome, including fever, dyspnea, and cough.[14] Along with other systematic damage like acute cardiac or kidney injury.[15]

Severe illness, of almost any etiology, is accompanied by a generalised host inflammatory response. This host immune response process is referred to as systemic inflammatory response syndrome. If this process is not controlled or is dysfunctional, it will lead to cytokine storm syndrome.[16] Cytokine storm is one of the possible mechanisms underlying rapid disease progression.[17] The COVID-19 risk is greater in older people, kids and the patients having other health problems like lung diseases, heart diseases, diabetes, and cancer.[18]

2.2 Epidemiology

Global numbers of cases and deaths continued to decrease over the past week (14-20 June 2021) with over 2.5 million new weekly cases and over 64 000 deaths, a 6% and a 12% decrease respectively, compared to the previous week. While the number of cases reported globally now exceeds 177 million, last week saw the lowest weekly case incidence since February 2021. This week, the Americas and Western Pacific Regions reported numbers of new weekly cases similar to the previous week, while the South-East Asia and the European Regions reported a decline in the number of new cases. The African Region recorded a marked increase in the number of weekly cases as compared to the previous week. Globally, mortality remains high with more than 9000 deaths reported each day over the past week, however, the number of new deaths reported in the past week decreased across all Regions except for the Eastern Mediterranean and the African Regions.[17,18]

2.3 Transmission

Many domestic and wild animals, including camels, cattle, cats, and bats, may serve as hosts for coronaviruses.[19] However, there are exceptions, such as SARS and MERS, which are mainly spread though close contact with infected people via respiratory droplets from cough or sneezing. With regard to COVID-19, early patients were reported to have some link to the Hunan Seafood Market in Wuhan, China, suggesting that these early infections were due to animal-to-person transmission. However, later cases were reported among medical staff and others with no history of exposure to that market or visiting Wuhan, which was taken as an indication of human-to-human transmission.[19]

Respiratory viruses are transmitted in three main ways

1. Contact transmission, where someone comes into direct contact with an infected person or touches a surface that has been contaminated.
2. Through droplet transmission of both large and small respiratory droplets that contain the virus, which would occur when near an infected person.
3. Through airborne transmission of smaller droplets and particles that are suspended in the air over longer distances and time than droplet transmission. However, latest research suggests that this is unlikely to be a major route of transmission as although SARS-CoV-2 can persist for days on inanimate surfaces, attempts to culture the virus from these surfaces were unsuccessful. Infection control guidelines have stated that most respiratory virus transmission occurs from large infected droplets produced by coughing, sneezing, and breathing in close proximity to another person. This understanding has led to social distancing being the cornerstone of public health advice, but confusion exists as to the safe distance required between people to reduce transmission with the WHO suggesting 1 meter (Medicine, 2020).

2.4 Classification of coronavirus

Coronaviruses belong to the subfamily *Coronavirinae* in the family *Coronaviridae* and the order *Nidovirales*. *Coronaviridae* are further subdivided phylogenetic ally into five genera.

(*Alpha coronavirus*, *Beta coronavirus*, *Gamma coronavirus*, *Delta coronavirus* and *Mu coronavirus*)

Alpha coronaviruses and *Beta coronaviruses* are found in mammals, whereas *Gamma coronaviruses* and *Delta corona viruses* are primarily found in birds. International Committee of Taxonomy of Viruses (ICTV) in 2018 claimed that *Beta coronavirus* lineage was reclassified into five subgenera, namely *Embecovirus*, *Sarbecovirus*, *Merbecovirus*, *Nobecovirus*, and *Hibecovirus*.HCoV-

229 E in the subgenus *Duvinacovirus* and HCoV-NL63 in the subgenus *Setracovirus* belong to the *Alpha coronavirus* genus. HCoV-HKU1 and OC43 in the subgenus *Embecovirus*, MERS-CoV in the subgenus *Merbecovirus*, SARS-CoV and SARS-CoV-2 in the subgenus *Sarbecovirus* belong to the genus *Beta coronavirus.*[20]

5. Coronaviruses structure

Coronaviruses (CoVs) are enveloped viruses with a single-strand, positive-sense RNA genome approximately 26–32 kb in size, which is the largest known genome among all RNA viruses. The term 'coronavirus' refers to the appearance of CoV virions considering the protruding spike proteins on their surface that look like a crown under electron microscopy ("*corona*" means crown). The first coronavirus is an infectious bronchitis virus and was isolated from chicken embryos in 1937, along with subsequent viral isolations in rodents, domestic animals, and humans. Coronaviruses have been identified in many mammalian animals including humans and avian species and can induce various severe diseases involving respiratory, gastrointestinal, enteric, and neurological systems.[21]

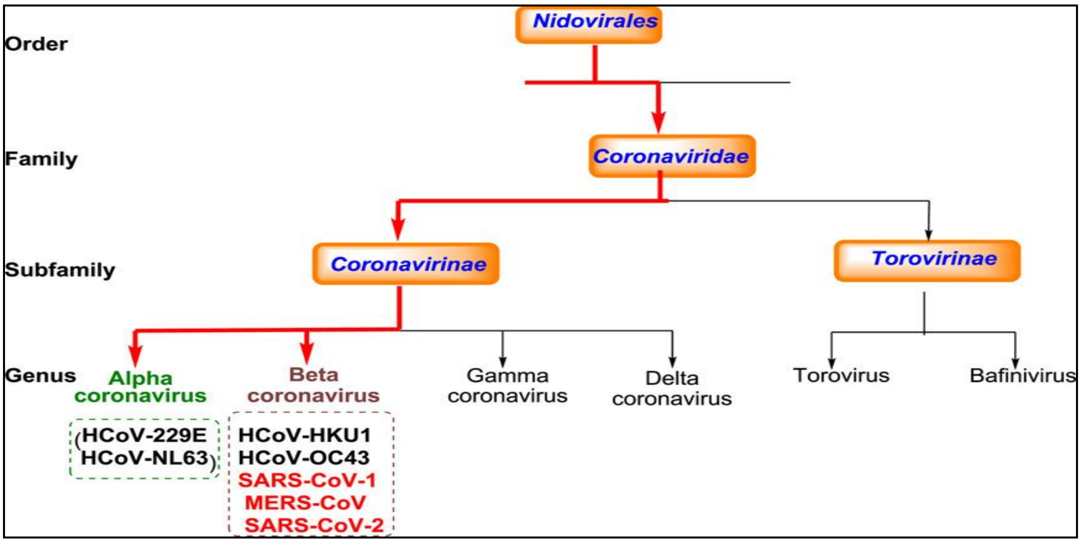

Figure 1.1: Coronaviruses classification (21).

5.1. Spike (S) protein

The coronavirus spike (S) protein is a large glycosylated transmembrane protein ranging from about 1452 to 1162 amino acid residues The S protein is the most outward envelope protein of the coronaviruses. The S glycoprotein plays critical roles in mediating virus attachment to the host cell receptors and facilitating fusion between viral and host cell.[22]

5.2. Membrane (M) protein:

Coronavirus M proteins represent the major protein component of the viral envelope. They play an essential role during viral assembly by interacting with all of the other structural proteins. Its length ranges from 217 to 230 amino acid residues in most coronaviruses.[23]

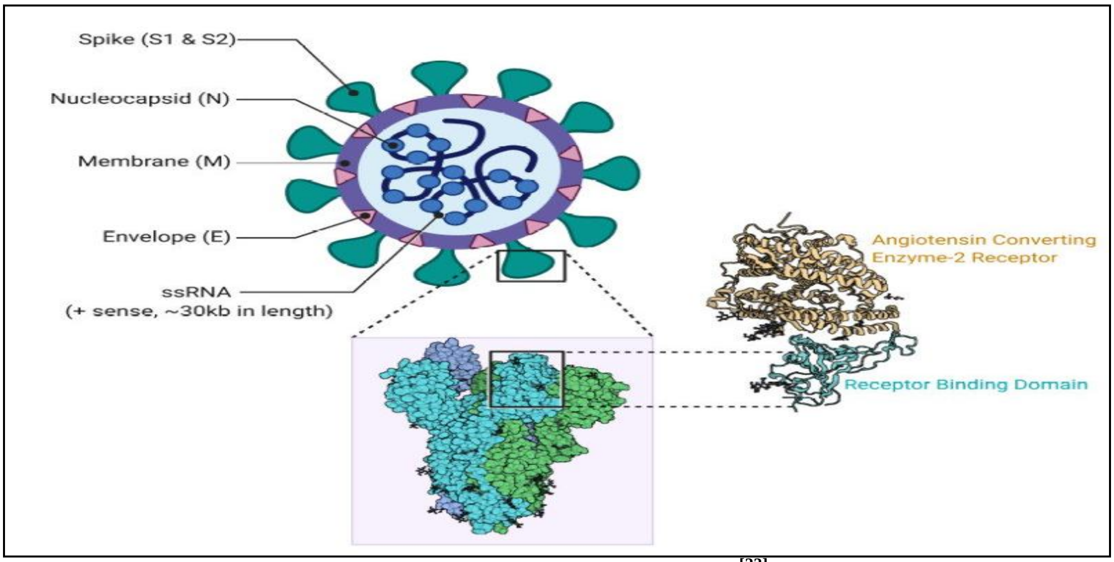

Figure 1.2: Coronavirus structure.[22]

5.3. Envelope (E) protein

The envelope (E) protein is a small integral membrane polypeptide, ranging from 76 to 109 amino acid residues with molecular weight of 8.4–12 k Da. The E protein plays important roles in a number of aspects of the coronavirus replication cycle, such as assembly, budding, envelope formation, and pathogenesis.[23]

5.4. Nucleocapsid (N) protein

The coronavirus nucleocapsid (N) protein is a structural phosphoprotein of 43–46 KDa, a component of the helical nucleocapsid. The main function of the N protein is to package the viral genome into a ribonucleoprotein (RNP) particle in order to protect the genomic RNA and for its incorporation into a viable virion. The N protein is thought to bind the genomic RNA in a beads-on-a-string fashion. In addition, it also interacts with the viral membrane protein during virion assembly and plays a critical role in improving the efficiency of virus transcription and assembly.[24]

6. Replication of coronaviruses

6.1 Attachment and entry

Several steps are necessary to start and complete the coronavirus infective cycle.

1. Recognition and binding to the cellular receptor(s).
2. Changes in the conformation and proteolysis of S protein.
3. Fusion to cellular membrane.
4. Entry of the virus into the host cells by endocytosis**.**

The genome of the coronavirus encodes a number of structural proteins that facilitate cellular entry and assembly of virions, of which the spike protein S appears to be critical for cellular entry. The spike protein guides the virus to attach to the host cell. The spike protein contains a receptor-binding domain (RBD), a fusion domain and a transmembrane domain. The RBD of spike protein S binds to Angiotensin Converting Enzyme 2 (ACE2) to initiate cellular entry.[23]

6.2 Genome replication/transcription and virion assembly and release Virus:

After entry, genomic RNA (g RNA) is translated by host ribosomes in poly protein pp1a and pp1b, which are auto-cleaved to form non-structural protein (NSP). These NSPs induce a rearrangement of cellular membrane to form double-membrane vesicles where the viral replication complexes are anchored.[23]

Using the g RNA as a template, the coronavirus replicase synthesizes full-length negative sense (−) RNA, which, in turn, serves as a template for the synthesis of new genomic (+) g RNA and a set of different sg RNA, synthetized by discontinuous transcription. These sg RNAs encode viral structural and accessory proteins.[25]

Although genome replication/transcription is mainly mediated by the viral replicase, other host factors have been involve, as an example, coronavirus N protein, known to act as an RNA chaperone to facilitate template switching, and the enzyme glycogen-synthase-kinase-3 (GSK3).[26] Finally, RNA helicases (NSP13) represent the second most conserved subunit of the RNA synthesis machinery in (+) RNA coronaviruses and are involved in diverse steps of their life cycle. They utilize the energy derived from the hydrolysis of nucleoside triphosphates to unwind double-stranded RNA.[27]

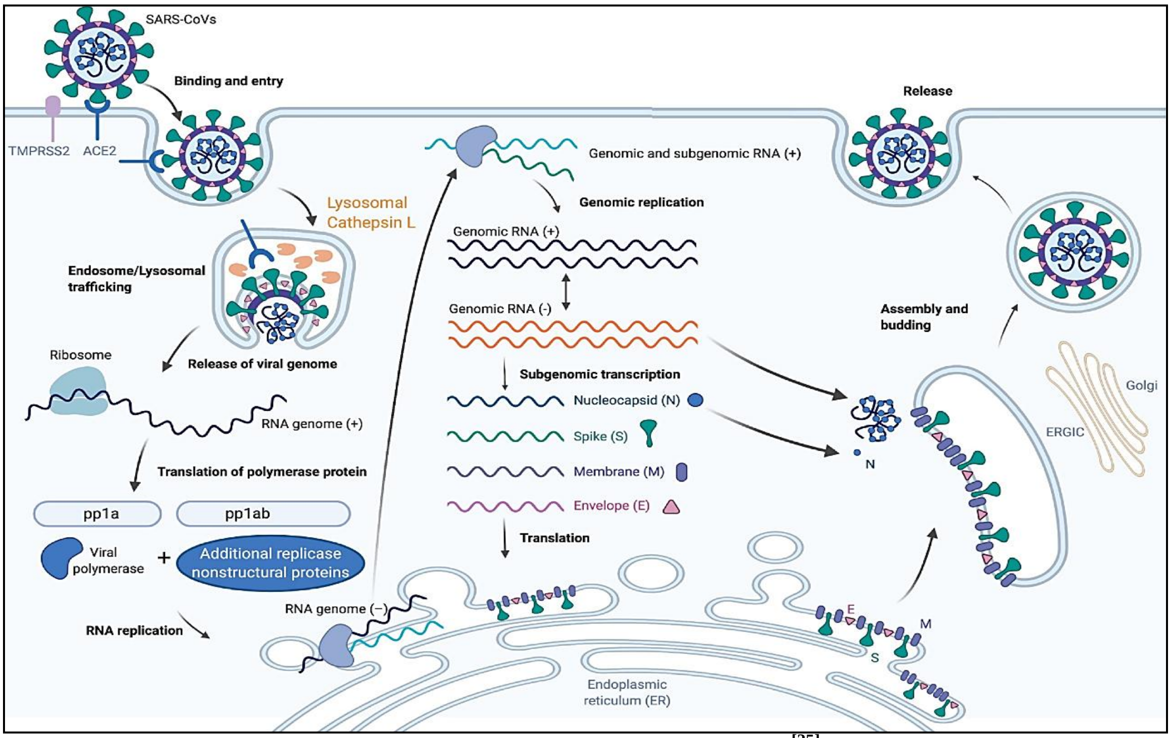

Figure 1.3: Life cycle of coronaviruses.[25]

The assembly of viral particles takes place in the ER-Golgi intermediate complex under the control of M protein through homotypic interactions. In this phase, M protein acts as a scaffold for virus assembly because the interactions between S and M and M and N proteins allow the recruitment of structural proteins to the assembly site. E protein contributes in this phase interacting with M and inducing membrane curvature.[26] Finally, mature virions are released in smooth-walled vesicles via the secretory pathway and released by exocytosis.[27]

7. Pathogenesis of COVID-19

7.1 Coronavirus entry and replication

Coronavirus S protein has been reported as a significant determinant of virus entry into host cells (De Wit *et al*., 2016). The envelope spike glycoprotein binds to its cellular receptor, ACE2 for SARS-CoV. The entry of SARS-CoV into cells was initially identified to be accomplished by direct membrane fusion between the virus and plasma membrane.[28] This a critical photolytic cleavage event occurred at SARS-CoV S protein at position (S2′) mediated the membrane fusion and viral infectivity.[29]

Besides membrane fusion, the clathrin-dependent and -independent endocytosis mediated SARS-CoV entry too. After the virus enters the cells, the viral RNA genome is released into the cytoplasm and is translated into two poly proteins and structural proteins, after which the viral genome begins to replicate.[30] The newly formed envelope glycoproteins are inserted into the membrane of the endoplasmic reticulum or Golgi, and the nucleocapsid is formed by the combination of genomic RNA and nucleocapsid protein. Then, viral particles germinate into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). At last, the vesicles containing the virus particles then fuse with the plasma membrane to release the virus.[31]

7.2 Antigen presentation in coronavirus infection

While the virus enters the cells, its antigen will be presented to the antigen presentation cells (APC), which is a central part of the body's anti-viral immunity. Antigenic peptides are presented by major histocompatibility complex (MHC); or human leukocyte antigen (HLA) in humans and then recognized by virus-specific cytotoxic T lymphocytes (CTLs). Hence, the understanding of antigen presentation of SARS-CoV-2 will help our comprehension of COVID-19 pathogenesis. Unfortunately, there is still lack of any report about it, and we can only get some information from previous researches on SARS-CoV and MERS-CoV. The antigen presentation of SARS-CoV mainly depends on MHC I molecules, but MHC II also contributes to its presentation.[32]

7.3 Humoral and cellular immunity

Antigen presentation subsequently stimulates the body's humoral and cellular immunity, which are mediated by virus-specific B and T cells. Similar to common acute viral infections, the antibody profile against SARS-CoV virus has a typical pattern of IgM and IgG production. The SARS-specific IgM antibodies disappear at the end of week 12, while the IgG antibody can last for a long time, which indicates IgG antibody may mainly play a protective role.- Comparing to humoral responses, there are more researches on the cellular immunity of coronavirus. The latest report shows the number of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of SARS-CoV-2-infected patients significantly is reduced.[34]

7.4 Cytokine storm in COVID-19

Cytokine storm is a hyper-inflammatory, pathological state that results from a sudden increase in certain circulating pro-inflammatory cytokine levels, which leads to overwhelming systemic inflammation, exacerbating viral pathogenesis and causing sepsis, ARDS, and multi-organ failure.[35] The cytokine storm has also been observed in SARS, MERS, H5N1 influenza, and H7N9 influenza, and with other respiratory viruses.[36] In severe SARS-CoV-2 infection, the total T cell counts as well as CD4+ and CD8+ T cell counts were all significantly lower than that in more moderate cases.[37]

7.5 Mechanisms of cytokine storm in covid-19:

After entering respiratory epithelial cells, SARS-CoV-2 provokes an immune response with inflammatory cytokine production accompanied by a weak interferon (IFN) response**,** this is followed by the infiltration of macrophages and neutrophils into the lung tissue, which results in a cytokine storm.[38] Particularly, SARS-CoV-2 can rapidly activate pathogenic Th1 cells to secrete pro-inflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6). GM-CSF further activates $CD14^+CD16^+$ inflammatory monocytes to produce large quantities of IL-6, tumor necrosis factor-α (TNF-α), and other cytokine. Neutrophil extracellular traps, the extracellular nets released by neutrophils, may contribute to cytokine release.[39] The cytokine storm in COVID-19 is characterized by a high expression of IL-6 and TNF-α.[40]

8. Bio chemical markers in COVID-19

8.1. D. dimer

D-dimer originate from the lysis of cross-linked fibrin with rising levels indicating the activation of coagulation and fibrinolysis.**[41]** D-dimer levels are commonly elevated in patients infected with SARS-CoV-2. Significantly higher levels are found in those with critical illness and may be used as a prognostic marker for in hospital mortality.[42]

D-dimer was associated with mortality and severe COVID-19. This finding supports the hypothesis that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection could induce the dysfunction of the hemostatic system, leading to a hypercoagulable state, a condition which we commonly encounter in sepsis.[43] Recent evidence of lung pathology dissection has shown occlusion and micro-thrombosis formation in pulmonary small vessels of patients critically ill with COVID-19.[44]

8.2. S. ferritin

Ferritin is a useful marker to predict the outcomes in COVID-19. Hyperferritinemia can activate macrophages, which increases the secretion of pro-inflammatory cytokines, and the subsequent inflammation is mainly responsible for organ damage. Although ferritin is known as a positive acute phase reactant and serum level of ferritin intracellular protein increases during inflammation, dying cells may also release the ferritin.[45]

8.3. C. reactive protein (CRP):

C. reactive protein (CRP) is an acute phase inflammatory protein produced by the liver that may be elevated in several conditions, such as inflammation, cardiovascular disease, and infection.[46] CRP has been suggested to be used as a prognostic marker, and higher levels of CRP indicating increased risk of disease progression.[47] Increased CRP levels might be early indicators of nosocomial infections in COVID-19 patients.[48]

8.4. Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is a glycolytic cytoplasmic enzyme present in virtually every tissue. In general, its elevation suggests tissue injury. Possible subclinical tissue damage was indicated by our observation of increased LDH in the early stage of extreme COVID-19 cases.[49] Elevated LDH has been associated with a higher risk of ARDS, need for intensive care and mortality.[50]

8.5. Liver enzymes in COVID -19

SARS-CoV-2 virus may bind to angiotensin-converting enzyme 2 (ACE2) on cholangiocytes, leading to cholangiocyte dysfunction and inducing a systemic inflammatory response leading to liver injury.[51] Cytokine storm caused by excessive immune response induced by the virus may also be one of the pathways of liver damage.[52] However, ALT, AST, total bilirubin and other liver function indices were significantly increased in patients with severe COVID-19 compared to patients with mild COVID-19, and the liver function indices gradually returned to normal during recovery.[53]

9. Immunological markers

9.1 Interleukin -6 (IL – 6)

Interleukin -6 (IL-6) is an important member of the cytokine network and plays a central role in acute inflammation.[54] Is a multifunctional cytokine that plays an important role in human metabolism, autoimmune cell differentiation, disease treatment, etc.[55] IL-6 is a small polypeptide consisting of four α helices. It has a molecular weight of 19–28 K Da and comprises 184 amino acid residues, usually in monomer form, with an isoelectric point of 5.0, glycosylation sites and two disulfide bonds.[56] IL-6 can be produced by almost all stromal cells and immune system cells, including B-lymphocytes, T-lymphocytes, macrophages, monocytes, dendritic cells, mast cells, and other non-lymphocytic cells such as fibroblasts, endothelial cells, keratinocytes, glomerular mesangial cells and tumor cells.[57]

The main activators of IL-6 expression are IL-1β and tumor necrosis factor-alpha (TNFα). However, there are also other ways to promote the synthesis of IL-6, such as Toll-like receptors (TLRs), prostaglandins, adipokines, stress response and other cytokines.[58] Interleukin 6 (IL-6) were found in the acute stage associated with lung lesions in SARS-CoV-1 patients. IL-6 can induce the hyper-innate inflammatory response due to the SARS-CoV-1 invasion of the respiratory tract.[59]

This happens also with SARS-CoV-2 in COVID-19 patients: some retrospective and meta-analysis studies show how elevated IL-6 and C-reactive protein (CRP) correlate with mortality and severe disease in comparison to moderate disease.[60, 61] IL-6 levels in patients with severe COVID-19, and this viral load is associated with ARDS severity and lung tissue damage.[62]

Shock and organ failure in several organs, such as the kidneys, heart, lungs, and liver, are severely damaged by cytokine storms caused by an increase in inflammatory cytokines, including IL-6, IL-1β, TNF-α, IL-8, IL-2, IL-17, G-CSF, GMCSF, in patients with COVID-19.[63,64] These cytokines can also cause extensive pulmonary damage through the accumulation of neutrophils and macrophages in lung tissue, leading to the development of hyaline membranes and diffuse thickening of the alveolar barrier and ultimately diffuse alveolar damage.[65]

9.2 Tumor necrosis factor alpha:

Tumor necrosis factor (TNF)-α, an important member of the TNF superfamily of ligands, is a pleiotropic pro-inflammatory cytokine.[66] In monocytes and macrophages, lipopolysaccharide (LPS) induces TNF-α expression by activating early growth response factor-1 (Egr-1), activator protein-1 (AP-1) and nuclear factor-κB (NF-κB).[67, 68, 69]

TNF-α is synthesized as a monomeric Type II protein containing 233 amino acid (27 K Da), and then it is arranged in stable homotrimers as transmembrane TNF-α. Three monomers associate around a 3-fold axis to form a compact bell-shaped trimer. This structure is typical for most members of the TNF family but comparison to known protein structures also shows structural homology to several viral coat proteins.[70, 71]

TNF-α plays key roles in immune responses and tissue homeostasis. Despite the role of TNF-α signaling in combating viral infections, high levels of TNF-α in some diseases like influenza and COVID-19 are associated with lung injuries.[72-74] Studies have demonstrated that high levels of TNF- α are observed in plasma and alveolar fluid lavage of patients with ARDS. Elevated levels of cytokines lead to increased endothelial permeability and decreased alveolar fluid clearance caused by down-regulation of sodium channels in the epithelium.[75-78] The main sources of TNF-α are activated monocytes, fibroblasts, and endothelial cells.[79]

CONCLUSIONS

The mean age of the mild COVID-19 patients is lower than the mean age of the moderate and severe cases. Lymphopenia and neutrophilia in addition to increased total blood cell counts appears in the most severe cases (moderate to severe) but not in the mild cases. Only C.reative protein is shown to be elevated in mild cases in addition to other severity groups. Whereas other biomarkers such as D. dimer, S. ferritin were elevated in the more severe forms of the COVID-19 disease. The levels of both of IL-6 and TNF-α were elevated in all severity groups, however, moderate and severe forms characterized by higher levels of the cytokines. There was a positive correlation between IL-6 and neutrophils indicating that neutrophils production is affected by this pro-inflammatory cytokine. A negative correlation between TNF-α and lymphocytes may indicate that this cytokine possibly has a direct effect on lymphocyte production in bone marrow.

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