Original Research Article

ASSESSMENT OF SOME INFLAMMATORY CYTOKINES OF INTEREST IN IN PATIENTS WITH SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 ADMITTED IN ISOLATION CENTERS IN PORT-HARCOURT

Comment [S1]: This manuscript is one of the important topics that need many studies:
The language needs to be revised

ABSTRACT

Coronavirus disease 2019 (COVID-19) is a novel and highly contagious viral infection caused by sars-cov-2, and has been associated with hyper-inflammatory immune response. This research aimed at assessing alterations in some inflammatory cytokines as clinical biomarkers to help with the management of COVID-19 progression effectively in Port Harcourt. A case control study design was employed in this study where a total of one hundred and ten (110) subjects were recruited comprising of fifty five (55) COVID-19 positive subjects and fifty five (55) COVID-19 negative subjects (control) within the ages of twenty (20) to seventy (70) year old and were both male and female subjects. Ten millilitres (5ml) of whole blood was collected using standard venipuncture technique with sterile hypodermic syringes and needles aseptically and dispensed into a plain bottle for the analysis of inflammatory cytokines. For the confirmation of COVID-19 positive, nasopharyngeal swab was collected aseptically using RT-PCR technique. The results in this study revealed a significantly increased level of IL-6 (p=0.0399) among subjects with COVID-19. The results from this study indicate significant alterations in inflammatory cytokines among subjects with COVID-19. It is necessary that tertiary health care settings and isolation centers should consider for effective management of patients from mild to severe COVID-19 with the following parameters; IL-6.

INTRODUCTION

1.1 Background of the Study

Since December 2019, there has been a serious pandemic hit worldwide by the coronavirus disease (commonly known as COVID-19), which has presented several challenges on the livelihood of individuals, health systems and socioeconomic aspects of life (WHO, 2020a; Jayasinghe*et al.*, 2020). These challenges may be attributed to the uniqueness and dynamics in which the disease is transmitted, as well as the symptoms and immune response associated with the disease (UNICEF, 2020).

Coronaviruses belong to a large family of single-stranded RNA, enveloped and non-segmented positive-sense viruses capable of infecting animals and humans, thereby causing diseases of the respiratory and gastrointestinal tracts, liver, and nerves (Weiss and Leibowitz, 2013). Coronaviruses are enveloped and non-segmented positive-sense RNA viruses that belong to the Coronaviridae family in the order of Nidovirales. They have a wide-spread in humans and other mammals (Richman *et al.*, 2016).

They are the largest known RNA viruses to have existed, and are grouped into four genera, including alpha-coronaviruses, beta-coronaviruses, gamma-coronaviruses, delta-coronaviruses and Omicron coronaviruses (Yang and Leibowitz, 2015). About six different types of human coronaviruses have been identified which include alphacoronaviruses such as NL63 and 229E, betacoronaviruses such as OC43, and HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV) (Zaki*et al.*, 2012; Drosten*et al.*, 2020). In humans also, a periodic emergence of new types of coronaviruses have been reported, and this may be attributed to the wide-spread and high prevalence of the coronaviruses; it may also be due to the fact that these viruses have a large genetic diversity, with their genomes undergoing regular recombination, and also due to an increase in human to animal interface activities (Cui *et al.*, 2019; Zhu *et al.*, 2020).

In the later part of December 2019, many individuals were reported by some local health authorities to have developed pneumonia whose origin was unknown; this was later discovered to be related to a seafood market in Wuhan, Hubei Province, China (Zhu *et al.*,

2020), followed by the identification of a novel coronavirus known as severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) by local hospitals (Li *et al*., 2020a; Zhu *et al*., 2020). However, on the 30th day of January 2020, COVID-19 was declared by the World Health Organization as a "public-health emergency of international concern" (Li *et al*., 2020b). On the 2nd day in the month of December 2020, over 64 million cases worldwide and less than 1.5 million deaths were recorded (Beaney*et al.,* 2022).

Coronaviruses have their genome wrapped by a capsid that is shaped like a helix, and an envelope made of lipoprotein; the envelope contains several spicules of glycoprotein making the virus appear like a crown (Li, 2016). The "term corona" was originated in Latin, meaning crown (Li, 2016). After infecting humans, coronaviruses undergo an incubation of about 2 to 5 days, after which they induce various diseases such as common cold (due to infection of the upper respiratory tract), liver disease, enteric fever or enteritis, and neurological diseases **(Cucinotta and Vanelli 2020).** Also, pneumonia and bronchitis may occur due to infection of the lower respiratory tract, and severe acute respiratory syndrome (SARS) (Schoeman and Fielding, 2019; Woo *et al*., 2009). Other symptoms of COVID-19 may include fever, dry cough, headache, myalgias, dyspnoea and fatigue (Jayasekara*et al*., 2020). The severe acute respiratory syndrome coronavirus (SARS-CoV) (Cheng *et al*., 2007); the coronavirus of the Middle East respiratory syndrome (MERSCoV) (Chan *et al*., 2015), or the coronavirus of severe acute respiratory syndrome 2 (SARS-CoV-2) (Zhu *et al*., 2020) have been implicated in the causation of severe acute respiratory syndrome coronavirus (SARS).

Most of the infections caused by coronaviruses in humans seem to be mild, however over ten thousand cases within the past two decades were caused by the two betacoronaviruses namely SARS-CoV-2 (Li, 2016) and Middle East respiratory syndrome coronavirus (MERS-CoV) (Hui, 2005) with a death rates of 10 percent and 37 percent respectively (Chan *et al*., 2015; Zhu *et al*., 2020). The incubation period and the clinical course of MERS are similar to that of SARS, except that a larger percentage of cases progress to respiratory deterioration and distress in MERS (Pal *et al.,* 2020).

Infection with the SARS-CoV-2 has been reported to induce alterations in the physiology of the human body including inflammatory and pro-inflammatory cytokines (Rahman *et al*., 2021).

In order to effectively control the spread of the coronavirus, and offer appropriate treatment to infected patients, laboratory testing must be conducted. The most appropriate samples used include nasopharyngeal and oropharyngeal swabs, which are high priority specimens. Others are lower priority specimens, which include broncho-alveolar lavage, tracheal aspirates, and sputum (CDC, 2020). Currently, the gold standard method of laboratory molecular diagnosis of SARS-CoV-2 infection is the real-time reverse transcriptase-polymerase chain reaction (RT-PCR), which is used for qualitative and quantitative detection of the viral nucleic acids (Rahbari*et al.,* 2021). Other relevant laboratory methods may include enzyme-linked immunoassays (EIA) for detecting viral antibody/antigen, and serum viral neutralization (SVN) assay for determining antibody neutralization (D'Cruz*et al.,* 2020).

1.2 Statement of the Problem

There has been discrepancies on reports from previous studies on COVID-19 pandemic as it affects the inflammatory cytokines of patients suffering from the disease. However meta-analysis performed on some of the reports revealed a significant increase in inflammatory cytokines. And most of these reports were from China, United States, Spain, Italy, Germany, France, Iran, Turkey and United Kingdom.

In order to effectively control the spread of the corona-virus and offer appropriate treatment to infected patients, physicians and the world health organization are consciously interested in getting data from different parts of the world on the pathogenicity and pathophysiology of the COVID-19.

This study considered data from Port Harcourt, Rivers State to evaluate how COVID-19 infection affects the above parameters in the patient's resident in the area. The observed changes adding to what has previously been reported would lead to finding out what is responsible for the changes.

1.3 Justification of the Study

Several studies related to this research topic showed conflicting reports; some reported significant increases in serum IL-6, IL-1β and TNF-α. Also, there is paucity of data for the assessment of some inflammatory cytokinesin subjects with severe acute respiratory syndrome coronavirus 2 in Port-Harcourt City, hence the need for the study.

1.4 Significance of the Study

The findings from this study will help give a presentation of the inflammatory cytokines in patients infected with SARS-CoV-2, which will further help clinically in managing these patients effectively.

1.5 Aim of the Study

The aim of the study is to assess some inflammatory cytokines in subjects with severe acute respiratory syndrome coronavirus 2 in Port-Harcourt

1.6 Objectives of the Study

The objectives of this study are to:

1. Determine the serum levels of IL-1β in subjects with COVID-19 infection and control subjects.
2. Determine the serum levels of IL-6 in subjects with COVID-19 infection and control subjects.
3. Determine the serum levels of TNF-α in subjects with COVID-19 infection and control subjects.
4. Compare the levels of the inflammatory cytokines among subjects according to sex
5. Compare the levels of the inflammatory cytokines among subjects according to age

1.7 Scope of the Study

This study is limited to patients with confirmed cases of COVID-19 on admission in the isolation centers and at home care treatment in Port Harcourt, Rivers State with or without symptoms with special consideration of the following variables inflammatory cytokines (IL-1β, IL-6 and TNF-α).

1.8 Research Questions of the study

1. Are there alterations in serum IL-1β among subjects with COVID-19 infection?
2. Are there alterations in serum IL-6 among subjects with COVID-19 infection?
3. Are there alterations in serum TNF-αamong subjects with COVID-19 infection?
4. Are there alterations in some inflammatory parameters among subjects with COVID-19 infection according to sex?
5. Are there alterations in some inflammatory parameters among subjects with COVID-19 infection according to age

1.9 Research Hypotheses

The null and alternative hypothesis of this study is as follows:

1. Ho: There is no significant difference in serum IL-1β among subjects with COVID-19 infection compared of the control subjects.
   H_1: There are significant differences in serum IL-1β among subjects with COVID-19 subjects compared with the control subjects.
2. Ho: There is no significant difference in serum IL-6among subjects with COVID-19 infection compared of the control subjects.
   H_1: There are significant differences in serum IL-6 among subjects with COVID-19 subjects compared with the control subjects.
3. Ho: There is no significant difference in serum TNF-αamong subjects with COVID-19 infection compared of the control subjects.
   H_1: There are significant differences in serum TNF-αamong subjects with COVID-19 subjects compared with the control subjects.
4. Ho: There is no significant difference in some inflammatory parameters among subjects with COVID-19 infection compared of the control subjects according to sex.
   H_1: There are significant differences in some inflammatory parameters among subjects with COVID-19 subjects compared with the control subjects according to sex.
5. Ho: There is no significant difference in someinflammatory parameters among subjects with COVID-19 infection compared of the control subjects according to age.
   H_1: There are significant differences in some inflammatory parameters among subjects with COVID-19 subjects compared with the control subjects according to age

LITERATURE REVIEW

2.1 Epidemiology of the COVID-19 Outbreak

In some hospitals in the city of Wuhan in China, several cases of pneumonia without explainable cause were reported since December in 2019 (Wu*et al.,* 2020) History-taking revealed that the cases were as a result of exposure to a seafood market located in the city of Wuhan, in Hubei province, in China. Soon enough, there was a confirmation that the pneumonia was an acute infection of the respiratory tract induced by a novel coronavirus. This disease continued to spread rapidly throughout the city of Wuhan to the whole of China, and then to other parts of the world, such that, advancement of this disease was reported where there was an emergence of some confirmed cases with no history of transit to the city of Wuhan or visit to the seafood market in Wuhan (Jin*et al.*, 2020; Stoeckli*net al*., 2020). On the 2nd day of March 2020, the National Health Commission of the People`s Republic of China reported a total of 80, 302 patients who had tested positive to the SARS-CoV-2 in China with 2947 (3.66 percent) mortality (Wu*et al.,* 2020). Also, it was reported that as of the 11th day of February 2020, about 1715 medical workers had been infected with the SARS-CoV-2, with 5 mortalities (WHO, 2020b). However, 10,415 COVID-19 cases were been confirmed internationally (outside of China) in 66 countries and 6 continents (WHO, 2020b). On the 14th day of April 2020, the WHO declared SARS-CoV-2 as a pandemic, with a record of 1,844,683 confirmed cases and 117,021 mortality all over the globe (Kumar *et al.*, 2020). In order to characterize the novel coronavirus, swabs from both the throat and Bronchoalveolar lavage fluid were obtained from nine patients who visited the Wuhan seafood market during the previous outbreak. The obtained samples were inoculated into special pathogen-free human airway epithelial (HAE) cells (used to isolate the virus) through the apical surfaces; these HAE cells were then monitored for cytopathic effects, and supernatant was collected to perform real-time polymerase chain reaction assays. However, phylogenetic analysis revealed that SARS-CoV-2 originated from bats (Andersen *et al.*,

2020). Contrarily, some studies suggest that the origin of SARS-CoV-2 is related with pangolins (Li *et al.* 2020a; Shereen*et al*., 2020).

2.2 SARS-CoV-2 Morphology

Using electron microscopy, it was revealed that the size of the SARS-CoV-2 ranges between 70 and 90 nm (Park *et al.*, 2020). The structure of SARS-CoV-2 is said to be the same with that of SARS-CoV due to high similarity in sequencing (Kumar *et al.*, 2020). The surface of the virus contains spike (S) proteins, which give them the appearance of a crown. Other structural proteins present on the surface of the SARS-CoV-2 include the envelope (E), membrane (M), nucleocapsid (N), and internal (I) protein (Weiss, 2011). However, the surface of the spike proteins, membrane, and envelope of the SARS-CoV-2 are embedded in the lipid-bilayer derived from the host membrane which encapsulates the helical nucleocapsid comprising the viral RNA (Finlay *et al.*, 2004). The structure of the SARS-CoV-2 is represented in Figure 1.

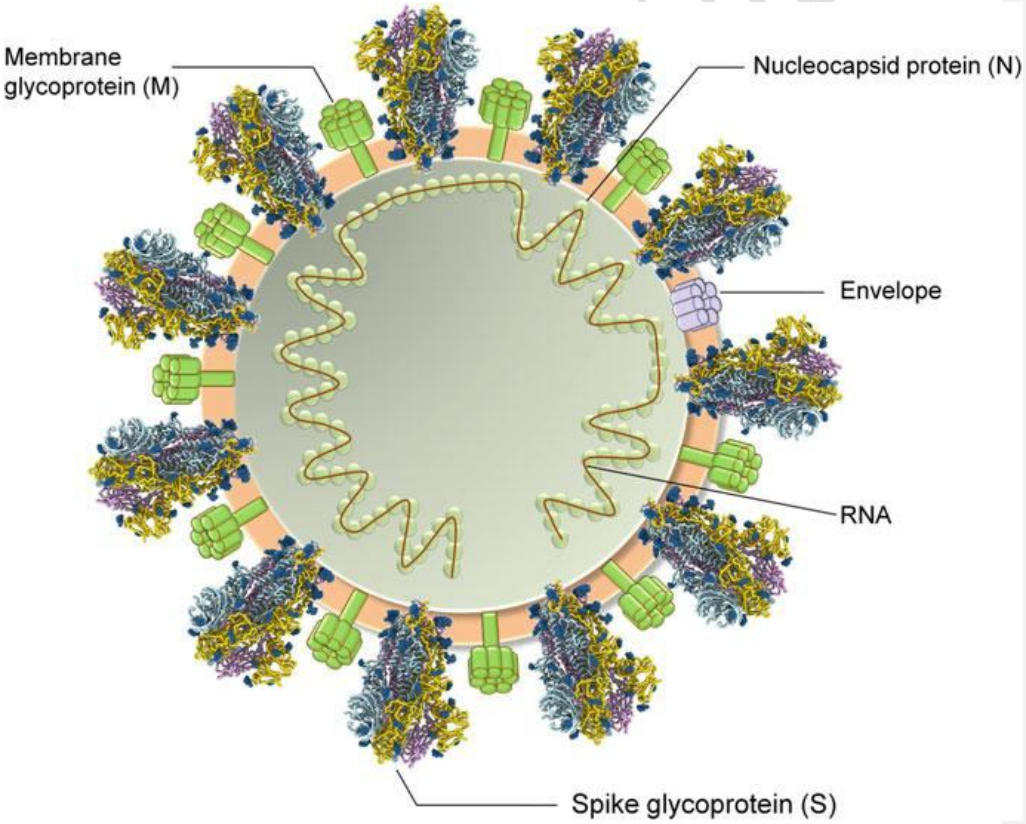

Figure 1: Structure of SARS-CoV-2 (Saxena*et al.*, 2020)

2.3 The Organization of the Genome of SARS-CoV-2

The genome of SARS-CoV-2 ranges between 26 and 32kb in size, and consists of about six to eleven open reading frames (ORF) which encode poly-proteins of 9680 amino acids (Luo *et al.*, 2020). About 67 percent of the genome is contained in the first open reading frame; this genome encodes sixteen non-structural proteins. On the other hand, accessory and structural proteins are encoded by the remaining oral reading frames. Unlike some other types of coronaviruses, SARS-CoV-2 does not have the haemagglutinin esterase gene, but it contains two flanking regions that are not translated at 50 and 30 ends of 265 and 358 nucleotides respectively (Luo *et al.*, 2020). The non-structural proteins (nsps) present are two cysteine proteases of the virus, such as the protease that is like a papain (nsp3), protease that is like chymotrypsin, protease that is like 3C, or main protease (nsp5), RNA-dependent RNA polymerase (nsp12), helicase (nsp13), and others with possible likelihood of getting involved in the transcription and replication of SARS-CoV-2 (Chan *et al.*, 2020).

In addition to the presence of non-structural proteins, 4 main structural proteins are encoded by open reading frames; these proteins include envelope (E) protein, nucleocapsid (N) protein, membrane (M) protein, surface spike (S) glycoprotein and accessory proteins. Both membrane and envelope proteins are needed for morphogenesis, assembly, and budding of the virus, while the S glycoprotein consists of two subunits, namely S1 and S2; the S1 and S2 subunits share 70 percent and 99 percent sequence similarity with bat SARS-like coronavirus and human SARS-coronavirus respectively (Chan *et al.*, 2020).

The S1 subunit is made up of signal peptide, N-terminal domain (NTD), and receptor-binding domain (RBD) (Walls *et al.*, 2020), while the S2 subunit is made up of two heptad repeat regions called HR-N and HR-C, which form the coiled structures enveloped by the protein ecto-domain (Coutard*et al.*, 2020).

2.4 The Entry and Replication of SARS-CoV-2 in Host Cells

Coronaviruses are spherical in shape, and are single-stranded RNA with a diameter ranging between 80 and 220 nm (Kamimura*et al.,* 2021). SARS-CoV-2 is transmitted either via exposure to micro-droplets from infected persons or by getting direct contact with contaminated fomites (Samprathi and Jayashree, 2021).

Coronaviruses enter into the target cells of the host by binding the host cellular receptor with the spike (S) glycoprotein; this is followed by priming of the spike glycoprotein by proteases of the host cells. Both SARS-CoV and SARS-CoV-2 use angiotensin-converting enzyme (ACE) 2 receptor for entrance into the host cells, and transmembrane serine protease 2 (TMPRSS2) for the priming of S protein (Hoffmann *et al.*, 2020). Angiotensin-converting enzyme-2 (ACE-2) is a membrane carboxypeptidase present in distal airways and alveoli, especially type 2 pneumocytes (which contains the largest ACE-2 expression together with alveolar macrophages and dendritic cells). Severe acute respiratory syndrome coronavirus 2 (ARS-CoV-2) may be seen in tissues outside the lungs, and this may be so because the ACE2 receptor is widely spread in those tissues; Agrawal *et al.* (2020) reported that ACE-2 is also expressed on the vascular endothelium, nasal, oral, nasopharyngeal and oropharyngeal epithelia, gut epithelia, cardiac pericytes, renal proximal tubular cells and in the skin, reticuloendothelial and the central nervous system. However, the expression of ACE-2 is dependent on age, gender, genetic factors, and presence of comorbid conditions such as obesity, chronic cardiopulmonary disease, cancer, and use of immunosuppressive drugs.

The virus enters the smaller airways and alveoli, and targets the bronchial and alveolar epithelial cells. Wrapp*et al.* (2020) reported that the spike glycoprotein of SARS-CoV-2

reveals higher affinity of about 10 to 20 times when compared to that of SARS-CoV. Therefore, when the spike protein gets bound to the ACE2 receptor, the virus enters into the host cell via the endosomal pathway, leading to some conformational changes in the spike glycoprotein, which then causes the envelop protein of the virus to fuse with the membrane of the host cell (Coutard*et al*., 2020). Then, the RNA of the virus becomes released into the cytoplasm of the host; this viral RNA then gets translated to generate replicase poly-proteins pp1a and pp1b that, in turn become broken down into small proteins catalyzed by proteinases encoded by the virus. Furthermore, the virus gets replicated though a process that involves the ribosomal frame shifting during the translation process; this produces genomic and subgenomic (multiple copies) species of RNA by discontinuous transcription that encodes for relevant viral proteins. The assembly of virion occurs through interaction of viral RNA and protein at the endoplasmic reticulum (ER) and Golgi complex; these virions are subsequently released out of the cells via vesicles (Hoffmann *et al.*, 2020). The diagrammatic representation of the entry and replication of SARS-CoV-2 in host cells is shown in Figure 2.

The alveolar epithelial cells, lymphocytes, and vascular endothelial cells are the primary targets of the virions. The virus inhibits the production of interferons which are part of cellular defense mechanisms. Viral replication releases a large number of virions leading to infection of neighboring target cells and viremia, which then cause an exaggerated pulmonary and systemic inflammatory response respectively (Samprathi and Jayashree, 2021).

2.5 Pathogenesis of SARS-CoV-2

Patients infected with SARS-CoV-2 share similar pathological findings with those infected with SARS-CoV and MERS-CoV(Rabaan*et al.,* 2021). There was a significant decrease in CD4 and CD8 T cell counts following cytometric analysis of peripheral blood samples (Zhang *et al.,* 2008). Also, chest X-ray images revealed a rapidly progressing pneumonia with apparent variations between the right and left lung; a biopsy of the lung was used for histological analysis, and the result revealed cellular fibromyxoid exudates with bilateral diffuse alveolar damage (Averyanov*et al.,* 2020). In the right lung, pneumocytes were prominently desquamated, and a hyaline membrane was formed, which indicates signs of acute respiratory distress syndrome (ARDS). In the left lung however, a hyaline membrane was formed accompanied by pulmonary edema (Xu *et al.*, 2020). Additionally, in both lungs, lymphocytes were found to dominate interstitial mononuclear patchy inflammatory infiltrates (Tian *et al.*, 2020).

The spaces within the alveolar were said to contain syncytial cells containing multiple nucleus, with irregular enlarged pneumocytes revealing cytopathic effect induced by the virus (Xu *et al*., 2020). A biopsy of the liver of patients infected with SARS-CoV-2 was used for histological analysis, and revealed moderate steatosis in microvesicles, and mild portal and lobular activity, which suggest the presence of the virus-induced or drug-induced injury. A biopsy of the heart tissue was also used for histological analysis, and the result revealed the presence of few interstitial mononuclear inflammatory infiltrates in the heart tissue (Xu *et al.*, 2020).

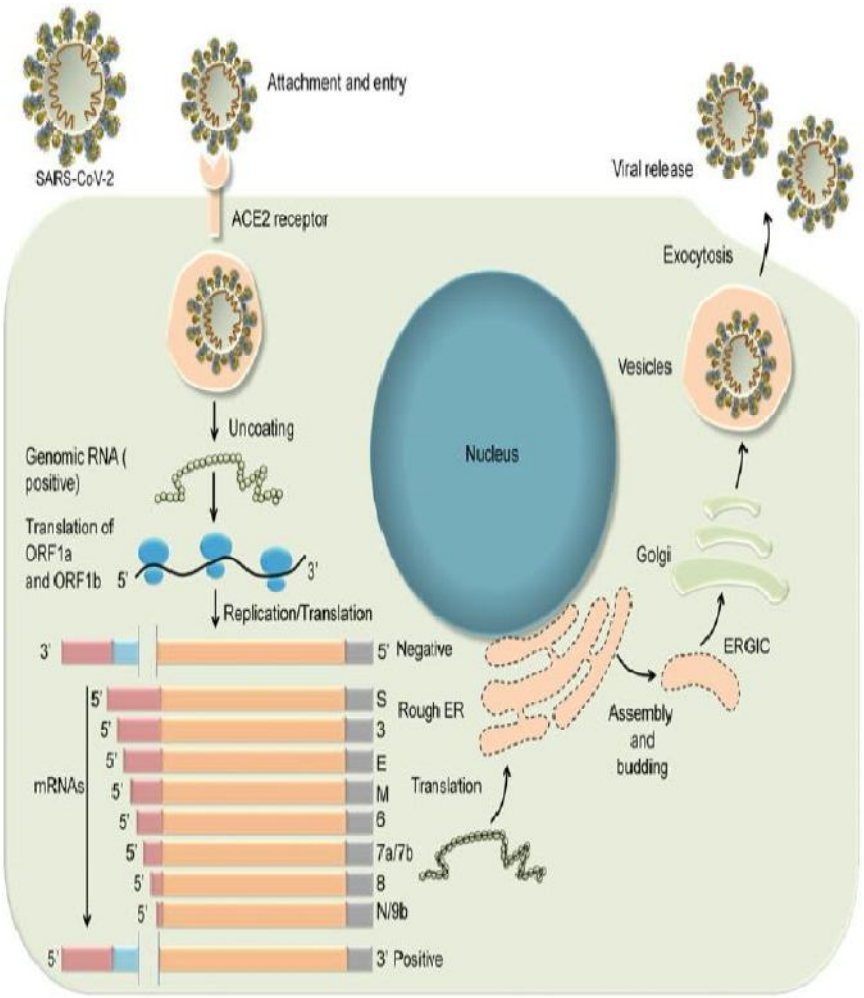

Figure 2: Entry and Replication of SARS-CoV-2 in Host Cells (Saxena*et al.*, 2020)

2.6 Clinical Manifestations of SARS-CoV-2 Infection

Patients with SARS-CoV-2 present with several clinical manifestations ranging from mild, moderate, and severe. However, most of the patients with SARS-CoV-2 were relatively normal and mild, with lower mortality compared to those with SARS-CoV and MERS-CoV (Wu *et al.*, 2020). The clinical manifestations of COVID-19 are not specific and the disease presentation can range from no symptoms (asymptomatic) to severe pneumonia and death. The incubation period of SAR-CoV-2 is between 2 to 5 days. The infections caused by this virus include mild upper respiratory symptoms (like the "common cold"), lower respiratory tract infections (such as bronchitis and pneumonia), fever, headache, and myalgias. Also, respiratory symptoms such as cough and dyspnoea often develop from several days to a week after the onset of the illness; pneumonia and respiratory deterioration takes place in 20 to 30 percent of cases (Li *et al.*, 2020a).

Interestingly, the incubation period and the clinical course of MERS are similar to that of SARS, except that a larger percentage of cases progress to respiratory deterioration and distress. The incubation period and clinical course of SARS-CoV-2 infection are probably similar to that of SARS (Huang *et al*., 2020; Wang *et al*., 2020). Individuals with severe SARS-CoV-2 infection may present with shortness of breath, moist rales in lungs, weakened breath sounds, dullness in percussion, and increased or decreased tactile speech tremor (Wu *et al*., 2020).

X-ray examination of the chest during the early stages of pneumonia cases, reveals multiple small patchy shadows and interstitial changes (Chaolin*et al*., 2020), remarkable in the lung periphery (Fuk-Woo *et al.*, 2020). Severe cases can further develop to bilateral multiple ground-glass opacity, infiltrating shadows, and pulmonary consolidation, with infrequent pleural effusion. A *computerized tomography*(CT) scan of the chest reveals pulmonary lesions more clearly than does X-ray examination, as well as ground-glass opacity and segmental consolidation in bilateral lungs, especially in the lung periphery. In children with severe infection, multiple lobar lesions may be present in both lungs (Wu *et al*., 2020).

2.7 Relationship between Inflammatory Cytokines and SARS-CoV-2 Infection

There are two phases of immune response to COVID-19 infections: the first phase entails the onset and viral incubation (non-severe) stages, in which a specific adaptive immune response is essential to control proliferation of the virus, eradicate the virus, and prevent progression of the disease. The second phase entails, release of cytokines in certain patients (with co-morbidities, of older age, and probably with specific genetic background), in which the cytokine release syndrome (so-called cytokine storm) enhances the progression of the disease from mild to severe, leading to organ damage (Shi *et al.*, 2020).

SARS-CoV-2 infection induces activation of the immune system through different receptors including the Toll-like receptors (TLR-3, TLR-4, and TLR-7) **(Osorio *et al.*, 2020).** When bound to the Toll-like receptors, SARS-CoV-2 activates the formation of active interleukin (IL)-1b and IL-6; these two cytokines are the central pro-inflammatory molecules that enhance the systemic clinical symptoms (such as malaise, fever, myalgia), leading to inflammation of the lungs (Conti *et al.*, 2020).

Inflammatory cytokines and chemokines, such as interleukin (IL)-2R, IL-6, IL-8, IL-10, and tissue necrosis factor (TNF)-alpha were elevated in severe cases of SARS-CoV-2 infection compared to mild infection (Chen *et al*., 2020; Gong *et al.*, 2020).

2.7.1 Role of Interleukin-1 in COVID-19 Infection

The cytokine storm following hyperactivated immune responses due to SARS-CoV-2 infection is probably the crucial source of severe pneumonia that leads to acute lung injury, systemic inflammatory response syndrome, or acute respiratory distress syndrome, and finally multiple organ dysfunction syndromes, as well as death in many cases (Mardi *et al.,* 2021). Several studies revealed that interleukin (IL)-1b levels were elevated during COVID-19 infection (Mardi *et al.,* 2021). In addition, the IL-1 cytokine family has a pivotal role in the induction of cytokine storm due to uncontrolled immune responses in COVID-19 infection.

Interleukin (IL)-1b is the most investigated member of the IL-1 family (IL-1F) because of its functions in regulating autoinflammatory diseases. Interleukin 1 beta (IL-1b) has an effective pyrogenic effect. It stimulates immune cells and increases the upregulation of adhesion molecules on endothelial cells, thus promoting activated immune cells such as neutrophils to migrate to the infection sites (Newton and Dixit, 2012). Interleukin 1 beta (IL-1b) can be produced from several cell types. Blood monocytes, dendritic cells, and tissue macrophages are the main sources of IL-1b in the body (Ma *et al.,* 2003). Interleukin 1 beta (IL-1b) is also produced by NK cells and B lymphocytes (Netea*et al.*, 2002). Interleukin 1 beta (IL-1b) can induce the expression of multiple genes that regulate fever, hypotension, pain threshold, and

vasodilatation (Mardi *et al.,* 2021). Unlike other cytokines, IL-1F members act indirectly in the immune system. For example, IL-1b promotes the induction of type 2 phospholipase A, cyclooxygenase type 2, inducible nitric oxide (NO) synthase, which accounts for platelet-activating factor, prostaglandin E2, and NO synthesis, respectively (Dinarello 2002). During the activation of pattern recognition receptors (PRRs), which recognize pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), IL-1b is produced by tissue-resident macrophages (Krishnaswamy*et al.,* 2013). The most important PRR in macrophages is the intracellular nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3), Nucleotide-binding oligomerization domain-like receptors(NOD-like), acts as a latent monomer in inactive cells. Several signals are necessary for the activation of the NLRP3 inflammasome. Once stimulated, NLRP3 employs procaspase 1 and the adapter protein ASC to create a functional NLRP3 inflammasome complex through oligomerization, demonstrating a potential pathway of cytokine overproduction response in sepsis (Yaqinuddin and Kashir, 2020). The inflammasome can cleave and convert the inert IL-1b precursor into an active secreted cytokine following conversion of inactive procaspase 1 to active caspase 1 (by autocatalysis). Finally, IL-1b is released into the extracellular space. IL-1 plays central roles in a variety of human diseases, including autoinflammatory and autoimmunity diseases such as rheumatoid arthritis. Unbalanced release of IL-1b is attributed to autoinflammatory diseases such as cryopyrinassociated periodic syndrome (CAPS), familial Mediterranean fever (FMF), and tumor necrosis factor (TNF) receptor associated periodic syndrome (TRAPS), which are triggered by mutations (Mardi *et al.*, 2021).

According to numerous studies, IL-1 takes an essential part in the induction of cytokine storm due to an uncontrolled immune response in COVID-19 infection (Mardi *et al.,* 2021). Cytokine storm has been found to result in acute lung injury (ALI), systemic inflammatory response syndrome (SIRS), or acute respiratory distress syndrome (ARDS), and may also be associated with the severity of multiple organ failure conditions, ultimately resulting in death (Mardi *et al.,* 2021).

The majority of patients infected with COVID-19 have normal or reduced white cell counts and lymphocytopenia, and those with severe disease have shown significantly elevated levels of neutrophils, D-dimer, and urea in blood, with a continuing decrease in lymphocytes (Costela-Ruiz *et al.,* 2020). Because immune genes are mostly located on the X chromosome, COVID-19 mostly affects men (Toniato*et al.,* 2020). The RNA of SARS-CoV-2 expresses at least 27 proteins, including 15 non-structural, 4 structural, and 8 auxiliary proteins (Divani*et al.,* 2020). All coronaviruses have a structural glycoprotein called the spike (S) protein, which binds to angiotensin converting enzyme 2 receptors (ACE-2Rs) on host cells. This interaction is critical for viral entry into the host cells (Ceribelli*et al.,* 2020). ACE-2Rs are expressed by mature lung epithelial cells, intestinal enterocytes (Mardi *et al.,* 2020), neurons and glial cells (Duong *et al.*, 2020), endothelial cells, and kidney proximal tubular cells (Batu and Ozen, 2020). Given the expression of ACE-2R by neurons and glial cells, SARS-CoV-2 may show extensive neurological manifestations, including stroke (Divani*et al.,* 2020), which is probably related to higher D-dimer levels (Manji, et al 2020). The extensive expression of ACE-2R on the respiratory system epithelium clarifies the involvement of the lung tissue in SARS-CoV-2 infection (Divani*et al.,* 2020). Upon ACE-2R binding, lysosomal proteases initiate cleavage of the S protein resulting in the signal peptide release that helps viral entry into the host cell. This pathway can be targeted and blocked by a therapeutic agent such as chloroquine, an antimalarial drug; the primary results revealed its clinical advantage in COVID-19 management (Ceribelli*et al.,* 2020). Lymphopenia is one of the most important indicators of SARSCoV-2 infection, which is observed in almost 80% of cases (Jamilloux*et al.,* 2020). Since lymphocytes do not express any ACE-2Rs, the virus cannot attack lymphocytes directly and may lead to lymphocytopenia by the destruction of lymphocytes

through cytokine storm (Mardi *et al.*, 2021). However, another study determined that viruses can directly infect T lymphocytes, but are not able to replicate inside them (Jamilloux*et al.*, 2020). Infection of T cells may lead to cell death by apoptosis, necrosis, or pyroptosis (Jamilloux*et al.*, 2020), or T cells (especially CD4+ T cells) are probably destroyed by the immune system (Duong *et al.*, 2020).

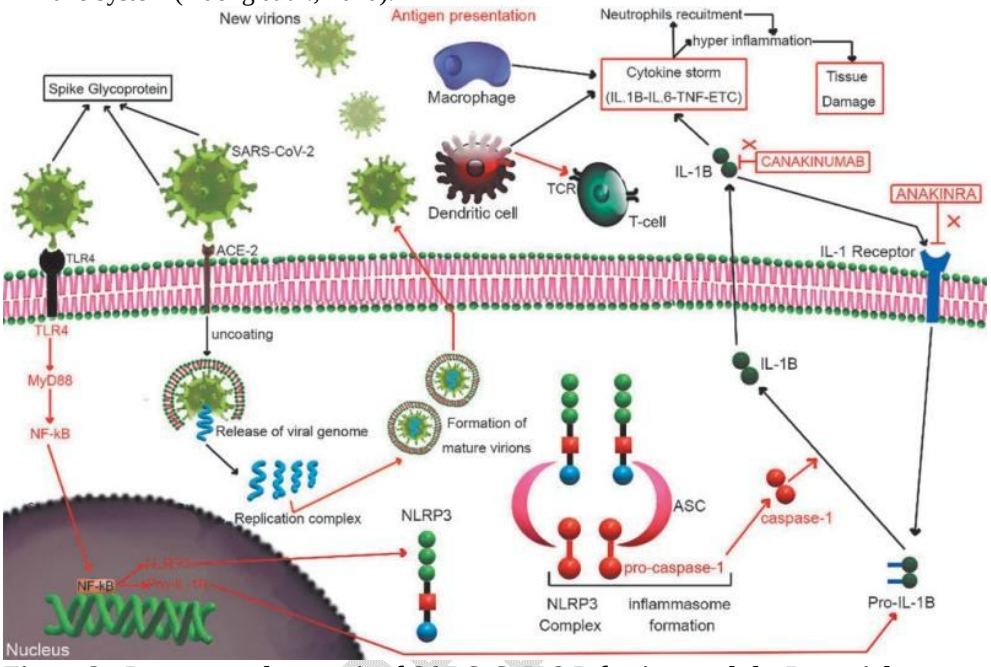

Figure 3: Immunopathogenesis of SARS-CoV-2 Infection, and the Potential Role of IL-1 in COVID-19 Pathogenesis(Mardi *et al.*, 2021).

2.7.2 Role of IL-6 in COVID-19 Infection

SARS-CoV-2 induces a dysregulated hyperinflammatory response in later stages as the virus was found to infect monocytes, macrophages, and dendritic cells (DCs) that increase the secretion of pro-inflammatory cytokines including interleukin-6 (IL-6) (Gautret*et al.*, 2020).

IL-6 is one of the most important pro-inflammatory cytokines (Kishimoto, 2003) and was discovered in late '80s (Potere*et al.*, 2021). After its molecular cloning, IL-6 was found to be identical to other proteins with different functions, indicating its pleiotropic nature. The IL-6/IL-6 receptor (IL-6 R) axis is known to be involved in the pathophysiology of many diseases and its inhibition has been already proven to be beneficial in rheumatoid arthritis,

Castleman disease, and the cytokine release syndrome following chimeric antigen receptor (CAR) T cell therapy, among others (Kang et al., 2020)

In COVID-19, IL-6 is believed to drive multi-organ injury, the most severe form of the illness (Copaescu et al., 2020; Potere*et al.*, 2021). To this end, IL-6 blockade was postulated to help reducing the inflammatory burden of COVID-19 in the setting of a cytokine storm and improve the clinical status of patients (Cavalli*et al.*, 2020; Horby*et al.*, 2021). In this narrative review, we summarize basic concepts about IL-6 biology and currently approved therapeutic indications for IL-6 blockade. Then, we discuss in detail the relevance of IL-6 in the pathophysiology of COVID-19 along with its prognostic implications. The safety and efficacy of IL-6 pathway inhibition in COVID-19 is also extensively covered. Finally, we provide future perspectives about the role of IL-6 based on contemporary evidence.

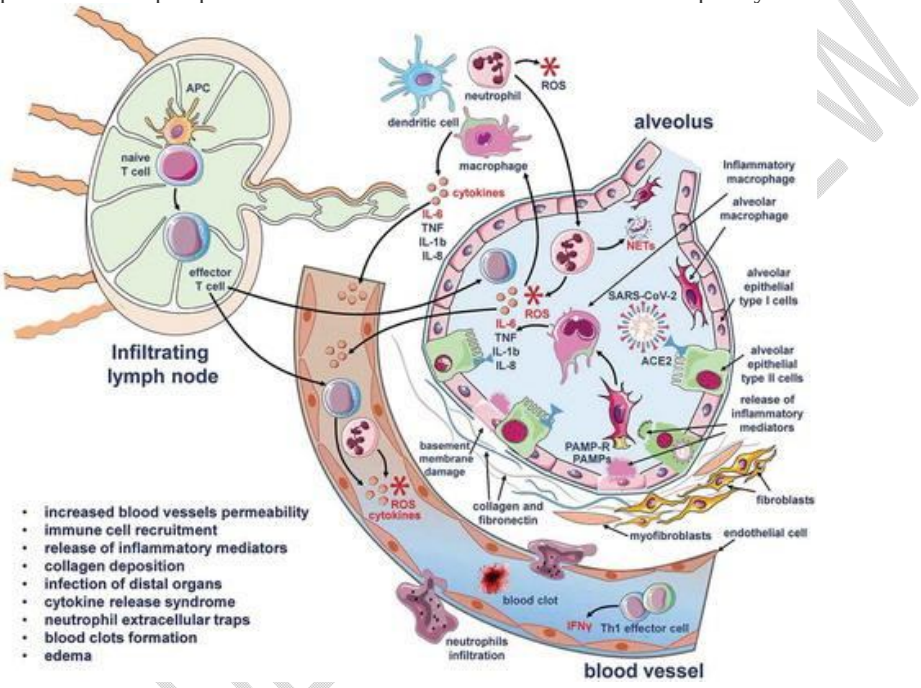

Figure 4: SARS-CoV-2 Induces a Deregulated, Hyperinflammatory Response Mediating Organ Injury (Potere*et al.*, 2021).

After binding its receptor - angiotensin-converting enzyme 2 (ACE2) - SARS-CoV-2 enters type II pneumocytes and replicates. Following viral invasion, macrophages, neutrophils, and dendritic cells activate to capture SARS-CoV-2. Damaged cells release pathogen-associated molecular patterns (PAMPs) stimulating further recruitment of immune cells, that in turn release a large amount of pro-inflammatory cytokines, including IL-6. These mediators are

responsible for the increased permeability of alveolar vessels and further immune cell recruitment to the site of infection, thus sustaining the positive, hyperinflammatory loop. Because of lung vessel permeability, SARS-CoV-2 can spread to other organs rich in ACE2, such as kidney, intestine, and pancreas, explaining clinical manifestations other than respiratory ones.

Very well-known Angiotensin-converting enzyme 2 (ACE2), antigen presenting cell (APC), interferon (IFN), interleukin (IL), neutrophil extracellular trap (NET), pathogen-associated molecular pattern (PAMP), reactive oxygen species (ROS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and tumor necrosis factor (TNF) Reproduced with permission from Gubernatorova *et al.*, 2021 'IL-6: Relevance for immunopathology of SARS-CoV-2' (Potere*et al.*, 2021).

2.7.2.1 Pathophysiological role of IL-6 in COVID-19-related Dysregulated Cytokine Response

According to the clinical-therapeutic staging of COVID-19 (Siddiqi and Mehra, 2020). Interleukin-6 (IL-6) plays a pivotal role in the third stage that is characterized by an abnormal systemic hyper inflammatory response. The dysregulated cytokine release is clinically responsible for severe COVID-19, whose main marker is abnormal IL-6 levels (Polidoro*et al.*, 2020; Harold *et al.*, 2020). Interleukin 6 (IL-6) is produced by a subset of highly inflammatory macrophages (Potere et al., 2021), but not by alveolar macrophages, which are low or absent in the bronchoalveolar fluid of severely ill patients (Bost*et al.*, 2020). Of interest, while IL-6 absence at early phases of a viral infection was shown to depress T follicular helper cell maturation thus reducing antiviral response, COVID-19 patients admitted to the intensive care unit (ICU) show a negative correlation between IL-6 and other cytokines, as well as $CD4^+$ and $CD8^+$ T cells (Diao*et al* 2020). This indicates that aberrant IL-6 production has a negative impact on adaptive immunity.

An exaggerated hyperinflammatory response was reported by early studies in China in patients with COVID-19, which described markedly increased levels of several inflammatory mediators, including – but not limited to – IL-6, IL-1β, IL-18, IL-8, granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Potere*et al.*, 2021).

It appears now clear that patients with higher levels of inflammatory mediators experience worse outcomes during SARS-CoV-2 infection (Ruan*et al.*, 2020). In particular, COVID-19 patients progressing to ARDS showed increased concentrations of IL-6, IL-1β, and tumor necrosis factor (TNF)-α (Huang *et al.*, 2020). This abnormal increase in cytokine levels, described as a cytokine storm, is responsible for an exaggerated activation of the immune system that, in turn, promotes further production of cytokines and chemokines. Importantly, dysregulated inflammation seems to be associated with abnormalities in the coagulation cascade, finally leading to immunothrombotic processes (McFadyen*et al.*, 2020), which are also responsible for organ damage.

SARS-CoV-2 infects preferentially type II pneumocytes and alveolar macrophages within the lungs (Grant *et al.*, 2021; Hu *et al.*, 2021). Recently, Patra *et al*. (2020), showed that the SARS-CoV-2 spike protein can trigger an angiotensin II type 1 (AT1) receptor-mediated signaling cascade, finally increasing IL-6 release, which is down-regulated by the AT1 receptor antagonist candesartan. In the lungs, the virus replicates and alters the lung epithelial layer thus entering underlying tissues, where immune cells – namely neutrophils, macrophages, and dendritic cells (DCs) – capture the pathogen (Gubernatorova*et al.*, 2020). In lung samples of patients who died because of COVID-19-related ARDS, SARS-CoV-2 was found to trigger the activation of the NACHT, LRR, and PYD domains-containing

protein 3 (NLRP3) inflammasome in monocytes (Potere*et al.,* 2021), leading to the production and release of IL-1β, which is upstream of IL-6.
Injured pneumocytes can release danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), that trigger the activation of the lung epithelium and resident immune cells (Grant *et al.,* 2021). Activation of neutrophils and macrophages, antigen presentation by DCs, and local SARS-CoV-2 replication lead to increased production of inflammatory cytokines, especially IL-6, IL-1β, and TNF-α, finally contributing to organ damage, especially the lungs, as this uncontrolled inflammatory response is able to self-propagate. Indeed, a correlation between IL-6 and viral load was described, with the latter being associated with ARDS severity and lung damage. Finally, during infections, increased vessel permeability allows immune cell infiltration and viral spread, followed by the release of inflammatory mediators, such as IL-6, that exacerbate the hyperinflammatory environment (Potere*et al.,* 2021).
IL-6 is also involved in the COVID-19-associated coagulopathy as it is known to interfere with the coagulation cascade through the generation of tissue factor and thrombin, to stimulate platelet activity, and induce endothelial dysfunction. With this regard, tocilizumab seems to improve the hypercoagulable state in COVID-19 patients, irrespective of prophylactic or therapeutic dose of anticoagulant therapy, and was associated with a parallel improvement in respiratory function (Potere*et al.,* 2021). Recently, Canzano *et al*., 2021 provided evidence that COVID-19 coagulopathy may be supported by diffuse cell activation mediated by tissue factor produced by platelets, granulocytes, and microvesicles on the common background of endothelial dysfunction, with all of these events strongly sustained by increased levels of IL-6. Indeed, IL-6 blockade with tocilizumab and antiplatelet drugs (aspirin or P2Y12 inhibitors) were found beneficial in blunting these effects (Potere*et al.*, 2021).

2.7.3 Role of Tumor Necrosis Factor in COVID-19 Disease

Tumor necrosis factor (TNF), a 17 kDa protein consisting of 157 amino acids, is a homotrimer in solution that is mainly produced by activated macrophages, T lymphocytes, and natural killer (NK) cells (Atzeni*et al.,* 2013).
Proinflammatory cytokines such as TNF and interleukin (IL)-1β play a key role in the pathogenesis of rheumatoid arthritis (RA). TNF has major effects on bone remodelling: it regulates the bone marrow levels of osteoclast precursors directly by up regulating c-fmsexpression, and activates osteoclasts by enhancing the signalling mechanisms of the receptor activator of NF-κB (RANK). It also plays an important role in controlling infection (Atzeni*et al.,* 2013).

The macrophage release of TNF seems to be crucial for the formation and maintenance of granulomas, and plays a critical role in defending intracellular organisms against invasion. TNF is also involved in leukocyte trafficking and immune complex (IC) clearance. Large quantities are produced in the heart and, although not entirely clear, the mechanisms by which TNF mediates cardiac injury once again seem to depend on its levels (Atzeni*et al.,* 2013).
Tumor necrosis factor (TNF) promotes dyslipidemia and insulin resistance, both of which are traditional risk factors for atherosclerotic processes. Tumor necrosis factor (TNF) is a pleiotropic cytokine involved in multiple homeostatic and pathological mechanisms (Atzeni et al., 2013).
Respiratory distress and activation of blood clotting in severe COVID-19 cases result in unusually high mortality rates, particularly among people of advanced age and those that have comorbidities-cardiovascular or pulmonary disease, obesity, and diabetes (Ablamunits and Lepsy, 2022). Severe disease is associated with “cytokine storm”, a delayed onset burst

of pro-inflammatory cytokines in circulation. The cytokines associated with fatalities are TNF, IL-6, IL-8, IFNγ and possibly others (Costela-Ruiz *et al*, 2020). It is difficult to identify the pivotal cytokine(s) in this process, but some facts argue in favor of TNF.

Numerous pathologies are associated with elevated TNF levels, from autoimmune disorders to sepsis and cancer. In the respiratory system, TNF causes bronchial hyperreactivity, narrowing of the airways, damage to the respiratory epithelium, stimulation of collagen synthesis and fibrosis (Malaviya*et al.*, 2017). Chronic obstructive pulmonary disease (COPD) is a known risk factor for severe COVID-19 disease (Leung *et al.*, 2020). Circulating TNF levels are increased in COPD (Yao *et al.*, 2019).

The role of TNF in COVID-19 disease has been suggested, and TNF inhibition was shown effective in lowering the incidence of hospitalization in one study (Suissa*et al.*, 2008), but did not improve health status and lung function in the other (Ablamunits and Lepsy, 2022). However, TNF blockage in COVID-19 patients with COPD may be advocated as a measure to reduce additive damage to already compromised lungs. In addition, pulmonary fibrosis is observed in a significant proportion of patients after acute COVID-19 pneumonia (George et al 2020). Although the role of TNF in this process is not established, there is evidence for TNF involvement in a closely related idiopathic pulmonary fibrosis (Lechowicz*et al.*, 2020). Administration of anti-TNF drugs during the acute phase of infection may subsequently alleviate development of this complication.

The effects of TNF on the cardiovascular system are also well known. Tumor necrosis factor (TNF) significantly contributes to the development of heart failure by direct negative inotropic and pro-apoptotic effects on cardiomyocytes, and by other mechanisms (Hanna and Frangogiannis, 2020). TNF is also elevated in patients with hypertension (Ablamunites and Lepsy, 2022). Moreover, TNF levels are increased in obesity, and TNF is considered to play a role in insulin resistance (Harder-Lauridsen*et al.*, 2014). All these conditions are risk factors for development of severe COVID-19 disease and associated mortality or long-term complications.

The ability of TNF to activate tissue factor on endothelial cells and monocytes and induce severe blood clotting during infection has been well documented (Ablamunites and Lepsy 2022). Tumor necrosis factor (TNF) also inhibits fibrinolysis by increasing plasminogen activator inhibitor (Ablamunites and Lepsy, 2022). Reports on pro-coagulant activities induced by IL-6 are scarce (Kang *et al.*, 2020). Increased blood clotting observed in COVID-19 patients is a well- documented complication requiring anti-coagulant therapy.

Both TNF and IL-6 levels are elevated with age: this chronic inflammation termed inflammaging is suggested to serve as a biomarker of frailty and mortality in elderly population (Kang *et al.*, 2020). Age-related loss of muscle mass and strength is particularly attributed to the action of TNF (Reid and Li, 2001) and exposure of human cells to TNF in vitro can induce cell senescence (Mavrogonatou*et al.*, 2018). Strong association of TNF with ageing may explain, to some extent, higher incidence of severe COVID-19 disease in patients of advanced age. Interestingly, mTOR inhibitor has been suggested recently for treatment of severe disease based on its ability to alleviate cytokine storm (Omarjee*et al.*, 2020). The drug is also known to improve longevity and reverse age-related immunosenescence in experimental animals, and its use in older adults may prevent age-associated complications of COVID-19 by poorly understood “rejuvenating” mechanisms (Bischof*et al.*, 2021). On the other hand, the effects of mTOR inhibition may be reduced to a direct inhibition of TNF synthesis or signal transduction (Ablamunits and Lepsy, 2022).

Pro-inflammatory cytokines TNF, IL-6 and others are elevated in major depressive disorder, which is strongly associated with COVID-19 infection. On the other hand, a number of reports demonstrate anti-inflammatory effect of various antidepressants (Ablamunites and Lepsy, 2022). Of interest is a retrospective multicenter study reported by a French group

demonstrating that antidepressants reduce the risk of intubation and death in hospitalized patients with COVID-19 (Ablamunites and Lepsy, 2022). At least two clinical trials are currently underway to investigate the impact of this class of drugs on the disease outcomes (NCT04342663, NCT04377308) (Ablamunites and Lepsy, 2022).

2.8Mechanism of Coagulopathy in COVID-19

COVID-19 pneumonia appears to have distinguishing features compared to conventional pneumonia. It is evident that COVID-19 patients develop dysregulated uncontrolled host response, that results in excessive release of many inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6 and IL-8 (Hadid, *et al.,* 2021). The release of these molecules induces macrophage activation syndrome-like picture, which triggers the endothelial cells, macrophages and neutrophils to express tissue factor within the lungs, which in turn initiates and further augments pulmonary coagulopathy and microvascular thrombosis (Ablamunits and Lepsy, 2022).

Interleukin 6 (IL-6) is a key cytokine that is markedly elevated in severe COVID-19 infection and is a key activator of coagulopathy by inducing tissue factor expression and increasing production of fibrinogen and platelets (Hadid*et al.,* 2021). In COVID-19 patients requiring mechanical ventilation, median IL-6 is reported to be at 121-218 pg/mL (Hadid*et al.,* 2021). This significant difference in IL-6 level in critically ill patients is likely directly induced by COVID-19 infection, which may explain the significant difference in the pattern of coagulopathy in these patients. In addition, there is cumulative evidence implicating endotheliitis in COVID-19 pathogenesis (Hadid, *et al.,* 2021). A recent postmortem series showed evidence of a direct multi-organ infection of the endothelial cells with COVID-19 with an associated diffuse inflammation. Apoptosis and pyroptosis were suggested as possible mediators of endothelial injury in these patients (Hadid*et al.,* 2021). Notably, this inflammatory endothelial cascade can directly result in microvascular dysfunction and occlusion but can also induce hypercoagulable state, resulting in microvascular thrombosis. Moreover, hypoxia, a frequent feature of severe COVID-19 is a prominent stimulant of thrombosis via expression of hypoxia-inducible transcription factors, which in turn target several genes that regulate thrombosis (Serebrovska*et al.,* 2020). Also a preclinical model showed that SARS-CoV results in disruption of the fine balance between plasmin and the urokinase pathway, resulting in fibrin accumulation (Gralinsk*et al.,* 2013). Dysregulation of the urokinase pathway is likely in part responsible for the coagulopathy encountered in COVID-19, which is more magnified than that seen in conventional sepsis. Together, these events result in extensive microvascular thrombosis within the lungs, an entity referred to as diffuse pulmonary intravascular coagulopathy (PIC). The elevation of D-dimer and FSP in COVID-19 patients reflect the immunothrombosis induced by PIC (Hadid*et al.,* 2021).

2.9 Laboratory Diagnosis of SARS-CoV-2

Laboratory diagnosis of the virus is essential in distinguishing it from other known viruses of pneumonia, and from non-infectious diseases, such as vasculitis, dermatomyositis, and organizing pneumonia (Jin*et al.,* 2020). It involves isolation of the virus, followed by detection of the viral nucleic acid; Koch`s postulates states that, virus isolation is the "gold standard" for virus diagnosis in the laboratory (Yu *et al*., 2020).

It must be of note that no matter how accurate and fast laboratory testing methods are, the diagnosis of viral pneumonias such as those caused by SARS-CoV-2, involves obtaining the appropriate specimen from the patient at the right time. Samples collected from the upper and lower respiratory tracts are used to detect human coronavirus (HCoV); notably, nasopharyngeal swabs are high priority specimens when SARS-CoV-2 is to be detected, while others such as oropharyngeal swabs, broncho-alveolar lavage, tracheal aspirates, and

sputum are lower priority specimens (CDC, 2020). The United States Centers for Disease Control recommend the use of upper respiratory nasopharyngeal swab (which is a high priority specimen), and that, collection of an oropharyngeal specimen is of lower priority, and, if collected, it should be combined in the same tube (containing a viral transport medium) as the nasopharyngeal swab (CDC, 2019). Also, nasopharyngeal aspirates are specimens appropriate to detect human Coronaviruses (HCoVs).

There are different methods used in the diagnosis of SARS-CoV-2, which include the following:

2.9.1 Real-Time Polymerase Chain Reaction (RT-PCR)

Using this method, the isolated viral RNA is re-transcribed to copy DNA (cDNA) and then amplified using a RT-PCR technique. World health organization (WHO) reported different probe and primer sets for SARS-CoV-2 developed previously in China, Hong Kong, Germany, Japan, USA and Thailand (WHO, 2020b). There are several segments of the genetic sequence of the virus, such as the envelope (E) gene, RNA-dependent RNA polymerase (RdRp) gene and the nucleocapsid (N) gene (Chu *et al.*, 2020, Corman*et al*., 2020, WHO 2020b), and these segments are targeted by primers. However, Corman*et al*. (2020) reported highest sensitivity when the E gene was targeted and the next was the RdRp gene which is commonly referred to as a confirmatory gene. Furthermore, multiple probe and primer sets found at various regions in the genome of the SARS-CoV-2 have been integrated (referred to as multiplexed PCR tests) by some laboratories, such that these primer sets may have the potential to simultaneously target many genes (RdRp/hel, S, N) (Chan *et al*., 2020b), or to target several regions in a single gene (for instance, the N gene) (U. F. A. D Administration, 2020). However, in cases where there is viral RNA degradation or loss during sample collection and/or extraction of nucleic acid, or in the case of mutation in the viral genome, the use of multiplexed assays helps to promote sensitivity of the test. These methods make use of RNA (that was synthesized in vitro) obtained from transcripts as positive controls and to produce standard curves. An internal control using RNAse P (RP) enhances the authentication of the presence and quality of nucleic acid in samples, and molecular-grade nuclease-free water is used as a negative amplification control. Sample from a negative patient serves a negative extraction control for monitoring cross contamination across samples and for test reagents validation (Roshan *et al*., 2020).

2.9.1.1 Advantages of Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR being the gold standard technique used for the diagnosis of COVID-19, it possesses the potential of testing thousands of samples daily, and reveals a high testing sensitivity of about 95 percent (Corman*et al*., 2020). From a study conducted recently, the limit of detection of the virus with this technique is less than 10 copies per reaction (Chu *et al*., 2020), which implies that, it can detect early infection, as well as low viral load.

2.9.1.2 Disadvantages of Real-Time Polymerase Chain Reaction (RT-PCR)

When the RT-PCR technique is used, a cross-reaction between the primers and nucleic acids arising from co-infection with other microbes, may lead to the production of false-positive results, in which the detected pathogen may not have caused the disease. Also, when the RT-PCR primers and probes of SARS-CoV-2 are matched with reliable libraries (for example, BLAST), homology with other coronaviruses like SARS-CoV or other pathogens (for example *Staphylococcusaureus* and *Candidaalbicans*) is usually ruled out. Also, the occurrence of false positive results may be attributed to the contamination of reagents in the laboratory, especially with the huge testing rate faced during a pandemic; however, a negative patient's sample is useful in diagnosing or ruling out this anomaly (Corman*et al*., 2020).

In furtherance with the aforementioned, false positive result may also be attributed to mutations in the primer and probe target regions in the genome of the SARS-CoV-2.

However, negative results do not rule out possibility of COVID-19 infection, therefore, re-analysis should be carried out using different sets of primer against the same target gene, in combination with patient's medical history and other clinical details to accurately ascertain patient's infection status (Corman*et al.*, 2020).

MATERIALS AND METHODS

3.1 Study Design

A case control study was employed for the assessment of some inflammatory cytokines, haematologic and haemostatic parameters in subjects with severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) in Port-Harcourt city. The study considered inflammatory cytokines like (IL-1β, IL-6 and TNF-α)

3.2 Study Area

This study was carried out in Port-Harcourt Metropolis and Eleme Local Government Area, Rivers State, Nigeria, and the subjects was recruited at the Port-Harcourt and Eleme COVID-19 isolation centres. Port Harcourt metropolis, which is the capital of Rivers state is located between Latitude 4°53'N and Latitude 4°23'N, and Longitude 6°54'E and Longitude 6°18'E in Rivers State (Baeka*et al.,* 2021). It is a city in the Niger Delta region of Nigeria which lies at the mouth of Bonny River in Rivers State. It is located at about 25 km from the Atlantic Ocean and is situated between the Dockyard creek/Bonny River and the Amadi creek also lies at an average altitude of about 12m above mean sea level (Akukwe and Ogbodo, 2015). Port Harcourt metropolis spans over two local government areas (LGAs) viz Port Harcourt and Obio/Akpor. According to census 2016, the Port Harcourt urban area has an estimated population of 1,865,000 inhabitants, up from 1,382,592 as of 2006 with land mass of 360km^2 and Obio/Akpor local government area recorded a population of 878,890 and land mass of 260 km^2 (Akukwe and Ogbodo, 2015), whereas Eleme Local Government Area. According to Wikipedia, it has a land mass of 138 sq. km with a population of 190,884 people (2006 Census) (Dsw and Wuwu, 2021).

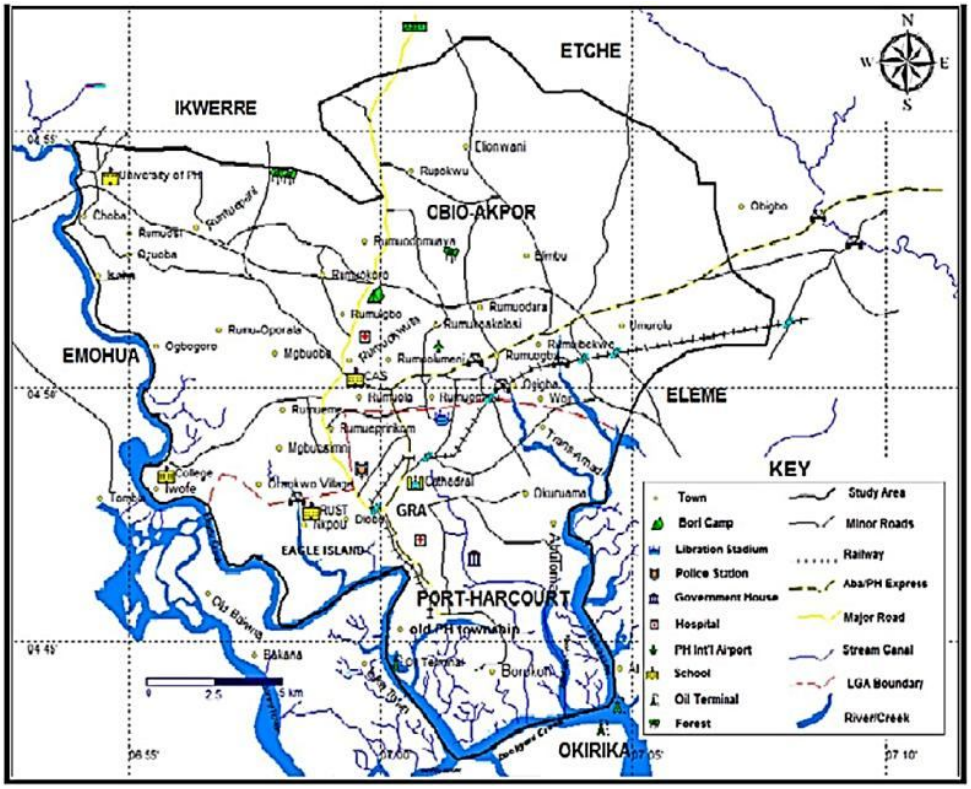

Figure 5. Map of Port Harcourt metropolis.
Source. Adapted from Google Earth (2012)

3.3 Study Population

This is a case-control study, in which a total of 110 subjects (aged within 20 to 70 years) was recruited for the study; 55 subjects were tested positive, and confirmed COVID 19-positive subjects, while the remaining 55 subjects were apparently healthy COVID-19-negative subjects. This recruitment was taken after spoken to and intimated with the full details of the study, such that those who gave both informed and written consent will be recruited and a well-structured questionnaire was administered to every subject to gather information on their name, sex and age.

3.3.1 Sample size was determined using Cochran's Formula (Kotrlik*et al.*, 2001)

$$n = \frac{z^2 (pq)}{e^2}$$

Where n = sample size
z = standard error with the chosen level of confidence (typically 1.96)
p = Prevalence (taken from previous studies)
q = 1-p
e = Acceptable sample error (0.05)

The sample size was calculated based on the prevalence of COVID-19 in Rivers State which was reported as 6% (Chiedozie et *al.*, 2020)
Using the formula;
Sample size for COVID-19 in Rivers State $(n) = \frac{1.96^2 x 0.06 x 0.6}{0.05^2} = 55.3$ i.e. **n = 55**

At the time of sample collection for COVID-19 testing for this study there was scarcity of positive COVID-19 subjects, fifty (55) subjects were recruited overall after the third wave of the pandemic which was used for this study.

3.4 Ethical Consideration/ Informed consent

Ethical approval was obtained from the Ministry of Health, Port-Harcourt, with a clearance from Rivers State hospital management board, Rivers state, Nigeria as shown in appendix.

3.5 Eligibility Criteria

3.5.1 Inclusion Criteria

Subjects (males or females) who tested positive to COVID-19, confirmed for the disease, and currently at the isolation center will be included for the study. Also, those who tested negative for the disease was recruited for the study.

3.5.2 Exclusion Criteria

However unconscious subjects or those experiencing severe difficulty in breathing as a result of COVID-19 was excluded from the study as obtaining consent from them was difficult.

3.6 Blood Sample Collection

About 5mls of whole blood sample was collected from each subject with sterile hypodermic syringes and needles using standard venepuncture technique. The blood sample collected was dispensed into plain bottle, which was spun to obtain the serum that was then used for the analysis of inflammatory cytokines (IL-1, IL-6 and TNF-alpha) using ELISA test kit.
Nasopharyngeal swab was collected from the subjects and RNA extraction was done on them by kiagene viral RNA extraction kit manual extraction process using standard protocols and the extracted RNA was taken for real-time RT-PCR.

3.7 Sample Analysis

3.7.1 Coronavirus Analysis

3.7.1.1 Method: Manual RNA extraction and RT-PCR, (kit supplied by Liferiver Lot Number ZJ0009) as Described by Arya *et al.* (2005).

3.7.1.2 Principle

The principle of the real-time detection is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities in real time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification. Real time reverse-transcription polymerase chain reaction (real-time RT-PCR) is used when

the starting material is RNA. In this method, RNA is first transcribed into the complementary DNA (cDNA) by reverse transcriptase from total RNA. The cDNA is then used as a template for the real time PCR.

3.7.1.3 Reagent preparation

It was ensured that kit were stored at -20±5℃ after receipt, reagents were kept in a clean environment and store at -20±5℃. Reagents were properly thawed and mixed before use. Reagent was Sub-aliquoted to reduce the times of multiple freeze-thaws and the likelihood for contamination, 5 sets RT-PCR Buffer, Enzyme mix and Reaction Mix was aliquoted into labeled centrifuge tubes and stored at - 20±5℃ without light. Then 75μL/tube RT-PCR Buffer was Sub-pack into 5 centrifuge tubes, 50μL/tube Enzyme Mix was Sub-pack into 5 centrifuge tubes, 40μL/tube SARS-CoV-2 Reaction Mix was Sub-pack into 5 centrifuge tubes and then 35μL/tube RNase-free Water was Sub-pack into 5 centrifuge tubes then the RNase-free water and Positive Control was carefully Processed in the specimen processing area to avoid contamination. It was ensured repeated freeze-thaw was avoided and Blank Control and Positive Control were stored at -20±5℃ or ≤-70℃, after extraction Blank Control and Positive Control were Stored at -20±5℃ or ≤-70℃.

3.7.1.4 Procedure

The volumes of Super Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls and samples prepared. Molecular Grade Water was used as the negative control. For reasons of imprecise pipetting, an extra virtual sample was added then sample was mixed completely and then spun down briefly with a centrifuge then 20μL master mix with micropipettes of sterile filter tips was pipetted to each of the Real Time PCR reaction plate/tubes then 5μL template (nucleic acid extracted from negative control and specimen, positive control without extraction) was separately added to different reaction plates/tubes then the plates/tubes was immediately close to avoid contamination then to collect the Master Mix and template in the bottom of the reaction tubes it was Spun down briefly then the instrument of MIC POC DX48 was used to Perform protocols as instructed by the manufacturer, it was ensured that for the MIC POC DX48 system “none” was selected as passive reference and quencher to avoid any errors.

3.7.1.5 Data Analysis and Interpretation

Table 1 below, lists the expected results for the SARS-CoV-2 Real-Time Multiplex RT-PCR Kit. If results are obtained that do not follow these guidelines, re-extract and re-test the sample.

Table 1.Expected Results for SARS-COV-2 Real-Time Multiplex RT-PCR Kit.

Ct value				Result interpretation[a]
ORF1ab	N	E	IC	
+	+	+	/	SARS-CoV-2 detected
+	—	+	/	
+	+	—	/	
—	—	—	+	SARS-CoV-2 not detected[b]
—	—	—	—	Invalid; Repeat testing or collect a new specimen from the patient.
—	+	+	/	Inconclusive
—	—	+	/	
+	—	—	/	
—	+	—	/	
"+"represents a positive detection signal, which is defined as Ct≤41; "-" represents a negative detection signal, which is defined as Ct>41; "/" represents no requirement. Detection of Internal Control is not required if result positive in any of the other three detection channels.				

3.7.2 Determination of Inflammatory Cytokines

3.7.2.1 Interleukin (IL)-1β

3.7.2.1.1 Method of assay: Enzyme-Linked Immunosorbent Assay (ELISA) as described by (Fristiohady *et al.,* 2020) was used

3.7.2.1.2 Principle

Sandwich-Elisa principle is applied in the IL-IB. The micro Elisa plate provided is precoated with an antibody specific to human IL-IB. samples or standards are added to the micro Elisa plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for human IL-IB and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to reach micro plate well and incubated. Free components are washed away. The substrate solution is added to each well.

Only those wells that contain Human IL-IB, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450±2mm. The OD value is proportional to the concentration of the Human IL-IB in the samples is calculated by comparing the OD of the samples to the standard curve.

3.7.2.1.3 Procedure

Wells was determined for diluted standard and samples 100µl of diluent was added for standard, blank and samples into the appropriate wells respectively, and done in duplicate, plates were covered with sealer provided in the kit, and was incubated for 90 mins at 37^{o}C. Liquid from each well was discarded and avoided washing, immediately added 100µl of Biotinylated Detection Antibody working solution to each well. Plates was covered again with a new sealer and incubated for 1 hour at 37^{o}C. Solution from the wells again was discarded, and added 350µl of wash buffer to each well and soaked for 1min and discarded the solution from each well and patted it dry against clean absorbent paper. This process was repeated for 3 times. 100µl of HRP conjugate working solution was added and plates covered with new sealer and incubated for 30mins at 37^{o}C. The solution from each well was discarded and washed again for 5 times by adding 350µl of wash buffer to each well and soaked for 1 min and aspirated the solution from the well and patted it dry against clean absorbent paper. 90µl of substrate reagent was added to each well and plates was covered with new sealer and incubated for 15mins at 37^{o}C and protected the plates from the light. 50µl of stop solution

was added to each well. The optical density of each well was determined at once with a micro-plate reader set to 450nm

3.7.2.2 Interleukin (IL)-6

3.7.2.2.1 Method of assay: Enzyme-Linked Immunosorbent Assay (ELISA) as described by (Fristiohady*et al.*, 2020) was used.

3.7.2.2.2 Principle

Sandwich-Elisa principle is applied in the IL-6. The micro Elisa plate provided is precoated with an antibody specific to human IL-6. Samples or standards and Horseradish Peroxidase (HRP) linked antibody specific for human IL-6 areadded to the micro Elisa plate wells and human IL-6 in samples or standard combines with the coated antibody and HRP linked detection antibody special to human IL-6. Excess conjugate and unbound sample or standard are washed from the plate. The substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450±2mm. The OD value is proportional to the concentration of Human IL-6. The concentration of Human IL-6 in the samples is then determined by comparing the OD of the samples to the standard curve.

3.7.2.2.3 Procedure

Wells was determined for diluted standard, blank and samples 50µl of diluent was added for standard, blank and samples into the appropriate wells respectively, and done in duplicate, immediately 50ul of HRP linked antibody working solution was added to each well. Plates was covered with sealer provided in the kit, and was incubated for 60 mins at 37^{o}C. Solution from the wells was decanted, and added 350ul of wash buffer to each well and soaked for 1min and decanted the solution from each well and patted it dry against clean absorbent paper. This process was repeated for 5 times. 90µl of substrate reagent was added to each well and plates was covered with new sealer and incubated for 15mins at 37^{o}C and protected the plates from the light. 50µl of stop solution was added to each well. The optical density of each well was determined at once with a micro-plate reader set to 450nm.

3.7.2.3 Tissue Necrotic Factor (TNF)-Alpha

3.7.2.3.1 Method of assay: Enzyme-Linked Immunosorbent Assay (ELISA) as described by (Adias*et al.*, 2018) was used.

3.7.2.3.2 Principle

Sandwich-Elisa principle is applied in the TNF-α. The micro Elisa plate provided is precoated with an antibody specific to human TNF-α. Samples or standards are added to the micro Elisa plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for human TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to reach micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450±2mm. The OD value is proportional to the concentration of the Human TNF-α in the samples and is calculated by comparing the OD of the samples to the standard curve.

3.7.2.3.3 Procedure

Wells was determined for diluted standard, blank and samples, 100µl 0f diluent was added for standard, blank and samples into the appropriate wells respectively, and done in duplicate,

plates were covered with sealer provided in the kit, and was incubated for 90mins at 37^{o}C. Liquid from each well was discarded and avoided washing, immediately added 100µl of Biotinylated Detection Antibody working solution to each well. Plates was covered again with a new sealer and incubated for 1hour at 37^{o}C. Solution from the wells again was discarded, and added 350 of wash buffer to each well and soaked for 1min and aspirated or discarded the solution from each well and patted it dry against clean absorbent paper. This process was repeated for 3 times. 100µl of HRP conjugate working solution was added to each well and plates covered with new sealer and incubated for 30mins at 37^{o}C. The solution from each well was discarded and washed again for 5 times by adding 350µl of wash buffer to each well and soaked for 1min and aspirated the solution from the well and patted it dry against clean absorbent paper. 90µl of substrate reagent was added to each well and plates was covered with new sealer and incubated for 15mins at 37^{o}C and protected the plates from the light. 50µl of stop solution was added to each well. The optical density of each well was determined at once with a micro-plate reader set to 450nm.

RESULTS

This study was conducted to assess the effect of SAR-Cov-2 infection on some inflammatory cytokines, haematologic and haemostatic parameters in Port Harcourt, Nigeria.

4.1: Distribution of Demographic Characteristics of Study Population by COVID-19 Test Results

There were 55 COVID-19 positive subjects, comprising 35 males and 20 females, and another 55 COVID-19 negative subjects comprising 23 males and 32 females. The subjects were categorized according to their age ranging from <30, 30-39, 40-49 and 50+ years for both COVID-19 positive and negative (control) subjects with the following number of participant for COVID-19 positive 9, 22, 12 and 12 respectively according to age group and 32, 15 and 8 respectively for COVID-19 negative subjects as shown in figure 6

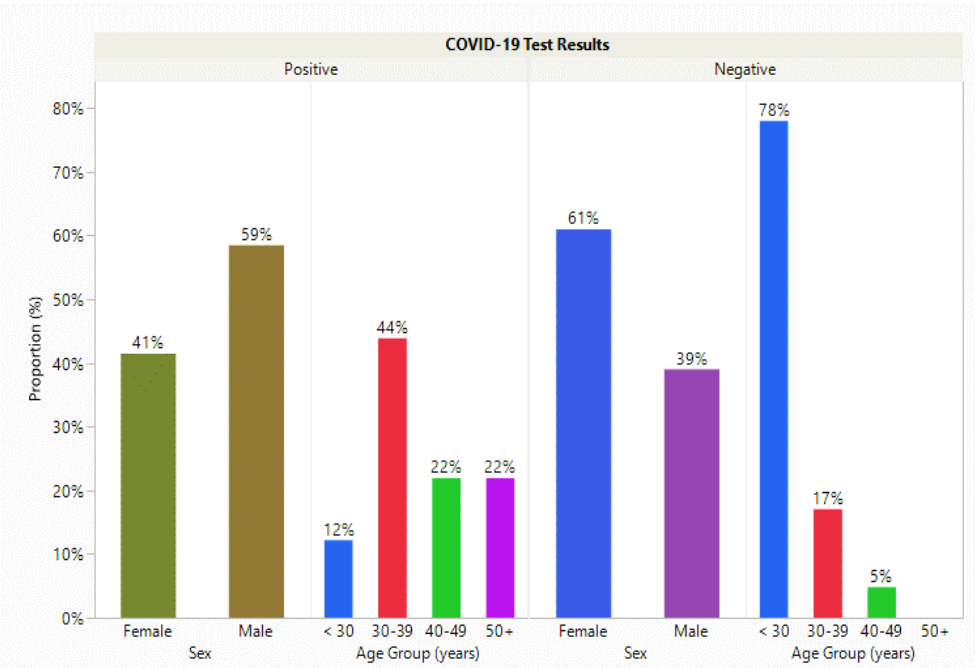

Figure 6 : Distribution of Demographic Characteristics of Study Population by COVID-19 Test Results

4.2 Comparison of inflammatory cytokines

The mean values of IL-6 was highly statistically and significantly increased in SAR-Cov-2 positive patients (21.62±8.85 pg/ml) as against 2.84±0.07 pg/ml in the control (Negative) subjects (t=-2.1235; P=0.0399). No statistically significant differences were observed in the mean values of IL-1β pg/ml and TNF-α pg/ml 5.357±0.574 and 2.246±0.097 respectively in the positive patients when compared with 7.213±0.841 and 2.279±0.057 in the same order in the negative subjects as shown in table 2

Table 2: Comparison of inflammatory cytokines

Parameter	COVID-19	Test Statistics

	Positive (n=55)		Negative (n=55)			
	Mean	SEM	Mean	SEM	t-Ratio	Prob>|t|
IL-1β (pg/ml)	5.357	0.574	7.213	0.841	1.8233	0.0725 [ns]
IL-6 (pg/ml)	21.62	8.85	2.84	0.07	-2.1235	0.0399*
TNF-α (pg/ml)	2.246	0.097	2.279	0.057	0.2913	0.7718 [ns]

*Abbreviations: SEM: Standard Error of Mean; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor Alpha.Significance Level: *=p<0.05; ns=Not Significant (p>0.05).*

4.6 Comparison of Inflammatory Cytokines by Sex

The interleukin 1 Beta, interleukin 6 and tumor necrosis factor alpha mean values for female SARS-Cov-2 positive patients were 3.97±1.11, 7.53±9.61 and 2.246±0.124 and male positive patients were 6.34±0.94, 31.61±8.09 and 2.246±0.104 respectively when compared with the negative female subjects 7.26±0.92, 2.67±7.93 and 2.215±0.102 and male subjects 7.13±1.15, 3.10±9.91 and 2.378±0.128 in the same order. Sex was not found to exert any significant influence on the inflammatory cytokines (p >0.05) as shown in table 3.

Table 3: Comparison of Inflammatory Cytokines by Sex

COVID-19 Results	Sex	n	IL-1β (pg/ml) Mean ± SEM	IL-6 (pg/ml) Mean ± SEM	TNF-α (pg/ml) Mean ± SEM
Positive	Female	20	3.97±1.11	7.53±9.61	2.246±0.124
	Male	35	6.34±0.94	31.61±8.09	2.246±0.104
Negative	Female	32	7.26±0.92	2.67±7.93	2.215±0.102
	Male	23	7.13±1.15	3.10±9.91	2.378±0.128
Test Statistics	F Ratio		1.4665	1.7532	0.4980
	P-value		0.2296 [ns]	0.1893 [ns]	0.4825 [ns]

*Abbreviations: SEM: Standard Error of Mean; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor Alpha. Significance Level: ****=p<0.0001; ns=Not Significant (p>0.05).*

4.10 Comparison of Inflammatory Cytokines by Age Groups

The mean values of SARS-Cov-2 positive patients categorized as <30, 30-39, 40-49 and 50+ for IL-1β (pg/ml) were 4.564±1.637, 4.413±0.863, 6.536±1.220 and 6.506±1.220 respectively, for IL-6 (pg/ml) 16.96±10.68, 4.90±0.63, 27.29±23.15 and 52.01±31.97, for TNF-α (pg/ml) 2.310±0.278, 2.224±0.147, 2.008±0.207 and 2.492±0.207, respectively. The mean values of negative subjects categorized as <30, 30-39 and 40-49 in the same order for IL-1β were 7.531±0.971, 6.244±2.075 and 5.525±3.882, for IL-6 2.83±0.08, 2.97±0.07 and 2.60±0.13, for TNF-α 2.278±0.061, 2.130±0.131 and 2.810±0.245, respectively. There was no influence of age on the mean values of the inflammatory cytokines of patients with SARS-Cov-2 positive and the negative controls (p>0.05) as shown in table 4.

Table 4: Comparison of Inflammatory Cytokines by Age Groups

COVID-19 Results	**Age Group (Years)**	**n**	**IL-1β Mean ± SEM**	**IL-6 Mean ± SEM**	**TNF-α Mean ± SEM**
Positive	< 30	9	4.564±1.637	16.96±10.68	2.310±0.278
	30-39	22	4.413±0.863	4.90±0.63	2.224±0.147
	40-49	12	6.536±1.220	27.29±23.15	2.008±0.207
	50+	12	6.506±1.220	52.01±31.97	2.492±0.207
Test Statistics	*F Ratio*		*1.0834*	*1.4791*	*0.9362*
	P-value		*0.3681* ^{ns}	*0.2361* ^{ns}	*0.4329* ^{ns}
Negative	< 30	32	7.531±0.971	2.83±0.08	2.278±0.061
	30-39	15	6.244±2.075	2.97±0.07	2.130±0.131
	40-49	8	5.525±3.882	2.60±0.13	2.810±0.245
	50+	----	----	----	----
Test Statistics	*F Ratio*		*0.2570*	*0.6067*	*3.0063*
	P-value		*0.7747*^{ns}	*0.5504* ^{ns}	*0.0614*^{ns}

Abbreviations: SEM: Standard Error of Mean; IL-1β: Interleukin-1β; IL-6: interleukin-6; TNF-α: Tumor Necrosis Factor Alpha. Significance Level: ns=Not Significant (p>0.05).

DISCUSSION

Coronavirus disease 2019 (COVID-19) remains an ongoing global pandemic. It is assumed that in severe COVID-19 patients, inflammatory cytokines and coagulation cascade, including D-dimer are activated.

This study was carried out to evaluate some inflammatory cytokines in subjects with SARS-COV-2 in Port Harcourt. Data from the study revealed a significantly elevated level of an inflammatory cytokines involving IL-6 (p=0.0399) among subjects with COVID-19. A known study by Chen *et a*l., (2020) indicated a similar assumption with this study and pointed at the elevated level of IL-6 among individuals with COVID-19 compared with apparently healthy individuals, another study by meta-analysis reported by Henry *et al.*, 2020 confirmed an exaggerated elevation of IL-6 and IL-10 throughout the severe level of COVID-

19 infection. This elevation of IL-6 and other similar findings by Potere*et al.*,(2021), add to link the immunological features of severe-to-critical COVID-19 to those of cytokine storm syndromes as it is associated with coagulopathy known to interfere with the coagulation cascade through the stimulation of tissue factor and thrombin to stimulate platelet activity and induce endothelial dysfunction. More also this study revealed that there were no significant changes in the other inflammatory cytokines such as IL-1β and TNF-α (p=>0.05) of COVID-19 subjects when compared with COVID-19 negative subjects as control.

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Cytokine concentrations are elevated in patients with severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), given this findings, cytokine storm is problematic and alterative mechanisms of COVID-19 induced organ dysfunction are worth considering. The elevation of IL-6 is linked with the immunological features of severe-to-critical COVID-19 to those of cytokine storm syndromes as it is associated with coagulopathy known to interfere with the coagulation cascade through the stimulation of tissue factor and thrombin to stimulate platelet activity and induce endothelial dysfunction. Measurement of serum IL-6 should be performed extensively upon admission to clinic or isolation centres which will be key to identify patients with greater risk of progression to severe disease and therapy to adopt necessary precautionary measures.

6.2 Recommendation

The research on the assessment of some inflammatory cytokines in subjects with severe acute respiratory syndrome coronavirus 2 in Port-Harcourt determined that the most effective parameters to predict managements of COVID-19 patient on admission and severity are IL-6. Close monitoring of these parameter and early intervention in alteration are of vital importance.

6.3 Contribution to Knowledge

4. The study revealed thatthe presence of COVID-19 had more impact on IL-6 levels than in COVID-19 negative subjects.

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