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# Assessment of Some Inflammatory Cytokines of Interest in Patients with Severe Acute Respiratory Syndrome Coronavirus 2 Admitted in Isolation Centres in Port-Harcourt

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Coronavirus disease 2019 (COVID-19) is a novel and highly contagious viral infection caused by Sars-cov-2 and has been associated with a hyper-inflammatory immune response. This study aimed to evaluate changes in some inflammatory cytokines as clinical biomarkers to aid in the effective management of COVID-19 progression 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 fifty-five (55) COVID-19 positive subjects and fifty-five (55) COVID-19 negative subjects (controls), all between the ages of twenty (20) and seventy (70) years old, and both male and female subjects. Five milliliters (5 ml) of whole blood was collected using standard venipuncture

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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 positivity, a nasopharyngeal swab was collected aseptically using the RT-PCR technique. The results of 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 centres consider IL-6 as a biomarker for effective management of patients with COVID-19 disease.

*Keywords: COVID-19; cytokines; RT-PCR technique; pneumonia.*

## 1. INTRODUCTION

### 1.1 Background of the Study

The coronavirus illness, also known as COVID-19, has been causing significant pandemics over the world since December 2019. This has brought a number of issues to people's quality of life, health systems, and economical elements of life [1,2]. These difficulties could be explained by the peculiarities and dynamics of the disease's transmission as well as its symptoms and immune response [3].

The broad family of single-stranded RNA, non-segmented, enveloped coronaviruses can infect both humans and animals and cause disorders of the liver, nerves, gastrointestinal tract, and respiratory systems [4]. Positive-sense RNA viruses with an envelope and non-segments make up the family Coronaviridae of the order Nidovirales. They affect a lot of mammals, including people [5].

They are classified into five genera, including alpha, beta, gamma, delta, and Omicron coronaviruses, and are the largest known RNA viruses [6]. There are about six different types of human coronaviruses known, including alphacoronaviruses, betacoronaviruses, and a coronavirus that is in association with severe acute respiratory syndrome (SARS-CoV), as well as MERS-CoV, a virus that causes the Middle East respiratory illness [7,8]. The widespread and high prevalence of coronaviruses, as well as the viruses' ability to undergo diversification with respect to their genes, as well as their ability to regular undergo genetic recombination, together with an increase in activities involving human-to-animal contact, may all contribute to the periodic emergence of new coronavirus types in humans [9],(Zhu et al. 2020);

Many people were reported to have developed pneumonia in the late part of December 2019 by some local health authorities; this was later linked to a seafood market in Wuhan, Hubei Province, China (Zhu et al. 2020), and local hospitals then discovered a novel coronavirus known as severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) (Zhu et al. 2020) [10]. However, in the later part of January 2020, there was a declaration by the World Health Organization which referred COVID-19 to being a "public health emergency of international concern" [11]. On the 2nd day of December 2020, there were over 64 million cases worldwide and fewer than 1.5 million fatalities [12].

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 [13]. The "term corona" was originated in Latin, meaning crown [13]. 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 [14]. A lower respiratory tract infection may also manifest in pneumonia, bronchitis, and severe acute respiratory syndrome (SARS) (Schoeman and Fielding, 2019) [15]. The COVID-19 virus can also cause fever, a dry cough, headaches, myalgias, dyspnea, and exhaustion [16]. The Middle East respiratory syndrome coronavirus (MERSCoV), the coronavirus of severe acute respiratory syndrome 2 (SARS-CoV-2) [17], and that of severe acute respiratory syndrome (SARS-CoV) (Zhu et al. 2020) have all been linked to the disease (SARS). The majority of coronavirus infections in humans appear to be mild, but over 10,000 cases of the two betacoronaviruses SARS-CoV-2 [13] and Middle East respiratory syndrome coronavirus (MERS-CoV) [18] within the past 20 years have been reported, with death rates of 10% and 37%, respectively (Zhu et al. 2020) [17]. The period of incubation and clinical outcomes are similar with the Middle East respiratory Syndrome (MERS);

however, most cases of MERS proceed to impairment of the respiratory tract (Pal et al. 2020) [19].

The physiology of the human body is said to change as a result of SARS-CoV-2 infection, including the production of pro-inflammatory and inflammatory cytokines [20].

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, sputum, and aspirates from the trachea [21]. Currently, real-time reverse transcriptase PCR which qualitatively and quantitatively identifies viral genetic material, is the gold standard approach of laboratory molecular diagnosis of infection caused by the SARS-CoV-2 [22]. Other pertinent laboratory techniques may include serum viral neutralization (SVN) assays for measuring antibody neutralization and enzyme-linked immunoassays (EIA) for identifying viral antibody/antigen [23].

## 1.2 Statement of the Problem

There are discrepancies in reports from previous studies on the 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, the United States, Spain, Italy, Germany, France, Iran, Turkey, and the United Kingdom.

To effectively control the spread of the coronavirus and provide appropriate treatment to infected patients, physicians and the World Health Organization are consciously interested in gathering data on the pathogenicity and pathophysiology of the COVID-19 from around the world.

This study considered data from Port Harcourt, Rivers State, to evaluate how COVID-19 infection affects the above parameters in the patient's fellow residents in the area. The observed changes, added 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 a paucity of data for the assessment of some inflammatory cytokines in subjects with severe acute respiratory syndrome caused by 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-1 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 serum TNF levels in COVID-19-infected and uninfected 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 included patients with confirmed COVID-19 cases admitted to isolation centres and receiving home care in Port Harcourt, Rivers State, with or without symptoms, with a focus on inflammatory cytokines (IL-1, IL-6, and TNF-α).

### 1.8 The study's Research Questions

1. Are there alterations in serum IL-1 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-α levels among COVID-19 infected individuals?
4. Does sex influence the inflammatory parameters among subjects with COVID-19 infection?
5. Does age influence the inflammatory parameters among subjects with COVID-19 infection?

### 1.9 Research Hypotheses

The null and alternative hypotheses of this study are as follows:

1. Ho: There is no significant difference in serum IL-1 among subjects with COVID-19 infection compared to the control subjects.
   $H_1$: There is a significant differences in serum IL-1 among subjects with COVID-19 compared with the control subjects.
2. Ho: There is no significant difference in serum IL-6 among subjects with COVID-19 infection compared to the control subjects.
   $H_1$: There is a significant differences in serum IL-6 among subjects with COVID-19 compared with the control subjects.
3. Ho: There is no statistically significant difference in serum TNF levels between COVID-19 infections and controls.
   H1: Serum TNF levels differ significantly between COVID-19 patients and controls.
4. Ho: There is no statistically significant difference in some inflammatory parameters between subjects with COVID-19 infection and control subjects based on sex.
   $H_1$: There is a significant influence of sex in the inflammatory parameters among subjects with COVID-19 when compared with the control subjects.
5. Ho: There is no significant difference in some inflammatory parameters between subjects with COVID-19 infection and control subjects based on age.
   $H_1$: There is a significant influence of age in the inflammatory parameters among subjects with COVID-19 compared with the control subjects.

## 2. LITERATURE REVIEW

### 2.1 Epidemiology of the COVID-19 Outbreak

Since December of 2019, several cases of pneumonia with no known cause have been reported in some hospitals in the Chinese city of Wuhan [24]. History-taking revealed that the cases were as a result of being exposed to a market where seafoods were sold; this market was however situated in Wuhan, China. Soon enough, there was confirmation that the pneumonia was an acute infection of the respiratory tract induced by a coronavirus they termed “novel”, which kept on spreading rapidly throughout the city of Wuhan to the whole of China and then to other parts of the world, such that the 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 [25,26]. In the earlier part of March 2020, there was a report of a total of 80, 302 SARS-CoV-2 positive patients in China with 2947 (3.66 percent) mortality [24]. Also, as of the 11th of February 2020, approximately 1715 medical workers had been infected with SARS-CoV-2, with 5 fatalities [27]. However, 10,415 COVID-19 cases were confirmed internationally (outside of China) in 66 countries and 6 continents [27].

On the 14$^{th}$ day of April 2020, there was also a declaration by the World Health Organization, referring to SARS-CoV-2 as a pandemic, with a record of 1,844,683 confirmed cases and 117,021 deaths all over the globe [28]. In order to characterize the novel coronavirus, swabs from both the throat and bronchoalveolar lavage fluid were obtained from 9 patients who had visited the seafood market in Wuhan within the period of the first outbreak. The obtained samples were inoculated into special pathogen-free human airway epithelial (HAE) cells via the apical surfaces. This was followed by monitoring of the HAE cells for the presence of cytopathic effects and consequent collection of supernatants for analysis using the real-time polymerase chain reaction. Some studies have reported that SARS-CoV-2 originated from bats [29], while other studies reported its relationship with pangolins [10,30].

### 2.2 SARS-CoV-2 Morphology

It was discovered by electron microscopy that the SARS-CoV-2 is between 70 and 90 nm in size

[31]. Because of their highly comparable sequences, SARS-CoV-2 and SARS-CoV are believed to share the same structural characteristics [28]. Spike (S) proteins on the surface of the virus give it the appearance of a crown. The envelope protein (E), membrane protein (M), nucleocapsid protein (N), and internal protein (I) are additional structural proteins found on the surface of the SARS-CoV-2 virus [4]. The lipid-bilayer produced from the host membrane, which encases the helical nucleocapsid containing the viral RNA, is embedded in the surface of the spike proteins, membrane, and envelope of the SARS-CoV-2 [32]. The structure of the SARS-CoV-2 is represented in Fig. 1.

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

The genome of SARS-CoV-2 ranges between 26 and 32 kb in size and consists of about six to eleven open reading frames (ORF), which encode poly-proteins of 9680 amino acids [34]. 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 the 50 and 30 ends of 265 and 358 nucleotides, respectively [34]. The non-structural proteins (nsps) present are two cysteine proteases of the virus, such as the protease that is like papain (nsp3), the protease that is like chymotrypsin, the protease that is similar to 3C, or the primary protease itself (which is nsp5), RNA-dependent RNA polymerase (nsp12), and lots more with a possible likelihood of getting involved in the transcription and replication of SARS-CoV-2 (Chan et al. (2020).

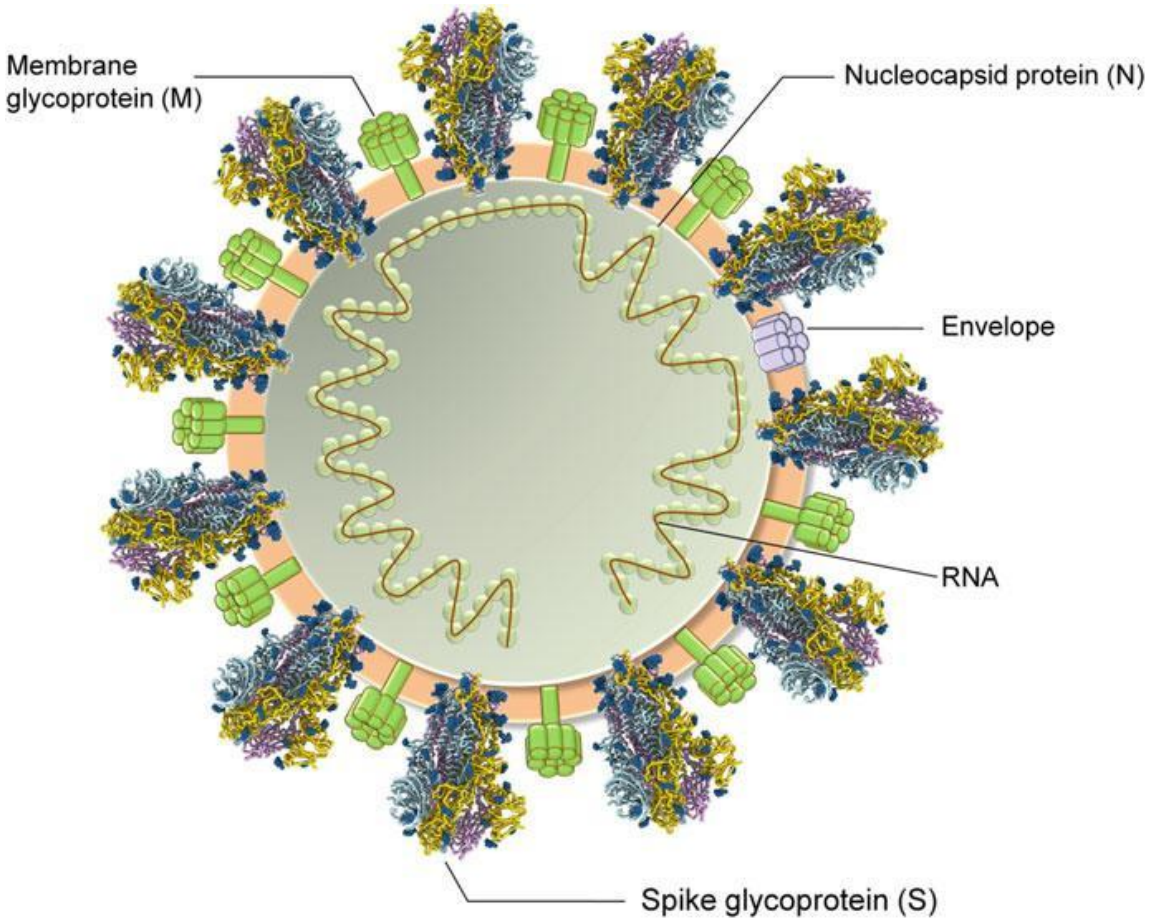


**Fig. 1. Structure of SARS-CoV-2 [33]**

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) [35], 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 [36].

## 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 [37]. SARS-CoV-2 is transmitted either via exposure to micro-droplets from infected persons or by getting direct contact with contaminated fomites [38].

As shown in Fig. 2 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 [39]. 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). Severe acute respiratory syndrome coronavirus 2 (ARS-CoV-2) may be seen in tissues outside the lungs, and this may be so because there is a wide-spread of the ACE2 receptors in those tissues; Agrawal et al. [40] 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. [41] 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 [36]. 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 [39]. The diagrammatic representation of the entry and replication of SARS-CoV-2 in host cells is shown in Fig. 2.

Interferons are part of cellular defense mechanisms, which are inhibited by the SAR-CoV-2. Notably, the primary targets of the virions happen to be the lymphocytes, vascular endothelial cells, and alveolar epithelial cells. During the replication of the virus, a large number of virions are released which then proceed to infect nearby target cells, which in turn, results in an exaggeration of pulmonary and systemic inflammatory responses [38].

## 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 [42]. There was a significant decrease in CD4 and CD8 T cell counts following cytometric analysis of peripheral blood samples [43]. Also, chest X-ray images revealed a rapidly progressing pneumonia with

apparent variations between the left and right lungs; a biopsy of the lung was used for histological analysis, and the result revealed damage in the alveolus [44]. In the right lung, pneumocytes were prominently desquamated, and a hyaline membrane was formed, which is indicative of acute respiratory distress syndrome (ARDS). In the left lung however, a hyaline membrane was formed accompanied by pulmonary edema [45]. Additionally, in both the left and right lungs, lymphocytes were seen to have dominance [46].

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 [45]. 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 [45].

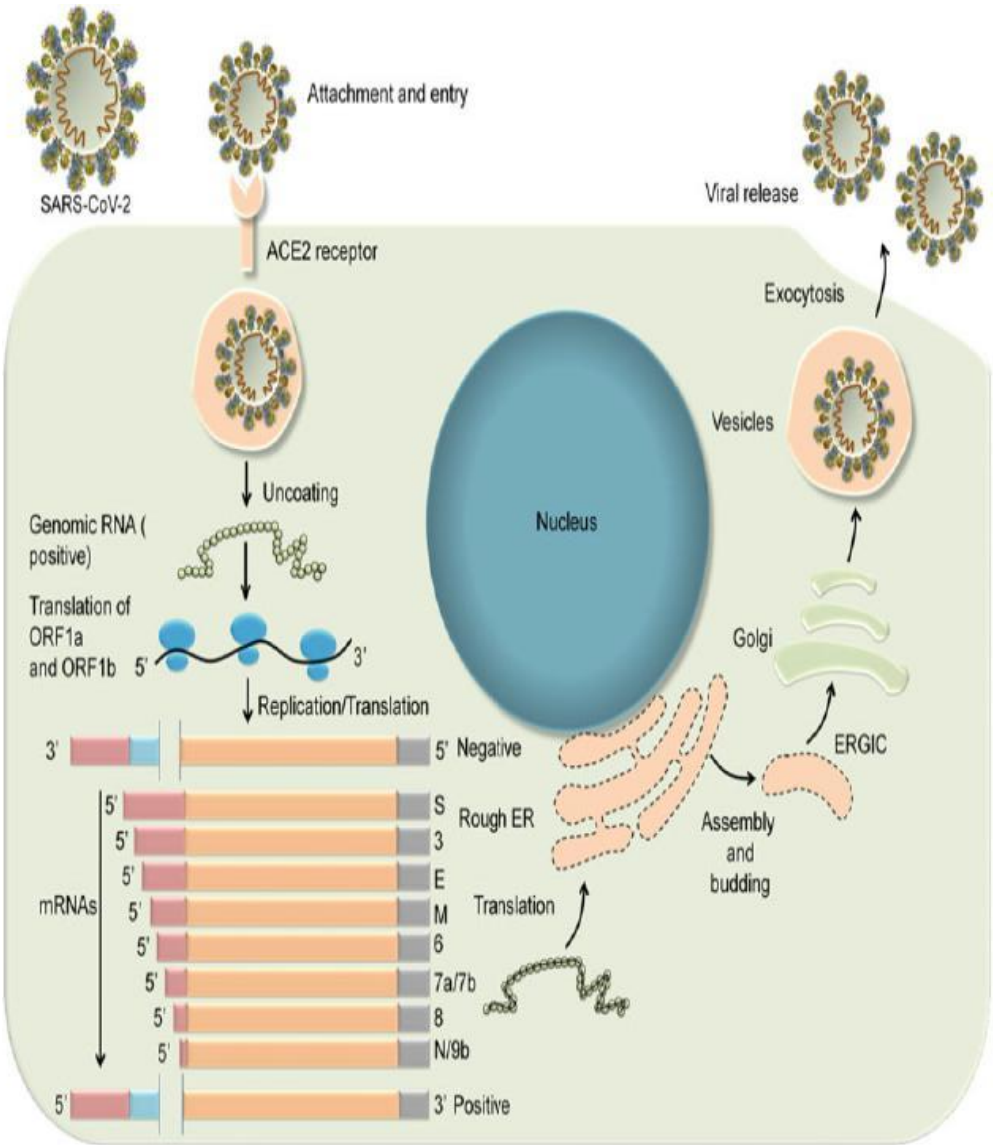


**Fig. 2. Entry and Replication of SARS-CoV-2 in Host Cells [33]**

## 2.6 Clinical Manifestations of SARS-CoV-2 Infection

Patients with SARS-CoV-2 present with several clinical manifestations ranging from mild to moderate to 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 [24]. The clinical manifestations of COVID-19 are not specific, where the disease presents as being asymptomatic to having severe symptoms of pneumonia, and consequently mortality. The incubation period of SAR-CoV-2 is between 2 and 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), headache, and fever. Similarly, cough and difficulty in breathing usually develop as symptoms of respiratory distress, followed by pneumonia and respiratory deterioration in about 30 percent of cases [10].

Interestingly, there is a similarity in the period of incubation period and the clinical course between those of MERS and those of SARS, with the exception of the fact that more cases undergo progression to deterioration of the respiratory tract. Similarly, there may be a similarity in the period of incubation and clinical course between SARS-CoV-2 and SARS infections [47,48]. Individuals with severe SARS-CoV-2 infection may present with dyspnoea with breath sounds that are weak, increased or decreased tactile speech tremor [24], and others.

X-ray examination of the chest during the early stages of the pneumonia-like symptoms reveals several patchy shadows and changes in interstitial cells present in the lung periphery [49]. Severe cases can further develop into bilateral multiple ground-glass opacities, infiltrating shadows, and pulmonary consolidation with infrequent pleural effusion. A *computerized tomography* (CT) scan of the chest reveals pulmonary lesions more clearly than does an X-ray examination, as well as ground-glass opacity and segmental consolidation in bilateral lungs, especially in the lung periphery.

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

There are two phases of the immune response to COVID-19 infections: the first phase entails the onset and incubation period of the virus; during this period, the proliferation and elimination of the virus was done by specific adaptive immune responses, and as such, the disease is prevented from progressing. In the second phase, certain cytokines are released in some patients, which in turn, promote the disease progression from being mild to being severe, with consequent damaging of organs [50].

SARS-CoV-2 infection induces activation of the immune system via varying receptors such as the Toll-like receptors [51]. 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, and myalgia), leading to inflammation of the lungs [52].

Inflammatory cytokines and chemokines, such as interleukin (IL)-2R, IL-6, IL-8, IL-10, and tissue necrosis factor (TNF)-alpha, were elevated in cases where the SARS-CoV-2 infection is severe compared to mild infection [53], (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 [54]. Several studies revealed that interleukin (IL)-1b levels were elevated during COVID-19 infection [54]. 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 [55]. As shown in Fig. 3, 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 [56]. Interleukin 1 beta (IL-1b) is also produced by NK cells and B lymphocytes [57]. Interleukin 1 beta (IL-1b) can induce the expression of multiple genes that regulate fever, hypotension, pain threshold, and vasodilatation [54]. 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 [58]. 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 [59]. 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 [60]. 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 cryopyrin associated periodic syndrome (CAPS), familial Mediterranean fever (FMF), and tumor necrosis factor (TNF) receptor associated periodic syndrome (TRAPS), which are triggered by mutations [54].

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 [54]. 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 [54].

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 [61]. Because immune genes are mostly located on the X chromosome, COVID-19 mostly affects men [62]. The RNA of SARS-CoV-2 expresses at least 27 proteins, including 15 non-structural, 4 structural, and 8 auxiliary proteins [63]. 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 [64]. ACE-2Rs are expressed by mature lung epithelial cells, intestinal enterocytes (Mardi et al. 2020), neurons and glial cells [65], endothelial cells, and kidney proximal tubular cells [66]. Given the expression of ACE-2R by neurons and glial cells, SARS-CoV-2 may show extensive neurological manifestations, including stroke [63], which is probably related to higher D-dimer levels [67]. The extensive expression of ACE-2R on the respiratory system epithelium clarifies the involvement of the lung tissue in SARS-CoV-2 infection [63]. 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 [64]. Lymphopenia is one of the most important indicators of SARS-CoV-2 infection, which is observed in almost 80% of cases [68]. 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 a cytokine storm [54]. However, another study determined that viruses can directly infect T lymphocytes but are not able to replicate inside them [68]. Infection of T cells may lead to cell death by apoptosis, necrosis, or pyroptosis [68], or T cells (especially CD4+ T cells) are probably destroyed by the immune system [65].

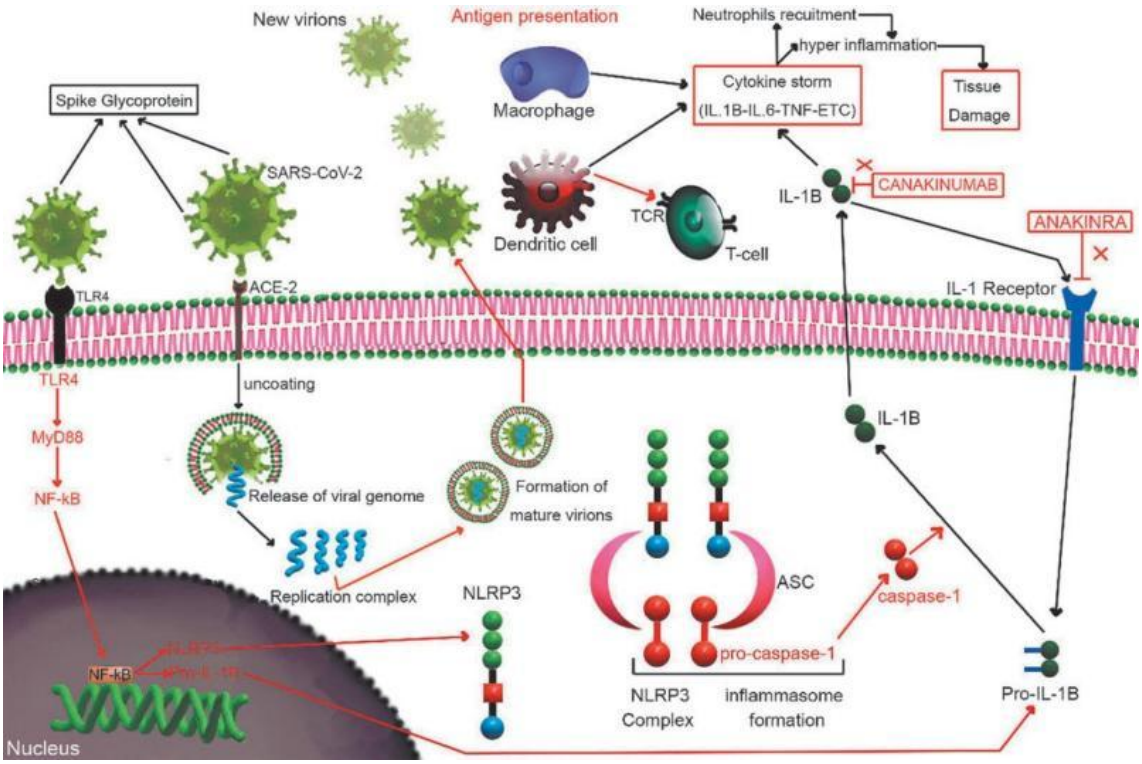


**Fig. 3. Immunopathogenesis of SARS-CoV-2 Infection, and the Potential Role of IL-1 in COVID-19 Pathogenesis**

*[54]*

### 2.7.2 Role of IL-6 in COVID-19 infection

Usually in the later stages of the infection, hyperinflammatory responses are dysregulated by SARS-CoV-2. This is due to the fact that the virus infects monocytes, macrophages, and dendritic cells, which then results in an elevated secretion of inflammatory cytokines such as interleukin-6 (IL-6) [69]. This IL-6 has been reported to be the most essential inflammatory cytokines [70,71]. This cytokine was found to similar to other proteins which have different activity which is an indication of its pleiotropic nature; this was reported after its molecular cloning. However, the receptor for IL-6 has been reported to be highly involved in the pathophysiology of many diseases, and thus inhibition of this receptor has also been reported to be of benefit to humans, particularly in diseases such as rheumatoid arthritis and Castleman disease [72].

In COVID-19, IL-6 is believed to drive multi-organ injury, the most severe form of the illness [73,71]. To this end, IL-6 blockade was postulated to help reduce the inflammatory burden of COVID-19 in the setting of a cytokine storm and improve the clinical status of patients [74,75]. In this narrative review, the basic concepts of IL-6 physiology and pathophysiology were discussed, as well as approval of therapeutic indications for IL-6 blockade. Consequently, details involving the importance of IL-6 in the pathophysiology of COVID-19 alongside its prognostic implications were discussed.

SARS-CoV-2 enters into the host cells through binding to its receptor angiotensin-converting enzyme 2 (ACE2), after which it undergoes a replication process. On entrance into human cells, macrophages, neutrophils, and dendritic cells become activated, so as to capture the virus. Cells which have been damaged release pathogen-associated molecular patterns (PAMPs), which in turn, trigger the stimulation of more immune cells to be recruited, and then release of IL-6 and other inflammatory cytokines, which work together to induce the alveolar vessels to be more permeable, and more recruitment of immune cells to the site of infection; this sustains the positive, hyperinflammatory loop. Due to permeability of lung vessel, the virus spreads to other organs that have increased number of ACE2, such as the kidney, intestine, and pancreas as shown in Fig. 4.

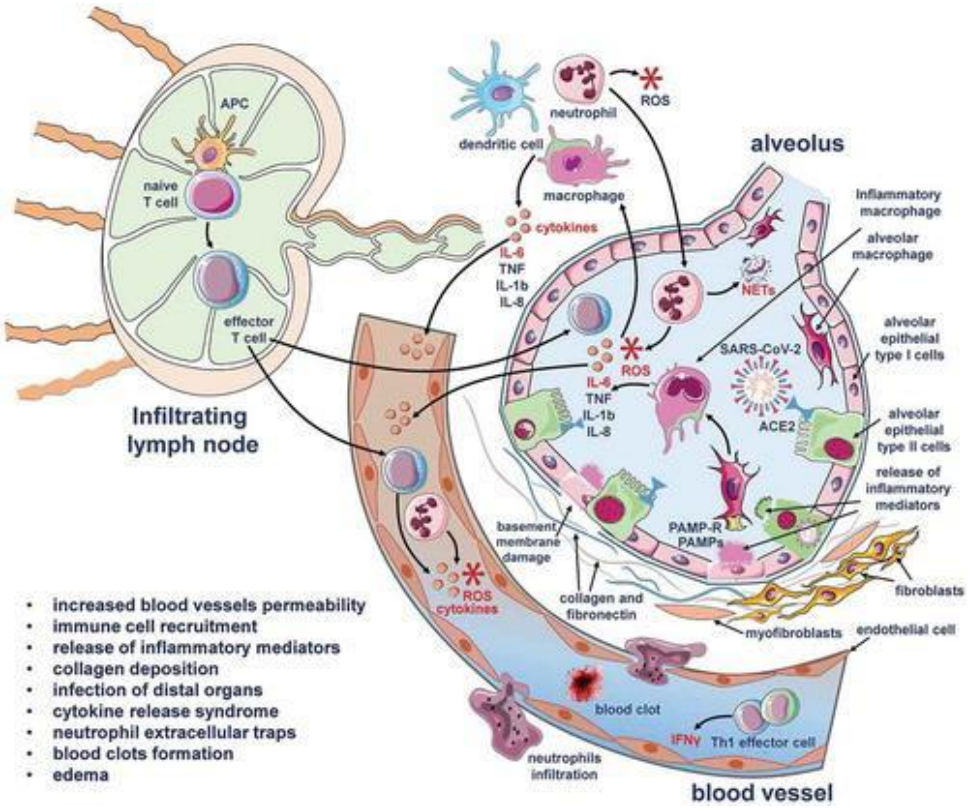


**Fig. 4. Induction of a Deregulated Hyperinflammatory Response by SARS-CoV-2 [71]**

*2.7.2.1 Pathophysiology of interleukin-6 in COVID-19*

Interleukin-6 has been reported to play a crucial role in the third stage of the clinical-therapeutic staging of COVID-19 [76]; this third stage is characterized by abnormal systemic hyper-inflammatory response, and dysregulated cytokine release which is clinically responsible for severe COVID-19, in which case, the levels of IL-6 is significantly elevated [77,78]. Some highly inflammatory macrophages (with the exception of alveolar macrophages) are responsible for producing IL-6 [79].

At the early phase of the viral infection, IL-6 is absent, and it was revealed that this absence resulted in the depression of T follicular helper cell maturation, which in turn resulted in a decline in antiviral response. As reported by Diao et al. [80], patients who were admitted at the intensive care unit (ICU) due to COVID-19 had a negative correlation between IL-6 and other cytokines, as well as $CD4^+$ and $CD8^+$ T cells [80]. This may be suggestive of aberrant IL-6 production, which in turn, has a negative impact on adaptive immunity.

According to Potere et al. [71], hyperinflammatory response that was typically exaggerated was reported from studies in China, which noted elevated levels of several inflammatory mediators, such as IL-6, IL-1β, IL-18, IL-8, granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF). Also, Ruan et al. [81] reported that increased levels of inflammatory mediators in COVID-19 patients were associated with worse outcomes. Huang et al. [47] revealed that patients with COVID-19 who are progressing to ARDS had elevated levels of cytokines such as IL-6, IL-1β, and tumor necrosis factor (TNF)-α. This abnormally elevated cytokine levels are responsible for the induction of the exaggerated activation of the immune system that, in turn, enhances further release of cytokines and chemokines.

Also, it has been reported an association between the dysregulated inflammation and abnormalities in the coagulation cascade, which has also been reported to cause immune thrombotic processes; these processes are thus responsible for organ damage [82]. SARS-CoV-2 preferentially infects type II pneumocytes and

alveolar macrophages within the lungs [83], (Hu et al. 2021). Recently, Patra et al. [84] 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 [85]. 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 [71], leading to the production and release of IL-1β, which is upstream of IL-6.

There is usually a release of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) from damaged pneumocytes, which in turn enhance the activation of the epithelium of the lung, and resident immune cells [83]. When activated, neutrophils, antigen-presenting cells, and macrophages together with the local replication of SARS-CoV-2 induce elevated release of cytokines, particularly IL-6, IL-1β, and TNF-α, which then result in organ damage, especially the lungs; this is owing to the fact that the uncontrolled inflammatory response undergoes self-propagation.

Additionally, IL-6 is said to be correlated with viral load, where the viral load is associated with the severity of ARDS and lung damage. Also, Potere et al. [71] has reported elevated permeability of blood vessels during infections, which permits the infiltration of immune cell and spread of the virus; this is often accompanied by the production of inflammatory mediators, such as IL-6, that further increase the severity of the hyper-inflammatory environment [71].

IL-6 has been reported to interfere with the coagulation cascade via the generation of tissue factor and thrombin, and stimulate platelet activity, and induce endothelial dysfunction; thus, it is said to be involved in coagulopathy associated with COVID-19. Therefore, the hypercoagulable state reported in COVID-19 could be improved with the use of tocilizumab [71]. Recently, an evidence was provided by Canzano et al. [86] which reported that coagulopathy in COVID-19 may get contribution from cells that have been activated, and is modulated by tissue factor produced by platelets, granulocytes, and micro vesicles due to endothelial cell dysfunction; these events are however, sustained by elevated levels of IL-6. However, using tocilizumab and antiplatelet drugs to block the IL-6 were reported to be beneficial in eliminating these effects [71].

### 2.7.3 Tumor necrosis factor's role in COVID-19 disease

Tumor necrosis factor (TNF), a 17 kDa protein consisting of 157 amino acids, is a hormone in solution that is mainly produced by activated macrophages, T lymphocytes, and natural killer (NK) cells [87].

Proinflammatory cytokines such as TNF and interleukin (IL)-1 play a key role in the pathogenesis of rheumatoid arthritis (RA). TNF has major effects on bone remodeling: it regulates the bone marrow levels of osteoclast precursors directly by up-regulating c-fms expression and activates osteoclasts by enhancing the signaling mechanisms of the receptor activator of NF-B (RANK). It also plays an important role in controlling infection [87]. 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 [87].

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 [87].

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 [88]. Severe disease is associated with a "cytokine storm," a delayed-onset burst of pro-inflammatory cytokines in circulation. The cytokines associated with fatalities are TNF, IL-6, IL-8, IFN, and possibly others [61]. 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 [89]. 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 [90].

The role of TNF in COVID-19 disease has been suggested, and TNF inhibition was shown to be effective in lowering the incidence of hospitalization in one study [91], but did not improve health status or lung function in the other [88]. 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 [92]. Although the role of TNF in this process is not established, there is evidence for TNF involvement in a closely related form of idiopathic pulmonary fibrosis [93]. Administration of anti-TNF drugs during the acute phase of infection may subsequently alleviate the development of this complication.

The effects of TNF on the cardiovascular system are also well known. Tumor necrosis factor (TNF) plays an important role in the development of heart failure through direct negative inotropic and pro-apoptotic effects on cardiomyocytes, as well as through other mechanisms [94]. TNF is also elevated in patients with hypertension [88]. Moreover, TNF levels are increased in obesity, and TNF is considered to play a role in insulin resistance [95]. All these conditions are risk factors for the development of severe COVID-19 disease and its 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 [88]. Tumor necrosis factor (TNF) also inhibits fibrinolysis by increasing the plasminogen activator inhibitor [88]. Reports on pro-coagulant activities induced by IL-6 are scarce [72]. 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 the elderly population [72]. Age-related loss of muscle mass and strength is particularly attributed to the action of TNF [96], and exposure of human cells to TNF in vitro can induce cell senescence [97]. TNF's strong association with aging may explain, to some extent, the higher incidence of severe COVID-19 disease in elderly patients. Interestingly, mTOR inhibitors have been suggested recently for the treatment of severe disease based on their ability to alleviate cytokine storms [98]. 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 [99]. On the other hand, the effects of mTOR inhibition may be reduced to a direct inhibition of TNF synthesis or signal transduction [88].

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 the anti-inflammatory effect of various antidepressants [88]. 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 [88]. At least two clinical trials are currently being conducted to investigate the impact of this class of drugs on disease outcomes (NCT04342663, NCT04377308) [88].

## 2.8 Coagulopathy Mechanism in COVID-19

COVID-19 pneumonia appears to have distinguishing features compared to conventional pneumonia. COVID-19 patients clearly develop a dysregulated, uncontrolled host response, resulting in the overproduction of many inflammatory cytokines and chemokines such as TNF-, IL-1, IL-6, and IL-8 [100]. The release of these molecules induces a 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 [88].

Interleukin-6 (IL-6) is a key cytokine that is significantly elevated in severe COVID-19 infection and acts as a key activator of coagulopathy by inducing tissue factor expression and increasing fibrinogen and platelet production [100]. In COVID-19 patients requiring mechanical ventilation, median IL-6 is reported to be between 121-218 pg/mL [100]. 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 endothelitis in COVID-19 pathogenesis [100]. A recent postmortem series showed evidence of a direct multi-organ infection of the endothelial cells with COVID-19 and an associated diffuse inflammation. Apoptosis and pyroptosis were suggested as possible mediators of endothelial injury in these patients [100]. Notably, this inflammatory endothelial cascade can directly result in microvascular dysfunction and occlusion but can also induce a hypercoagulable state, resulting in microvascular thrombosis. Moreover, hypoxia, a frequent feature of severe COVID-19, is a prominent stimulant of thrombosis via the expression of hypoxia-inducible transcription factors, which in turn target several genes that regulate thrombosis [101]. 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 [102]. 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 reflects the immune thrombosis induced by PIC [100].

## 2.9 Laboratory Identification of SARS-CoV-2

Laboratory diagnosis of the virus is essential in distinguishing it from other viruses that cause pneumonia, and from diseases that are non-infectious, including vasculitis and dermatomyositis [25]. It involves isolation of the virus, followed by detection of the viral nucleic acid; As described by Yu et al. [103], Koch`s postulated a theory which emphasized on the need for the isolation of the virus, which is regarded as the “gold standard” for the identification of viruses in the laboratory. However, in other for SARS-CoV-2 to be diagnosed, the appropriate sample must be collected from the patient at the appropriate 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 [27]. 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) [104,27], and these segments are targeted by primers. However, Corman et al. [104] 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) [105]. 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 [104]. 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 *Staphylococcus aureus* and *Candida albicans*) 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 [104].

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 [104].

## 3. 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 and Eleme Isolation Centres. 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) [106].

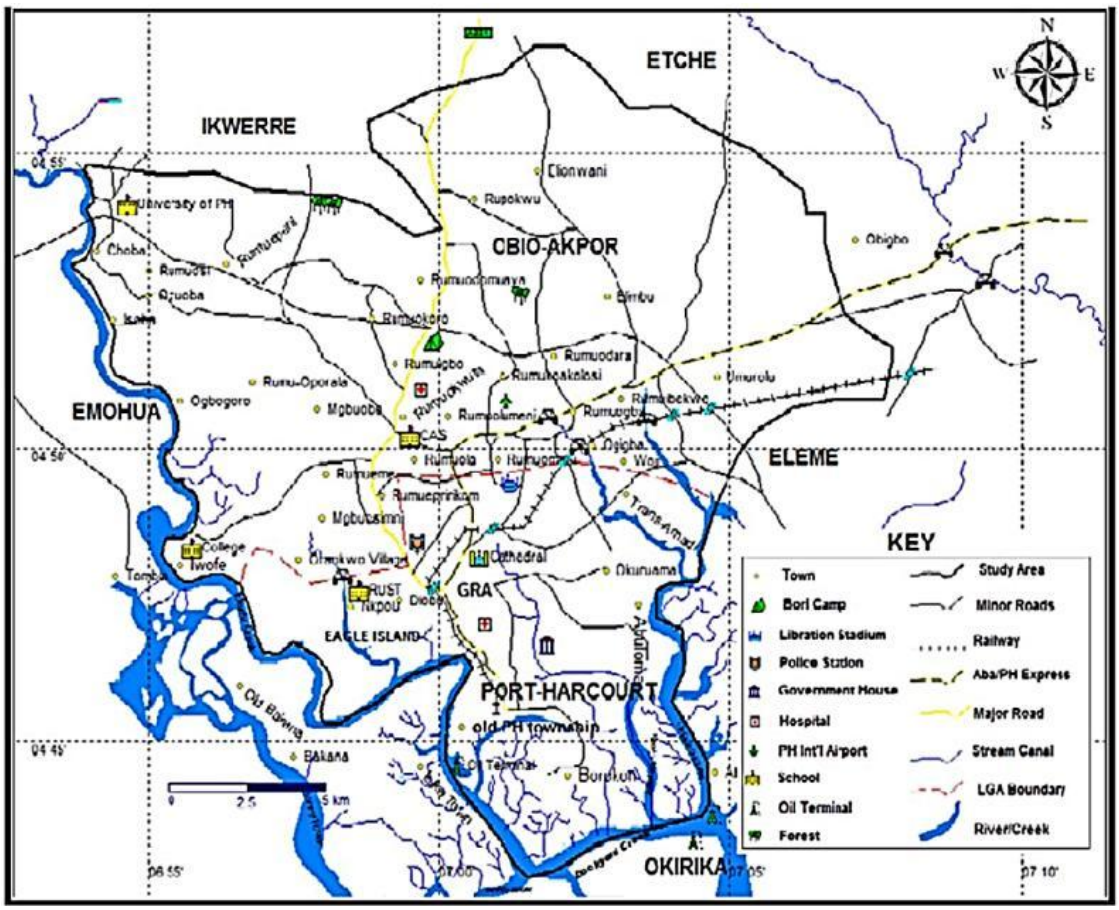


**Fig. 5. Map of Port Harcourt metropolis**
*Source. Adapted from Google Earth (2012)*

## 3.3 Study Population

A total of 110 subjects comprising of 58 males and 53 females 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. Those who gave both informed and written consent were recruited and a well-structured questionnaire was administered to every subject to obtain their demographic information.

### 3.3.1 Sample size calculation

Sample size was determined using Cochran's Formula [107]

$$n = \frac{z2\ (pq)}{e2}$$

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% [108].

Using the formula;

Sample size for COVID-19 in Rivers State **(n) = $\underline{1.96^2 x 0.06 x 0.6}$ = 55.3 i.e. n = 55 $0.05^2$**

## 3.4 Eligibility Criteria

### 3.4.1 Inclusion criteria

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

### 3.4.2 Exclusion criteria

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

## 3.5 Blood Sample Collection

About 5ml 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.

## 3.6 Sample Analysis

### 3.6.1 Coronavirus analysis

*3.6.1.1 Method*

Manual RNA extraction and RT-PCR, (kit supplied by Liferiver Lot Number ZJ0009), as described by Arya et al. [109] was employed.

*3.6.1.2 Principle*

The principle of 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 in real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected (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 complementary DNA (cDNA) by reverse transcriptase from total RNA. The cDNA is then used as a template for the real-time PCR.

*3.6.1.3 Reagent preparation*

After receipt, the kit was kept at -205 °C, and the reagents were kept in a clean environment and kept at -20±5 °C. Reduce the times of multiple freeze-thaws and the likelihood of contamination, reagents were properly thawed, mixed before use, and sub-aliquoted. Five sets of RT-PCR buffer, enzyme mix, and reaction mix were aliquoted into labeled centrifuge tubes and stored at -20±5°C without light. Then 75μL/tube of RT-PCR buffer was sub-packed into 5 centrifuge tubes, 50μL/tube. Enzyme mix was sub-packed into 5 centrifuge tubes, 40μL/tube. The SARS-CoV-2 Reaction Mix was divided into 5 centrifuge tubes, with 35μL/tube. To avoid contamination, RNase-free Water was sub-packed into 5 centrifuge tubes, and the RNase-free Water and Positive Control were carefully processed in the specimen processing area. It was ensured that 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℃.

*3.6.1.4 Procedure*

The number of samples, including the number of prepared samples and controls, multiplies the quantities of Super Mix and Enzyme Mix per reaction. 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 various reaction tubes, the tubes were immediately sealed to prevent contamination. The reaction tubes were swiftly spun down to collect the master mix and template, after which the instrument MIC POC DX48 was employed. 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.

**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 detected [b] |
| - | - | - | _ | Invalid;<br>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 C> 41;*
*"/" represents no requirement, Detection of internal control is not required if result positive in any of the other three detection channels.*

*3.6.1.5 Data analysis and interpretation*

Table 1 lists the expected results for the SARS-CoV-2 Real-Time Multiplex RT-PCR Kit. For all results that did not follow these guidelines, samples were re-extracted and re-tested.

### 3.6.2 Determination of inflammatory cytokines

*3.6.2.1 Interleukin (IL)-1β*

3.6.2.1.1 Method of assay

Enzyme-Linked Immunosorbent Assay (ELISA) as described by (Fristiohady et al. 2020) was used

3.6.2.1.2 Principle

Sandwich-Elisa principle was 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 were added successively to reach micro plate well and incubated. Free components were 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 appeared blue in color. The enzyme substrate reaction were 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.

3.6.2.1.3 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 were 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

*3.6.2.2 Interleukin (IL)-6*

3.6.2.2.1 Method of assay

Enzyme-Linked Immunosorbent Assay (ELISA) as described by (Fristiohady et al. 2020) was used.

3.6.2.2.2 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 are added 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 were 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 were then determined by comparing the OD of the samples to the standard curve.

3.6.2.2.3 Procedure

Wells were 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 were 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 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.

*3.6.2.3 Tissue Necrotic Factor (TNF)-Alpha*

3.6.2.3.1 Method of assay

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

3.6.2.3.2 Principle

Sandwich-Elisa principle was applied in the TNF-α. The micro Elisa plate provided is precoated with an antibody specific to human TNF-α. Samples or standards were 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 were 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.

3.6.2.3.3 Procedure

Wells were 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 were 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 were 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.

## 4. 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

There were 55 COVID-19 positive subjects, comprising of 35 males and 20 females, and another 55 COVID-19 negative subjects comprising of 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 in the same order according to age group and 38, 10 and 7 in the same order for COVID-19 negative subjects as shown in Fig. 6.

### 4.2 Comparison of Inflammatory Cytokines among the Study Participants

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

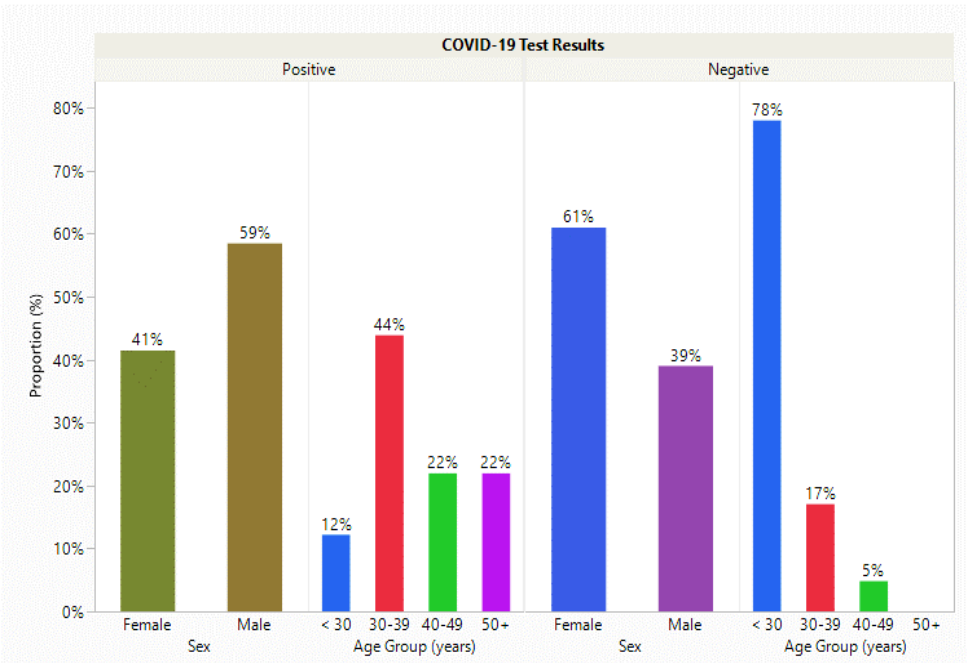


**Fig. 6. Distribution of demographic characteristics of study population**

**Table 2. Comparison of inflammatory cytokines among the study participants**

| 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)*

**Table 3. Comparison of inflammatory cytokines by sex among the study subjects by sex**

| COVID-19 Results | Sex | n | IL-1β (pg/ml) | IL-6 (pg/ml) | TNF-α (pg/ml) |
|---|---|---|---|---|---|
| | | | Mean ± SEM | Mean ± SEM | 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 |
| | Female | 32 | 7.26±0.92 | 2.67±7.93 | 2.215±0.102 |
| Negative | 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)*

### 4.3 Comparison of Inflammatory Cytokines among the Study Subjects 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±1.11pg/ml, 7.53±9.61pg/ml and 2.246±0.124pg/ml and male positive patients were 6.34±0.94pg/ml, 31.61±8.09pg/ml and 2.246±0.104 in the same order when compared with the negative female subjects 7.26±0.92pg/ml, 2.67±7.93pg/ml and 2.215±0.102pg/ml and male subjects 7.13±1.15pg/ml, 3.10±9.91pg/ml and 2.378±0.128pg/ml 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.

### 4.4 Comparison of Inflammatory Cytokines among Study Subjects by Age Groups

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

## 5. DISCUSSION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) remains an ongoing global pandemic. There is an assumption that the haemostatic cascade and inflammatory cytokines become activated in patients with COVID-19 [110-112].

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 increased level of an IL-6 (p = 0.0399) among subjects with COVID-19. This study agrees with the findings of Chen et al. [53] which showed an elevated level of IL-6 among individuals with COVID-19 compared with apparently healthy individuals. This study is also in agreement with Henry et al. (2020) which confirmed an exaggerated increase in IL-6 and IL-10 throughout the severe stage of COVID-19 infection. This elevation of IL-6 and other similar findings by Potere et al. [71] add to the link between features of the immunological reactions involved in COVID-19 and the cytokine storm syndrome, which is said to be in association with coagulopathy, which in turn is said to induce interference in the cascade of coagulation by enhancing platelet function and endothelial dysfunction via the activation of tissue factor and thrombin.

Furthermore, when COVID-19 subjects were compared to COVID-19 negative subjects as controls, there were no significant differences in other inflammatory cytokines including IL-1 and TNF-α (p > 0.05). In this study, it was also observed that age and gender have no statistically significant influence on the inflammatory cytokines of patients with COVID-19 when compared to the control subjects (p > 0.05).

**Table 4. Comparison of inflammatory cytokines by age groups**

| COVID-19 Results | Age Group (Years) | n | IL-1β | IL-6 | TNF-α |
|---|---|---|---|---|---|
| | | | Mean ± SEM | Mean ± SEM | 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)*

## 6. CONCLUSION AND RECOMMENDATION

### 6.1 Conclusion

Cytokine concentrations are increased in the patients with SARS-COV-2; given these findings, cytokine storm is problematic, and alternative mechanisms of COVID-19-induced organ dysfunction are worth considering. This elevation of IL-6 and other similar findings by Potere et al. [71] add to the link between features of the immunological reactions involved in COVID-19 and the cytokine storm syndrome, which is said to be in association with coagulopathy, which in turn is said to induce interference in the cascade of coagulation by enhancing platelet function and endothelial dysfunction via the activation of tissue factor and thrombin. Measurement of serum IL-6 should be performed extensively upon admission to a clinic or isolation centres, which will be key to identifying patients with a greater risk of progression to severe disease and guiding therapy to adopt the necessary precautionary measures [113,114].

### 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 the management of COVID-19 patients on admission and severity are IL-6. Close monitoring of these parameters and early intervention in the event of changes are critical.

### 6.3 Contribution to Knowledge

The presence of COVID-19 had a greater impact on IL-6 levels in positive subjects than in negative subjects, according to the study.

## ETHICAL CONSIDERATION AND 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.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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