Comparative analysis of expression pattern of *IL-10, IL-6, IL1β* and *TNF-α* genes and genetic variation of *ACE2* gene among COVID-19 patients cohort from Western India

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by

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CERTIFICATE

This is to certify that this dissertation entitled **Comparative analysis of expression pattern of *IL-10, IL-6, IL1β* and *TNF-α* genes and genetic variation of *ACE2* gene among COVID-19 patients cohort from Western India** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Taku Aammee at Indian Institute of Science Education and Research under the supervision of Dr. HariOm Singh, Department of Molecular Biology, ICMR-NITVAR during the academic year 2024-25.

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This thesis is dedicated to my parents, who always motivated me and assured me to not give up on myself and others.

DECLARATION

I hereby declare that the matter embodied in the report entitled "**Comparative analysis of expression pattern of *IL-10, IL-6, IL1β* and *TNF-α* genes and genetic variation of *ACE2* gene among COVID-19 patients cohort from Western India**" are the results of the work carried out by me at the Department of molecular biology, ICMR-NITVAR, Pune, under the supervision of Dr. HariOm Singh and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Taku Aammee

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Abstract

Background: Host factors ranging from cell surface receptor to immune and metabolic regulators, play an important role in deciding the fate of COVID-19 disease. Genetic variants tune our immune systems differently. Cellular factors (ACE2, TMPRSS2), detected in nasal and bronchial epithelia play a crucial role in cellular entry and HLA elicits specific antiviral immunity, thus contribute in immune system protection. There is a limited data on genetic differences, which contribute to individual variations in the susceptibility to SARS-CoV-2 infection and disease severity. Following exposure to SARS-CoV-2 leads Chronic inflammation in the lungs, persistent inflammation in the lungs is associated with increased level of inflammatory cytokines, IL-6, which impair the SARS-CoV-2 induced pathology. Immune associated genes play a role in innate and antiviral immune response. Cellular factors are involved in spread of virus and its pathogenesis. Clinical outcomes of SARS-CoV-2 infection are varied from individual to individual and population to population. This variation is linked with genetic background. Difference in responses of individuals to the infection and disease condition may influence by genetic variants of ACE 2, TMPRSS2, FUR and Immune and inflammation -related genes and different levels of expression and function of *these* proteins. Hence, we aimed to examine the genetic variation and expression of cellular factor and inflammation -related genes among COVID-19 patients.

Objective: To examine the genetic variation of cellular factors (*ACE2*) and, expression of inflammation -related genes (*IP-10, IL-6, IL1β* and *TNF-α*) in COVID-19 patients and healthy individuals.

Method: Examination of genetic variation of *ACE2* gene was done in DNA samples of 201 COVID-19 patients and 200 healthy controls using PCR-RFLP method. Expression of inflammation -related genes (*IP-10, IL-6, IL1β* and *TNF-α*) was done in RNA samples of 14 COVID-19 patients and 14 healthy controls using RT-PCR.

Results: *ACE2* rs2106809 GA genotype and rs2106809A allele were associated with the reduced risk of SARS-CoV-2 infection (27.5% vs. 41.8%, P=0.001, OR=0.49, 95%CI: 0.31-0.77). *ACE2* rs210680GG genotype and rs210680G allele were associated with impaired CRP level (11.5% vs. 2.2%; P=0.009, OR=6.67, 95%CI: 1.44-34.73; 27.0% vs. 15.1%, P=0.007, OR=2.08, 95% CI: 1.20-3.69). *ACE2* rs210680AG and rs210680GG genotypes were associated with reduced risk of impaired ferritin level (10.5% vs. 40.4%, P=0.001, OR=0.16, 95%CI: 0.17-0.36; 2.3%

vs. 7.0%, P=0.06, OR=0.20, 95%CI: 0.03-1.07). *ACE2* -8970GA genotype was associated with higher risk of mild and moderate disease stage of COVID-19 patients (OR=3.09, P=0.05, OR =3.17, P =0.0003). *ACE2* -8970A allele were associated with the risk of mild and moderate disease stage (P =0.007, OR =1.98, P =0.05, OR =1.80). We compared the expression level *IL-1β, IL-6, TNF-α* and *IL-10* genes between COVID-19 patients and healthy controls. The expression of *IL-1β* gene was significantly higher in COVID-19 patients than healthy controls (4.72 vs. 1.53, 3.08 fold). The expression of *IL-6* gene was also higher in COVID-19 patients than healthy controls but could reach statistical significance (1.62 vs. 1.44, 1.12 fold). Similarly expression of *TNF*-α gene was higher in COVID-19 patients than healthy controls (37.67 vs. 23.00, 1.64-fold). However, expression of *IL-10* gene was decreased in COVID-19 patients than healthy controls (0.28 vs 3.38, 0.08-fold).

Conclusions: *ACE2* rs2106809 G/A may assist the increase the risk for SARS-CoV-2 infection. *ACE2* rs210680GG genotype may facilitate to increase CRP level and reduce the risk to increase ferritin level. *ACE2* -8970GA genotype may contribute the mild and moderate disease stage. A higher expression of *IL-1β* may facilitate the COVID-19 disease progression.

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Contributions

Contributor name	Contributor role
Dr. HariOm Singh	Conceptualization Ideas
Dr. HariOm Singh	Methodology
Dr. HariOm Singh	Software
Dr. HariOm Singh	Validation
Dr. HariOm Singh	Formal analysis
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Dr. HariOm Singh	Resources
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Taku Aammee	Writing - original draft preparation DR
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Dr. HariOm Singh	Visualization
Dr. HariOm Singh	Supervision
Dr. HariOm Singh	Project administration
Dr. HariOm Singh	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy.

CHAPTER 1

Introduction

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the coronavirus disease (COVID-19) pandemic. During the 2021 Delta variant wave, approximately 40.9% of Indians were infected with SARS-CoV-2 (Liu et al., 2024). According to Liu et al. (2024), the real occurrence of infection was estimated to be 30 times higher than that based on already documented cases. According to reports, 22% of people have long COVID-19 (Naik et al., 2021). As per Coronavirus Tracker report on April 13, 2024, 704,753,890 infected cases, 7,010,681 deaths were recorded. A large variety of clinical symptoms, from asymptomatic to severe, are displayed by COVID-19 patients. The clinical symptoms of COVID-19 range widely from asymptomatic or slightly symptomatic to severe or critical illness, (Liao et al., 2020, Covino et al., 2020). About 10% of the infected patients who developed symptoms advanced to a severe or critical stage necessitating intensive care or mechanical ventilation assistance, but the majority of those who did so initially showed signs of mild disease (Andrade *et al.*, 2023, Di Pietro *et al.*, 2023, Wang et al., 2021). Older age (Zhou *et al.*, 2020), menopause (Liu *et al.*, 2023), Males (Attaway *et al.*, 2021), smoking (Zheng *et al.*, 2020), alcohol use (Wei *et al.*, 2023), and underlying comorbidities e.g., hypertension (Zheng *et al.*, 2020, Gao *et al.*, 2020), cardiovascular disease, diabetes (Zheng *et al.*, 2020, Gao *et al.*, 2020, Docherty *et al.*, 2020), obesity (Zheng *et al.*, 2020), chronic pulmonary disease (Gao *et al.*, 2020, Docherty *et al.*, 2020, Nakanishi *et al.*, 2023), cancer (Zheng *et al.*, 2020), and immunodeficiencies (Zheng *et al.*, 2020), are some known risk factors that may be responsible for the variation in COVID-19 symptoms or severity. Even though comorbidities (Kompaniyets et al., 2021) , menopause (Liu *et al.*, 2023) and older age (Zhou *et al.*, 2020), were linked to the severity of the illness, these risk factors by themselves were unable to explain why some young, healthy people (Machado *et al.*, 2023) experienced a severe or life-threatening condition. Additionally, genetic underliers that confer inter-individual variations in susceptibility to COVID-19 infection and disease severity may be partially accountable for this aberrant event (Niemi *et al.*, 2021, Anastassopoulou *et al.*, 2020, Elhabyan *et al.*, 2020, Grolmusz *et al.*, 2021).

The spike (S) protein binds to a particular cellular receptor (ACE2) in order for coronaviruses to enter cells, and cellular proteases then primes the S protein (TMPRSS2). (Markus Hoffmann et al., 2020, Matsuyama et al., 2010). It was found

that the binding affinity of the S protein and ACE2 was a major factor in the replication rate of SARS-CoV and disease severity (Zhou et al., 2020, Hoffmann et al., 2020). The *ACE2* and *TMPRSS2* gene expression has been observed to occur largely in alveolar epithelial type II cells (Zhao et al., 2020, Zou et al., 2020, Qi et al., 2020), which are central to SARS-CoV pathogenesis. At initial infection stage *TMPRSS2*, may be a limiting factor for viral entry (Waradon Sungnak et al., 2020). Along the respiratory system, the spatial distribution of receptor accessibility determines the viral transmissibility (Waradon Sungnak et al., 2020).

In recent studies, it is found that during viral infections *ACE2* negatively regulates inflammatory responses (Yang et al. 2014; Sodhi et al., 2019). Therefore, the susceptibility, symptoms, and outcome of 2019-nCoV/SARS-CoV-2 infection may be significantly influenced by the expression level and pattern of human ACE2 in various tissues. (Li et al., 2005, Porzani et al., 2021). According to a study, Asian men might exhibit more ACE2 (Zhao et al., 2020). Four German cases with moderate clinical symptoms and no serious disease were reported in recent research done in Munich (Rothe et al., 2020). However, the genetic basis of *ACE2* expression and function in different populations is still largely unknown (Rothe et al., 2020). In the ChinaMAP, just one ACE2 singleton truncating variation (Gln300X) was found. (Zhao et al., 2020). The coding-region variants of *ACE2* genes affect the expression of *ACE2*. According to Cao et al. (2020), there was no direct proof found that coronavirus S-protein binding-resistant ACE2 mutants existed genetically in various populations.

The risk of lung damage from SARS-CoV-2 is increased by the persistant inflammation in the lungs. Patients with severe COVID-19 disease were associated with higher levels of inflammatory markers (IL2R, IL-6, IL-7,IL-8, IL-10, GSCF, IP10, MCP1, MIP1, TNF-α, IL-1β,CCL2/MCP-1,CXCL10/IP-10,CCL3/MIP-1A and CCL4/MIP1B,PD-1) and decreased level of immune cells (CD4+T-cells and CD8+T-cells, CD3,CD4,CD8,NK cells) and IFN-γ than mild or moderate (Diao et al., 2020, Pederson et al., 2020).

Some people's genes slow down the synthesis of ACE2, or they produce protein locks which makes it more difficult for COVID-19 to select (Chan et al., 2021). Therefore, depending on our DNA, some of us may have cells that are more susceptible to infection and, as a result, experience more severe disease, while others provide fewer targets for viruses and manage to avoid getting seriously ill.

The virus may enter cells with greater ease or difficultly depending on variations in the ACE2 gene which modifies the receptor. The genetic susceptibility to COVID-19 is most strongly associated with the human immune and genetic systems (Sun & Xi, 2014).

The cytokine storm, which is characterized by a significant activation of systemic inflammatory pathways, is the most prominent characteristic of severe COVID-19 (Ladds *et al.*, 2020). SARS-CoV-2 triggers a dysregulated hyper-inflammatory immune response called as cytokine release syndrome (CRS), which is present in patients with severe and complex forms of COVID-19 (Coomes and Haghbayan, 2020, Broman *et al.*, 2020, Potere *et al.*, 2021, Liu *et al.*, 2020). Plasma levels of Interleukin (IL)-6 (> 200 mg/L) and IL-8 (1500 pg/mL) were significantly higher in severe cases than those of mild cases. Therefore, it has been suggested that IL-6 and IL-8 play an important role in CRS, multi-organ failure, respiratory failure, and shock (Coomes and Haghbayan, 2020, Potere *et al.*, 2021, Abu-Farha *et al.*, 2020). It is evident that cytokines, including interleukin-8 (IL-8) and interleukin-1α (IL-1α), play a role in this inflammatory process (Vendramini *et al.*, 2010). The susceptibility and severity of COVID-19 disease in patients have been considered to be determined by host genetic variations, including those related to immunological responses (Van Der Made *et al.*, 2022).

Pathogenesis

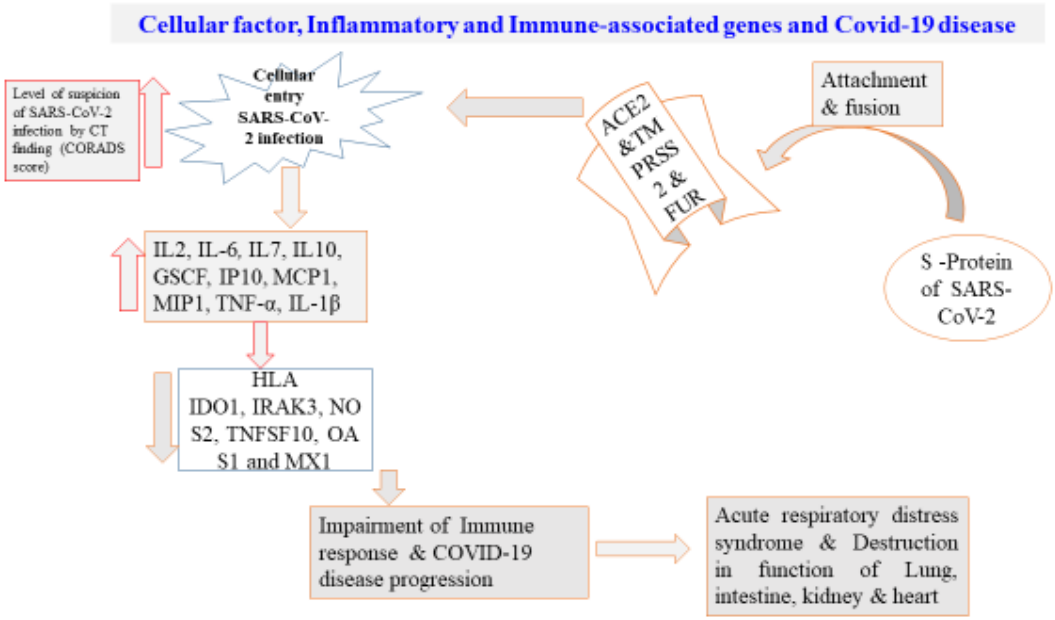

Figure 1: Pathogenesis of cellular factor, Inflammatory and immune-associated genes associated with COVID-19

Following exposure of SARS-CoV-2, Furin enzyme cleave S protein to S1 and S2 protein. S1 protein attach to *ACE2* receptor of host. TMPRSS2 play a role to fuse the membrane of host and virus. After fusing virus enter in cytosol of host and host get infection. After infection of SARS-CoV-2, at one side level of inflammatory protein goes up while protein of immune repose related goes down as *ACE2* is expressed in various tissues including oral and nasal mucosa, nasopharynx, lung, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney, brain, heart, endothelial cells, and vascular smooth muscle cells. Hence S1 protein attaches to *ACE2* and binds with *ACE2* of various organs and may lead to multiorgan failure.

Hypothesis

Hypothesis

Following the exposure of SARS-CoV-2 infection, some people get infection or severe COVID-19 disease, while others that are exposed identically do not get infection or severe disease because production of *ACE2* is slowed down by regulatory genes, which reduces the availability for binding of viral protein to cellular receptor and restrict the entry to cytosol. Following exposure to SARS-CoV-2 leads chronic inflammation in the lungs, persistent inflammation in the lungs is associated with an increase in the in level of inflammatory cytokines, IL-6, which impair the SARS-CoV-2 induced pathology (Gubernatorova et al., 2020). Immune associated genes has a role in innate and antiviral immune response. Cellular factors are involved in spread of virus and its pathogenesis. Clinical outcomes of SARS-CoV-2 infection are varied from individual to individual and population to population. This variation is linked with genetic background. Difference in responses of individuals to the infection and disease condition may be influenced by genetic variants of ACE 2, TMPRSS2, Furin and immune-associated genes and different levels of expression and function of *these* proteins. Evidence suggested that variation in cellular factor (ACE2, TMPRSS2, FUR) and Immune- and inflammation-related genes (*HLA-A, B* and *IL2, IL-6, IL7, IL10, GSCF, IP10, MCP1, MIP1, TNF-α, IL-1β*)) genes affect the susceptibility to infection and clinical outcome of disease. So far, limited data available on genetic variation & expression of these genes which contribute the individual variations in susceptibility to SARS-CoV-2 infection and clinical outcome.

Hence we **hypothesized** that SARS-CoV-2 infection and disease severity are influenced by cellular factor (ACE2) and inflammation-related genes (*IL-6, IL10, IP10, MIP1, and TNF-α, IL-1β*) genes.

Aim & Objectives

Aim:

To examine the genetic variation of cellular factor (*ACE2*) and expression of and inflammation- related genes (*IL-6, IL10, IP10, MIP1, and TNF-α, IL-1β*) in COVID-19 patients

Objectives

1. To examine the genetic variation of *ACE2* gene in COVID-19 patients and healthy controls
2. To examine the expression of inflammation- related genes (*IL-6, IL10, IP10, MIP1, and TNF-α, IL-1β*) in COVID-19 patients and healthy controls

Review of literature

Review of literature

Coronaviruses are comprised of a varied group of viruses that infect numerous animal species, that leads to respiratory infections that can range from mild to severe. In years 2002 and 2012, two highly pathogenic variants of coronaviruses, known as SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), were found in humans, resulting in lethal respiratory diseases that highlighted the threat posed by emerging coronaviruses to public health (Zhong et al., 2003, Zaki et al., 2012). By the time the year 2019 was ending, a novel coronavirus was identified as SARS-CoV-2 that came out of Wuhan, China, that initiated an outbreak of abnormal viral pneumonia. With its high rate of transmission, this new coronavirus disease, also known as coronavirus disease 2019 (COVID-19), has rapidly spread across the world (Hu et al., 2020). SARS-CoV-2 is part of the Coronaviridae family and belongs to the Betacoronavirus genus. It appears as a spherical envelope containing characteristic club-shaped glycoprotein spikes that project from its lipid bilayer. This enveloped, single-stranded positive RNA virus has attracted a lot of attention due to its worldwide spread and its significant effect on public health (Zhu et al., 2020). Prior to the World Health Organization (WHO) announcing the conclusion of the COVID-19 global health emergency in May 2023, SARS-CoV-2 infection was associated with an estimated 15 million excess fatalities during 2020 and 2021 alone (Msemburi et al., 2023). As SARS-CoV-2 evolves toward becoming endemic, it continues to be a significant cause of illness globally. The clinical features of COVID-19 range from being asymptomatic or exhibiting mild respiratory issues to severe pneumonia, multi-organ failure, acute respiratory distress syndrome (ARDS), and death (WHO, 2020). The abnormal immune response in the host also creates a prothrombotic environment, increasing the likelihood of thromboembolic issues such as pulmonary embolism and disseminated intravascular coagulation (DIC) (Connors & Levy, 2020). Factors that increase the risk of severe disease include older age, gender, existing health conditions like cardiovascular disease, chronic obstructive pulmonary disease obesity, diabetes, and immunocompromised status. The cytokine storm, marked by uncontrolled immune responses and heightened inflammation, plays a significant role in disease severity and associated complications. The complications arising from the infection involve a complex array of issues, creating difficulties across multiple physiological systems both in the acute phase and during the prolonged recovery period. (Ruan et al., 2020). COVID-19

patients shows a tendency for extra-pulmonary effects, highlighted by its ability to cause cardiovascular complications. Myocardial damage, indicated by increased cardiac biomarkers and myocarditis, has become a significant issue, leading to arrhythmias, heart failure, and myocardial infarction (Guo et al., 2020; Shi et al., 2020). Moreover, neurological complications represent a significant aspect of COVID-19 issues, ranging from acute cerebrovascular incidents like ischemic strokes to encephalopathies and Guillain-Barré syndrome (Ellul et al., 2020; Mao et al., 2020). Mechanistically, the virus's ability to invade the nervous system, immune-related damage, and increased blood clotting combine to create a variety of neurological problems, highlighting the importance of monitoring and treating neurological signs in COVID-19 patients (Han et al., 2021). Diagnostic methods for SARS-CoV-2 include several techniques like reverse transcription-polymerase chain reaction (RT-PCR), antigen detection, and serological tests to identify viral RNA, proteins, or antibodies in clinical samples such as nasopharyngeal swabs, saliva, or blood (WHO, 2020).

1.1. Structure of SARS-CoV-2 Virus

SARS-CoV-2 is a single-stranded RNA virus that is enveloped and possesses a positive sense, classified under the Coronaviridae family. Its structural elements are made up of spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S protein binds to the angiotensin converting enzyme 2 (ACE2) receptor and facilitates the entry of the virus into host cells thereby aiding in viral attachment and fusion (Belouzard et al., 2012). The E protein plays a role in the assembly and budding of the virus, while the M protein is crucial for maintaining the integrity of the viral envelope. The N protein attaches to the viral RNA, resulting in the formation of the helical nucleocapsid core. The genome of SARS-CoV-2 spans approximately 30 kb in length and encodes numerous structural and non-structural proteins. It features a 5’ cap and a 3’ poly-A tail, which are characteristic of eukaryotic mRNA. The viral genome contains multiple open reading frames (ORFs), with ORF1a encoding for pp1a and ORF1b encoding for polyproteins pp1ab. These polyproteins are processed by viral proteases through proteolytic cleavage, generating a total of 16 non-structural proteins (nsps) (Hardenbrook & Zhang, 2021). These nsps are vital for processes such as viral replication, transcription, and the evasion of host immune defenses. The replication of SARS-CoV-2 takes place in the cytoplasm after the

virus enters the cell. The viral RNA is translated into polyproteins pp1a and pp1ab, which are then processed into NSPS by the action of viral proteases. Together, these nsps make up the replicase-transcriptase complex (RTC), which is in charge of synthesizing viral RNA. The viral genome acts as a template for both genomic RNA replication and the production of sub genomic mRNAs (sgRNAs). These sgRNAs, which encode structural and accessory proteins, are produced through a process of discontinuous transcription, a distinguishing feature of coronaviruses. (D.A. Jackson n Jr. et al, 2022)

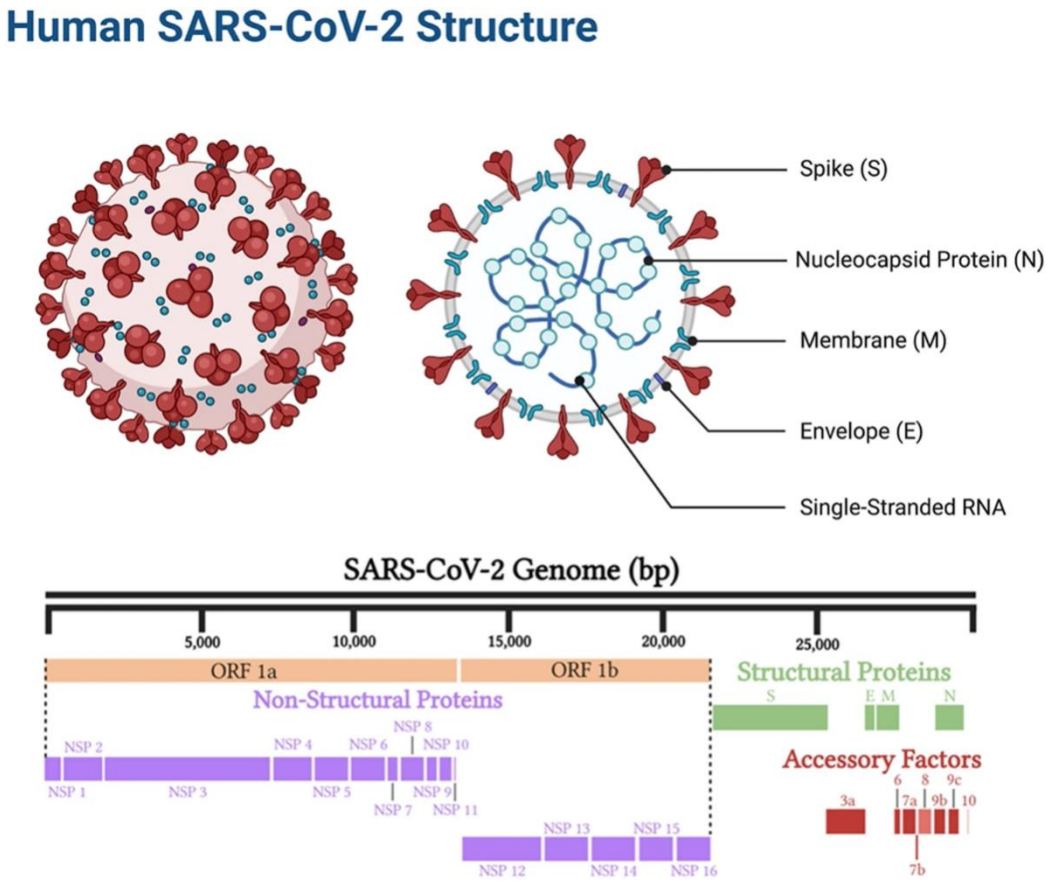

Figure 1: Structure and viral genome of SARS-CoV-2 virus (D.A. Jamison n Jr. et al, 2022)

1.2. Pathogenesis of COVID-19 Disease

SARS-CoV-2 primarily infects respiratory epithelial cells, using *ACE2* as its main receptor for entry (Lee & Choi, 2021, Zhu et al., 2020). Once it attaches and penetrates, the viral genome is released into the host cell's cytoplasm, where it is translated into replicase polyproteins. Subsequently, proteolytic cleavage occurs, generating non-structural proteins crucial for transcription and the replication of viral RNA. Viral particle assembly occurs in the endoplasmic reticulum-Golgi intermediate

compartment (ERGIC), followed by exocytosis and the release of mature virions via vesicular transport. This cycle enhances viral entry into host cells, initiating a sequence of events that leads to inflammation, tissue damage, and systemic issues. In the cell, the virus releases its RNA which is then translated into viral proteins, including non-structural proteins (NSPs) that hinder host immune responses (Gordon et al., 2020). The replication of the virus causes cell lysis, which releases the pro-inflammatory cytokines such as interleukin-6 (*IL-6*) and tumor necrosis factor-alpha (*TNF-α*), drawing immune cells to the site of infection (Chen et al., 2020). In response to the viral invasion, innate neutrophils and monocytes become activated and migrate toward the infection site, secreting cytokines and chemokines to brings in more immune cells (Merad and Martin, 2020). Dysfunction in neutrophils can lead to tissue damage through the release of reactive oxygen species (ROS) and proteases, exacerbating inflammation and tissue injury (Barnes et al., 2020). However, in severe cases of COVID-19, dysregulated immune responses can trigger a cytokine storm, identified by the over-the-top production of pro-inflammatory cytokines and extensive tissue damage (Mehta et al., 2020).

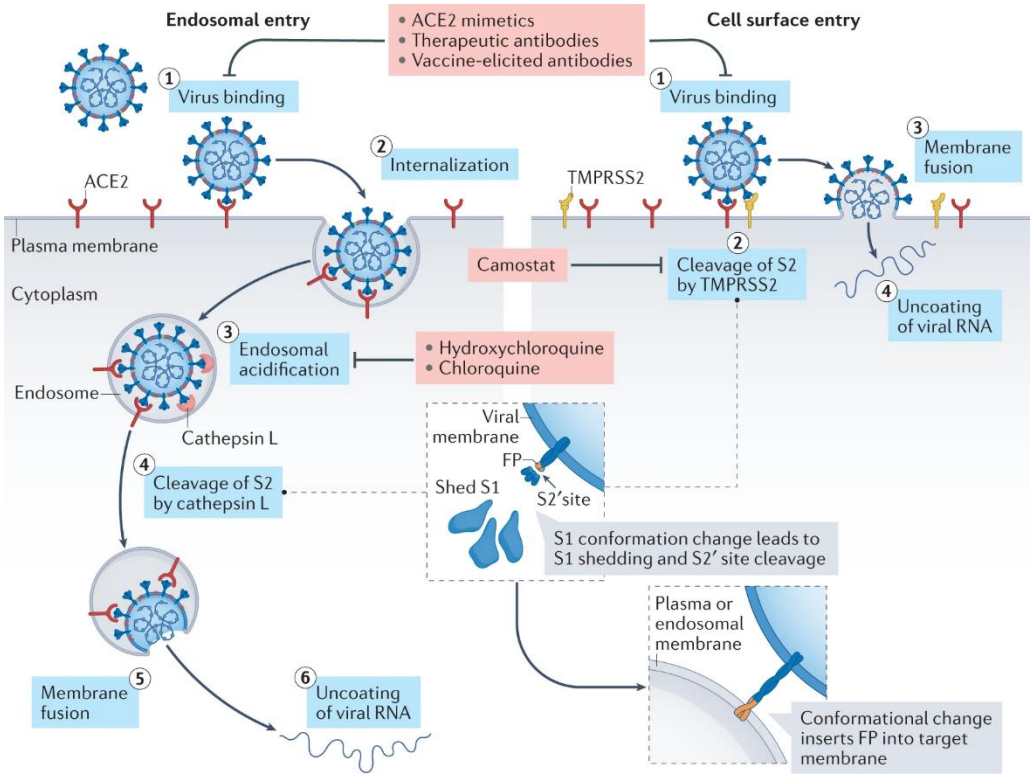

Figure 2: Pathogenesis of SARS-CoV-2 in the host cell (Jackson et al. 2022)

1.3. Cytokine storm

The cytokine storm seen in COVID-19, commonly known as hypercytokinemia, signifies an unchecked and vigorous immune response triggered by the SARS-CoV-2 virus. This event leads to an overproduction of both pro-inflammatory as well as anti-inflammatory cytokines and chemokines, resulting in widespread inflammation and tissue damage, which aggravates the intensity and morbidity of the infection.

After the virus's enter in the body, SARS-CoV-2 primarily attacks the cells with the help of angiotensin-converting enzyme 2 (*ACE2*) receptors, predominantly found in respiratory epithelial cells, but it is also present in various other tissues. A crucial component of the cytokine storm related to COVID-19 is the increased expression of genes such as *tumor necrosis factor-alpha* (*TNF-α*), *interleukin-6 (IL-6),* and *interleukin-1 beta (IL-1β),* with many other pro-inflammatory cytokines (Faraj & Jalal, 2023). These cytokines facilitate the recruitment and activation of immune cells, leading to inflammation and damage in the lungs and other organs. For instance, *IL-6*, exacerbates the inflammatory response by promoting the production of acute-phase reactants and additional cytokines that are pro-inflammatory, worsening tissue damage and creating a cycle of cytokine amplification (McGonagle et al., 2020). Simultaneously, *IL-1β* enhances vascular permeability and allows more inflammatory cells to infiltrate the damaged tissues (Jose and Manuel, 2020). Notably, CXCL10 has been correlated with the recruitment of T cells and monocytes in the lungs, thereby perpetuating local inflammation and tissue damage (Xu et al., 2020). The unregulated expression of various additional inflammatory genes, including *IL-10, IL-18, IL-1B,* and *IL-12,* underscores the fundamental pathophysiology of the cytokine storm, ultimately leading to systemic inflammation, endothelial dysfunction, and multi-organ injury.

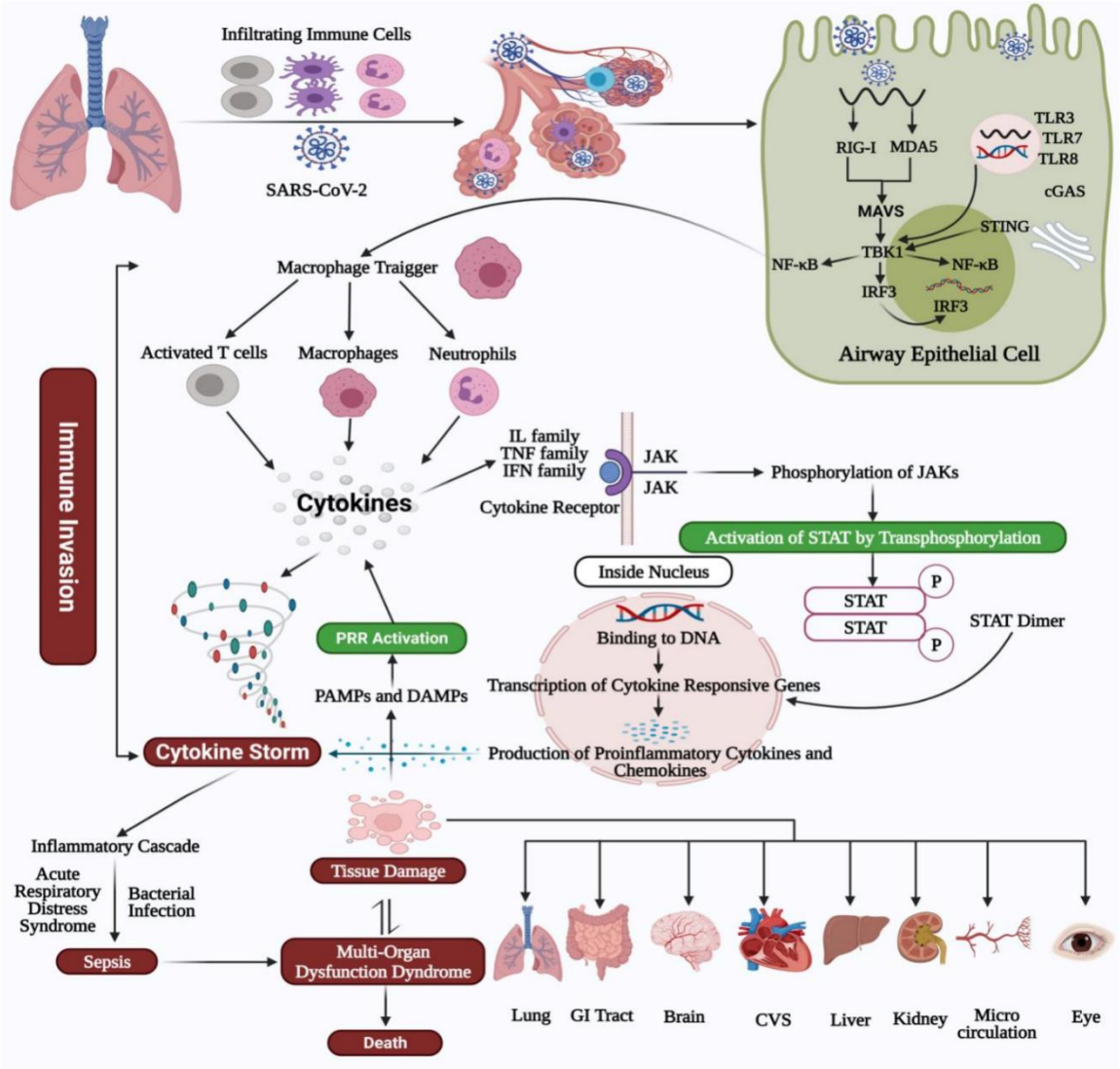

Figure 3: Molecular mechanisms, immune functions, and immunopathology of inflammatory cytokines in COVID-19 disease. (Rabaan et al., 2021)

1.4. Pathway and Inflammatory genes

1.4.1. *ACE-2*

Angiotensin converting enzyme 2 (ACE2) plays a role to attaches to the target cells. ACE2 protein is present in oral and nasal mucosa, nasopharynx, lung, stomach and various other organs (Hamming et al., 2004) and reported in hypertension, diabetes, dyslipidemia, cancer, heart failure and COVID-19. Higher expression of *ACE2* is related to the faster replication of SARS-CoV-2 and the severity of COVID-19 disease. *HLA* gene present on cell surface molecule (APC) plays a role to increase the binding specificity of S protein with *ACE2* receptor leading to progression of COVID-19 disease.

ACE2 gene polymorphism, rs2285666 or 8790G/A, has been examined for its ability to affect COVID-19 susceptibility and severity. Studies show that this gene polymorphism can potentially affect how different individuals respond to SARS-CoV-2 infection.

A study found that subjects with the GG genotype of rs2285666 had about two times higher risk of SARS-CoV-2 infection and three times the higher risk of severe COVID-19 or death (Möhlendick et al.2021). The correlation remained significant even after adjustment for known risk factors such as male sex and cardiovascular disease (Sahranavard-Pirbazari et al., 2023). A research article for the Indian population revealed a robust positive association of the alternative allele (T or A) of rsID 2285666 with low infection rates and case fatality (Srivastava et al.2020). This supports the possibility that the frequency of this allele is able to modify COVID-19 outcomes in diverse populations.

1.4.2. *IL-10*

Interleukin-10 (IL-10) plays an important role in moderating immune responses and maintaining immune homeostasis. The *IL-1*0 gene, located on chromosome 1q31-32, encodes *IL-10*, which is produced by various immune cells, including T cells, B cells, monocytes, and macrophages (Iyer, S. S., & Cheng, G.2012). In COVID-19, *IL-10* has a dual role in immune regulation. On one hand By preventing the synthesis of pro-inflammatory cytokines and preventing immune cell activation, *IL-10* reduces inflammation, thereby mitigating excessive inflammation and tissues can also avoid getting damaged (Al-Qahtani et al., 2024). On the other hand, elevated *IL- 10* levels may contribute to immune dysregulation and impaired viral clearance, leading to disease progression and severe outcomes in COVID-19 patients (Carlini et al., n.d.). In addition to its role in immune regulation, *IL-10* may also influence the thrombotic and coagulation pathways implicated in COVID-19 pathogenesis. It is shown to inhibit the production of pro-thrombotic factors and attenuate endothelial dysfunction, suggesting a potential protective role against COVID-19-associated thrombosis and vascular complications (McGonagle et al., 2020). Recent studies have also highlighted the significance of *IL-10* in the pathogenesis of COVID-19. Patients with severe COVID-19 show irregular immune responses indicated by elevated levels of *IL-10*, which may also be responsible for the cytokine storm and systemic

inflammation as seen to be in severe cases of the disease (Majeed et al., 2023, Zhao et al., 2020).

1.4.3. *IL-1 β*

Interleukin-1β (*IL-1β*), an important pro-inflammatory cytokine, playing a critical role in mediating immune responses and inflammatory processes (Kaneko et al., 2019). In response to a variety of stimuli, including infection, injury, and inflammation, activated macrophages and monocytes release IL-1β, which is encoded by the IL-1β gene on chromosome 2q14 (Dinarello, 2009). In COVID-19, IL-1β plays a pivotal role in driving inflammation and cytokine release syndrome (CRS), which are the features of increased disease severity. SARS-CoV-2 infection can trigger an overactive immune response called as a "cytokine storm," indicated by the excessively released cytokines, which includes *IL-1*β(Fajgenbaum & June, 2020, Anaeigoudari et al., 2021). This hyperinflammatory state is one of the precursors to acute respiratory distress syndrome (ARDS) and multi-organ failure noticed in severe COVID-19 cases (Conti P. et al., 2020). Elevated *IL-1β* levels have been observed in individuals suffering from severe COVID-19 and are associated with disease severity and poor clinical outcomes (Huang et al., 2020). Recent studies have told us the significance of IL-1β in COVID-19 pathogenesis and that *IL-1β* can be used as potential therapeutic target by inhibition of it's signalling pathways. Possible emerging treatment approach for reducing inflammation and cytokine storm in severe COVID-19 disease is *IL-1β* blockade. Clinical trials that have investigated the efficacy of *IL-1β* inhibitors, such as anakinra and canakinumab, have shown promising results in reducing inflammation and improving clinical outcomes in COVID-19 patients (Cavalli et al., 2020).

1.4.4. *IL-6*

This gene can act as both a pro-inflammatory cytokine and an anti-inflammatory myokine. *Interleukin (IL)-6* is a pleiotropic cytokine that plays various roles in the regulation of the inflammation, immune response, and haematopoiesis (Kishimoto, 2010). Two receptor chains and downstream signalling molecules comprise the IL-6 receptor signalling system (Kishimoto et al. 1992). The signal-transducing chain is made up of gp130, whereas the IL-6-binding chain is made up of the *IL-6* receptor (IL-6R), which comes in two forms: 80 kDa transmembrane and 50–55 kDa soluble

IL-6R (sIL-6R). Both proteins have a Trp-Ser-X-Trp-Ser motif and are members of the cytokine receptor family (Yamasaki et al. 1988; Hibi et al. 1990).

According to the clinical and therapeutic diagnosis of COVID-19, IL-6 has a major role in the third stage, which is indicated by an unusual systemic hyperinflammatory response (Di Spigna et al., 2023). Because of Il-6's dual nature high detection of this gene implies that person's immune system is dysregulating and that there is a long term inflammation due to various reasons. Generally, studies have shown post COVID-19 infection patients had higher average *IL-6* levels than the healthy population. *IL-6* was also identified as a potential biomarker of long-term neuropsychiatric symptoms of the disease as well. (Kow et al., 2023).

1.4.5. *TNF-α*

Tumour necrosis factor-alpha (TNF-α) has also been associated with neuroinflammatory disorders, where it is able to be involved in influencing neuronal viability and function, and thus potentially in diseases such as multiple sclerosis. (Caldito, 2023; Costantini et al., 2018). Anti-TNF-α monoclonal antibodies and soluble TNF-receptors are suggested to be provided to neutralize the activity of *TNF-α* and thereby suppress inflammation and disease symptoms. A basis for *TNF-α* studies is served by the link shown with poorer asthma functioning. A study has measured the levels of *IL-6, IL-8, TNF-α*, and *IL-1β* in serum from 1,484 hospitalized COVID-19 patients at initial presentation. It concluded that increased levels of *TNF-α* was independent and powerful predictors of survival in patients and indicated that higher levels of *TNF-α* are associated with higher risk of death in patients (Valle et al., 2020). A study examined patients with COVID-19 who did not respond to tocilizumab, an *IL-6* receptor antagonist. The patients in this study exhibited high levels of *TNF-α*, indicating that it may be involved in disease severity, especially when treatments targeting *IL-6* are unsuccessful. (Danlos et al., 2021)

Another research study investigated the potential therapeutic use of TNF-α inhibitors in seriously sick patients with COVID-19. The researchers hypothesized that these inhibitors could neutralize the cytokine storm of critical illness and reduce mortality. (Jang et al., 2021; Sun et al., 2020)

Together, these studies point to the central position of *TNF-α* in COVID-19 pathophysiology and the therapeutic potential of *TNF-α*-targeted solution for severe disease.

Table 1: Candidate genes and their functions

Proteins	Nature	Produced by	Function
Interleukin-1β (IL-1 β)	Proinflammatory	Monocytes, Macrophages	Stimulates proliferation of Th2 Cells, Inhibitor of gastric acid secretion.
Interleukin-10 (IL-10)	Antiinflammatory	Macrophages, T h2 Cells	Inhibits production of Th1 Cells and Macrophages function
ACE2	proiinflammatory	Epithelial cells of longs, nasal and oral mucosa etc	Acts as cell surface receptor for SARS-CoV-2
IL-6	Proinflammatory and antiinflammatory	Macrophages, monocytes and stromal cells	Act as both an anti-inflammatory myokine and pro-inflammatory cytokine plays a role in acute lung injury. Secretion of cytokine, such as IL1B, IL1RA, IL6, IL7, IL8, known as cytokine storm, is associated with disease severity

TNF-α	Pro-inflammatory	Macrophages	central mediator in acute inflammation

CHAPTER 2

Materials and Methods

Methodology:

Methodology was taken from previous work done in the lab.

a. **Patients and Clinical material:**

Subjects:

There were total of two hundred COVID-19 patients in this case-control observational study, of whom 200 had negative RT-PCR results from the Government Medical College's molecular laboratory in Aurangabad, India, and 9 had critical, 71 had severe, 86 moderate, and 34 mild cases. The study was administered to those who agreed to participate.

The inclusion criteria for these patients were:

A. Critical cases:

Inclusion criteria: Critically ill patients with any of the following:

1. ratio of partial pressure of arterial oxygen to fractional concentration of oxygen inspired air ≤200 mm Hg;
2. Shock (Requiring Vasopressor to maintain a MAP >= 65mmHg or MAP below 65)
3. Respiratory failure in which mechanical ventilation is required, septic shock, or non-respiratory organ failure that require monitoring and treatment in the ICU)

Exclusion criteria : 1) Any SARS-CoV-2 infected individual with concurrent conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol daily and 3) patients that used recreational drugs.

B. Severe cases of COVID-19

Inclusion criteria: 1) COVID-19-positive patients proven by real time PCR;

2) Patients who had any of the following features at the time of, or after, admission were classified as severe cases: (a) respiratory distress (≥30 breaths per min); (b) oxygen saturation at rest ≤93%; (c) ratio of partial pressure of arterial oxygen to fractional concentration of oxygen inspired air ≤300 mm Hg; or.

Exclusion criteria: 1) Any infected individual with concurrent conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol daily and 3) patients that used recreational drugs.

C. Moderate cases:

Inclusion criteria: 1. RT-PCR confirmed patients admitted with COVID-19 illness who has symptoms such as fever and respiratory tract symptoms with pneumonia manifestations which can be seen on imaging;

2. Has any of the two

a. PaO2/ FiO2: 200-300

b. Respiratory Rate > 24/min and SaO2 < 93% on room air

Exclusion criteria: 1) any SARS-CoV-2 infected person with coexisting conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol every day and 3) patients who used recreational drugs

D. Mild Cases of COVID 19:

Inclusion criteria: 1. Patients admitted for COVID-19 illness confirmed with RT-PCR.

Has the following signs & symptoms: Fever, loss of sense of smell, tiredness & sore throat and no pneumonia manifestation can be found on imaging;

Exclusion criteria: 1) any SARS-CoV-2 infected person with coexisting conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol every day and 3) patients who used recreational drugs.

E. Asymptomatic cases of COVID-19

Inclusion criteria: 1) COVID-19 positive patients confirmed by real time PCR; 2) Patients clinically do not show clinical signs and symptoms of COVID.

Exclusion criteria: 1) Patients with co-morbid conditions like Hypertension, diabetes and cardiac or respiratory illnesses.

2) any SARS-CoV-2 infected person with coexisting conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol every day and 3) patients who used recreational drugs.

All the five categories of COVID case presentations will be included in the study with 50 patients in each category

Healthy Individuals: This will be divided into two groups:

With co morbidity and without comorbidity like diabetes, hypertension, cardiac or respiratory illness with 100 subjects in each category.

Inclusion criteria:1) Individuals who were negative on real time PCR for SARS-CoV-2 infection 2) Age and gender matched with cases of COVID-19

Exclusion criteria: 1. any SARS-CoV-2 infected person with coexisting conditions such as tuberculosis, Hepatitis B/C 2) patients who consumed alcohol every day and 3) patients who used recreational drugs

Ethical committees at the Government Medical College in Aurangabad, India, and the National AIDS Research Institute in Pune, India, approved this study.
All eligible participants were also asked to provide written informed consent

Method

DNA and RNA was extracted from a total of two hundred COVID-19 patient's samples with critical, serious cases and mild, moderate or no disease cases and two hundred healthy individuals. DNA was used for analysis of genetic variation of above mentioned genes in all 200 patients and 200 controls using PCR-RFLP and sequencing method.

Detail of the work carried out during the period

a. **Subjects**

A total of 200 COVID-19 patients, out of whom 9 had critical, 71 had severe, 86 moderate, 34 mild and 200 individuals those who have been tested negative on RT-PCR from molecular laboratory of Government Medical college, Aurangabad, India. The mean ages of 200 COVID-19 patients, and 200 healthy controls were 50.92 ± 6.34yrs and 47.74 ± 7.45 years, respectively.

Out of 200 COVID-19 patients, 64% were males and 36% were females. The data for age, sex, CT Score, D Dimer, CRP level, Ferritinin level, SGPT, Serum Bilirubin, Blood Urea, creatinine level, TLC, Platelet count & HB level were collected from case record of Department of medicine, GMC, Aurangabad. A total of 10 ml of blood was drawn; 5 ml blood was collected in an EDTA vial for DNA and RNA extraction and 2.5 ml for plasma separation; and 2.5 ml for serum separation from COVID-19 patients and healthy controls. The sample was kept frozen at -70 °C.

DNA Isolation

Frozen blood samples were allowed to thaw at room temperature before proceeding with DNA extraction. High-molecular-weight DNA was isolated from peripheral blood using the Qiagen kit. Both DNA and RNA extractions were performed on samples obtained from COVID-19 patients and healthy control subjects.

Quantification and Storage of DNA

The concentration and purity of the extracted DNA and RNA were determined by measuring optical density (OD) at 260 nm and 280 nm. The absorbance ratio (260/280 nm) for DNA ranged between 1.7 and 1.9, while for RNA, it was between 1.8 and 2.01. The integrity and purity of the samples were further assessed through 0.8% agarose gel electrophoresis using a 1x TBE buffer system. DNA samples were stored at -20°C for long-term preservation.

Genotyping

Genotyping of ACE2 -8970G/A (rs2106809A/G) polymorphisms was conducted using the PCR-restriction fragment length polymorphism (PCR-RFLP) technique. The primers used for gene amplification are detailed in Table 1. PCR was carried out in a total reaction volume of 20 µL, which included genomic DNA (100-150 ng), 10 pmol of each primer, 10 mM deoxynucleotide triphosphates (dNTPs), PCR reaction buffer containing 100 mM Tris-HCl, 1.5 mM MgCl2, and 1 unit of Taq DNA polymerase (Bangalore Genei, India).

For ACE2 rs2106809A/G, the thermal cycling conditions were as follows: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles comprising denaturation at 95°C for 1 minute, annealing at 33°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The amplified products were digested with the restriction enzyme TaqI (MBI Fermentas Inc., Hanover, MD, USA) and analyzed on a 6% agarose gel with molecular weight markers. Ethidium bromide staining was used for visualization. Based on sequence variations, the genotypes for ACE2 rs2106809A/G were determined as follows: 207 bp for the GG genotype, 207 bp, 183 bp, and 24 bp for the AG heterozygous genotype, and 183 bp & 24 bp for the AA genotype. The PCR reactions were performed in a Veriti 96-well Thermal Cycler (Applied Biosystems, USA), and PCR products were visualized using a 2% agarose gel electrophoresis system.

For ACE2-8790G/A, the thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The amplified products were digested with the restriction enzyme AluI (MBI Fermentas Inc., Hanover, MD, USA) and analyzed on a 6% agarose gel using molecular weight markers. Ethidium bromide staining was used for visualization. Based on SNP sequence and location, genotype assignment was as follows: 467 bp for the GG genotype, 467 bp, 281 bp, and 185 bp for the GA heterozygous genotype, and 281 bp & 185 bp for the AA genotype. The PCR reactions were conducted in a Veriti 96-well Thermal Cycler (Applied Biosystems, USA), and PCR products were visualized on a 2% agarose gel.

Real-time PCR

Unlike traditional PCR, real-time PCR allows the quantification of DNA in real-time during the amplification process. Fluorescent Dye: TB GreenPremix Ex Taq II an alternative to SYBR green is used, it is a dye that binds to the double-stranded DNA. As the amount of DNA increases during PCR, the fluorescence intensity increases proportionally. The fluorescence emitted by TB GreenPremix Ex Taq II is measured at the end of each PCR cycle, providing a real-time readout of the amount of DNA. Here we have used 2 genes, one of which is our target gene and another is a housekeeping gene; this expression of the housekeeping gene is used against the target gene for the comparative analysis of the data. GAPDH was used for the study of the following target genes: *IL-10, IL-6, IL1β* and *TNF-α*

In this study we compared the gene expressions of above mentioned four inflammatory associated genes between 14 healthy controls and 14 COVID-19 patients. For cDNA synthesis PrimeScript RT Reagent Kit (Perfect Real Time) from TaKaRa Bio was used. Gene expression was done by Real time PCR using TB Green *Premix Ex Taq* II (Tli RNase H Plus) by TaKaRa Bio. MS excel was used for data analysis of the fold change.

Data Analysis

The standard deviation was calculated for the mean age variable. The Hardy-Weinberg equilibrium in the control group was assessed using the Chi-square ($\chi 2$)

goodness-of-fit test. Additionally, genotype frequencies in COVID-19 patients and healthy controls were compared using the Chi-square test. Multivariate unconditional binary logistic regression was applied to estimate odds ratios (ORs) and 95% confidence intervals (CIs) using SPSS software version 17.0 (SPSS Inc., 2008). The data were analyzed using SPSS Statistics Version 17.0 for Windows (Chicago: SPSS Inc.). Statistical significance was considered for P-values less than 0.05 in a two-tailed analysis.

CHAPTER 3

Results

Results

3.1 Demographic profile

A total of 200 COVID-19 patients, and 200 healthy controls were analyzed. The mean age and standard deviation (Years ± SD) were 50.92 ± 6.34, 46.74 ± 7.45 respectively. The characteristics profiles of COVID-19 patients and healthy controls are shown in table 1.1.

3.2. Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with COVID-19

ACE2 -8970G/A, rs2106809A/G polymorphisms was observed to follow the hardy Weinberg equilibrium in healthy control population (P= 0.95, 0.80). Occurrence of *ACE2* -8970GG genotype and -8970G allele found to be lesser in COVID-19 patients than healthy controls (52.0% vs 60.7%; 73.3% vs. 77.9%). Frequency of *ACE2* -8970GA, -8970AA genotype and -8970A allele were found to higher in COVID-19 patients than healthy controls (42.5% vs. 34.3%, P=0.09, OR=1.45, 95%CI: 0.94-2.23; 5.5% vs. 5.0%, P=0.74, OR=1.29, 95%CI: 0.49-3.44; 26.7% vs. 22.1%, P=0.15, OR=0.15, 95%CI: 0.92-1.80).

Frequency of *ACE2* rs2106809 AA genotype and rs2106809 A allele were higher in COVID-19 patients than healthy controls (67.5% vs. 50.2%; 81.3% vs. 71.1%). Frequency of *ACE2* rs2106809 GG genotype was lesser in COVID-19 patients than healthy controls (5.0%vs. 8.0%; P=0.10, OR=0.47, 95%CI: 0.19-1.15). *ACE2* rs2106809 GA genotype and rs2106809 A allele were associated with the reduced risk of SARS-CoV-2 infection (27.5% vs. 41.8%, P=0.001, OR=0.49, 95%CI: 0.31-0.77).

3.3 Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired CRP Level

ACE2 -8970GG genotype and -8970G allele were found to be higher in impaired CRP level than normal among COVID-19 patients (59.0% vs. 48.9%; 78.7% vs. 70.9%). Occurrence of *ACE2* -8970GA, -8970AA genotype and-8970A allele were lesser in impaired CRP level than normal among COVID-19 patients (39.3% vs. 43.9%, P=0.43, OR=0.74; 95%CI: 0.38-1.45; 1.7% vs. 7.2%, P=0.16, OR=0.19; 95%CI: 0.01-1.54; 21.3% vs. 29.1%, P=0.13, OR=0.66, 95%CI: 0.39-1.12).

In COVID-19 patients, the occurrence of *ACE2* rs210680AA genotype and rs210680A allele were lesser in impaired CRP level than normal (57.4% vs. 71.9%; 73.0 vs 84.9%). *ACE2* rs210680AG genotype distributed lesser in impaired CRP level than normal (31.1% vs. 25.9%; P=0.30, OR=1.5, 95%CI: 0.37-3.13). *ACE2* rs210680GG genotype and rs210680G allele were associated with impaired CRP level (11.5% vs. 2.2%; P=0.009, OR=6.67, 95%CI: 1.44-34.73; 27.0% vs. 15.1%, P=0.007, OR=2.08, 95% CI: 1.20-3.69)

3.4 Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired Ferritin level

Frequency of *ACE2* -8970GG genotype and -8970G allele were found to be lesser in impaired ferritin level than normal among COVID-19 patients (45.3% vs. 57.0%; 69.8% vs. 75.9%). Occurrence of *ACE2* -8970GA was higher in impaired ferritin level than normal among COVID-19 patients ((48.8% vs. 37.7%, P=0.13, OR=1.63, 95%CI: 0.87-3.04). Occurrence of *ACE2* -8970AA genotype was almost similar in impaired ferritin level and normal among COVID-19 patients (5.8% vs. 5.3%, P=0.84, OR=1.39, 95%CI: 0.34-5.61). Occurrence of *ACE2* -8970A allele was higher in impaired ferritin level than normal among COVID-19 patients (30.2% vs. 24.1%; P=0.21, OR=1.36, 95%CI: 0.85-2.18) (Table 4).

In COVID-19 patients, occurrence of *ACE2* rs210680AA genotype and rs210680A allele were lesser in impaired ferritin level than normal (87.2% vs. 52.6%; 92.4% vs. 72.8%). *ACE2* rs210680AG and rs210680GG genotypes were associated with impaired ferritin level (10.5% vs. 40.4%, P=0.001, OR=0.16, 95%CI: 0.17-0.36; 2.3% vs. 7.0%, P=0.06, OR=0.20, 95%CI: 0.03-1.07). *ACE2* rs210680G allele was lesser in impaired ferritin level than normal (7.6% vs. 27.2%, P=0.92, OR=0.9, 95%CI: 0.44-1.90) (Table 4).

3.5 Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired D Dimer level

Frequency of *ACE2* -8970GG genotype and -8970G allele were found to be lesser in impaired D Dimer level than normal among COVID-19 patients (77.8% vs. 50.8%; 88.9% vs. 72.5%). Occurrence of *ACE2* -8970GA genotype and -8970A allele were higher impaired D Dimer level than normal among COVID-19 patients (22.2% vs. 43.5%, P=0.28,OR=0.33, 955CI:0.05-1.82, 11.1% vs. 27.5%, P=0.20, OR=0.33, 95%CI:0.05-1.53). (Table 5).

In COVID-19 patients, occurrence of *ACE2* rs210680AA genotype and rs210680A allele were lesser in impaired D Dimer level than normal (44.4% vs. 68.6%; 72.2% vs. 81.7%). *ACE2* rs210680AG and rs210680G allele were higher in impaired D Dimer level than normal (55.6% vs. 26.2%, P=0.15, OR=3.28, 95%CI: 0.73-15.26; 27.8% vs. 18.3%; P=0.48, OR=1.71, 95%CI: 0.51-5.39) (Table 5).

3.6 Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms among different stages of COVID-19

ACE2 -8970GA genotype was associated with higher risk of mild and moderate disease stage of COVID-19 patients (OR=3.09, P=0.05, OR =3.17, P =0.0003) when compared between mild, moderate disease stage and healthy controls. Similarly, *ACE2* -8970A allele were associated with the risk of mild and moderate disease stage (P =0.007, OR =1.98, P =0.05, OR =1.80) (Table 6).

ACE2 rs210680AG genotype was associated with the risk of severe stage of COVID-19 patients when compared between severe stage and healthy controls (P=0.005, OR=2.30) (Table 6).

3.7 Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired Platelet count

Frequency of *ACE2* -8970GG, -8970 GA, -8970 AA genotypes and -8970G , -8970A alleles were found to be almost similar in impaired platelet count and normal among COVID-19 patients (50.7% vs. 52.7%;43.7% vs. 41.9%;5.6% vs. 5.4% and 72.54% vs. 73.64%, 27.46% vs. 26.36%) (Table 7). In COVID-19 patients, the occurrence of *ACE2* rs210680AA, rs210680AG, rs210680GG genotypes and rs210680A, rs210680G alleles were almost alike in impaired platelet count and normal (69.0 vs. 66.7%, 26.8% vs. 27.9%,4.2% vs 5.4% and 82.39% vs. 80.62, 17.61% vs. 19.38%) (Table 7).

3.8 Expression Analysis level of *IL-1β, IL-6, TNF-α* and *IL-10* genes

We compared the expression level *IL-1β, IL-6, TNF-α* and *IL-10* genes between COVID-19 patients and healthy controls. The expression of *IL-1β* gene was significantly higher in COVID-19 patients than healthy controls (4.72 vs. 1.53, 3.08 fold). The expression of *IL-6* gene was also higher in COVID-19 patients than healthy controls but could not reach statistically significant (1.62 vs. 1.44, 1.12 fold).

Similarly expression of *TNF*-α gene was higher in COVID-19 patients than healthy controls (37.67 vs. 23.00, 1.64-fold). However, expression of *IL-10* gene was observed to be decreased in COVID-19 patients than healthy controls (0.28 vs 3.38, 0.06-fold).

Representative gel pictures

Representative gel pictures

ACE2 rs2106809A/G (INS/DEL)

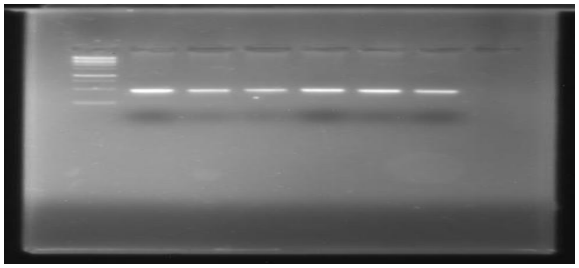

Figure 4: Gradient PCR set from 50°C to 55°C

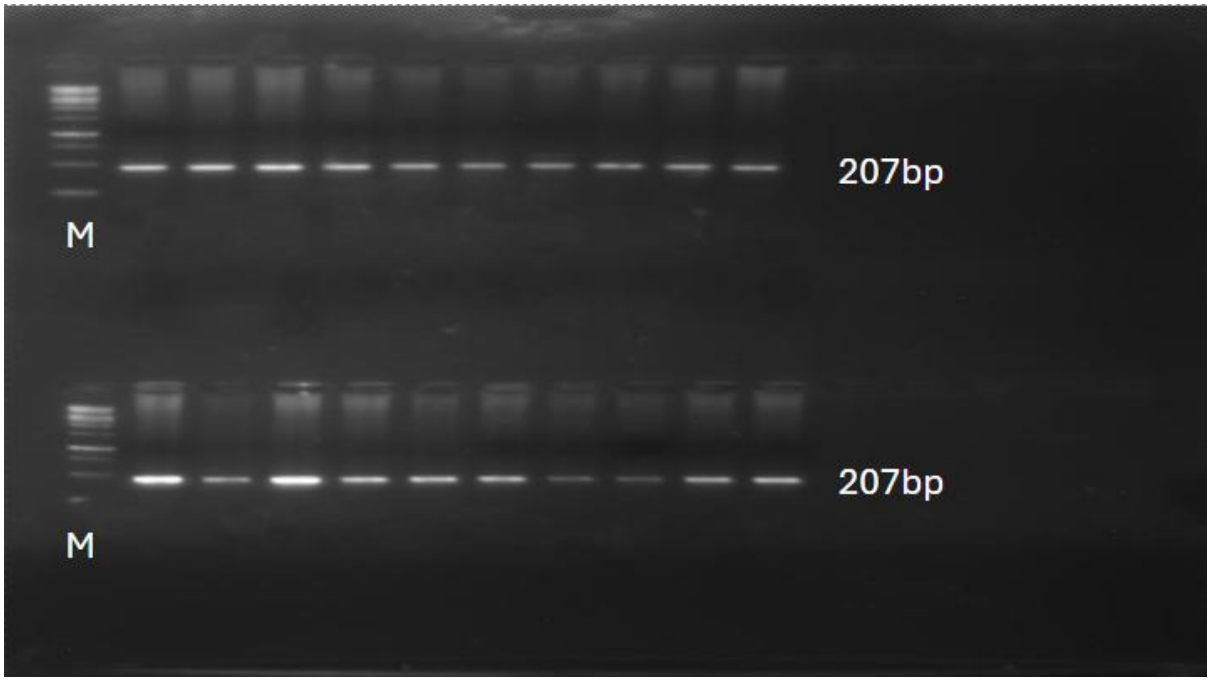

Figure 5: Representative gel picture of PCR

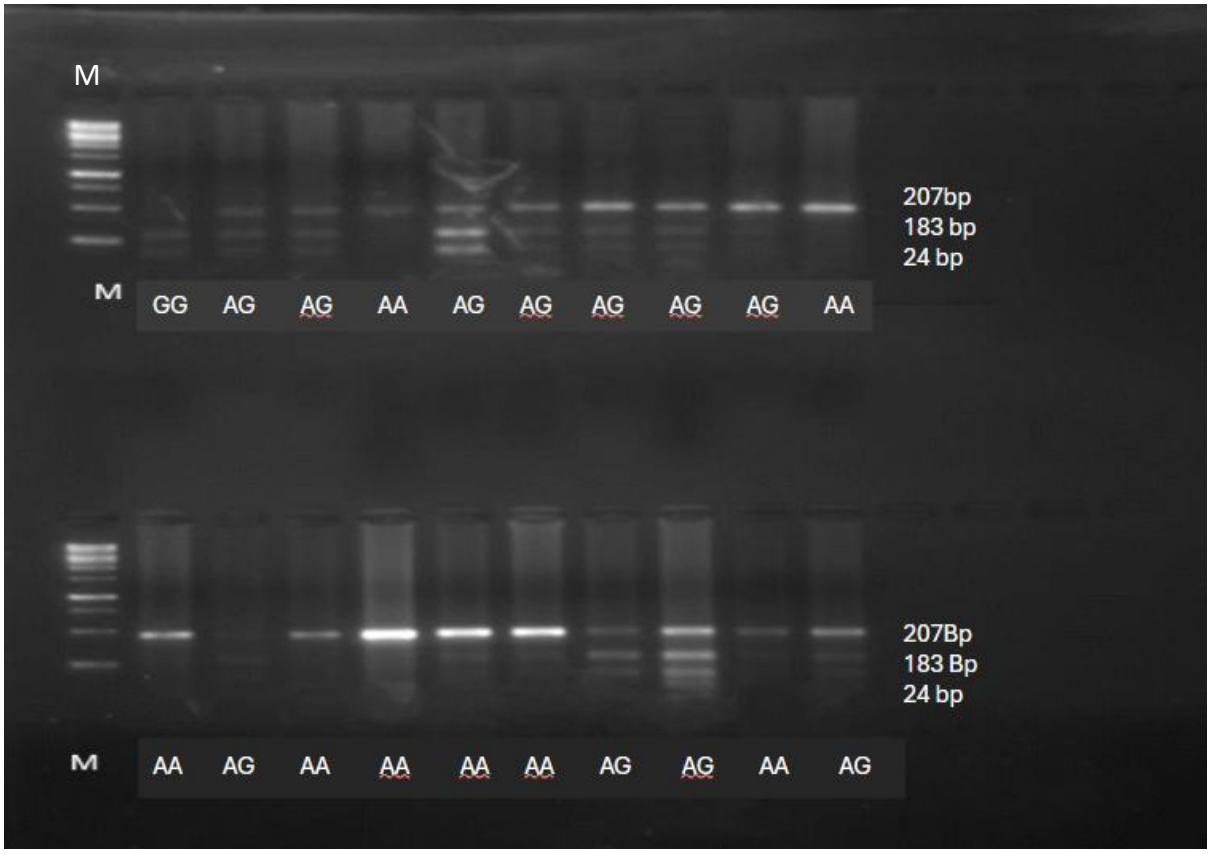

Figure 6: Representative gel picture of Genotyping of *ACE2 rs2106809A/G* polymorphism

***ACE2* -8790G/A (rs2285666) polymorphisms**

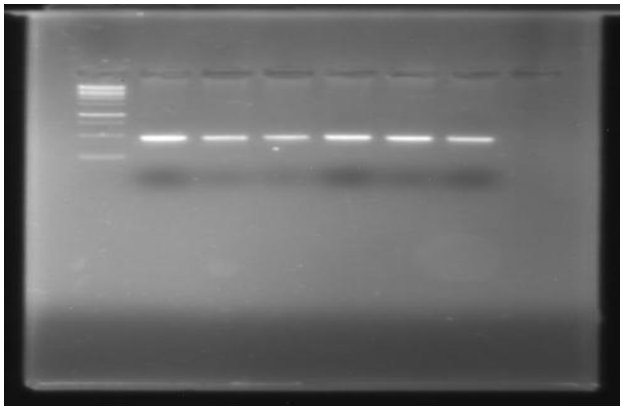

Figure 7: Gradient PCR set from 50°C to 55°C

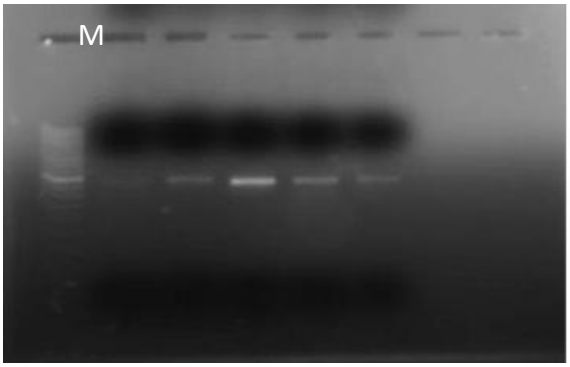

Figure 8: Representative gel picture of PCR

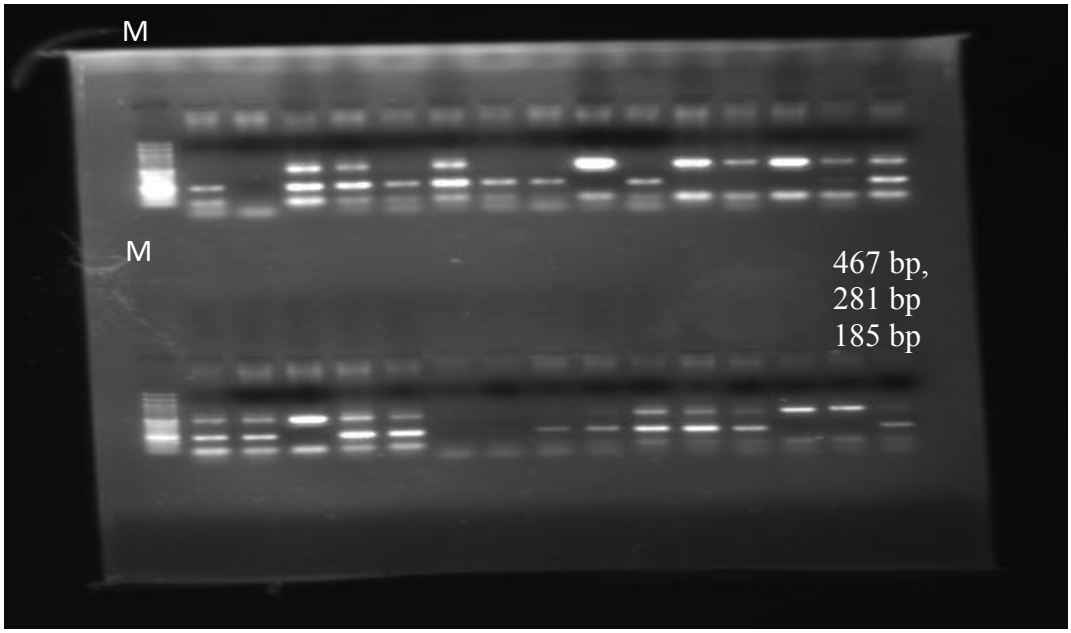

Figure 9: Representative gel picture of Genotyping of *ACE2 rs2106809A/G* polymorphism

M: 50bp ladder

PCR product: 467 bp for *ACE2*-8790GG genotype, 467 bp, 281 bp & 185 bp for the *ACE2*-8790GA heterozygous genotype, and 281 bp & 185 bp for the ACE2-8790AA genotype

PCR product: 207 bp for *ACE2 rs2106809AA* genotype, 207 bp, 183 bp & 24 bp for the *ACE2 rs2106809*GA heterozygous genotype, and 183 bp & 24 bp for the *ACE2 rs2106809GG* genotype

Summary

Summary

In the present study,
ACE2 rs2106809 GA genotype and rs2106809A allele were associated with the reduced risk of SARS-CoV-2 infection.

ACE2 rs210680GG genotype and rs210680G allele were associated with impaired CRP level

ACE2 rs210680AG and rs210680GG genotypes were associated with reduced risk of impaired ferritin level.

ACE2 -8970GA genotype was associated with higher risk of mild and moderate disease stage of COVID-19 patients.

ACE2 -8970A allele were associated with the risk of mild and moderate disease stage.

The expression of *IL-1β* gene was significantly higher in COVID-19 patients than healthy controls. *IL-1β* may have a role in COVID-19 disease progression.

CHAPTER 4

Conclusion

It is possible that *ACE2* rs2106809 G/A contributes to the elevated risk of SARS-CoV-2 infection. The genotype of *ACE2* rs210680GG may help to raise CRP levels and lower the chance of raising ferritin levels. Mild to moderate illness stages may be influenced by the *ACE2*-8970GA genotype. An increase expression of *IL-1β* could help the COVID-19 disease progression.

Limitations and Future Direction

Certain limitations need to be addressed for further research. The small sample size might limit the generalizability of the findings of RT-PCR and further studies with but larger population needs to be done to confirm these observations and to get better results.

In future polymorphism of immune associated genes can be can be checked and investigate how these polymorphisms alter protein function resulting in the adversity of the disease.

RTPCR of the immune associated genes can be done to observe their expression levels can be done with a larger sample size to get better and significant results.

List of Abbreviations

Sl. No.	Abbreviations	Full form
1	PCR	Polymerase chain reaction
2	RFLP	Restriction fragment length polymorphism
3	SNPs	Single nucleotide polymorphism
4	RTPCR	Reverse transcription polymerase chain reaction
5	ACE2	Angiotensin converting enzyme 2
6	IL	Interleukin
7	EtBr	Ethidium bromide
8	SD	Standard deviation
9	TAE	Tris acetate EDTA
10	DNA	Deoxyribonucleic acid
11	RNA	Ribonucleic acid
12	dNTP	Deoxynucleotide triphosphate
13	MGW	Molecular grade water
14	SARS	Severe acute respiratory syndrome
15	TMPRSS2	Transmembrane protease, serine 2
16	WHO	World health organization
17	ARDS	Acute respiratory disorder
18	ORF	Open reading frame
19	OR	Odds ratio
20	HLA	Human leukocyte antigen
21	UTR	Un-translated region
22	MERS	Middle east respiratory syndrome
23	DIC	Disseminated Intravascular Coagulation
24	COVID	Corona virus disease
25	HWE	Hardy Weinberg equilibrium
26	TNF	Tumor necrosis factor
27	RT	Reverse transcriptase

28	UV	Ultra-violet
29	HC	Healthy controls
30	CV	Covid samples
31	FP	Forward primer
32	RP	Reverse primer
33	SYBR green	Synergy brands green
34	TB green	Takara bio green
35	EDTA	Ethylenediaminetetraacetic acid
36	Kda	Kilodalton
37	CoV2	Corona virus 2
38	rsID	Reference SNP cluster ID

Reference

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Appendix

Results:

Table 1: Name of gene polymorphism, primer sequence, annealing temperature, Restriction enzyme, genotypes for PCR-RFLP

S. No.	Gene Name	Primer Sequence	Annealing Temperature	Restriction Enzyme	Genotype
1	*ACE2-8790G/A (rs2285666)*	FP-5' CATGTGGTCAAAAGGATATCT3' RP-5' AAAGTAAGGTTGGCAGACAT 3'	52°C	*AluI*	GG: 467 bp GA: 467, 281, 185 bp AA: 281 & 185 bp
2	ACE2 A/G (rs2106809)	FP-5' GAAAGCCAGATGCTTTAACAAG 3' RP-5' TTTTTCCATATCTCTATCTGATCG 3'	53°C	TaqI	AA: 207bp AG: 207 bp, 183 & 24 bp GG: 183 bp & 24bp

Table 1.1. Demographic profile of COVID-19 patients and healthy controls

Subjects	**COVID-19 patients**				**Healthy controls**
Total Number	200				200
Mean age and standard deviation (Years ± SD)	50.92 ± 6.34 yrs				46.74 ± 7.45
Females	79 (39.5%)				60(29.9%)
Males	121(60.5%)				141 (70.1%)
Ethnicity	Western India				Western India
Disease stages (CT Score)	Critical <8	Severe 9-15	Moderate 16-25	Mild >26	

	9(4.5%)	71(35.5%)	86(43%)	34(17%)	
D, Dimer	Normal		Impaired		
	191(95.5%)		9(4.5%)		
CRP level	Normal 0.3 mg/dl		Impaired >1.0 mg/dl		
	139(69.5%)		61(30.5%)		
Ferritin in level	Normal (<300 mg/dl)		Impaired (>300 mg/dl)		
	114(57%)		86(43%)		
Creatnin level status	Normal (0.7-1.3)		Impaired (>1.3)		
Blood Urea	Normal (1.8-7.1mmol/L)		Impaired (>7.1mmol/L)		
	153(76.5%)		47(23.5%)		
SGPT	Normal (8-45units/L)		Impaired (<45units/L)		
	119(59.5%)		81(40.5%)		
Serum Bilirubin	Normal (0.2-1.2 mg/dL)		Impaired (<1.2 mg/dL)		
	154		46		
HB	Normal (M=14-18 mg/dL), (F= 12-16 mg/dL)		Impaired (M=>14 mg/dL), (F= >12mg/dL)		
	105(52.5%)		95(47.5%)		
Platelet count	Normal (For men= 135,000 to 317,000 per microliter and for women=157,000 to 371,000/microliter)		Impaired (For men= <135,000 to 317,000 per microliter and for women=<157,000 to 371,000/microliter		
	129(64.5%)		71(35.5%)		
TLC count	Normal (*4,000*		Impaired (>*4,000*		

	and 11,000 µL)	*and 11,000 µL)*	
	141(70.5%)	59(29.5%)	

Table 2: Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with COVID-19

Genotypes ACE2 -8970G/A	COVID-19 patients N= 200(%)	Healthy controls N= 201(%)	*P*-Value	OR(95%CI) HW=0.95
GG	104(52.0%)	122(60.7%)	1	Reference
GA	85(42.5%)	69(34.3%)	0.09	1.45 (0.94-2.23)
AA	11(5.5%)	10(5.0%)	0.74	1.29(0.49-3.44)
Alleles	**COVID-19 patients N= 400(%)**	***Healthy controls* N= 402(%)**	***P*-Value**	**OR(95%CI)**
G	293(73.3%)	313(77.9%)	1	Reference
A	107(26.7%)	89(22.1%)	0.15	1.28(0.92-1.80)
Genotypes ACE2A/G (rs2106809)	COVID-19 patients N= 200(%)	Healthy controls N= 201(%)	P-Value	OR(95%CI) HW=0.80
AA	135(67.5%)	101(50.2%)	1	Reference
AG	55(27.5%)	84(41.8%)	**0.001**	**0.49(0.31-0.77)**
GG	10(5.0%)	16(8.0%)	0.10	0.47(0.19-1.15)
Alleles	COVID-19 patients N= 400(%)	Healthy controls N= 402(%)	P-Value	OR(95%CI)
A	325(81.3%)	286(71.1%)	1	Reference
G	75(18.7%)	116(28.9%)	**0.001**	**0.57(0.40-0.80)**

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

Table 3: **Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired CRP Level**

Genotypes ACE2 -8970G/A	**Impaired CRP level N=61 (%)**	**Normal CRP level N=139 (%)**	***P*-Value**	**OR(95%CI)**
GG	36(59.0%)	68(48.9%)	1	Reference
GA	24(39.3%)	61(43.9%)	0.43	0.74(0.38-1.45)
AA	1(1.7%)	10(7.2%)	0.16	0.19(0.01-1.54)
Alleles	**Impaired CRP level N=122 (%)**	**Normal CRP level N=278 (%)**	***P*-Value**	**OR(95%CI)**
G	96(78.7%)	197(70.9%)	1	Reference
A	26(21.3%)	81(29.1%)	0.13	0.66(0.39-1.12)
Genotypes ACE2A/G (rs2106809)	**Impaired CRP level N=61 (%)**	**Normal CRP level N=139 (%)**	***P*-Value**	**OR(95%CI)**
AA	35(57.4%)	100(71.9%)	1	Reference
AG	19(31.1%)	36(25.9%)	0.30	1.51(0.37-3.13)
GG	7(11.5%)	3(2.2%)	**0.009**	**6.67(1.44-34.73)**
Alleles	**Impaired CRP level N=122 (%)**	**Normal CRP level N=278 (%)**	***P*-Value**	**OR(95%CI)**
A	89(73.0%)	236(84.9%)	1	Reference
G	33(27.0%)	42(15.1%)	**0.007**	**2.08(1.20-3.69)**

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

Table 4: Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired Ferritin level

Genotypes ACE2 -8970G/A	**Impaired Ferritin level N=86 (%)**	**Normal ferritin level N=114 (%)**	***P*-Value**	**OR(95%CI)**
GG	39(45.3%)	65(57.0%)	1	Reference
GA	42(48.8%)	43(37.7%)	0.13	1.63(0.87-3.04)
AA	5(5.8%)	6(5.3%)	0.84	1.39(0.34-5.61)
Alleles	**Impaired Ferritin level N=172 (%)**	**Normal Ferritin level N=228 (%)**	***P*-Value**	**OR(95%CI)**
G	120(69.8%)	173(75.9%)	1	Reference
A	52(30.2%)	55(24.1%)	0.21	1.36(0.85-2.18)
Genotypes ACE2A/G (rs2106809)	Impaired Ferritin level N=86 (%)	Normal Ferritin level N=114 (%)	P-Value	OR(95%CI)
AA	75(87.2%)	60(52.6%)	1	Reference
AG	9(10.5%)	46(40.4%)	**0.001**	**0.16(0.17-0.36)**
GG	2(2.3%)	8(7.0%)	0.06	0.20(0.03-1.07)
Alleles	Impaired Ferritin level N=172 (%)	Normal Ferritin level N=228 (%)	P-Value	OR(95%CI)
A	159(92.4%)	166(72.8%)	1	Reference
G	13(7.6%)	62(27.2%)	0.92	0.91(0.44-1.90)

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

Table 5: **Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with impaired D Dimer level**

Genotypes	**Impaired D**	**Normal D Dimer**	***P*-Value**	**OR(95%CI)**

ACE2 -8970G/A	**Dimer status N=9 (%)**	**status N=191 (%)**		
GG	7(77.8%)	97(50.8%)	1	Reference
GA	2(22.2%)	83(43.5%)	0.28	0.33(0.05-1.82)
AA	0(0.0%)	11(5.8%)	-	-
Alleles	**Impaired D Dimer status N=18 (%)**	**Normal D Dimer status N=382 (%)**	*P*-Value	OR(95%CI)
G	16(88.9%)	277(72.5%)	1	Reference
A	2(11.1%)	105(27.5%)	0.20	0.33(0.05-1.53)
Genotypes ACE2A/G (rs2106809)	**Impaired D Dimer status N=9 (%)**	**Normal D Dimer status N=191 (%)**	*P*-Value	OR(95%CI)
AA	4(44.4%)	131(68.6%)	1	Reference
AG	5(55.6%)	50(26.2%)	0.15	3.28(0.73-15.26)
GG	0(0.0%)	10(5.2%)	-	-
Alleles	**Impaired D Dimer status N=18 (%)**	**Normal D Dimer status N=382 (%)**	*P*-Value	OR(95%CI)
A	13(72.2%)	312(81.7%)	1	Reference
G	5(27.8%)	70(18.3%)	0.48	1.71(0.51-5.39)

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

Table 6: Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms among different stages of COVID-19

Genotypes ACE2 -8970G/A	***Healthy controls* N=200(%)**	**Critical disease stage**		**Severe disease stage**		**Moderate disease stage**		**Mild disease stage**	
		N=9	OR(P)	N=71	OR(P)	N=86	OR(P)	N=34	OR(P)

GG	122(60.7%)	9 (100%)	1(Ref)	54 (76.05%)	1(Ref)	29(33.72%)	1(Ref)	12(35.29%)	1(Ref)
GA	69(34.3%)	0 (0%)	-	12(16.90%)	0.39(0.01)	52(60.46%)	**3.17(0.0003)**	21(61.76%)	**3.09(0.05)**
AA	10(5.0%)	0(0%)	-	5(7.04%)	1.13(0.93)	5(5.81%)	2.10(0.33	1(2.94%)	1.02(0.59)
ACE2 -8970G/A Allele	***Healthy controls* N=402(%)**	**Critical disease stage**		**Severe disease stage**		**Moderate disease stage**		**Mild disease stage**	
		N=18	OR(P)	N=142	OR(P)	N=172	OR(P)	N=68	OR(P)
G	313(77.9%)	18 (100%)	1(Ref)	120(84.50%)	1(Ref)	110 (63.92%)	1(Ref)	45(66.17%)	1(Ref)
A	89(22.1%)	0(0%)	-	22 (15.49%	0.64(0.11)	62 (36.04%)	**1.98(0.007)**	23 (33.82%)	**1.80(0.05)**
Genotypes ACE2 rs2106809 A/G	***Healthy controls* N=201(%)**	**Critical disease stage**		**Severe disease stage**		**Moderate disease stage**		**Mild disease stage**	
		N=9	OR(P)	N=71	OR(P)	N=86	OR(P)	N=34	OR(P)
AA	101(50.2%)	3(33.33%)	1(Ref)	24(33.80%)	1(Ref)	74 (86.04%)	1(Ref)	34(100%)	1(Ref)
AG	84(41.8%)	1(11.11%)	0.40(0.76)	46 (64.78%)	**2.30(0.005)**	8(9.30%)	0.13(0.000)	0(0%)	-
GG	16(8.0%)	5(55.55%)	10.52(0.002)	1(1.40%)	0.26(0.31)	4(4.65%)	0.34(0.09)	0 (0%)	-
ACE2 rs2106809 A/G Allele	***Healthy controls* N=402(%)**	**Critical disease stage**		**Severe disease stage**		**Moderate disease stage**		**Mild disease stage**	
		N=18	OR(P)	N=142	OR(P)	N=172	OR(P)	N=68	OR(P)
A	286(71.1%)	7(38.88%)	1(Ref)	94(66.19%)	1(Ref)	156(90.69%)	1(Ref)	68(100%)	1(Ref)
G	116(28.	11(61.1	3.87(0	48(33.80	13.28	16(9.30	0.25(0.	0	-

	9%)	1%)	.07)	%)	(0.00)	%)	00)		

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

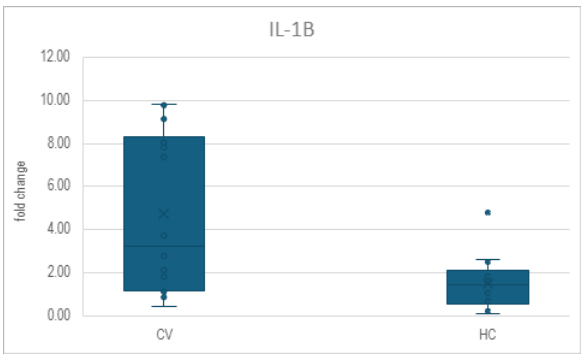
Table- 7: Association of *ACE2 -8970G/A, rs2106809A/G* polymorphisms with Platelet count

Genotypes ACE2 -8970G/A	**Impaired Platelet count N=71 (%)**	**Normal Platelet count N=129 (%)**	***P*-Value**	**OR(95%CI)**
GG	36(50.7%)	68(52.7%)	1	Reference
GA	31(43.7%)	54(41.9%)	0.91	1.08(0.57-2.06)
AA	4(5.6%)	7(5.4%)	0.82	1.08(0.25-4.50)
Alleles	**Impaired Platelet count N=142 (%)**	**Normal Platelet count N=258 (%)**	***P*-Value**	**OR(95%CI)**
G	103(72.54%)	190(73.64%)	1	Reference
A	39(27.46%)	68(26.36%)	0.90	1.06(0.65-1.72)
Genotypes ACE2 rs2106809A/G	Impaired Platelet count N=71 (%)	Normal Platelet count N=129 (%)	P-Value	OR(95%CI)
AA	49(69.0%)	86(66.7%)	1	Reference
AG	19(26.8%)	36(27.9%)	0.95	0.93(0.43-1.88)
GG	3(4.2%)	7(5.4%)	0.95	0.75(0.15-3.44)
Alleles	Impaired Platelet count N=142 (%)	Normal Platelet count N=258 (%)	P-Value	OR(95%CI)

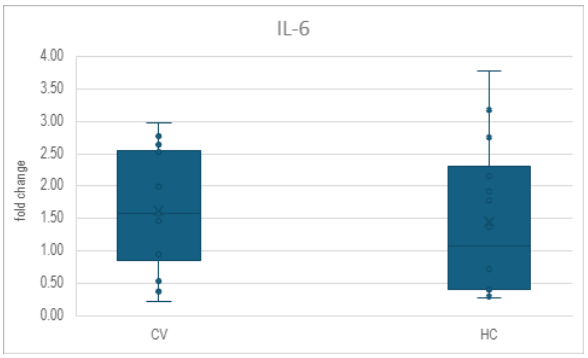
A	117(82.39%)	208(80.62%)	1	Reference
G	25(17.61%)	50(19.38%)	0.76	0.89(0.50-1.56)

N= Total no. of subjects, (%) = frequency of genotypes/alleles**,** (OR) and 95% CI confidence intervals (CI) were derived from logistic regression models comparing the homozygous wild-type genotype/allele (*GG genotype and G allele for ACE2* -8970G/A polymorphism, AA genotype and A allele for ACE2A/G (rs2106809) polymorphism were taken as reference) with other genotypes/alleles.

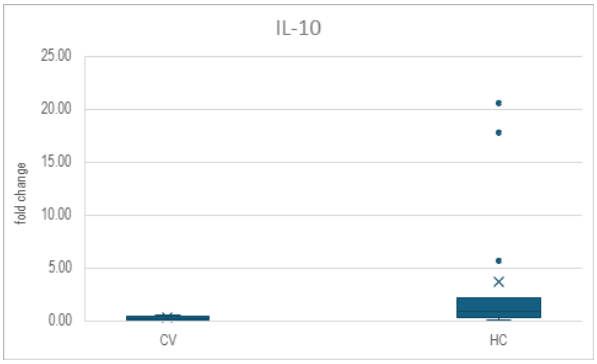
Bar diagram: expression levels of *IL-1B, IL-6, IL-10 and TNF-* α in COVID19 patients and healthy controls



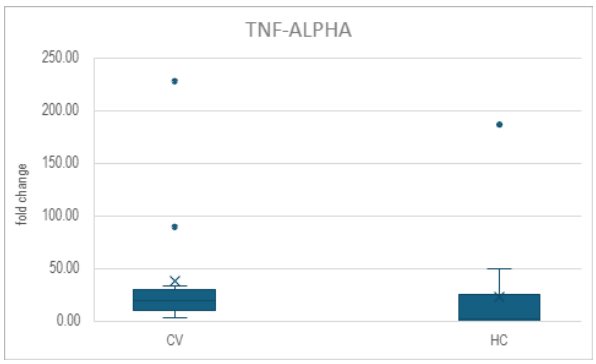
(a)



(b)



(c)



(d)

a) shows the levels of expression of *IL-1β* of COVID-19 patients compared to healthy controls.
b) shows the levels of expression of *IL-6* of COVID-19 patients compared to healthy controls.
c) shows the levels of expression of *IL-10* of COVID-19 patients compared to healthy controls.
d) shows the levels of expression of *TNF- α* of COVID-19 patients compared to healthy controls.