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STUDY IL6 174C/G AND IL10 1082T/G POLYMORPHISM IN PATIENTS WITH COVID 19

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February 2023

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ABSTRACT

STUDY IL6 174C/G AND IL10 1082T/G POLYMORPHISM IN PATIENTS WITH COVID 19

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Master of Science in Biology

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This study aims to determine whether COVID19 infection is associated with variations in the IL-6 and IL-10 genes. In the study, samples were obtained from 20 healthy individuals as a control group and 90 individuals (male and female) with COVID-19 infection. IL-6 levels are significantly higher in COVID-19 infected patients than in healthy individuals. However, the data showed that the level of IL-10 in infected patients was significantly higher than in healthy ones. The serum vitamin D3 levels of infected patients were significantly lower than those of healthy controls. However, the results showed that infected patients had significantly higher vitamin B12 levels than healthy people. The data suggested that IL6 cytokines should be considered as a crucial factor in achieving the therapeutic response in people infected with COVID-19. The results showed a strong correlation between the frequency of the SNP gene and the severity of COVID-19 cases. Overall, the study's findings confirm that the increase in IL-10 and the decrease in lymphocytes are reliable indicators of COVID-19.

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Keywords: COVID-19, IL-6 174C/G, IL-10 1082 T/C

ÖZET

KORONAVİRÜS (COVID-19) HASTALARINDA IL6 174C/G VE IL10 1082T/G POLİMORFİZMİ

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Bu çalışma, COVID19 enfeksiyonunun IL-6 ve IL-10 genlerindeki varyasyonlarla bağlantılı olup olmadığını belirlemeye yöneliktir. Çalışmada, kontrol grubu olarak 20 sağlıklı birey ile COVID-19 enfeksiyonu olan 90 bireyden (erkek ve kadın) numuneler elde edilmiştir. COVID-19 enfekte hastalarda IL-6 seviyeleri sağlıklı bireydekilere göre önemli ölçüde daha yüksek olduğu belirlendi. Bununla birlikte veriler, enfekte hastalarda IL-10 seviyesinin sağlıklı olanlara göre önemli ölçüde daha yüksek olduğunu göstermiştir. Enfekte hastaların serum vitamin D3 seviyeleri, sağlıklı kontrollerinkinden önemli ölçüde düşüktü. Ancak sonuçlar, enfekte hastaların B12 vitamini düzeylerinin sağlıklı insanlara göre anlamlı derecede yüksek olduğunu göstermiştir. Veriler, IL6 sitokinlerinin, COVID-19 ile enfekte kişilerde terapötik yanıtın gerçekleştirilmesinde çok önemli bir faktör olarak dikkate alınması gerektiğini ileri sürmüştür. Sonuçlar, SNP geninin sıklığı ile COVID-19 vakalarının şiddeti arasında güçlü bir ilişki olduğunu göstermiştir. Genel olarak, çalışmanın bulguları, IL-10'daki artışın ve lenfositlerdeki düşüşün COVID-19'un güvenilir göstergeleri olduğunu doğrulamaktadır.

2023, 59 sayfa

Anahtar Kelimeler: COVID-19, IL-6 174C/G, IL-10 1082 T/C

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LIST OF SYMBOLS

µg	Microgram
µL	Microliter
Cm	Centimeter
g	Gram
Kg	Kilogram
L	Liter
mg	Milligram
mL	Milliliter
Ng	Nano-Gram
Nm	Nanometer
^{o}C	Degree celsiu
Pg	Picogram

LIST OF ABBREVIATIONS

AB	Antibody
ACE2	Angiotensin-converting enzyme 2
AT1	Angiotensin II
CD	Closter identification
CD	Cluster of differentiation
COVID-19	Coronavirus disease 2019
CRP	C-reactive protein
CTL	Cytotoxic T-cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzy-linked ımmunosorbent assay
EPO	Erythropoietin
HGF	Hybridoma growth factor
IFNγ	Interferon-γ
IL	Interleukin
INF	Interferon
MAS	Macrophage activation syndrome
MCP-1	Monocyte chemoattractant protein-1
MERS	Middle east respiratory syndrome
NK	Natural killer
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sHLH	Secondary hemophagocytic lymphohistiocytosis
SST	Serum separator tubes
THPO	Thrombopoietin
TNF	Tumor necrosis factor
Tr1	Treg cells type 1
Treg	T regulatory cells

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1. INTRODUCTION

The history of cytokines may be traced back to the turn of the previous century, when the first instance of interferon was discovered. Leukocytes are just one type of cell that secretes cytokines into the bloodstream in order to control the immune system and the production of new blood cells (hematopoiesis) (Deverman and Patterson 2009).

They are separated into two distinct categories according to the cell type from which they originate: Th1 cells or Th2 cells. Recently, a third subgroup of T helper cells known as Th17 and T regulatory cells known as Treg have been characterized. These two cell types exhibit a distinct cytokine profile in comparison to Th1 and Th2 cells. It produces IL-17, IL-17F, IL-22, and IL25 in its secretions (Vitenberga *et al.* 2022).

The main cytokines that are secreted by regulatory T cells of type 1 (Tr1) are IL-10 and IFN-. They also produce a smaller amount of IL-5 and a negligible amount of TGF-β and IL-2. The Tr3 subset of regulatory T cells, often known as Th3 cells, is responsible for the production of mostly TGF-β and only a marginal quantity of IL-10 (Vitenberga *et al.* 2022).

As of right now, more than 225 nations have been impacted by COVID-19, with almost 140 million confirmed cases (WHO 2020) About 3 million fatalities documented globally as of April 15, 2021 (WHO 2020). The global vaccination campaign against COVID-19 has recently begun, and there have been some successful productions of vaccine candidates (Lauring and Hodcroft 2021).

Despite this, there is still a need for efficient treatments and medications to be developed in order to combat this pandemic virus (Sharun *et al.* 2020, He *et al.* 2021) have all presented significant hurdles. The worsening effects and severity of SARS- It has been estimated that the incubation period for the virus within the human body lasts anywhere from 5 to 14 days (Lauer *et al.* 2020).

When virus particles infiltrate the lung endothelial cells and attach themselves to the ACE2 receptors on the surface of the cells, this is the first step in the pathogenesis of SARS-CoV-2 (Keam *et al.* 2020).

This, in turn, causes immune cells such as macrophages, natural killer (NK) cells, or other immune cells to become hyperactivated, which results in the production of chemokines and cytokines by these cells. This is the initial stage in the progression of the SARS-CoV-2 pathogenesis (Keam *et al.* 2020).

It was aimed in this research:

1. Study the correlation of IL 6 174C / G polymorphism in patients with COVID-19.
2. Study the correlation of IL 10 1082A/G polymorphism in patients with COVID-19.
3. Estimate the concentrations of IL 6 in patients with COVID-19.
4. Estimate the concentrations of IL 10 in patients with COVID-19.
5. Estimate the concentrations of some vitamins (vitamin D and B12) in patients with COVID-19.

2. LITERATURE REVIEW

2.1 Cytokines

Their name derives from the Greek words for cell and movement: cyto- and -kine. It is possible that they are glycoproteins, peptides, or proteins. They are a large category of proteins with a molecular weight between (~5– 20 kDa) that play a significant role in cell signaling. Cytokines are proteins that are secreted by cells and have the ability to alter the behavior of neighboring cells and even the secreting cell itself (Weaver *et al.* 2007).

Cytokines regulate the maturity, proliferation, and responsiveness of specific cell populations as well as the balance between humoral and cell-based immune responses. They do this by acting through receptors, and their importance cannot be overstated when it comes to the immune system. In a variety of intricate ways, the actions of certain cytokines can be enhanced or inhibited by the actions of other cytokines (Lackie 2010, Ibelgaufts 2013).

Particular types of immune cells are controlled on and off by certain cytokines, which act as chemical switches. They feature a wide variety of interleukins, interferons, and growth factors among their constituents. They play an essential role in the host's response to infection, immunological responses, inflammation, trauma, sepsis, cancer, and reproduction. They also play a role in the development of illness (Schulte *et al.* 2013).

The production of T cells in the immune system is stimulated by interleukin 2, often known as IL-2. Because of its ability to strengthen one's immune system, IL-2 has long been considered a viable treatment for a variety of diseases. (Kierszenbaum and Sztein 1994).

2.2 Classification

The categorization that is based on the function or kind of cell from which they originate, their range of activity, the class of activity they affect, the cells they target, or specifics of their ligand-receptor connection (Cohen and Cohen 1996), Particularly in the human and mouse species, our understanding of the structural and functional characteristics of cytokines has advanced to an extraordinary degree (Tisoncik *et al.* 2012).

2.3 Classification According to Structure

Through the use of structural homology, researchers have been able to partially differentiate between cytokines that do not exhibit a significant amount of redundancy, allowing for the cytokines to be categorized into four distinct categories (Bodmer and Henderson 1996). Structures each of whose constituent parts consists of four bundles of -helices. This family is further subdivided into three subfamilies: the interferon (INF) subfamily, the IL-2 subfamily, and the IL-10 subfamily (Tonge and Moore 2007).

The first of these three subfamilies is the largest of the three, and it contains a number of cytokines that are not involved in immunology. These cytokines include erythropoietin (EPO) and thrombopoietin (THPO). IL-1 family, the most prominent members of which are IL-1 and IL-18. IL-17 family, which has not yet been fully described in its whole. On the other hand, it is common knowledge that they have the impact of encouraging the multiplication of T cells, which are responsible for cytotoxic effects (Rozwarski *et al.* 1994).

2.4 Classification According to Function

A classification of cytokines into those that favor antibody responses (TGF-, IL-4, IL-10, and IL-13, for example) and those that favor cellular immune responses (IFN-, TNF, for example) has proven to be more useful in both clinical and experimental settings

(Ouaguia *et al.* 2014). The fact that cytokines belonging to one of these two categories have a tendency to limit the actions of those belonging to the other has been a primary focus of research. The dysregulation of this propensity is currently the subject of extensive research due to the possibility that it plays a role in the etiology of autoimmune illnesses (Jin *et al.* 2007). Cytokines have solidified their crucial roles as therapeutic, prognostic, and diagnostic tools in human disease., making them a significant new frontier in the field of medicine (Dinarello 2007).

Which are required for the production of vaccinations and also form the basis of various vaccines. IL-1, owing to the fact that its effects ranged from those on the management of body temperature to those on the production of liver proteins to those on T-cell responses to antigens and mitogens (Dinarello 2007).

2.5 Cytokine Receptors

Through interactions with receptors that are located on the cell membranes of receptive target cells, cytokines are able to exert a variety of biological effects. These receptors contain a single domain that spans the plasma membrane in addition to an extracellular domain and a cytoplasmic domain. In addition to this, there are additionally two polypeptide chains present. One is a α signal transducing β subunit, while the other is a cytokine-specific subunit (Wilson *et al.* 2002).

The presence of particular membrane receptors is what establishes the type of target cell to which cytokines bind. Cytokines can only bind to certain types of cells. They are capable of autocrine, paracrine, and endocrine activities, and they operate as mediators of cellular communication (Wilson *et al.* 2002).

By influencing the activation, proliferation, and differentiation of specific cells in one of two ways, one can regulate the intensity or length of an immune response, and in turn, one can regulate the release of antibodies or other cytokines. In addition, cytokines often trigger the production of additional cytokines, setting off a chain reaction in which

the actions of later-produced cytokines modulate those of earlier-produced cytokines. Last but not least, they have a small window of effectiveness since their half-life in the blood and extracellular fluids is very low (Owen *et al.* 2013).

2.6 Interleukins

Interleukins, or ILs for short, are a family of proteins with a wide range of forms and functions that are produced from cells. These proteins interact with receptors and have a role in leukocyte communication. They have a close relationship with both the stimulation and inhibition of the immune system as well as the division of cells. Helper CD4+ T lymphocytes, monocytes, macrophages, and endothelial cells are responsible for the majority of the production of interleukins (Akdis *et al.* 2011).

Forty distinct interleukins have been identified to date, with some of these interleukins themselves being classified into subtypes (e.g., IL-1, IL-1). These ILs are organized into families according to the similarity between their sequences, receptor chains, and/or functions (Wang *et al.* 2016, Catalan *et al.* 2017).

2.7 Interleukin 6

L-6 is a pleiotropic soluble mediator with effects on hematopoiesis, inflammation, and the immune response. Different functions of IL-6 were identified at first, and their names were afterwards based on the particular biological processes they facilitated. Because of how it influences hepatocytes' production of acute phase proteins, this substance is known as hepatocyte-stimulating factor. Due to its capacity to accelerate the growth of fusion cells made up of plasma cells and fibroblasts, hybridoma growth factor (HGF) was given the term (Kishimoto 1985).

But in 1986 (Hirano *et al.* 1986), after the BSF-2 cDNA was successfully cloned, it was found that the molecules studied by several laboratories under various designations were actually similar, giving rise to the moniker IL6. The human IL-6 protein contains

212 amino acids in total including a signal peptide that is just 28 amino acids long, and the location of its gene on chromosome 7p21 has been determined. Natural IL-6 ranges in size from 21 to 26 kDa, despite the fact that the core protein is only 20 kDa. This is due to glycosylation (Kishimoto 1989).

At first, inflammation starts at a local lesion, where IL-6 is then created. From there, it is absorbed into the bloodstream and eventually reaches the liver. Then, a cascade of acute phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin, and a1-antichymotrypsin, are rapidly induced (Heinrich *et al.* 1990).

Proteins including fibronectin, albumin, and transferrin are normally produced by the body, but IL-6 prevents this from happening. At first, it was thought that the hepatocyte-affecting biological effects were caused by Hepatocyte stimulating factor, and studies were conducted to confirm this. Through the production of amyloid, which occurs when high levels of SAA are present for an extended period of time, a major complication of a number of chronic inflammatory disorders can be brought about amyloidosis (Gillmore *et al.* 2001).

Deposition of amyloid fibrils, which leads to gradual damage in a variety of organs, is the consequence of this process. Additionally, IL-6 plays a role in the modulation of serum iron and zinc levels by regulating the transporters that carry iron and zinc. Hepcidin is produced in response to IL-6 and inhibits the iron transporter ferroportin 1 in the stomach, resulting in decreased serum iron levels (Nemeth *et al.* 2004).

With this knowledge, we can infer that the hypoferremia and anemia associated with chronic inflammation are caused by the IL-6-hepcidin axis. Low zinc levels are related to inflammation, which is brought on by IL-6's capacity to boost the expression of the zinc importer ZIP14 on hepatocytes (Liuzzi *et al.* 2005). Platelets are made when IL-6 is transported to the bone marrow, where it stimulates the maturation of megakaryocytes (Figure 2.1) (Ishibashi *et al.* 1989).

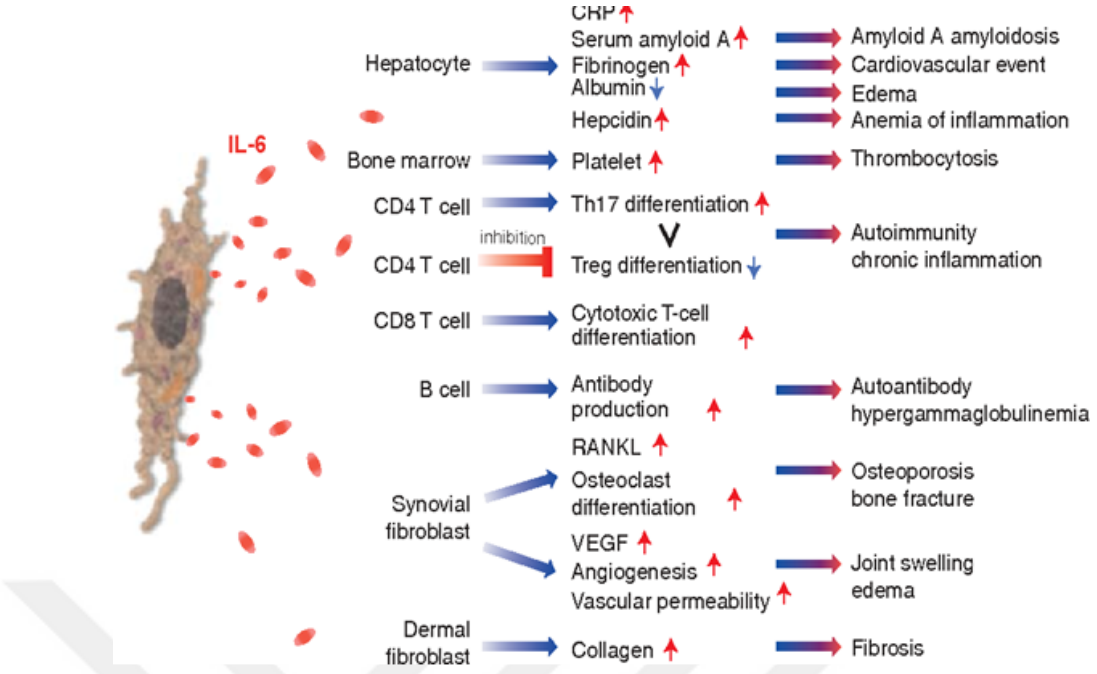

Figure 2.1 Interleukin 6 and its role in inflammation immunity and disease (Nguyen and Tewari 2014)

2.8 Interleukin 10

One of the most effective anti-inflammatory cytokines, interleukin 10 (IL-10) is crucial in warding off inflammatory and autoimmune diseases. Involvement of this kind is frequently required. Indeed, this sort of participation is frequently required. 3,4 Both a deficiency in IL-10 and an aberrant expression of the cytokine have been linked to increased inflammation in response to microbial danger, as well as the onset of inflammatory bowel disease and other autoimmune disorders (Garra *et al.* 2008).

Decreased pathogen clearance during an acute infection may be worth the increased immunopathology and tissue damage that results from a disturbance in IL-10 production or signaling (Li *et al.* 1999, Siewe *et al.* 2006, Sun *et al.* 2009). On the other hand, certain pathogens are able to use the immunosuppressive potential of IL-10 in order to reduce the immunological response of the host, which might result in prolonged infection (Brooks *et al.* 2006).

2.8.1 Interleukin 10 role during infection

Preliminary studies suggest that IL-10 deficiency is to blame for this phenomena. This could be due to a lack of IL-10 due to a mutation of the IL10 gene or a lack of IL-10 signaling due to an antibody blocking the IL-10 receptor (IL-10R). In the absence of IL-10, intracellular infections are typically more easily managed or eradicated more quickly. Suppressing IL-10 signaling is associated with increased survival post-infection and a heightened adaptive immunological response, including IFN- production by CD4 T cells (a response of 31) and persistent development of a pro-inflammatory milieu. Increased post-infection survival is the result. The cytokine interleukin-10 (IL-10) is an important predictor of poor health result in numerous parasite infections, including but not limited to Leishmania donovani, Yersinia pestis, and Yersinia enterocolitica (Leng and Denkers 2009).

Toxoplasma gondii, for instance, can block TLR4-mediated LPS communication in a way that reduces TNF-alpha expression while leaving IL-10 synthesis intact (Othieno *et al.* 1999). Patients who are infected with Mycobacterium tuberculosis display a pattern of expression that is quite similar to that of healthy individuals. On the other hand, despite the fact that an absence of IL-10 may at first be advantageous to the host, sustained IL-10 shortage may, in many cases, be deleterious over the course of time. In the context of an infection caused by bacteria, viruses, or fungi, an increase in the production of inflammatory cytokines that is sustained over time can result in septic shock (Li *et al.* 1999, Omer *et al.* 2003).

Infections cause apoptosis, although its severity can be mitigated by increasing IL-10 levels. This is due to the fact that inflammatory chemicals are frequently powerful activators of cell death. For example, mice that were exposed to a Chlamydia pneumoniae model had significant inflammation and saw raised levels of apoptosis. Excessive production of IL-10 can suppress the pro-inflammatory response to several pathogens, including Plasmodium species, Leishmania species, and Lymphocytic choriomeningitis virus, allowing the pathogens to evade immune control and cause

either acute and rapidly fatal infections or chronic, slowly progressing infections (Reed *et al.* 1994, Brooks *et al.* 2006, Belkaid 2007).

2.9 Causes and Effects of the Cytokine Storm

They are the masterminds behind everything that happens. A complex system of regulatory mechanisms makes sure that the production of pro-inflammatory and anti-inflammatory cytokines is in a healthy balance. This guarantees that the inflammatory response is minimized, proportional to the level of the pathogenic noxa, and that it is completely extinguished when the latter is eradicated. If any of these pathways break down, the immune system becomes overactive, producing excessive amounts of cytokines, which in turn triggers a systemic inflammatory reaction that can harm the entire body (Schulert and Grom 2015).

In contrast, autoinflammatory diseases and idiopathic inflammatory bowel disease may both initiate cytokine production after a non-pathogenic reason has been discovered. It is possible for a pathogen to either evade the immune system's defenses and spread throughout the body (as in sepsis) or to evade clearance and keep the immune system on high alert for an extended period of time (as seen in HLH associated to the Epstein-Barr virus). Finally, a genetic predisposition could be the root cause of immune hyperactivation (chimeric antigen receptor T-cell therapy) (Verbist and Nichols 2019).

Several pro-inflammatory cytokines can be generated by activated macrophages. These macrophages may contribute to the cytopenia frequently seen in patients with this illness (Fajgenbaum and June 2020).

Similarly, the activity of NK cells can become affected when there is an excess of IL-6 present. Since this reduces granzyme and perforin production, it may be supportive of certain cytokine storms. When activated by mediators like IL-6 and IFN-, endothelial cells begin to release more pro-inflammatory cytokines that can aid in the initiation of

coagulopathy. It is possible that endothelial cells contribute to the development of coagulopathy (Gust *et al.* 2017).

2.10 COVID-19 Related Cytokine Storm

The Coronaviridae are a group of enveloped viruses. The RNA in these viruses is single-stranded and positive-sense (RNA). The coronavirus family includes SARS-CoV-2, also known as severe acute respiratory syndrome coronavirus (Maiese *et al.* 2019). It is airborne and has a profound effect on the respiratory system. Previous studies have shown that the coronaviruses responsible for severe acute respiratory syndrome (SARS-CoV-1) and Middle East respiratory disease (MERS) can both trigger a cytokine storm (Maiese *et al.* 2019). Bioptic and autopsy samples from patients with COVID-19 have been used to confirm the presence of inflammatory infiltrates in a variety of tissues (Maiese *et al.* 2021, Maiese *et al.* 2021). Cytokine storms have been related to SARS-CoV-2 infections, and it is postulated that one of the processes that contributes to their creation may be an inadequate immunological response to the virus (Frisoni *et al.* 2022).

This is demonstrated by the fact that infected cells display a diminished ability to generate interferons (Blanco-Melo *et al.* 2020). In addition, a number of research organizations have discovered that patients with COVID-19 produce autoantibodies directed against a variety of immunomodulatory proteins. Anti-type I interferon antibodies in particular have been associated to serious sickness and death in this population (Wang *et al.* 2021, Bastard *et al.* 2021).

The findings of Lv *et al.* (2021) are in agreement with this idea. Results showed that SARS-CoV-2 may replicate within macrophages. The immune system would then be able to effectively battle the virus once more in the second phase, but due to the infection's unimpeded proliferation, an overreaction would ensue. This would happen because the virus has had time to fully duplicate. In addition, the renin-angiotensin-aldosterone system has been hypothesized to play a part by certain authors (RAAS) (Zanza *et al.* 2021).

3. MATERIALS AND METHODS

In this investigation, a total of 90 individuals suffering with COVID-19 infection, including both males and females, as well as 20 people serving as controls, were used. All of the subjects are adults ranging in age from 20 to 65 years old. During the time period of October 2021 to January 2022, the research was carried out at a variety of hospitals located within the city of Kirkuk.

3.1 Samples of Blood

Both healthy and sick people participated in the study, and after fasting for 10-12 hours, samples were collected. Blood samples were initially stored in Gold-top serum separator tubes (SST) and Ethylenediaminetetraacetic acid (EDTA) tubes before being centrifuged. Serum was separated into individual samples and deposited in Eppendorf tubes before being centrifuged at 3,500 rpm for 10 minutes. To prepare for biochemical analysis, all samples were dissolved after collection procedures were completed.

3.2 Biochemical

3.2.1 Interleukin-6 determination in serum (IL-6)

IBL International GmbH Kit Flughafenstr was used for a quantitative measurement of human IL-6 through enzyme immunoassay in human serum. Hamburg, Germany.

3.2.2 The testing principle

Coatings of anti-human interleukin-6 antibody are adsorbed onto microwells, Complexes are formed with the adsorbed antibodies when human interleukin-6 is present in either the sample or the standard. Once human IL-6 has been isolated, a second antibody is added to attach to it; in this case, an anti-human IL-6 antibody that has been biotinylated.

Following incubation, the unbound biotin-conjugated anti-human IL-6 antibody is removed by washing. In order to bind the biotinylated anti-human IL-6 antibody, streptavidin-HRP is applied to the antibody. After the wells have been incubated, a substrate solution reactive with HRP is added after they have been washed to eliminate any unbound Streptavidin-HRP.

The quantity of color in the final product is proportional to the amount of human IL-6 in the sample or the reference standard. The process is halted by adding acid, and its absorbance is read at 450 nm. Once the concentration of human IL-6 samples is known, a standard curve is built using seven standard dilutions of human IL-6 to establish a range of values.

3.2.3 Test protocol

The assay protocol papers were included in the kit, and the applications of the assay procedure are described in more detail below:

a) We aspirated the whole contents of the microwells twice before washing the strips in Wash Buffer at a rate of around 400 l per well. Approximately 10–15 seconds passed after the Wash Buffer was placed in the wells before the aspiration process commenced. (In this experiment, microplates were cleaned using a fully automated system.
b) A standard dilution was performed by adding 100 l of the assay buffer to each standard well of the microwell plate. After pipetting 100 of the prepared standard into each well, the standard dilutions were made by moving the 100 from one well to the next. Among the newest 100 wells, 100 were shut down (Table 3.1).

Table 3.1 Showing how the microwell strips can be set up with blanks, standards, and samples

	1		2
A	Standard 1	(100.00 pg/mL)	Sample 1
B	Standard 2	(50.00pg/mL)	Sample 2
C	Standard 3	(25.00pg/mL)	Sample 3
D	Standard 4	(12.50pg/mL)	Sample 4
E	Standard 5	(6.25pg/mL)	Sample 5
F	Standard 6	(3.13 pg/mL)	Sample 6
G	Standard 7	(1.56pg/mL)	Sample 7
H	Blank		Sample 8

a. The blank wells each received one hundred microliters of the assay buffer that was added.
b. The sample wells each received a total of fifty microliters of the assay buffer that was added.
c. In each of the sample wells, fifty microliters of the respective sample were deposited.
d. It was decided to prepare a biotin-conjugate.
e. All of the wells received an addition of fifty microliters of biotin-conjugate.
f. After applying an adhesive coating over the microwell strips, the plates were placed on a microplate shaker and incubated at room temperature (between 18 and 25 degrees Celsius) for two hours.
g. It was decided to make streptavidin-HRP.
h. Both the adhesive film and the wells had to be removed before they could be emptied. By making use of an automatic microplate washer, the microwell strips were washed a total of four times with wash buffer.
i. All of the wells received an additional 100 µL of diluted streptavidin-HRP..
j. A microplate shaker was used to incubate adhesive-coated microwell strips at room temperature for one hour at a rotational speed of 100 revolutions per minute (18 to 25 degrees Celsius).

k. Both the adhesive film and the wells had to be removed before they could be emptied. By making use of an automatic microplate washer, the microwell strips were washed a total of four times with wash buffer.

l. All of the wells were each given a total of one hundred microliters of the TMB substrate solution.

m. Approximately ten minutes were spent incubating the microwell strips at ambient temperature (18 to 25 degrees Celsius). Avoiding direct contact with strong light was a priority.

n. The enzyme reaction was effectively stopped after a volume of 100 µL of the stop solution was pipetted into each well.

o. A spectrophotometer was used to measure the absorbance of each microwell at 450 nanometers. By utilizing the blank wells, the plate reader was blanked in accordance with the instructions provided by the manufacturer.

3.2.4 Resulting calculations

Human IL-6 levels in the blood were measured against a standard graph (Figure 3.1).

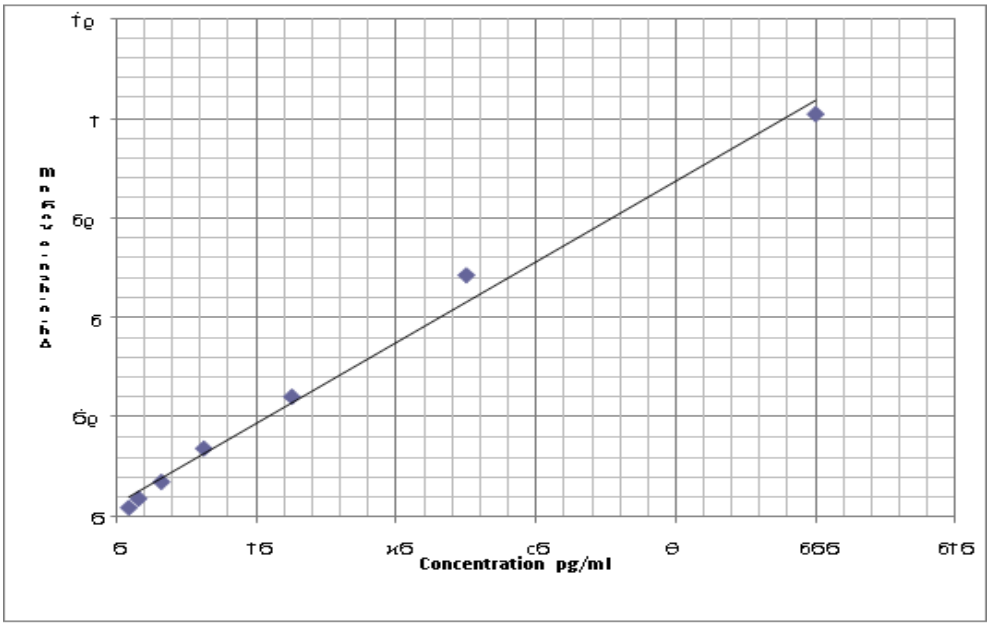

Figure 3.1 Standardized Curve for Interleukin-6

3.3 Interleukin 10 (IL-10)

The Human IL-10 R ELISA (Enzyme-Linked Immunosorbent Assay) kit is a rapid and sensitive in vitro enzyme-linked immunosorbent assay that can detect the presence of human IL-10 R in a variety of samples, including serum, plasma, cell culture supernatants, and urine. The kit contains everything needed to perform the measurement. An antibody that is specific for human IL-10 R beta is used in this experiment, and it is coated on a 96-well plate. Both standards and samples are pipetted into the wells, and the immobilized antibody binds any IL-10 R present in the samples.

3.3.1 Procedure

1. Before using any of the reagents or samples, make sure they are at room temperature (between 18 and 25 degrees Celsius). It is strongly suggested that at the very least, duplicate tests be performed on all of the standards and samples.
2. Mark the detachable strips of eight wells with the necessary labels for the experiment.
3. In the appropriate wells, add 100 µL of each standard (see to step 4 of the Reagent Preparation), as well as the sample. Incubate the mixture with gentle shaking for either twelve hours at a temperature of four degrees Celsius or for two and a half hours at room temperature.
4. Throw out the solution and repeat the washing process four times using the 1x Wash Solution. To do the wash, use a multichannel pipette or an autowasher to fill each well to the top with 300 µL of Wash Buffer. It is crucial to good performance that all liquid be removed in its entirety at each phase. Aspirating or decanting any remaining Wash Buffer after the last wash is recommended. After washing, invert the plate and dry it with a towel.
5. Step 6 of the Reagent Preparation process calls for adding 100 µL of 1x produced biotinylated antibody to each well. Incubate at room temperature for one hour while gently shaking the container.

6. Toss out the proposed solution.
7. Add 100 µL of the streptavidin solution that has been made up to each well. For forty-five minutes, while gently shaking the jar, incubate at room temperature.
8. Toss out the proposed solution. Proceed with the washing process as in step 4.
9. In each well, add one hundred microliters of the TMB One-Step Substrate Reagent (Item H). Incubate in the dark at room temperature for a period of thirty minutes while gently shaking the container.
10. In each well, add 50 µL of the Stop Solution, which may be found in Item I. Immediate reading should be done at 450 nm.

3.3.2 Resulting calculations

With the help of a standard curve, the levels of IL-10 in the blood were determined for each sample (Figure 3.2).

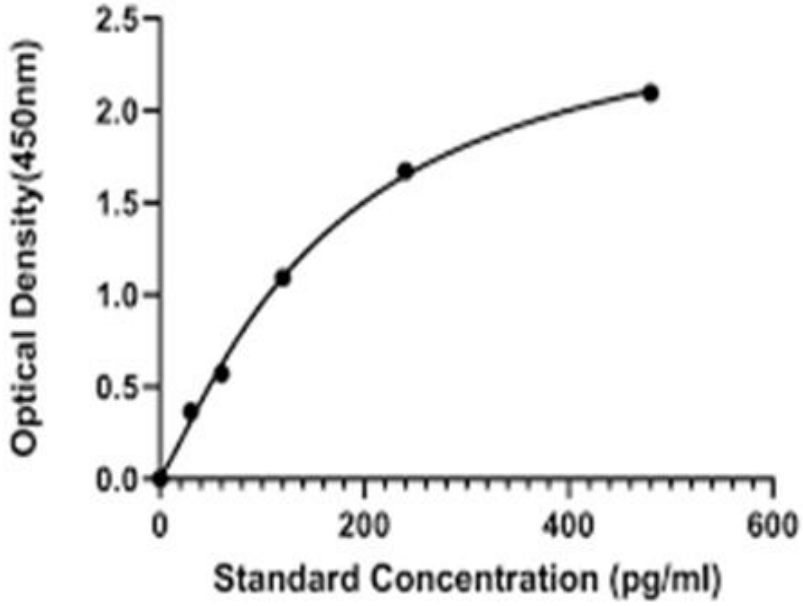

Figure 3.2 Interleukin-10 standard curve

3.4 Vitamin D

The active intestinal absorption of calcium and the homeostasis of calcium are both regulated by the steroid hormone known as vitamin D. There are two different types of vitamin D: vitamin D2 and vitamin D3. Vitamin D2 can be derived from dairy products, while vitamin D3 is synthesized by the body in response to exposure to UV light through sunbathing or tanning. Vitamin D is converted into 25-hydroxyvitamin D in the liver by a process called hydroxylation at carbon 25.

3.4.1 Procedure

1. In accordance with the instructions, 10 mL of 25(OH) D standards, controls, and samples were distributed into each well.
2. Each well received 200 mL of a 1x working solution of biotinylated 25 (OH) D reagent.
3. Plate shaker set between 200 and 400 revolutions per minute was used to mix the contents of the wells very carefully for twenty seconds (or equivalent motion). After that, they were taken from the shaker, and the plate was covered with an adhesive plate sealer, after which they ensured that a full seal was created over each well.
4. Incubation took place in a plate that was sealed for 90 minutes at room temperature (18-26 degrees Celsius).
5. After carefully removing the seal from the plate, the contents of each of the wells were emptied out and thrown away.
6. Following the dispensing of 300 mL of 1x Wash Buffer into each well, the previous contents of the wells were emptied out and set aside. Three times through the washing machine for a total of three times. The wells were tapped on some absorbent paper.
7. After dispensing 200 mL of enzyme conjugate (Streptavidin-HRP) into each well, the plates were left to incubate at room temperature (between 18 and 26 degrees Celsius) for thirty minutes.
8. The contents of the wells were emptied out and thrown away.

9. Following the dispensing of 300 mL of 1x Wash Buffer into each well, the previous contents of the wells were emptied out and set aside. Three times through the washing machine for a total of three times. The wells were tapped on some absorbent paper.
10. The TMB Substrate was administered into each well using a multichannel pipette, and the volume dispensed was 200 mL.
11. was allowed to incubate for a period of half an hour at room temperature, preferably in the absence of light.
12. To put a stop to the enzymatic reaction, each well received an injection of 50 milliliters of stop solution. The contents on the plate were thoroughly combined for twenty to thirty seconds.
13. After adding the stop solution, check the sample absorbance at 450 nm on the ELISA reader within 10 minutes.

3.4.2 Resulting calculations

According to the standard curve, the following values were calculated for the concentration of vitamin D in the human blood (Figure 3.3).

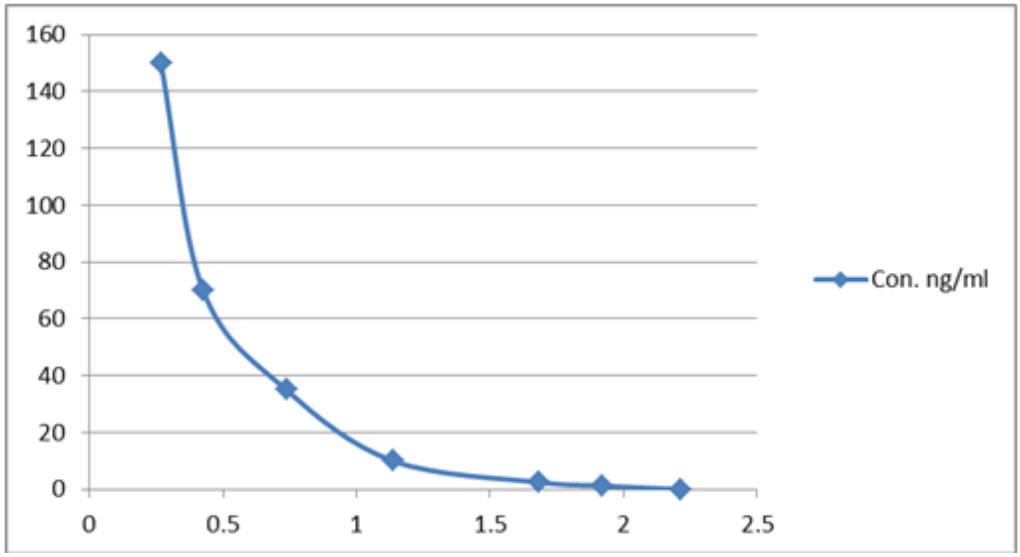

Figure 3.3 Vitamin D standard curve

3.5 Vitamin B12

Cyanocobalamin, often known as vitamin B12, is a water-soluble vitamin that is a member of the B12 vitamin family, which is sometimes referred to as the cobalamins family. It is most commonly found in foods derived from animals, particularly dairy products, meat, and eggs, among others. Applications can be found for it in the production of multivitamin supplements as well as food that has been fortified.

3.5.1 Procedure

Prepare samples as described above. In the relevant wells of the microtiter plate, pipette either 50 µL standard volumes or prepared sample volumes in duplicate. As soon as possible, inject 50 µL of the vitamin B12-peroxidase conjugate into each well. Incubate the microtiter plate with a plastic foil cover for a period of one hour at room temperature while using a microtiter plate shaker (or 90 minutes without shaker). After each cycle, wash the plate as follows: Empty the wells and throw away their contents (dump or aspirate). Each well should have 300 µL of the diluted washing solution pipetted into it. After the third cycle, remove any remaining liquid from the wells by tapping the plate on a paper towel, and then empty the wells once more. The washing process is an essential step. If you don't wash it thoroughly enough, you'll get inaccurate readings and erroneously high absorbencies. Each well should have 100 µL of the substrate solution pipetted into it. After 20 minutes have passed at room temperature, the reaction should be allowed to occur in the dark (for example, a cupboard or drawer, as the chromogen is light sensitive). In order to halt the enzyme reaction, 100 µL of stop solution containing 0.5 M H2SO4 was added to each well. After the addition of the yellow, the color will change to blue. After the mixture has been thoroughly combined, use an ELISA reader to determine the absorbance at 450 nm (the reference wavelength is 620 nm). The color won't change for the next half an hour.

3.5.2 Resulting calculations

From the standard curve, we were able to calculate the content of vitamin B12 in the blood of each sample (Figure 3.4).

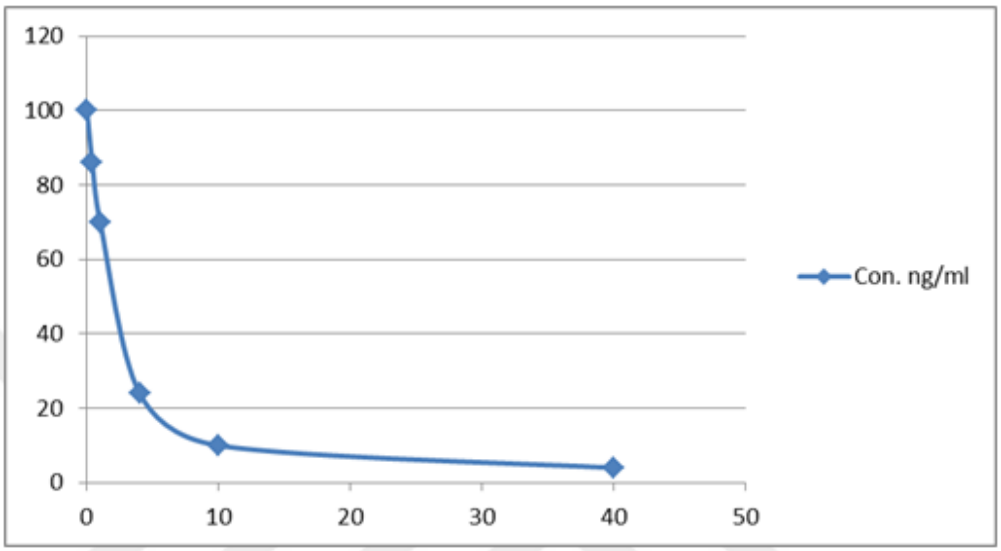

Figure 3.4 Vitamin B12 standard curve

3.6 Genetic Study

3.6.1 DNA isolation and genotyping

Two milliliters of venous blood from each subject was taken in an EDTA tube for DNA analysis. DNA isolation was performed using the salting-out method (Lahari *et al.* 1992). After centrifugation, the RBC pellet was prepared by lysing the red blood cells (RBC) in the blood sample with an equal volume of an RBC lysis solution containing Triton-X. After the pellet was lysed in WBC lysis solution containing 10% SDS, the protein fraction was separated by adding a large molar quantity of NaCl gradually. Finally, the DNA was extracted utilizing ice cold ethanol, before being isolated, resuspended in TE buffer, and stored at -200 degrees Celsius pending the PCR reaction. The IL6 polymorphism at position 174C/G rs1800795.15 was studied through the use of the polymerase chain reaction and the amplification refractory mutation system.

An allele-specific sense primer, 5'- IR4 GCAATGTGACGTCCTTTAGCT, and a general antisense primer, 5'- IF4 TCCCCCTAGTTGTGTCTTCCC 21', were employed in each reaction. Using specific primers, PCR was used to amplify a 426-base-pair internal control. The primers used were 5'- AAGACATGCCAAAGTGCTGAGTC-3' and 5'ATGAGCCTCAGACAT-CTCCAGTC-3'. As a result, the PCR amplification could be assessed.

The amplification refractory mutation system polymerase chain reaction method (ARMS PCR) was used to study the polymorphism in IL10 at position 1082T/C rs1800896. To sum up, we used a 5'-CCTCTTACCTATCCCTACTTCCACC-3' generic antisense primer and a 5'-GACAACACTACT-AAGGCTTCTTTGGTAA-3' sense primer for one of the two alleles in each reaction.

In a reaction mixture containing 40 ng of genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 400 mM dNTPs, 1.5 mM MgCl2, and 0.5 units of Taq polymerase, amplification was carried out, and 0.8 mM of each primer in a total volume of 20 L for the PCR incubation mixture. A 0.01% gelatin solution was included in the recipe. First, the DNA was denaturated at 95 degrees Celsius for one minute. Then, the amplification cycle began with 10 iterations of 15 seconds of denaturation at 95 degrees Celsius, 50 seconds of annealing at 62 degrees Celsius, and 40 seconds of extension at 72 degrees Celsius. The analysis was done on an ethidium bromide-stained 2% agarose gel.

3.7 Statistical Analysis

Minitab was used in conjunction with SPSS and Microsoft Excel XP to do statistical analysis on the collected data. The information was laid out clearly, with the average, standard deviation (SD), and extremes clearly shown.

Analysis of Variance (ANOVA) tests were used to examine the results and draw conclusions about the significance of the differences between the treatment and control groups. The means of the data were compared using Duncan's Multiple Range test.

4. RESULTS AND DISCUSSION

4.1 The Samples

The study included 110 blood samples, of which 90 blood samples (49 males and 31 females) were people infected with Coronavirus, as their average age was between (20 to 65) years, and the study included 20 blood samples (10 males and 10 females) for apparently healthy persons as a control group and their average age was between (30 to 55) years (Figure 4.1).

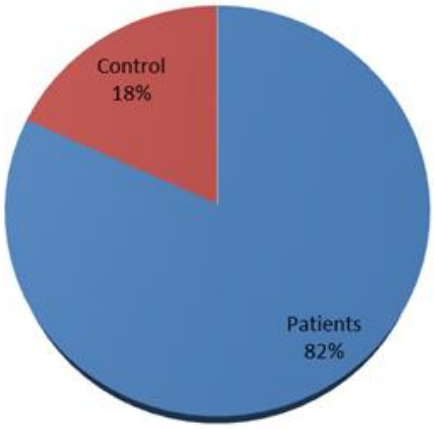

Figure 4.1 Percentage of study sample (patients and control)

4.2 Serological Study

The concentration levels of some cytokines and vitamins in the studied samples were estimated using the Enzy-Linked ImmunoSorbent Assay (ELISA) technique.

4.2.1 Interleukin 6 (IL-6)

Infected patients have a much greater IL-6 concentration than healthy people, as seen in Table 4.1. The concentration of IL-6 in the serum of infected patients was (7.63±0.21) pg/mL, while the concentration of IL-6 in the control sample was (2.95±0.15) pg/mL.

This indicates that the concentration of IL-6 in infected patients is significantly higher than that of healthy individuals (Figure 4.2). The findings of the statistical analysis revealed that there were significant changes in the concentration of IL-6 between those who had been infected with the Coronavirus and the sample that served as the control. These differences were significant at the <0.05 level of probability.

Table 4.1 Concentration of IL-6 in studied groups

Parameter	Patients (90) mean±SD	Healthy persons (20) mean±SD	P-Value
IL-6	7.63±0.21	2.95±0.15	0.000

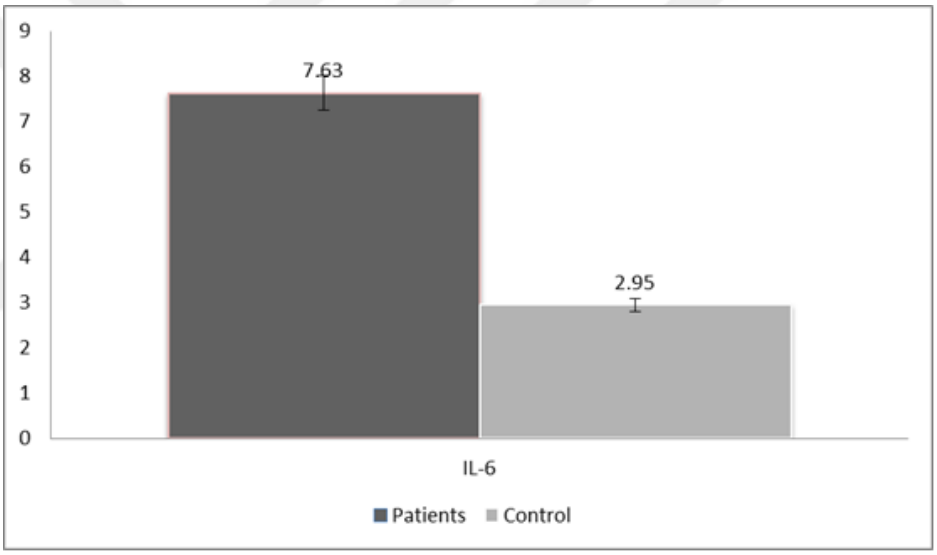

Figure 4.2 Concentration of IL-6 in studied groups

Our findings, which were consistent with those of more recent research, demonstrated that IL-6 levels were linked to the degree of COVID-19 infection. It is becoming more clearer that a dysregulated host immune response to invading pathogenic microorganisms plays a critical role in the emergence of target organ dysfunction and is a major cause of morbidity and mortality.

More people are coming to understand that this is the fact. It has been demonstrated, in particular, that the systemic inflammatory response in sepsis and the CRS are similar

39, 40. Furthermore, hyperactivation of the humoral immune system and a strong IL-6 response in patients with Covid-19 exacerbated by ARDS may indicate that part of the etiology of complicated disease involves an overactive and dysregulated host inflammatory response. This clinical profile resembles CRS, a condition for which tocilizumab's IL-6 receptor blockade has amply established efficacy (Le *et al.* 2018). It might be a subset of Covid-19 patients who are more seriously afflicted, requiring more intensive care and having worse clinical results (Mehta *et al.* 2020).

Previous research has shown that inflammation plays an important role in COVID-19's severity (Inciardi *et al.* 2020, Dietz *et al.* 2021). A'storm' of virus-induced cytokines has been linked to the clinical deterioration seen in certain infected patients (Ye *et al.* 2020).

SARS-CoV-2 infections were linked to increased IL-6 levels, which in turn correlated with patient symptoms such severe lung damage and inflammation (Lu *et al.* 2020). The protein suppressor of cytokine signaling-3 regulates and triggers the negative feedback mechanism of interleukin-6 (IL-6) and was found in low amounts in individuals with SARS-CoV-2 infection (Okabayashi *et al.* 2006).

Similar findings were found in another study, which suggested that elevated IL-6 levels in severely ill COVID-19 patients could be used as a basis for forecasting the onset of a severe illness (Wan *et al.* 2020). This is consistent with the current study's conclusion that IL-6 levels were greater in very ill patients with COVID-19.

Our findings indicate that higher levels of IL-6 correlate to a more severe disease and are particularly useful for identifying individuals who progressed to more advanced stages of COVID-19. Some previous research has examined IL-6's potential predictive value on several COVID-19 clinical characteristics. At least two of them showed that pre-admission IL-6 levels can be used to estimate the likelihood that a patient will require high-flow oxygen or mechanical ventilation during hospitalization (Liu *et al.* 2020, Herold *et al.* 2020).

Also, the results of an Italian investigation revealed that a score that included IL-6 in addition to the other six factors could be an effective predictor of a composite endpoint that included severe COVID-19 and/or mortality in the hospital (Grifoni *et al.* 2020).

Other research investigated the connection between IL-6 and the progression of lung damage as measured by CT scan (Liu *et al.* 2020). In one of these trials, the levels of IL-6 were measured during the patient's stay in the hospital. The researchers took into account the dynamic nature of the disease (Liu *et al.* 2020).

4.2.2 Interleukin 10 (IL-10)

Table 4.2, demonstrates that the concentration of IL-10 in infected patients is significantly higher than that of healthy persons. The concentration of IL-10 in the serum of infected patients was (35.27±2.12) pg/mL, while the concentration of IL-10 in the control sample was (26.05±3.08) pg/mL. This indicates that the concentration of IL-10 in infected patients is significantly higher than that of healthy persons (Figure 4.3).

The findings of the statistical analysis revealed that there were significant changes in the concentration of IL-10 between those who had been infected with Coronavirus and the sample that served as the control. These differences were significant at the P <0.05 level of probability.

Table 4.2 Concentration of IL-10 in studied groups

Parameter	Patients (90) mean±SD	Healthy persons (20) mean±SD	P-Value
IL-10	35.27±2.12	26.05±3.08	0.000

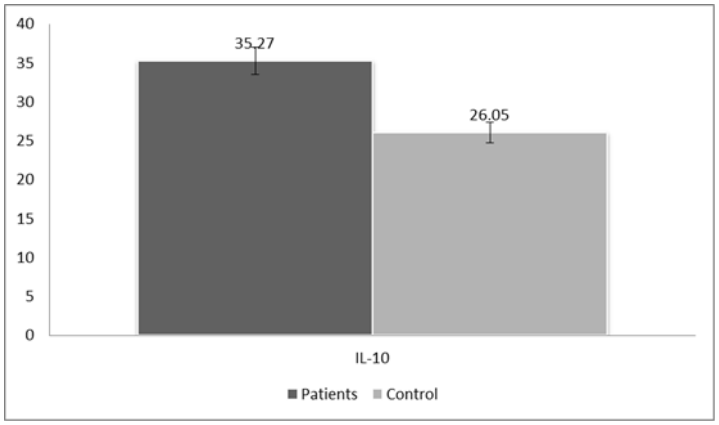

Figure 4.3 Concentration of IL-10 in studied groups

Interleukin-10, often known as IL-10, is a pleiotropic cytokine that is renowned for having powerful immunosuppressive and anti-inflammatory properties. IL-10 was first thought to be a product of T helper 2 cells; however, recent research has shown that it is really produced by a wide variety of myeloid- and lymphoid-derived immune cells that are involved in both adaptive and innate immunity (Saraiva *et al.* 2020).

On the other hand, IL-10 is a cytokine that works to reduce inflammation, and its levels were shown to be high in severe COVID-19 individuals (Chen *et al.* 2020, Qin *et al.* 2020).

Other studies have found that viral infection causes airway epithelial cell growth in two distinct conditions. One of these settings is high levels of viral-related acid inflammation with concomitant vascular leakage, which can be observed in SARS-CoV53 patients. This has the potential to be a catalyst for an inflammatory response in the future (Shaw *et al.* 2013, Channappanavar and Perlman 2017).

The serum levels of IL-10 in COVID-19 patients was tested using ELISA, and the results showed that serum levels of IL-10 in COVID-19 patients were much greater than those in non-COVID-19 patients. This difference was statistically significant. This finding is consistent with the findings of Lu *et al.* (2021), who reported that the serum levels of IL-1β, MCP-1, IL-6, and IP-10 in patients with COVID-19 were elevated, which was the same as having severe acute respiratory syndrome (SARS).

In another study that was carried out by Kathim *et al.* (2021), Baghdad city-Iraq, it was demonstrated that the level of IL-10 has been significantly elevated in newly diagnosed COVID-19 patients as compared to healthy controls. Control (38.184.57 vs.31.843.19 pg/mL) (P:0.01), and this finding is in agreement with the findings of the current study.

4.2.3 Vitamin D3

When comparing infected patients to healthy controls, Table 4.3, shows that the infected patients had a considerably decreased D3 concentration. The concentration of D3 in the serum of the infected patients was (10.34±4.61) ng/mL, while the concentration of D3 in the control sample was (22.95±3.82) ng/mL. This indicates that the concentration of D3 in the infected patients was significantly lower. Analysis using the P0.05 level of significance revealed a statistically significant difference in D3 levels between those infected with Coronavirus and a control group (Figure 4.4).

Table 4.3 Concentration of vit. D3 in studied groups

Parameter	Patients (90) mean±SD	Healthy persons (20) mean±SD	P-Value
Vit. D3	10.34±4.61	22.95±3.82	0.000

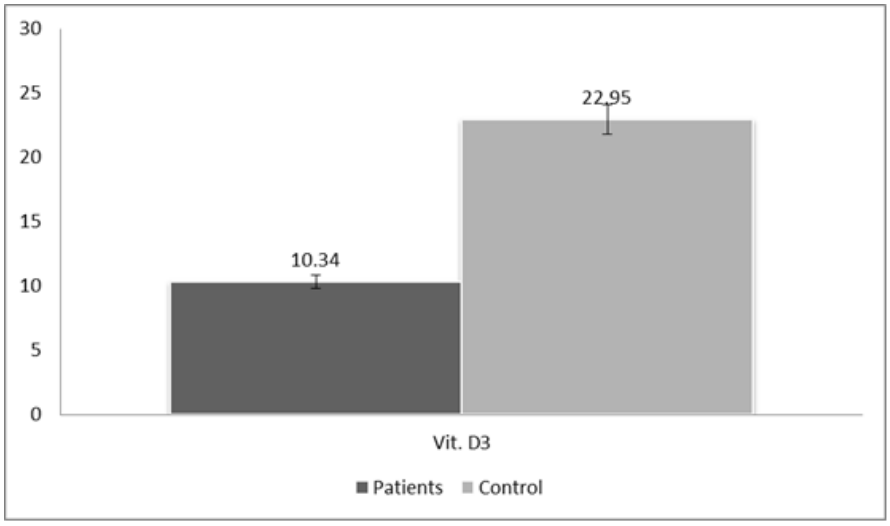

Figure 4.4 Concentration of vit. D3 in studied groups

Vitamin D, which has the function of a lipophilic hormone, can be gained in two ways: by dietary intervention and through exposure to sunlight. Vitamin D is implicated in the mechanisms of immune response in addition to its role in supporting normal bone homeostasis (White 2008).

Inhaled pathogens are challenged and prevented from entering the body by the respiratory alveolar epithelium, which is the first line of defense in the respiratory system. It plays a crucial role in the body's natural defenses, which also includes dendritic cells and alveolar macrophages. These cells, when activated, set off a chain reaction of intracellular signaling pathways that results in the creation of inflammatory mediators, specialized antimicrobial defenses, and adaptive immune responses (Basu and Fenton 2004).

Multiple pathways are hypothesized to be responsible for vitamin D's putatively beneficial effects on COVID-related health outcomes. Those mechanisms are thought to be the intermediaries of these advantages. Coronavirus activation of the ACE receptor results in a cascade of events, including upkeep of the pulmonary epithelial barrier, stimulation of epithelial repair, modulation of cytokine storm, modulation of neutrophil activity, and upkeep of the pulmonary epithelial barrier (Bilezikian *et al.* 2020, Quesada *et al.* 2020).

As a result of this process, vitamin D supplementation may have the beneficial effects that have been observed. Evidence from a small clinical research suggests that this is the case; patients with COVID-19 who took calcifediol supplements had a lower risk of being admitted to the intensive care unit (ICU) (Castillo *et al.* 2020).

According to findings published in by Lu *et al.* (2018) insufficient levels of 25(OH)D3 may be linked to an increase in the number of inflammatory cytokines as well as respiratory infections. Patients who had high amounts of C reactive protein were found to have blood 25(OH)D3 levels that were lower than 30 ng/mL, as reported by D'Avolio *et al.* (2020) in their study of 1377 control patients. In this regard, we discovered that patients who did not have COVID-19 had significantly greater serum levels of

25(OH)D3 compared to those without COVID-19, but no differences were found between those without COVID-19 and those with h-COVID-19.

In persons with HIV, low vitamin D levels have been related to increased levels of the inflammatory cytokine IL-6, according to recent studies (Liu *et al.* 2016, Manion *et al.* 2017). In accordance with this, experimental tests conducted on mice that vitamin D levels are inversely connected to the expression of IL-6 in the liver (Labudzynskyi *et al.* 2016). Synthesis of IL-6 is accelerated in the presence of low levels of serum 25(OH)D3, and vice versa.

These findings are consistent with those that were reported in earlier research regarding the increased risk of viral respiratory infections associated with vitamin D insufficiency, as well as the effectiveness of vitamin D supplementation in reducing that risk (Cannell *et al.* 2006, Grant 2010, Aranow 2011, Jolliffe *et al.* 2021). In fact, vitamin D's role in immunity is well established (Aranow 2011).

Finally, a higher level of vitamin D was found to be related with a reduced COVID-19 severity (n = 6/12, 50.0%) and a decreased mortality risk (n = 14/24, 58.3%) in approximately half of the trials. However, nine of the twenty-four studies (n=9/24, 37.5%) showed no association between levels of vitamin D and mortality, while one of the twenty-four studies (n=1/24, 4.1%) indicated that greater higher vitamin D levels were associated with a higher death rate (Grant *et al.* 2020).

According to a number of studies, those who have a vitamin D level that is higher than 95 nmol/L have a significantly lower chance of developing acute viral respiratory tract infections when compared to individuals whose vitamin D levels are lower than 95 nmol/L (Sabetta *et al.* 2010). Vitamin D supplements have been reported to be effective against and in treating COVID-19 due to their capacity to increase cellular immunity and stimulate the production of glutathione (Lei *et al.* 2017, Colunga *et al.* 2020). Vitamin D is a fat-soluble vitamin (Wimalawansa 2020).

4.2.4 Vitamin B12

Statistical analysis of Table 4.4, shows that the B12 levels of infected patients are higher than those of healthy individuals. Infected patients' serum B12 concentrations were 429.1632.57 ng/mL, while those of healthy controls were only 283.0228.41 ng/mL. This indicates that the concentration of B12 significantly increased in infected patients (Figure 4.5). The findings of the statistical analysis revealed that a substantial variation in B12 content was observed present in those who were infected with the Coronavirus as compared to the sample that served as a control, and this difference existed at a probability level of less than $P<0.05$.

Table 4.4 Concentration of vit. B12 in studied groups

Parameter	Patients (90) mean±SD	Healthy persons (20) mean±SD	P-Value
Vit. B12	429.16±32.57	283.02±28.41	0.000

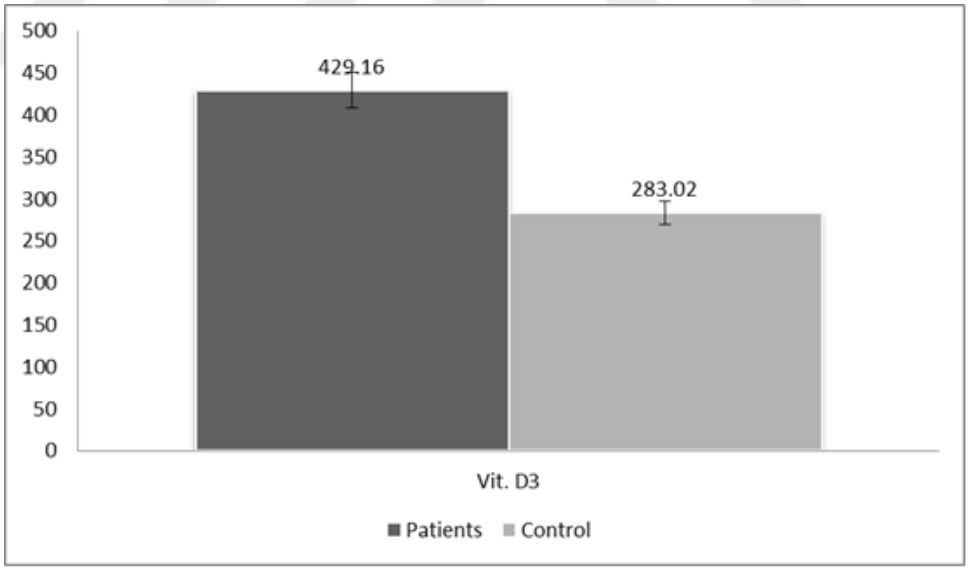

Figure 4.5 Vitamin B12 levels in the populations that were examined

High levels of vitamin B12 were found in our research population, which may be due to a variety of causes. B12 levels were significantly higher in infected patients compared to healthy individuals. Vitamin B12 was not cleared by the liver as effectively and was taken up by peripheral tissues to a lesser extent when carrier protein levels were high.

However, a possible role of high vitamin B12 in predicting sickness outcome is interesting to hypothesize about in light of the cytokine storm of the COVID-19 infection (Dalbeni *et al.* 2021).

Dos Santos (2020) offered this concept that vitamin B12 therapy can lessen serious damages caused by COVID-19 by improving circulation, reducing oxidative stress, acting as an anti-inflammatory, and providing analgesic relief.

We determined the serum levels of vitamin B12 in COVID-19 patients at the time of admission because of the antiviral and anti-inflammatory properties of vitamin B12 (Gombart *et al.* 2020). We discovered that patients who were admitted to the ICU and patients who were not admitted to the ICU had higher levels than healthy people. Tan et al. (2020), on the other hand, demonstrated that the administration of vitamin B12 in combination with magnesium and vitamin D in patients with COVID 19 reduced the proportion of patients whose clinical condition deteriorated to the point where they needed oxygen support and/or intensive care support.

Probiotics like bifidobacteria and lactobacilli have been shown to boost the immune system and ward off illnesses, including those of the respiratory system. Vitamin B12 not only helps maintain a healthy intestinal lining, but it also regulates the immune system in the colon. There is mounting evidence that probiotics can alter the immune response and provide protection against infections, suggesting that they may play a pivotal role in the immunity and protection against coronavirus (COVID-19) (Calder *et al.* 2020).

4.3 Molecolur Study

4.3.1 IL-6 gene

PCR optimization for rs1800795 detection using the IL6 174C/G primer pair was demonstrated in Figure 4.6. 60 degrees Celsius was the annealing temperature. The

emergence of the thickest DNA fragment at the determined size was used to establish these optimal conditions, with the goal of limiting the number of unspecific fragments.

IL-6 is a key regulator of immunological and inflammatory responses. IL-6 is generated by endothelium and smooth muscle cells, as well as adipose tissue (Fried *et al.* 1998, Papanicolaou 2000), which explains its pleiotropic role in the regulation of endothelial and adipose function and glucose and lipid metabolism (Khovidhunkit *et al.* 2004, Glund *et al.* 2007, Wannamethee *et al.* 2007).

This research yielded a number of noteworthy results. In IL-6 –174C/G polymorphisms, bearers of the G allele had higher t-tau levels. Vargas-Alarcón *et al.* (2016) discovered that SNPs in the IL-6 gene might affect transcription and, as a result, the amount of cytokines generated. Although Magalhães *et al.* (2017) found that a decrease in anti-inflammatory cytokines and an increase in pro-inflammatory cytokines leads to increased inflammation, which favors Alzheimer's disease progression.

Vargas-Alarcón *et al.* (2016) also demonstrated how SNPs in the IL-6 –174C/G gene might make certain persons more susceptible to neuroinflammation. The recognized function of IL-6 as a critical organizer for CD4 T cell fate suggests that the IL6 SNP might explain more about the COVID-19 pandemic.

Therefore, the current study suggests that IL6 SNPact as a biomarker for illness severity or to identify pathological changes produced by COVID-19 viral infections, including the capacity of some individuals to have asymptomatic infection or to be immune to the virus (Riazalhosseini *et al.* 2018, Liu *et al.* 2020). Jin and Wang summarize the significance of single nucleotide polymorphisms (SNPs) and their influence on immune response, a process known as immunogenetic profiling (Jin and Wang 2003).

Similarly, the severity of various lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, was diagnosed differently by SNP analysis (Kirtipal *et al.* 2020). The IL6-174 C/C genotype was also linked to the severity of respiratory

syncytial virus (RSV) infection (Doyle *et al.* 2010). Several studies have linked the C allele of the IL-6 –174 polymorphism to an increased risk of Alzheimer's disease (Mansoori *et al.* 2012, Rasmussen *et al.* 2013).

Meta-analyses examining the link between the IL-6 –174 polymorphism and Alzheimer's disease produced mixed results. Dai *et al.* (2012) and Qi *et al.* (2020) found that the CC IL-6 –174 genotype is linked to a lower risk of Alzheimer's disease, but Moudi *et al.* (2016) found that the IL-6 –174 polymorphism is not linked to the disease. Our findings back with previous research suggesting the CC IL-6 –174 genotype is linked to a lower risk of Alzheimer's disease (Qi *et al.* 2012, Dai *et al.* 2012, Flex *et al.* 2014).

Our findings identified a strong association between the case severity of COVID-19 and frequency of SNP gene, additionally present documents recommended to consider IL6 cytokines as a key factor to realize the therapy response in COVID-19 infected peoples. Also, show that carriers of different genotypes in the IL-6 –174C/G polymorphism may be varied amounts of Alzheimer's disease biomarkers.

These polymorphisms as possible genetic biomarkers for a disease should be examined to other biomarkers in a larger cohort of patients, as well as a comparison with biomarker neuroimaging. It should also be determined if various genotypes in these polymorphisms are to blame for the observed discrepancies in the amounts of these cytokines detected in bodily fluids, as well as their link to inflammasome and microglial activation.

Finally, the findings of this study imply that IL-6 –174C/G may render certain persons more prone to the development of neuroinflammation. These patients might be candidates for targeted anti-inflammatory therapy in Alzheimer's disease. Furthermore, our findings imply that differences in infected individuals are determined by the functional 174C/G at the IL6 locus (Figure 4.6).

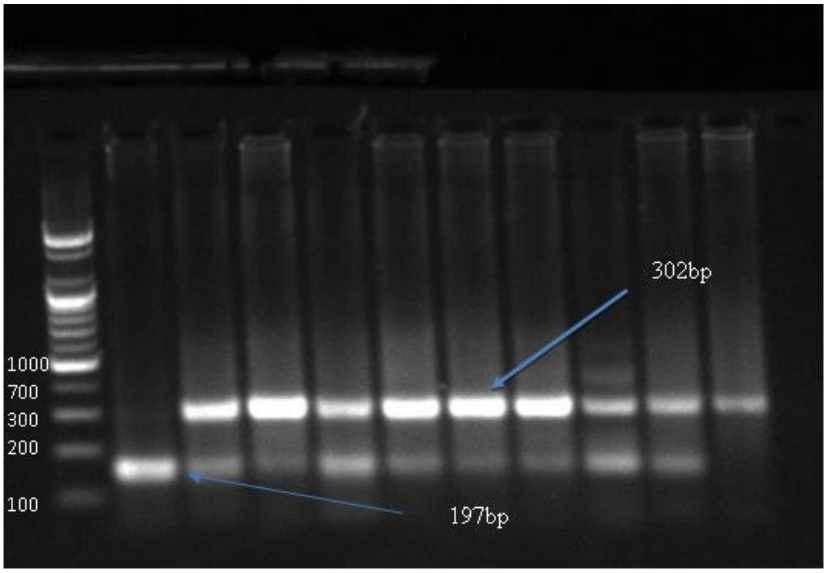

Figure 4.6 The electrophoresis of the IL6 gene to the 174C/G rs1800795 showing the two alleles C and G in infected samples

4.3.2 IL10 1082 T/C

Figure 4.7, illustrates the IL10 1082 T/C rs1800896 PCR optimization that was performed in order to discover rs1800896. The temperature during annealing was 61 degrees Celsius. The emergence of the thickest DNA fragment at the determined size was used to establish these optimal conditions, with the goal of limiting the number of unspecific fragments.

Cytokines (interleukins [ILs], growth factors, interferons, and others) are implicated in cancer formation and play a key role in controlling the inflammatory response and chronic inflammation (Hickman *et al.* 2008, Lee *et al.* 2015, Koscik *et al.* 2020). Cytokines are classified as proinflammatory or antiinflammatory depending on their inflammatory action (Su *et al.* 2016). Several cytokines, such as IL10, have a dual function. Interleukin 10, an antiinflammatory cytokine, has two functions: immunosuppressive (tumor suppressing). and immunostimulatory (tumor promoting), and hence may impact tumor susceptibility and progression (Lee *et al.* 2015, Braga *et al.* 2021).

Moudi *et al.* (2016) discovered a link between polymorphisms in the IL-10 promoter genes (-592 A/C, -819 T/C, -1082 A/G) and HBV susceptibility in Iranian populations. Persico, *et al.* (1998) describe the link between the CCG IL-10 haplotype and chronic liver illness in infected people with varying degrees of severity, including as hepatocyte lesions, liver cirrhosis, and hepatocellular cancer. Vargas-Alarcón *et al.* (2016) discovered a link between the A allele of the IL-10 –1082 polymorphism with an increased risk of Alzheimer's disease and the G allele with a lower risk. However, according to Bănescu *et al.* (2019) there is no link between IL10 rs1800872 and rs1800896 and the risk of acute myeloid leukemia.

A lethal cytokine storm has been discovered in vulnerable COVID-19 patients, according to new study. COVID-19 patients had higher levels of IL-6, IP-10, MCP-1, MIP-1A, and TNF-a in their blood (Wang *et al.* 2020). In another investigation, the levels of many cytokines were also assessed. According to certain studies, serum IL-6 and IL-10 levels were considerably higher in individuals with severe illness. As a result, serum IL-6 and IL-10 might be utilized to measure COVID-19 severity. Adaptive immune cells and innate immune cells both generate cytokines. Interleukin-6 is a well-known pro-inflammatory cytokine that also has anti-inflammatory properties (Scheller *et al.* 2011). Myeloid cells, T cells, smooth muscle cells, endothelial cells, and other cells produce IL-6 (Tanaka *et al.* 2014).

The substantial rise in IL-6 in COVID-19 patients was most likely caused by activated macrophages, according to another report3 and comparable to the IL-6 up-regulation seen in SARS patients (Wang *et al.* 2007). Interleukin-10, a cytokine produced by alternatively activated macrophages, T helper type 2 cells, Treg cells, and other cells, operates as an anti-inflammatory cytokine (Ouyang *et al.* 2005). The large rise in IL-10 in individuals with advanced illness might create a negative feedback loop on systemic and local inflammation. IFN-c, while being a critical antiviral cytokine generated by CD4+ T cells, CD8+ T cells, NK cells, and macrophages (Thäle and Kiderlen 2005, Kang *et al.* 2018) and having been documented to participate in the cytokine storm in SARS, remained at a low level in COVID-19 patients (Huang *et al.* 2005). Perhaps IFN-c in the blood stays the same while IFN-c in the lung tissue rises. To test this

notion, more research is required. In a lab research, IFN-c also suppressed the expression of the SARS coronavirus receptor angiotensin-converting enzyme 2 (de Lang *et al.* 2006).

The degree of IL-10 production controls immunological regulation and plays a crucial role in hepatitis development. It is generated by lymphocytes and monocytes. IL-10 expression is genetically regulated. The IL-10 gene polymorphism has been found to be a crucial role in differential IL-10 production, affecting the body's immunological response to HBV and susceptibility to HBV. As a result, these polymorphisms can contribute to individual variances in immune response and host immunological function (Höhler *et al.* 2005). As a result, determining the IL-10 polymorphism is critical for predicting susceptibility to liver infection. According to Shin *et al.* (2003), the IL-10 -592C allele (strong IL-10 production) speeds up chronic HBV infection. Individuals with the IL-10 -592A genotype (poor IL-10 production) have an increased vulnerability to HBV, according to Cheong *et al.* (2006) In addition, investigations have shown that the IL-10 -592AA genotype protects against HBV infection (Turner *et al.* 1997). HBeAg seroconversions in patients are caused by IL-10 intermediate producer genotypes or haplotypes, according to Peng *et al.* (2009).

Our findings suggest that a general reduction in lymphocytes, as well as an increase in IL-6 and IL-10, are valid indications of severe COVID-19. The IL-10 gene polymorphism has an effect on various infections, according to our research. The IL-10 -1082 polymorphism was found to have a substantial link to chronic liver disease susceptibility. The IL-10 -1082 was linked to a higher incidence of chronic hepatitis infection. The IL10 polymorphism found to be crucial in human HBV infection susceptibility (Figure 4.7).

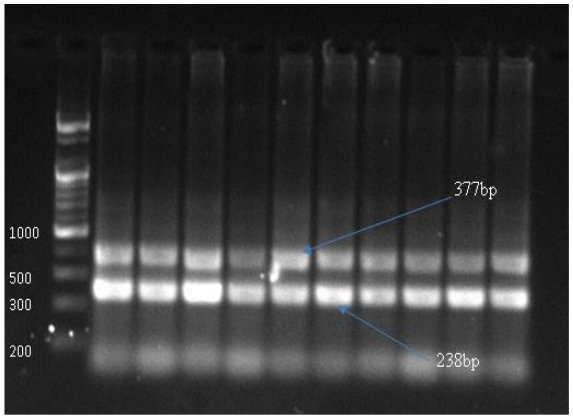

Figure 4.7 Infected samples show both T and C alleles of the IL10 gene at the 1082 T/C rs1800896, as shown by electrophoresis

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

1. In the current investigation, a significant difference ($P<0.05$) was found between the levels of IL-6 found in COVID-19 patients and those found in healthy people.
2. In the current investigation, a substantial rise in IL-10 concentration was found in COVID-19 patients when compared with healthy volunteers ($P<0.05$).
3. In the current investigation, a significant difference ($P<0.05$) was found between the levels of vitamin D3 found in patients with COVID-19 and those found in healthy people.
4. The current investigation found that COVID-19 patients had significantly higher concentrations of vitamin B12 compared with healthy people, as shown by a significance level of $P<0.05$.
5. Our research has shown that there is a significant correlation between the severity of COVID-19 cases and the frequency of SNP genes. In addition, recent publications have suggested that IL6 cytokines should be considered as an important factor in COVID-19 infected individuals' ability to respond favorably to treatment.
6. According to the results of our research, a general decrease in lymphocytes as well as an increase in IL-10 both appear to be valid indicators of severe COVID-19.

5.2 Recommendation

1. There is a need to know how many other types of cytokines are present in the serum of COVID-19 patients.
2. The need to know the amount of other types of hormones present in COVID-19 patients' serum.
3. The importance of examining the levels of various interleukins present in COVID-19 patients' serum in order to determine their concentrations.

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APPENDICES

APPENDIX 1. A list of the tools used in this study

APPENDIX 2. Provided reagents

APPENDIX 3. The Researches Committee decision

APPENDIX 1. A list of the tools used in this study

Instruments and glasses	Company	Country
ELISA	Labon	China
Spectrophotometer	Biobase	India
Centrifuge	Memmert	Germany
Light microscope	Olympus	Japan
Oven	Memmert	Germany
Water bath	Memmert	Germany
Shaking water bath	Memmert	Germany
Sysmex device	Sysmax	Japan
Pipette	BioSan	Germany
Different glasses	-----	China
Refrigerator	BEKO	Turkey

APPENDIX 2. Provided reagents

1. Packaged in an aluminum sleeve is a single microwell plate that has been coated with a monoclonal antibody specific to human interleukin-6.
2. One hundred milliliters of biotin-conjugated anti-human IL-6 monoclonal antibody.
3. One Streptavidin-HRP 150 mL vial
4. Two lyophilized vials of human IL-6 Standard, with a concentration of 200 pg/mL after reconstitution
5. One vial for the control of the high
6. One vial, set to low control
7. One vial containing 5 milliliters of the assay buffer concentrate (20x), which is PBS with 1% Tween 20 and 10% bovine serum albumin.
8. 50 mL of Wash Buffer Concentrate 20x (phosphate-buffered saline with 1% Tween 20).
9. Substrate Solution (tetramethyl-benzidine), 1 vial (15 mL) (tetramethyl-benzidine)
10. A 15-milliliter bottle of "Stop Solution" (1M Phosphoric acid)
11. Only a single vial (0.4 mL) Blue-Dye
12. One vial (0.4 mL) Green-Dye
13. There is only enough red dye for one vial (0.4 mL total volume)
14. Four sticky Films

APPENDIX 3. The Researches Committee decision