

## ***Association Of Hecpidin (HAMP) Gene Polymorphism And Cytokines, Iron, And Hb Levels In Covid-19 Patients***

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### **ABSTRACT**

**Introduction:** COVID-19 is a new type of coronavirus that infects humans and causes severe acute respiratory syndrome (SARS). Isolated in Wuhan City, China, in December 2019. Similar to discoveries relating to the coronaviruses that cause severe acute respiratory syndrome (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). Levels of cytokines (Interleukin-6, Interleukin-1  $\beta$ ), iron and hemoglobin changed during COVID-19 disease. Hecpidin is an iron regulatory protein that is encoded by the HAMP gene .

**Material and Methods :** Serum IL-6, IL-1  $\beta$  , Hecpidin are measured by an ELISA kit and Iron ,UIBC , TIBC are measured by colorimetric methods. The genotyping was done by the ARM-PCR technique with a specific set of primers.

**Results :** The difference in Hb and serum iron levels, UIBC, TIBC, IL-1, IL-6, and Hecpidin conc. between the patient and recovery groups is statistically significant at  $P \leq 0.05$  . No significant difference between the two groups in hecpidin gene polymorphism because of Polymorphisms of the hecpidin gene appeared in the two groups (in most samples).

**Conclusions:** The persons involved in this study are (most of them) heterozygous, and this indicates the presence of the mutation allele in the two groups , and it may be one of the reasons for the deficiency in hecpidin that appeared in this study.

**Keywords :** HAMP gene , coronavirus , IL-6 , IL-1 beta , Hemoglobin , Iron parameter .

### **Article Information**

Received: June 19, 2022; Revised: August 24, 2022; Online: September 4, 2022

### **INTRODUCTION**

COVID 19 is a consequence for a Severe Acute Respiratory Symptoms Coronavirus 2 (SARS- CoV 2) infection that was typically isolated and detected in individuals who were discovered in a Wuhan City seafood market , Hubei Province, China, in December 2019 (1). Corona-viruses are enveloped, single-stranded , positive, large RNA viruses that are capable of infecting both animals and humans. Genome sizes range between 26 and 32 kb (2). There are four main subgroups of coronaviruses ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) (3). In 1966, Tyrell with Bynoe were the first to characterize coronaviruses after isolating them from patients with the common cold (4). In accordance with

the Chinese National Health Commission's Assessment and Treatment of Novel Coronavirus Pneumonia , confirmed COVID-19 patients can be classified into four types depending on their clinical manifestations: mild, common , severe , and critical (5). Patients with such a fever, respiratory problems, and a CT scan finding of pneumonia are classified as having the common form.

Hecpidin is a 25-amino-acid produced predominantly by hepatocytes and Kupffer cells, which are strategically situated near portal veins which deliver dietary iron sensing microbes and recycling erythrocytes . Hecpidin

is also generated in less amounts by macrophages and adipocytes (6). Hepcidin is produced in hepatocytes as pre-prohepcidin and is expressed by a *HAMP* (hepcidin antimicrobial peptide) gene. The 84-amino-acid lengthy precursor form is quickly converted to the 60-amino-acid prohepcidin, which is then cleaved into the physiologically active 25-amino-acid peptide hormone hepcidin (7). Iron overload is caused by hepcidin deficiency in hereditary hemochromatosis, iron-loading anemia's and hepatitis C . Anemia of inflammation, chronic kidney illness, and iron-refractory iron deficiency anemia are all linked to high levels of hepcidin (8). Hepcidin is a 2.7-kD peptide that is not tightly linked to plasma proteins and is quickly removed by normal kidneys, where it is primarily reabsorbed and destroyed by the same proximal tubular mechanism that metabolizes other peptides. A little amount of hepcidin gets past this process and is identified in urine (9).

IL-1  $\beta$  is a pro-inflammatory cytokine that belongs to the IL-1 family. It is produced mostly by monocytes in the periphery, but also to a lesser extent by neutrophils, keratinocytes, endothelial and epithelial cells , smooth muscle cells, fibroblast and lymphocytes (10) , and is a powerful activator of the host immune response to both injury and infection (11). Interleukin-1 $\beta$  is a key cytokine that is released in response to inflammation as well as viral infection. Most viruses commence immune modulation by inducing IL-1 synthesis via NLR family. Pyrin domain containing 3 (NLRP3) inflammasome pathways (12). IL-1 $\beta$  is made up of a 31-kDa precursor peptide (pro Interleukin -1 $\beta$ ) that can be converted into 17-kDa mature forms (mIL-1 $\beta$ ) (10). Pro Interleukin -1 $\beta$  is physiologically inactive and requiring conversion to 17 kDa mIL-1 $\beta$  before it can be used . Caspases- 1 is involved in the synthesis and secretion of IL-1 $\beta$  from cells. The inflammasome, a multi-meric cytosolic protein complex, regulates caspase 1 activation (13).

IL-6 is a multifunctional cytokine that plays a role in immunological control, hematopoiesis, inflammation, as well as metabolic, proliferative, and regenerative

processes (14). Inducing the creation of Hepcidin , the main controller of iron homeostasis, IL-6 is a crucial facilitator of inflammation-driven iron management. Hepcidin regulates cellular iron eflux by degrading ferroportin 1 (FPN1), the sole cellular iron exporter, leading in iron retention in macrophage cells with reduced duodenal iron absorption (15).

Hemoglobin is a protein found in erythrocytes that delivers oxygen (O<sub>2</sub>) from the lungs toward the tissues. Every hemoglobin (Hb) molecule is a tetramer consisting of 4 globin polypeptide chains. Each subunit of globin has a heme moiety consisting of an organic proto-porphyrin ring and a ferrous iron ion (Fe<sup>+2</sup>). The ability of the iron molecule in each heme moiety to bind and unbind oxygen (O<sub>2</sub>) enables the delivery of oxygen (O<sub>2</sub>) throughout the body. In adults, the most prevalent kind of hemoglobin is (Hb-A), which is made up of two alpha ( $\alpha$ ) -globin and two beta ( $\beta$ ) -globin subunits (16) .

A side from its primary role of transporting oxygen, hemoglobin has a number of other functions . We'd like to attract attention to the following main functions:

a) erythrocyte metabolic regulation; b) heme oxidation and erythrocyte senescence; c) malaria resistance; d) molecular heat transducer; e) Enzymatic activity; f) Hemorphins, carbon monoxide, and nitric oxide (17).

Iron is a necessary component for growth and survival. Iron is required for oxygen transfer by hemoglobin, muscle oxygenation via myoglobin, cellular respiration via mitochondrial electron transport chain proteins, and DNA synthesis via ribonucleotide reductase. In the cytosol and other cellular compartments, iron is involved in various enzymatic activities. The average adult human body stores 3–5 g of iron, with the most majority (70%) being used in the erythron and the remainder being stored in the liver (18). When a human is infected with the virus, the liver produces hepcidin, which lowers serum iron levels and hence limits iron availability (19). Moreover, Iron excess also causes

oxidative stress, which leads to tissue damage (18). The maximum quantity of iron required to saturate plasma or serum transferrin (TRF), the principal iron-transport protein, is referred to as total iron-binding capacity (TIBC) (20).

The aim of this study was to genetically study hepcidin gene polymorphism , and its relationship to COVID-19 disease, as well as study the effects of COVID-19 disease on the levels of some biochemical markers ( Hb , Iron , UIBC ,TIBC , IL-6 , IL-1β and Hepcidin concentration).

**MATERIALS AND METHODS**

**Subjects :**

The study was conducted in Iraq – Al-Najaf Al-Ashraf city from November 2021 to March 2022 and it was a cross-sectional study . Participants in this study were 120 persons with the same age and gender . Divided into two groups 60 patients with Covid-19 (G1 ) and 60 recovery from Covid-19 (G2 - they had recovered from covid-19 disease for more than three months and had no residual complications from their infection with covid-19). Exclusion criteria included thalassemia (minor or major), iron deficiency anemia, and patients with autoimmune and chronic diseases such as rheumatoid arthritis, cancer, severe liver disease, and kidney disease. The study was approved by the Medical Human Research Ethics Committee at the Faculty of Medicine, University of Al-Qadisiyah, Iraq

**Blood sample collecting**

Five ml of venous Blood has been drawn from each subject and was put into a test tubes (1 ml in an EDTA tube for gene polymorphism , and 4 ml in a gel tube for biochemical examination) .

Whole blood is treated directly with the required tests. While blood samples that were put in gel tubes underwent centrifugation at 3000 rpm for 10 minutes to obtain serum, the obtained serum samples were stored in separated three Eppendorf tubes in the freezer at - 20 °C until the time of the test (21).

**Detection of serum IL-6 , Hepcidin , Iron , UIBC ,TIBC**

The Serum IL-6, IL-1 β and Hepcidin levels of all subjects measured by sandwich ELISA (Enzyme Linked Immuno-sorbent Assay) technique , and serum (Iron ,UIBC ) by using spectrophotometer, as well as serum TIBC calculated by bellow equation :

$$\text{TIBC Concentration} = \text{UIBC} + \text{serum iron concentration} \text{ (22).}$$

**DNA typing**

DNA was collected from blood samples of leukocytes using the favorgen biotech corp. kit and The genotyping of hepcidin gene tetra-arms PCR was carried out, four primers designed( Forward Outer, .Reverse Outer , Forward Inner, and Reverse Inner). The set of Primers. explained in table 2.1 (23).

**Table -1 : the set of primers that used in this study**

Gene	Type primer	Sequence
Hepcidin	Forward outer	5'- ATGCAGGGAGGTGTGT TAGGAGG-3'
	Forward inner	5'- CCATCTGCATTTTCTGC TGCGG-3'
	Reverse outer	5'- TGCAAGGCAGGGTCAG GACAAGC-3'
	Reverse inner	5'- CACTTTGATCGATGAC AGCAGT-3'

The SNP-containing gene segment was amplified using Outer and Reverse Inner primers; the mutant nucleotide was amplified using (.Forward Outer (FO) and Reverse Inner (RI) primers); and the wild nucleotide was amplified using (.Forward Inner (FI) and Reverse Outer (RO) primers).

A PCR mixture of 25 µl was made, consisting of 12.5 µl of green master mix, 1.5 µl of each reverse and forward primer and 6.5 µl of template DNA. The PCR amplification conditions were begins with initial denaturation at 94°C for 5 minutes for one cycle and followed by "denaturation at 94°C for 30 second , annealing at 56°C for 30 second, Extension at 72°C for 45 second " for thirty five cycles and final extension at 72°C for 5 minutes for one cycle. The amplified PCR product gene were run on 1.5% agarose gel in an electrophoresis technique .

**Statistical analysis**

All statistical analysis was conducted on windows ® using version 25 of the statistical package for the social sciences (SPSS) from IBM, US. All participant information was input and evaluated using acceptable statistical methods. The mean and standard deviation are used to represent continuous variables.

The G1 and G2 (continuous variable) was examined using the analysis of variance student t test; a P-value ≤ 0.05 indicated a statistically significant change. Allelic and genotypic frequencies were determined using the direct gene counting approach. All gene variations' Hardy– Weinberg equilibrium was tested using Chi-square testing , the odds ratio (OR) and a 95 percent confidence interval (CI), the link between covid-19 patients' genetic patterns and recovery groups was evaluated.

**RESULT**

As shown in table 2 , the (mean ± SD ) of ages and genders among G1 and G2 were not significant at P. value ≤ 0.05. The (mean ± SD ) of Hb and serum level of iron , UIBC , TIBC, IL – 1β , IL – 6 , and Hecpidin conc. for G1 and G2 were statistically significant at P. value ≤ 0.05.

**Table -2 :The Demographic Characteristics and the significant of some biochemical parameters of ( G1 and 60 G2 ) .**

Biochemical parameters	G1 No. 60 (Mean ±SD)	G2 No. 60 (Mean ±SD)	P. value
Age	52.54 ± 14.47	51.35 ± 11.98	0.670
Gender	1.56 ± 0.5	1.55 ± 0.5	0.925
Hb (g/dl)	12.38 ± 2.54	13.43 ± 1.66	0.027*
Iron (µg/dl)	61.84 ± 26.78	82.22 ± 33.02	0.002*
UIBC (µg/dl)	155.97 ± 36.34	190.63 ± 12.06	0.0001**
TIBC (µg/dl)	217.81 ± 45.68	272.86 ± 28.45	0.0001**
IL-1 β (pg/l)	496.03 ± 161.91	657.17 ± 197.92	0.0001**
IL-6 (ng/l)	47.76 ± 15.62	62.63 ± 8.18	0.0001**
Hecpidin concentration (pg/ml)	340.69 ± 135.73	529.1 ± 171.88	0.0001**

\*\*highly significant ≤0.01 , \* p. value significant ≤0.05 , SD (standard deviation),

Independent T test

**Results of gene polymorphism:**

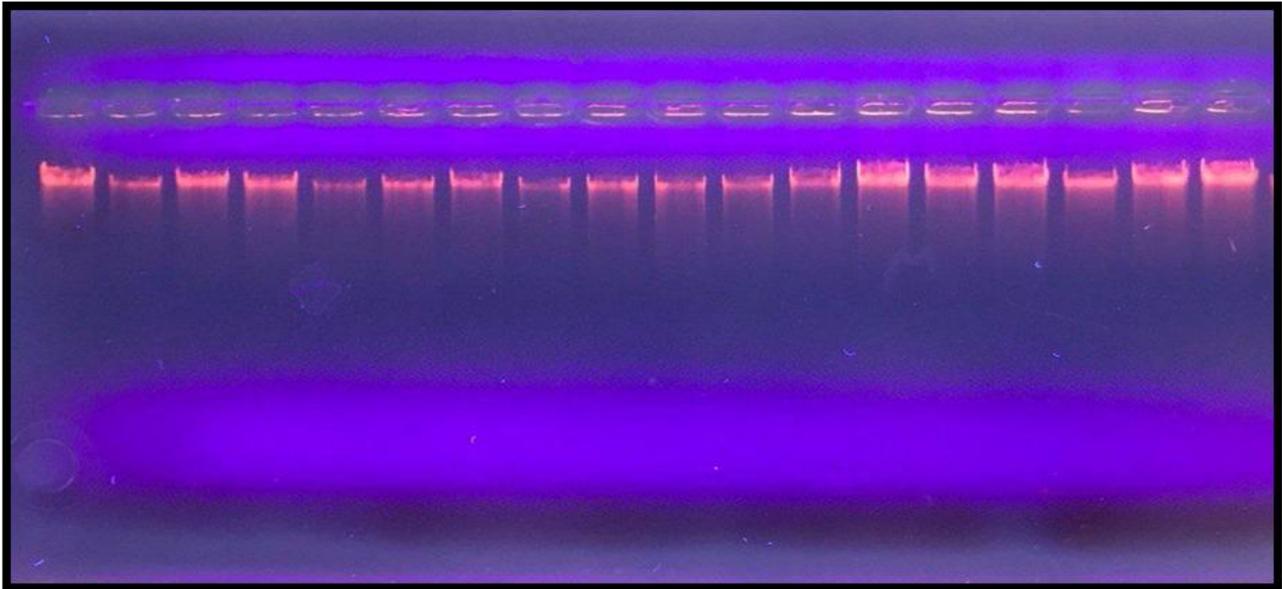
**DNA Detection:**

In order to verify the existence of a product and provide insight into the extraction process' accuracy, all of the extracted DNA samples were electrophoresed. Figures (1) illustrate the DNA bands of some of the analyzed samples.

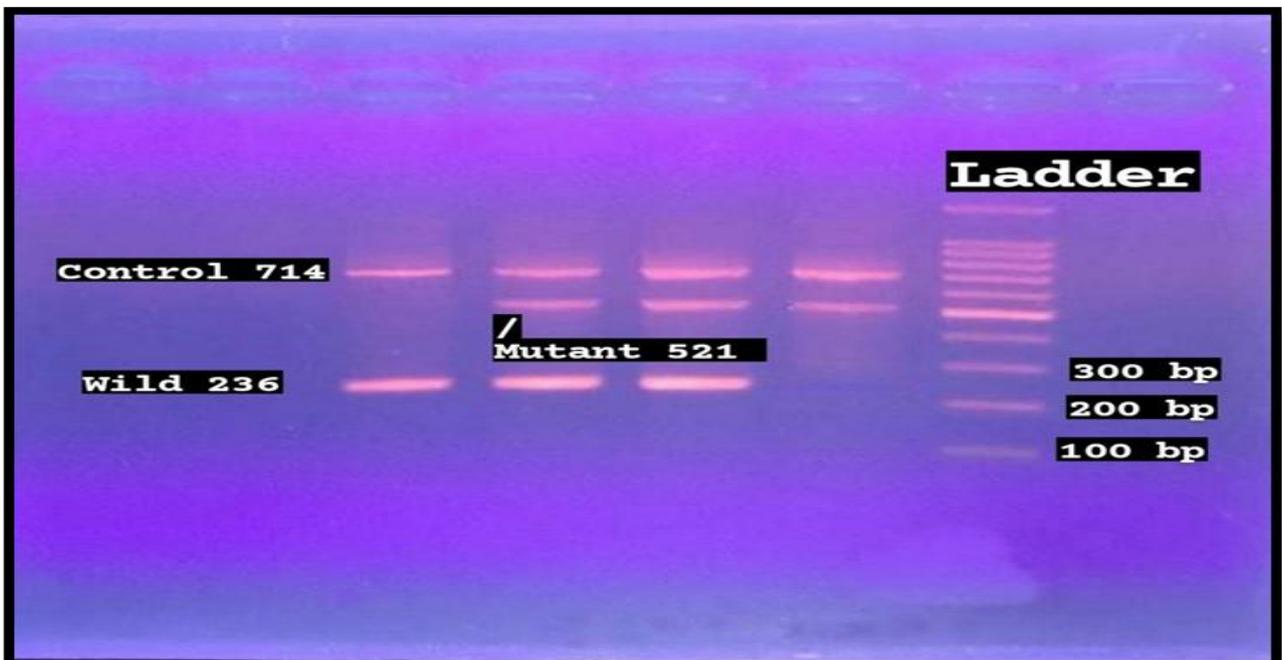
**Detection of hepcidin gene polymorphism :**

One hundred sample ( 50 patient , 50 recovery ) applied in gel electrophoresis after it was amplified by ARM-PCR technique. The results show in figure (2).

Polymorphisms of the hepcidin gene appeared in the two groups (in most samples), meaning that this result indicates that there is no significant difference through this gene in the two groups at  $P = 0.887$  and no significant association showed .



**Figure (1) :** depicts the electrophoresis of extracted DNA on 1.5 percent agarose at 85 V for 60 minutes with 5 µl of extracted DNA injected into each well, DNA isolation.



**Figure (2):** PCR product of hepcidin gene polymorphism , Ladder :100 bp.

**Table -3: The Allele and genotype frequency of hepcidin gene Polymorphism among Study Groups:**

Allele	Covid-19 patient		Recovery		OR (95% CI)	P. value
	N	%	N	%		
<b>G wild (236 bp)</b>	51	25.5	52	26	<b>1.041 (0.598-1.812)</b>	<b>0.887</b>
<b>A mutant (521 bp)</b>	49	24.5	48	24		
<b>Genotype</b>						
<b>GG</b>	1	1 %	2	2%	<b>2.042 (0.179 – 23.266)</b>	<b>0.558</b>
<b>GA</b>	49	49%	48	48%		
<b>AA</b>	0	-	0	-		

**DISCUSSION**

The mean variance of ages and genders among patient with covid -19 and recovery group are not significant at the  $P > 0.05$ . This matching is vital so as to exclude the difference in the results which will originate due to the variation in age and gender.

In this study , The hemoglobin level had been decreased in G1 compared to G2. The primary role of hemoglobin (Hb) is to carry oxygen, to deliver oxygen to different tissues of the body, to maintain healthy tissues, and to aid in the quick recovery from disease (24). The value of hemoglobin (Hb) in covid-19 patients decreases , and this is what was reached in this study , as it is identical to previous study (25) , and the low value of hemoglobin (Hb) can affect the severity of the covid -19 disease (26), In this study, we did not observe such an obvious effect of Hb . hemoglobin (Hb) can affect the severity of the disease when a person is in ICU or when the virus has infiltrated a person's lungs and causes a lack of oxygen, i.e. pneumonia (27). A decrease in hemoglobin absolute value or red blood cell count is used to diagnose anemia. Anemia is characterized by a hemoglobin level below 13.0 g/dl and has several causes, the

most significant of which is iron deficiency (28). Iron is a crucial metal necessary for several metabolic processes. In this study ,iron deficiency was found and the reason for the deficiency may be result from that Iron has a crucial role in the inhibition of infectious and inflammatory responses, and its levels may be diminished in febrile and autoimmune diseases (29,30), or may be Iron insufficiency is associated with skeletal muscular weakening and may reduce breathing capacity. Patients with Covid-19 may deteriorate and die as a result of a loss in their respiratory capacity (31,32).

Through this study ,a direct relationship appeared between iron and (UIBC , TIBC ) , where the lack of iron led to lack of the two mentioned markers . Covid-19 disease is associated with anemia and the type of anemia is anemia of inflammation . Immune disorders, cancer, chronic kidney disease, and infection can all cause anemia of inflammation (AI), which is also known as "chronic illness anemia" Mortality rates may be elevated in some disorders, and healthcare expenses rise drastically as a result. (33). In this type , the value of iron decreases ,and therefore the value of (UIBC , TIBC) decreases , and this was agreed by previous studies(34,35). Interleukin -6 is a key pro-inflammatory cytokine that impacts a wide range of biological processes, including immune system function, inflammation, hematopoiesis, metabolism, organ development, and the creation and maintenance of cancer cells. (36)

In this study , the value of IL-6 is higher in both groups ( covid-19 patient and recovery group ) than normal value of IL-6 . The reason for the lack of IL-6 in G1 compared to G2 may be due to the use of glucocorticoids during covid-19 infection and the use of this treatment leads to production in the macrophage to some extent. And therefore it may be less than what is in G2 (37), or may be In Covid-19 patients, an increase in blood pressure is observed, which was agreement by previous study (38), on the other hand *Vazquez* and coworker were founded the decrease in blood pressure leads to reduce in the amounts of IL-6 (39), and therefore it may be a reason for the lack of IL-6

compared to recovery group. There was a study founded that the value of IL-6 remains high after infection with Covid-19 disease, and the explanation for this might be that post-recovery declines in lymphocyte subsets and elevations in cytokines have been found as independent predictors of rehabilitation success (40).

Interleukin-1 Beta ( IL-1 $\beta$ ) is a cytokine belonging to the IL-1 family with significant proinflammatory characteristics. In this study, a different result was reached from what was mentioned in previous studies, which is that IL-1beta was not elevated in Covid-19 patients and the recovery group, and the value of IL-1 beta in both groups was within the normal limit. There was a difference between the two groups and the low value of IL-1 beta in Covid-19 patients compared to the recovery group may be the reason for the use of the beta-blocker treatment that patients use during infection to lower blood pressure and the effect of the treatment is the medication results in the stimulation of beta-adrenergic receptors, which adds to the release of inflammatory cytokines. Consequently, beta-blockers may be able to diminish cytokine storms in COVID-19 (38) . IL-6 is both a required and necessary cytokines for hepcidin induction. Hepcidin may be affected by different mediators during protracted inflammation, although interleukin-6 is a key regulator during acute inflammation (41). this is what was observed in this study, where the value of IL-6 and hepcidin was found in covid-19 patients less than in the recovered group.

As we explained previously , the type of anemia that occurs in covid-19 patients during this study is anemia of inflammation . hepcidin levels decrease during this type of anemia , Hepcidin is mostly suppressed by enhanced erythropoiesis or decreased serum or tissue iron due to increased iron use during anemia, according to these findings. (33) Another causes of decrease hepcidin levels in covid-19 patients may be probably secondary to iron deposition in hepatocytes, which reduces the production of hepcidin and thereby increases intestinal iron absorption (42).

Mutation in HAMP gene cause Hepcidin protein deficiency that can lead to iron overload or hepcidin level deficiency(43,44). In detection of hepcidin gene polymorphism among study groups (patients, recovery). we found that there was not significant variance between the both groups, as all alleles appeared in both groups . That is, the most persons involved in this study are heterozygous, and this indicates the presence of the mutation allele (521bp ) in the two groups, and it may be one of the reasons for the deficiency in hepcidin that appeared in this study.

## CONCLUSION

Covid-19 disease linked to anemia and type of anemia is (anemia of inflammation) where the infection has affected the value of hemoglobin (Hb) and which led to a decline in the value of Hb, as well as iron , UIBC , TIBC .

Elevation of some cytokines ( IL-6 ) in the recovery groups above normal range. Longitudinal research on a larger group of recovered Covid-19 patients for IL-6 are required to properly comprehend the implications of the illness.

In the study of hepcidin gene polymorphism, the appearance of the mutation allele in the both groups, it may be indicate one of the reasons for the deficiency in hepcidin that appeared in this study.

## Acknowledgement

Authors are grateful to the college of medicine/University of Al-Qadisiyah and all the staff of the Martyr Hassan Hallous Al-Hatami hospital in Al-Najaf Al-Ashraf Governorate (Iraq) for providing the research facilities .

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