Association of Hepcidin Gene Polymorphism and Iron Levels in Obese Patients with Covid-19

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Abstract

Covid-19 is a genus of enveloped, positive-sense, single-stranded RNA viruses with a high degree of genetic diversity. They induce a variety of disorders affecting the respiratory, gastrointestinal, hepatic, and nervous systems in humans and animals, with different manifestation and severity.

Obesity is the result of the accumulation of an excessive amount of fat in the body and the condition arises from an imbalance between the amount of energy stored by increased food intake and the amount of energy expended as physical activity.

The aims of study are evaluate the effect of covid-19 on iron levels in obese patients and study there correlations. As well as, study the association of hepcidin gene polymorphism to the risk of infection with covid-19 in obese patients.

This study is cross sectional. Which include conducted in isolation wards at Al-Amal Hospital and Al-Hakim hospital in Al-Najaf City for the period extending from November 2021 to the end of March 2022, 60 sample were collected from diagnosed patients with covd-19 and obese (BMI more than 30) . On the other hand 60 sample of individuals that suffering from obesity (BMI more than 30) and recovered from covid-19 for more than six months.

The variables was measured in this research are: ferritin, serum iron, unsaturated iron binding capacity (UIBC) and total iron binding capacity (TIBC). Also gene polymorphism of hepcidin hormone G71D (HAMP).The results showed that there were significant differences for (ferritin, serum iron, UIBC and TIBC), in obese patients with covid-19 compared to recovery from covid-19. The gene polymorphism of hepcidin show no significant difference between obese patients with covid-19 and obese recovery from covid-19.

the conclusion obese people are the most risk factor to covid-19 infection due to association of obesity with change in BMI and increase Adipose tissue mass in obese individuals, As well as Ferritin test is the most accurate biomarker to covid-19 infection while Gene polymorphism study of SNPs hepcidin G71D (HAMP) gene represent a weak maker for covid-19 disease.

Keywords: covid-19; polymorphism; hepcidin gene, iron levels

1. Introduction

Coronavirus disease 2019 (COVID-19), an emerging acute respiratory disease, is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1] This disease starts in Wuhan city in china, in the late December 2019 [2, 3]. A study of the first cases in China indicates the average incubation period between 2 to 7 days and the longest incubation period of 12.5 days [4]. The World Health Organization named the disease coronavirus disease 2019 (COVID-19) and subsequently declared it a pandemic due to the widespread infectivity and high contagion rate. Human coronaviruses typically cause respiratory and enteric infections [5] The new coronavirus has become a worldwide health threat [6], with sporadic cases reported globally From December 2019 till now, the disease, which resulted in several millions cases with many deaths [7]. The common symptoms include fever, dry cough, and tiredness though some individuals remain asymptomatic throughout the period. Asymptomatic patients are considered to be a potential source of infection [8] the key instruments for confirmed diagnosis of the infection are virus nucleic acid real-time polymerase chain reaction (RT-PCR), CT imaging, and certain hematology parameters [9].

several risk factors prognosticate an unfavorable disease course of COVID-19 are known, including higher age, male sex, type 2 diabetes mellitus, hypertension, coronary artery disease, and obesity (a higher body mass index (BMI)) [10, 11].

The worldwide prevalence of overweight and obesity has doubled since 1980 to an extent that nearly a third of the world population is now classified as overweight or obese [12] Obesity adversely affects nearly all physiological functions of the body and comprises a significant public health threat. It increases the risk for developing multiple disease conditions, such as diabetes mellitus,cardiovascular disease,several types of cancers,an array of musculoskeletal disorders, and poor mental health [13] and increase risk factor for covid-19 [14].

The World Health Organization (WHO) defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health [15].

The body mass index (BMI) is body assessment for obesity, calculated by dividing the body weight in kilograms by the square of height in meters, is a simple metric used to indicate overall body fatness;

Normal BMI range as 18.5 to 24.9

Overweight range as BMI ≥ 25 kg/m2

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Obese range as BMI ≥ 30 kg/m2
Severe obesity range as BMI ≥ 40 kg/m2 [16].
Measurements and comparisons of waist and hip circumference can also provide some information regarding risk factors associated with weight. The higher of the ratio, the greater chance for weight-associated complications
Iron (Fe) is crucially involved in multiple important biochemical pathways, including mitochondrial respiration, metabolic processes, hormone synthesis or deoxyribonucleic acid (DNA) synthesis [17].
Each of erythrocytes contains about 280 million hemoglobin molecules, which in turn contain four heme-subunits with central iron atoms. These iron atoms represent the binding sites for oxygen thus providing more than a billion oxygen binding sites per erythrocyte. The daily iron demand for metabolic processes in adult humans is about 20–30 mg [18].
Iron is absorbed in proximal duodenal endothelial cells either as heme iron via the heme transporter heme carrier protein 1 (HCP1) or as non-heme iron via the divalent metal transporter 1 (DMT1). Luminal non-heme iron (principally Fe3+ complex formations) is first reduced by the duodenal cytochrome B (DCYTB) to Fe2+ before it is transported across the cellular membrane by DMT1. Iron is either stored as mucosal ferritin in duodenal endothelial cells or transported to the bloodstream by ferroportin 1 (FPN1). FPN1 is the only known cellular iron exporter and not only responsible for iron export from duodenal enterocytes but also for iron mobilization from hepatocytes and erythrocyte recycling macrophages. After Fe2+ is exported by FPN1, it is oxidized to Fe3+ by hephaestin (HEPH) at the basolateral membrane of duodenal enterocytes and attached to transferrin. Contrary, mucosal ferritin is lost through shedding of mucosal endothelial cells over time. This is the only route for body iron elimination since there is no regulated iron excretion.
There are three component to the iron binding capacity of serum:
Serum iron is the concentration of iron present. Normally it is about 100 micrograms of iron per 100 milliliters of blood.
Total iron binding capacity (TIBC) is the maximum amount of iron that can be bound. Normally this is about 300 micrograms per 100 milliliters.
The unsaturated iron binding capacity (UIBC) is the difference between the TIBC and the serum iron. It is normally about 200 [19].
Hepcidin is the main systemic regulating hormone of iron metabolism and is primarily synthesized in the liver [20]. Hepcidin was discovered as the liver-expressed antimicrobial peptide in 2000. Hepcidin is produced primarily by the hepatocytes which are strategically located in the vicinity of portal veins (carrying dietary iron) as well as the Kupffer cells (sensing microbes and recycling erythrocytes). Hepcidin is also produced by macrophages and adipocytes in a small quantity [21].
Hepcidin is encoded by the hepcidin antimicrobial peptide (HAMP) gene on chromosome 19q13. It is initially synthesized as an 84 amino acid pre-pro-hepcidin. It is then processed to 60 amino acid pro-hepcidin and ultimately sliced to a mature C-terminal 25 amino acid active peptide.Hepcidin is a tightly folded peptide hormone that forms a simple hairpin structure stabilized by 4 disulfide bonds [22].
Mutations of the HAMP gene are associated with severe iron overload and hemochromatosis. Hepcidin expression is increased in iron overload and inflammation and is diminished in states of iron deficiency and hypoxia [23].
Ferritin is a hollow, globular protein of 480 kDa molecular weight. Classical ferritins consist of 24 subunits. Larger forms of ferritins consisting of 36 subunits are present in the heart and skeletal muscles, and microferritin with 12 subunits is produced by bacteria. Mitochondria and nucleus also contain ferritin, which consists of only FTH [24].
The main function of ferritin is to store iron. FTH has ferroxidase activity, which converts soluble ferrous iron into storable ferric iron. Then, ferric iron enters the cavity of ferritin and forms iron core under the action of the nucleation site of FTL. Ferritin rich in FTH can accumulate and release iron faster than that rich in FTL, and has a more active iron transport system. Ferritin rich in FTL has more iron than that rich in FTH, which plays an important role in iron storage [25].

2. Materials and Methods

This study is cross sectional; included 120 obese patients divided into two groups

G1, 60 individuals that suffering from obesity (BMI more than 30) and recovered from covid-19 for more than six months.
G2, 60 patients diagnosed with Covid-19 who were suffering from obesity (BMI more than 30), All patients were admitted to the quarantine section of Al-Amal Hospital and Al-Hakim hospital in Al-Najaf City for the period extending from November 2021 to the end of March 2022.
Ethical Issues: For this study, all samples were obtained from participants. All of the patients expressed their willingness for their specimens to be used in this study.
All ethical concerns were addressed, and the research was carried out with the permission of the hospitals. The names and characters, personal information, and even the illnesses and medical information of the patients were kept hidden.
Included criteria: Any person whose BMI more than 30 and infected with covid-19 has been confirmed by a PCR test or CT scan examination,or by a specialist doctors diagnosis of the disease.
Collection of Samples: Whole venous blood samples under aseptically collected by venous puncture using sterile 5ml disposable syringe. Blood divided to two parts;
Part one, 1.5 ml whole blood insert to EDTA plastic tube and stored in deep freezer in -20 ℃ until they used in T-ARMS-PCR assay.
Part two, 3.5 ml blood was evacuated in a gel and clot activator vacuum tubes that were centrifuged at 3000 rpm for 5 minutes. Sera were separate and immediately measure the ferritin, free iron and unbound iron binding

capacity (UIBC) by Architect c4000 technique.

Biochemical methodology: measure the concentration of ferritin, serum iron and unsaturated iron binding capacity (UIBC) in serum samples automatically, by using (Architect c4000 auto analyzer/ japan and C100 auto analyzer/ china). Total iron binding capacity (TIBC) measure by equilibrium (serum iron + UIBC = TIBC)

In this study, a Tetra amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) used for detection of polymorphisms of hepcidin hormone. This method is simple, rapid and sensitive for the detection of single nucleotide polymorphism [26]. The hepcidin genomic sequence was obtained from [27]. PCR amplification for genotyping T-ARMs PCR was carried out, briefly, four primers of each SNP were designed, Forward Outer, Forward Inner, Reverse Outer, and Reverse Inner. Forward and reverse primers of G71D (HAMP) were 5'-ATGCAGGGAGGTGTGTTAGGAGG-3' (Fo), 5'-CCA TCTGCATTTTCTGCTGCGG-3' (Fi), 5'-TGCAAGGCAG GGTCAGGACAAGC-3' (Ro) and 5'-CACTTTGATCG ATGACAGCAGT-3' (Ri). PCR mixture of 25 µl was made consisting of 12.5 µl master mix, 1.5 µl of each forward and reverse primer, 6.5 µl of template DNA, and without of ddH2O. The PCR amplification conditions were; initial denaturation 95ºC for 5 min, proceeding with 35 cycle of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s, extension 72ºC for 45 s, and final extension at 72ºC for 5 min. The amplified PCR products was run on 1% of agarose gel.

Statistical analysis: The data analysis for the results that included in this research was using the Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Results of all tests with p-values <0.05 (two-side) were considered to be statistically significant.

3. Results

the mean Age in G1 was 33.017 years, range (16 – 60 years) while in G2 was 56.708 years, range (16 – 85 years).The mean BMI in G1 was 41.683, rang (30 -52) while in G2 was 39.728. Range (30 - 52). As shown in table (1)

Table (1): Descriptive of the Demographic characteristics

	G1 (No. = 60)		G2 (No. = 60)	
	Mean ± SD	Range	Mean ± SD	Range
Age (years)	33.017±9.2306	16 -60	56.708±14.1844	16 -85
BMI	41.683±5.3226	30 -52	39.728±5.7048	30 -52

Biochemical study

This research was examined four biochemical markers (ferritin, iron, UIBC and TIBC) in serum of obese patients in G1 and G2 by using independent T-test, Markers were shown a significant difference as show in table (2).

The ferritin level in G2 was higher than its level in G1, Mean ± 2SD of ferritin level in G2 (478.581 ± 292.618 ng/ml) versus (64.747 ± 40.449 ng/ml) in G1 and the difference was highly significant (p<0.001). The iron level in G1 was higher than its level in G2, Mean ± 2SD of iron level in G1 (67.288 ± 19.467 mg/dl) versus (54.911 ± 21.460 mg/dl) in G2 and the difference was highly significant (p<0.001). The UIBC level in G1 was higher than its level in G2, Mean ± 2SD of UIBC level in G1 (181.717 ± 8.203 mg/dl) versus (168.909 ± 15.131 mg/dl) in G2 and the difference was highly significant (p<0.001). The TIBC level in G1 was higher than its level in G2, Mean ± 2SD of TIBC level in G1 (248.087 ± 21.451 mg/dl) versus (225.081 ± 25.532 mg/dl) in G2 and the difference was highly significant (p<0.001).

Table (2): The association some biochemical markers with covid-19 disease in G1 and G2

Variables	G1 (no.=60) Obese recovery from covid-19 Mean ± 2SD	G 2 (no.=60) Obese with covid-19 Mean ± 2SD	P-value
Ferritin	64.747 ± 40.449	478.581 ± 292.618	<0.001 [S]
Serum iron	67.288 ± 19.467	54.911 ± 21.460	0.001 [S]
UIBC	181.717 ± 8.203	168.909 ± 15.131	<0.001 [S]
TIBC	248.087 ± 21.451	225.081 ± 25.532	<0.001 [S]

Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant, independent T-test

The odd ratio of markers in both groups represent the risk factor of each independent variables and their clinical importance in covid-19 infection. Among covid-19 patients groups ferritin was shown a high risk factor in covid-19 infection. The logistic regression model was statistically significant, OR (1.039) and p < 0.001, as shown in table (3)

Table (3): The risk factor of some biochemicals markers by using binary logistic regression

Biomarkers	Odds Ratio (risk factor)	95% Confidence Interval Range (Lower-Upper)	P Value
Ferritin	1.039	(1.021 – 1.058)	< 0.001
Serum iron	0.970	(0.952 – 0.989)	0.002
UIBC	0.913	(0.878 – 0.950)	< 0.001
TIBC	0.957	(0.938 – 0.976)	< 0.001

In this research, the correlation between Ferritin levels and other biomarkers in obese patients infected with Covid-19 group was perfumed; a Pearson correlation coefficient was run to determine the relationship between them. There was a moderate to strong, negative correlation between UIBC and Ferritin, (r = -0.5), p <0.001). As shown in Figures (2). While there was a significant weak negative correlation between ferritin levels and serum Iron and TIBC. Results were shown the correlation coefficient r = (- 0.32 and -0.434) respectively and p-values were significant (<0.001) as shown in Figures (1 and 3)

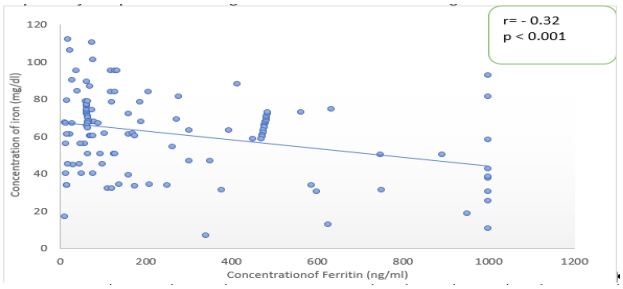

Figure (1): The corrlation between Ferritin levels and Iron levels in G2 by using Simple linear regression

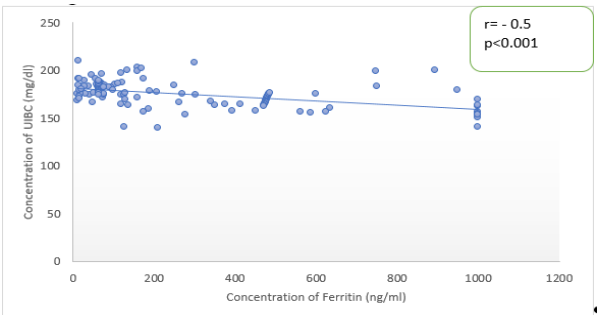

Figure (2): The corrlation between Ferritin levels and UIBC levels in G2 by using Simple linear regression

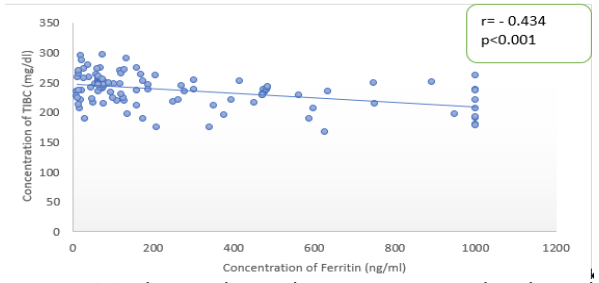

Figure (3): The corrlation between Ferritin levels and TIBC levels in G2 by using Simple linear regression

In each patients group (G1, G2), the Receiver operating characteristics (ROC) curves were performed for levels Ferritin, iron, UIBC & TIBC. The area under curve (AUC) and cut-off values were calculated according to their specificity and sensitivity as predictive factors. In G2, ferritin had the highest AUC, which was 0.98 [95% Cl= 0.964 – 0.998; Sensitivity% =98%; Specificity% =75%; Cut-off points =127.1 ng/ml], as shown in table (4).

Table (4): AUC, optimal threshold, Sensitivity and specificity of Ferritin levels obtained by the ROC curves for prediction of COVID-19 infection in G2

Test Variable	AUC	Sensitivity %	Specificity %	Cut-off points	Accuracy %	CI (95%)
Ferritin(ng/ml)	0.98	98%	75%	127.1	91.6%	0.964-0.998

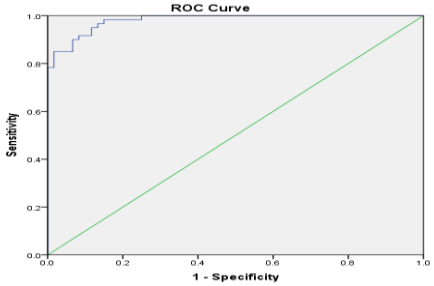

Figure (4) (ROC) curve of Ferritin levels in G2

While other markers (Iron, UIBC & TIBC) had high AUC, CI (95%), Sensitivity %, Specificity% and Cut-off points in G1 (table 5) .

Iron had the high AUC, which was 0.7 [95% Cl=0.583 - 0.773; Sensitivity% =83%; Specificity% =40%; Cut-off points =50.100]. UIBC had the high AUC, which was 0.8 [95% Cl=0.712 - 0.885; Sensitivity% =98%; Specificity% =53%; Cut-off points =167.5]. TIBC had the high AUC, which was 0.8 [95% Cl=0.703 - 0.867; Sensitivity% =90%; Specificity% =40%; Cut-off points =220.250].

Table (5): AUC, optimal threshold, Sensitivity and specificity of (iron, UIBC and TIBC) obtained by the ROC curves for G1

Test variables	AUC	Sensitivity %	Specificity %	Cut-off points	Accuracy %	CI (95%)
Iron(mg/dl)	0.7	83%	40%	50.100	61.66%	0.583 - 0.773
UIBC(mg/dl)	0.8	98%	53%	167.5	75.83%	0.712 - 0.885
TIBC(mg/dl)	0.8	90%	40%	220.250	65%	0.703 - 0.867

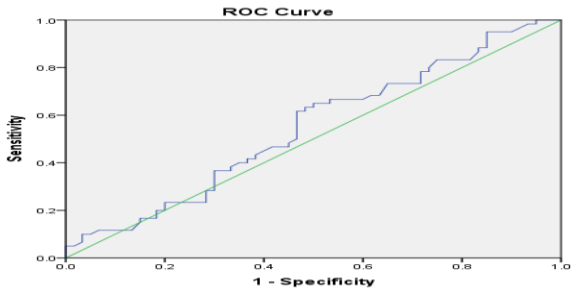

Figure (5) (ROC) curve analysis of serum Iron levels in G1

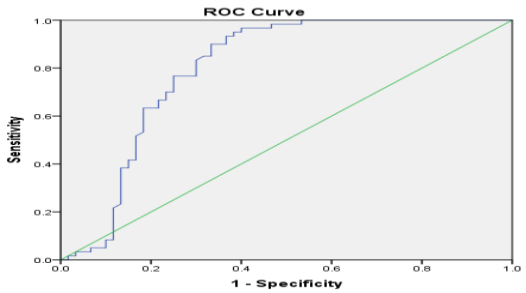

Figure (6) (ROC) curve analysis of UIBC in G1

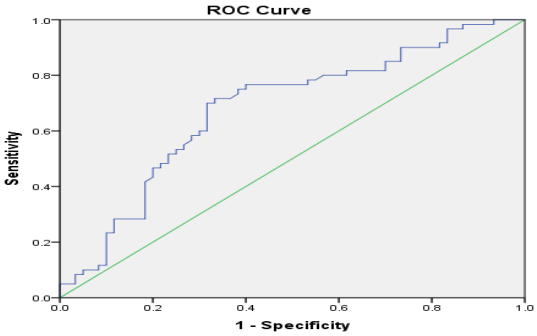

Figure (7) (ROC) curve analysis of TIBC levels in G1

The results of the PCR technique show the success of all DNA amplification processes extracted from the group 1 and group 2 of the hepcidin gene after Perform electrophoresis on the agarose gel. It showed the diagnostic gene for hepcidin at molecular weight 714 bp,

236 bp and 521 bp as revealed in figure (8).

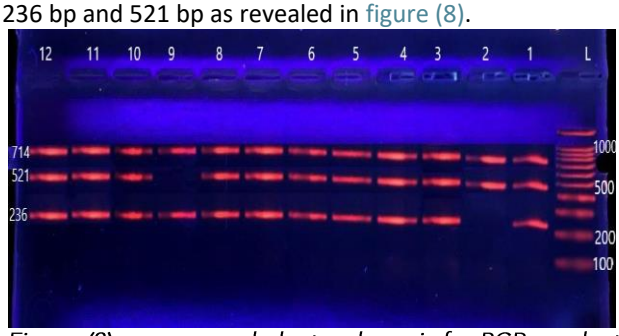

Figure (8): agarose gel electrophoresis for PCR product of hepcidin gene. product sizes were 714 bp for control band, 521 bp for A allele, and 236 bp for G allele. Lane number (1, 2, 3, 4, 5, 6) for group1 and lane number (7, 8, 9, 10, 11, 12) for group 2. Agarose 1.5% concentration, 75 V, stained with ethidium bromide. L, DNA ladder (100 bp)(promega company).

In table (6) illustrates the examination of 60 individuals of G1 and 60 individuals of G2. The result of PCR analysis for amplification of hepcidin gene polymorphism was 56(50.5%) for GA genotype, 3(42.9%) for GG genotype and 1(50%) for AA genotype in group 1 and 55(49.5%) for GA genotype, 4(57.1%) for GG genotype and 1(50%) for AA genotype in group 2. this result indicate there is no significance difference through this gene in two groups.

Table (6): Associated factors of Hepcidin Genotype (HAMP single-nucleotide polymorphisms) in G1 compered to G2.

Genotype	Group 1 N (%)	Group 2 N (%)	Odd Ratios	95% CI	P value
GA	56 (50.5)	55 (49.5)	0.737	0.158 - 3.445	0.698[NS]
GG	3 (42.9)	4 (57.1)	1a		-
AA	1 (50)	1 (50)	0.750	0.032 – 17.506	0.859[NS]

4. Discussion

The results indicated iron status in covid-19 obese patients and found a significant relationship between iron metabolism problems and the severity of covid-19 and the probability of various adverse outcomes. Covid-19 infection was linked to higher level of serum ferritin, lower levels of serum iron, TIBC, and UIBC levels.

The level of ferritin increase in obese patients with covid-19, this finding agree with many studies [28, 29] that give many explanations for this condition, recent studies suggest that increased levels of circulating ferritin levels may play a critical role in inflammation by contributing to the development of a cytokine storm [30] Another explanation for the increased levels of ferritin could be the role of iron metabolism in supporting the innate immune system to fight invading microorganisms. The innate immune system control over iron metabolism as a response to viral infections. For viral replication, enhanced cellular metabolism and optimal iron levels within host cells are necessary [31] Therefore, the innate immune system will decrease the bioavailability of iron to limit the replication of the virus during the acute phase of infection. In these conditions, through interleukin-6 and Toll like-receptor-4 dependent pathways, the levels of the liver derived iron hormone hepcidin-the master regulator of iron homeostasis could increase and block, the activity of the transporter ferroportin which carries iron out of the cells, and therefore decrease the amount of iron absorbed from the diet, causing cellular sequestration of iron (principally in hepatocytes, enterocytes, and macrophages) Increased intracellular iron sequestration will lead to an upregulation of cytosolic ferritin, which sequesters and stores iron to prevent iron-mediated free radical damage [32] The increase retention and storage of iron within ferritin in macrophages contribute to the characteristic fall in serum iron concentrations and an increase in serum ferritin concentrations [33] and as observed in this study.

Hepcidin is a peptide that regulates iron homeostasis by inhibiting iron absorption by the small intestine and release of iron from macrophages. Its production is stimulated by iron overload and by inflammation [34]. The our finding of hepcidin gene (HAMP) may be result from the fact of all patients diagnosed with sever covid-19 had higher hepcidin concentration [35] due to the inflammatory response changes iron homeostasis [36]. and Hepcidin levels are upregulated in inflammations commonly induced by infections [37].

A low serum iron, UIBC and TIBC was observed in G2; moreover, these patients presented with anemia. The observed changes in iron status could be due to the role of hepcidin as a negative regulator of intestinal iron absorption and macrophage iron efflux.

5. Conclusion

Ferritin test is the most accurate biomarker to covid-19 infection in obese individuals.

Our finding shown Link between inflammatory status and tissue iron reserves (ferritin level and iron over load) and risk of developing anemia and hypoxia, a subset of this research revealed the relationship is causal, which that having low iron in serum is enough to cause anemia and hypoxia in covid-19 disease.

Gene polymorphism study of SNPs hepcidin G71D (HAMP) gene represent a weak maker for covid-19 disease.

Recommendation

Clinicians caring for obese individuals that infected with covid-19 should aware of the inflammatory alteration that accompany with hormonal change.

Covid-19 infection is a leading cause to many other systemic diseases, so that must use suitable medicine thoroughly and individually assessed with special attention paid to the kind and route of administration.

More extended studies with other SNPs of hepcidin gene should be carried out to give more accurate picture about gene polymorphism studies.

Assessment of Gene expression of hepcidin gene that is an iron status indicator that allowing accurate assessment of iron status in covid-19 infected patients.

The soluble transferrin receptor is also an iron status indicator that is unaffected by the acute-phase response, allowing for an accurate assessment of iron status in obese individuals.

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