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Effect of Haptoglobin Genotype on Essential Mineral Levels in Patients With Sickle Cell Anemia, Hepatitis C: A comparative Analysis

Sally Salih Jumaa¹,

Afrah Abid Maktoof,²

Rasha Salih Nuhair²

sallysalih.bio@sci.utq.edu.iq

afrah.m_bio@sci.utq.edu.iq

rasha.n_bio@sci.utq.edu.iq

¹University of Thi-Qar/ Collage of Vet-Medicine.

² University of Thi-Qar/ Collage of Science.

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Abstracte

The current study was conducted in the laboratories of the Marsh Research Center, the Center for Genetic Diseases, and Al-Hussein Teaching Hospital during the period from June 2022 to January 2023, with the aim of studying the genetic polymorphism of the haptoglobin (Hp) gene and its relationship to levels of essential minerals in patients with sickle cell anemia. Hepatitis C, and sickle cell anemia with hepatitis C. a total of 130 participants were classified, (40) control groups, (40) patients with sickle cell anemia, (40) patients with hepatitis C, and (10) sickle cell patients with hepatitis C .

DNA was isolated and polymerase chain reaction (PCR) performed using genotype-specific primers for the three regions of the haptoglobin gene, and genotypes were determined after electrophoresis on agarose gels and determining the amplified fraction of each allele. Iron, copper, zinc, and chromium in blood serum were determined by flame atomic absorption spectrometry according to the manufacturer's procedures.

The study found that the Hp2-2 genotype was more common in patients with sickle cell anemia, hepatitis, and both sickle cell anemia and hepatitis compared to the healthy control group. However, there were no significant differences in this genotype between the three diseases. In all patients, the level of iron and copper in the serum was significantly higher compared to the control group. and In all patients, the study showed a significant depletion of serum zinc compared to the control group, And no significant difference in serum chromium concentration between the patients and the control group. Regarding the polymorphisms of haptoglobin, the study did not find a statistically significant relationship between these genetic variations and the levels of iron, copper, and chromium in any of the participant groups. However, the concentrations of zinc differed between the three types of haptoglobin.

Keywords: Haptoglobin polymorphism(Hp), Sickle Cell anemia, Hepatitis C(HCV).

.Introduction

Sickle cell disease (SCD) is a collection of autosomal recessive disorders that has a significant global impact, affecting approximately 20 to 25 million individuals worldwide, It is recognized as the most prevalent monogenic disorder, posing a substantial public health concern¹, is characterized by the presence of various mutations in the gene responsible for encoding the β -globin subunits of hemoglobin (HBB), resulting in the production of abnormal hemoglobin molecules that can polymerize and cause the red blood cells to become sickle-shaped², This leads to hemolytic anemia, a type of anemia characterized by increased red blood cell breakdown³.

Hepatitis C virus (HCV) infection is the primary cause of chronic liver disease in many industrialized nations⁴. The course of HCV infection can vary widely, with some individuals spontaneously clearing the virus, while others develop persistent infection that can progress to cirrhosis or hepatocellular carcinoma, a type of liver cancer⁵. Environmental and viral variables may act in conjunction with a person's vulnerability to cause liver damage to affect the clinical manifestation of HCV-related liver disease⁶. The role of the host's genetic background is anticipated to be significant in the context of HCV infection. Indeed, numerous studies investigating disease associations have provided substantial evidence suggesting the involvement of multiple genes in HCV infection⁷.

Haptoglobin is a type of alpha₂ globulin found in human plasma. Its primary function is to bind to the globin portion of free hemoglobin in the bloodstream⁸. Three distinct phenotypes, known as Hp 1-1, Hp 2-1, and Hp 2-2, can be identified since the protein is made up of two subunits that are expressed by two different genes. Haptoglobin functions as a positive acute phase protein and is capable of interacting with hemoglobin, There were functional variations among the Hp phenotypes⁹. The Hp 1-1 haptoglobin phenotype exhibits a greater capacity for binding to hemoglobin compared to the other phenotypes, On the other hand, Hp 2-1 and Hp 2-2 phenotypes have been associated with antibody-like properties. Additionally, these phenotypes have the ability to inhibit prostaglandin synthesis, making them acute phase reactants¹⁰.

According to some research, SCD patients with HP2-2 are more likely to experience disease-related complications¹¹. While, some research on SCD suggests that the HP 1-1 genotype is a risk factor for neurological or cardiovascular consequences compared to the HP 2-2 genotype, other research suggests that this genotype is protective against kidney injury and cardiovascular illness¹². Also, Some studies have shown an association between hepatitis C and Hp polymorphism, This finding suggests that the Hp phenotype may influence the clinical evolution of hepatitis C¹³.

Due to their involvement in numerous metabolic pathways, essential trace elements play critical roles in the preservation of health These metabolic problems¹⁴, such as poor glucose tolerance and dyslipidemia, are commonly brought on by a deficit or excess of certain trace elements, including iron, zinc, copper and chromium¹⁵. Since the liver controls a substantial portion of the metabolism of trace elements, a dysfunction of liver functions can lead to a variety of metabolic illnesses¹⁶.

Recent developments in molecular biology techniques have aided in elucidating the putative pathways by which anomalies in these trace elements cause liver illnesses to elicit metabolic problems. Genome-wide association studies found that a particular genetic variation influenced the metabolism of a trace elements¹⁷.

2. Materials and Methods:**Patients and controls groups:**

The study was approved by the Ministry of Health, Thi-Qar Health Department, 130 Samples were taken for the purpose of the study from health care centers in each of Al-Hussein Teaching Hospital, Al-Nasiriyah Teaching Hospital, Al-Shatrah General Hospital, Thi-Qar Gene bank, and some private clinics and laboratories, in addition to the center Genetic diseases in Thi-Qar province for the period from June 2022 to January 2023. Samples in this study are divided into four groups as in the following:

1-The first group: 40 is healthy control

2-The second group: 40 patients with sickle cell anemia

The third group : 40 patients with hepatitis C virus

4-The fourth group: 10 patients of sickle cell anemia with hepatitis C virus infection.

Blood collection:

Five ml of venous blood specimen were collected from patients and controls. Three milliliters of blood collected directly in a EDTA free plane tube and allowed to clot then serum was separated by centrifugation. After centrifugation serum was separated and stored at -20 C to be measure Fe, Zn, Cu, Cr, serum concentration. Two milliliters of blood collected EDTA tube for DNA extraction, by using Genomic DNA mini kit extraction (Geneaid- Tiwan).

DNA Extraction and Haptoglobin Genotypin : Genomic DNA was extracted from Blood isolates by using Geneaid Genomic DNA extraction Kit (Tiwan).

DNA Template and Polymerase Chain Reaction (PCR) : PCR technique was used to amplify the haptoglobine gene according to¹¹ and used the following Primers for the PCR technique. Primers (forward and reverse) as the table (1) the kit provide by Geneaid Genomic DNA extraction Kit company (Taiwan).

Table(1): The primers which used in this study.

Product Name	Product size (bp)	Annealing Temperature	Primer Name	Oligonucleotide sequence (5'-3')
Hp2	935	58	F3	CAGGAGTATACACCTTAAATG
			C42	TTACACTGGTAGCGAACCGA
Hp1S	1200	58	C51	GCAATGATGTCACGGATATC
			S2	TTATCCACTGCTTCTCATTG
Hp1F	1400	58	F3	CAGGAGTATACACCTTAAATG
			C72	AATTTAAAATTGGCATTTCGCC

Primers (forward, revers), D.W and DNA were mixed in master mix tube (13µl)

Table (2): PCR condition for amplification of HP gene :

Step	Temp.	Time	Cycle
Initial Denaturation	95	5.00 min	1 time
Denaturation	95	0.30 sec.	35 cycle
Annealing	58	0.45 sec.	
Extension	72	1.00 min	
Final Extension	72	10.00 min	1 time

All samples were submitted to three reactions (1, 2 and 3) and the genotypes were defined after electrophoresis on 1.5% agarose gel and identification of the amplified fragment for each allele.

Estimation of trace elements :

The estimation of Fe, Cu, Zn and Cr elements in the blood serum by Flame Atomic Absorption Spectrophotometer was done according to procedure of manufacturer.

3. Statistical Analysis

The analyses of data were expressed as mean \pm SD. The comparisons between each patients and healthy control groups it performed by two-way ANOVA along with Least significant differences (LSD) to calculate the significant differences among means. $P \leq 0.05$ is considered significant. All the statistical analyses were done by using computer through the (SPSS program) Statistical Package For Social Sciences (version-20).

4. Result

Molecular Analysis

Genomic DNA extraction

Genomic DNA was extracted by using Geneaid Genomic DNA extraction Kit (Tiwan) for patients and controls. All samples showed bands, which referred to the genomic DNA on the gel electrophoresis

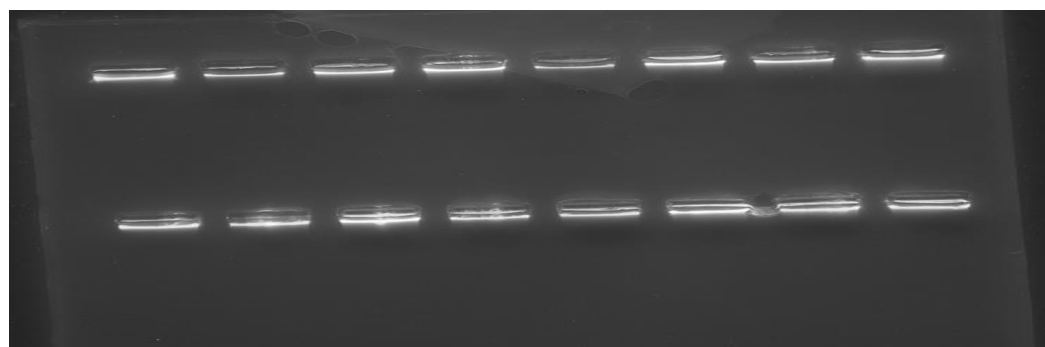


Figure (1): Gel-electrophoresis of DNA extracted from the whole blood sample, the fragments were fractionated by electrophoresis on (1%) agarose and 1X TAE (1h /90v).

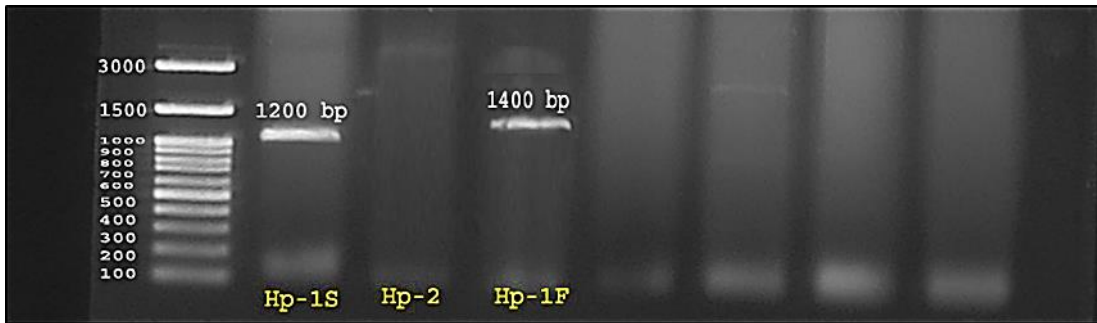
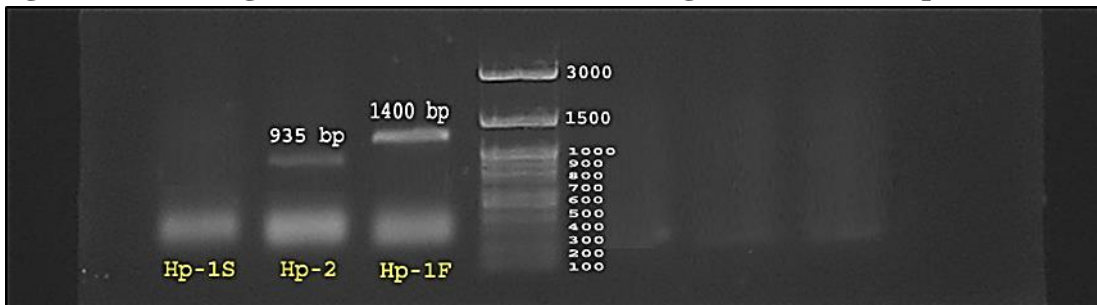
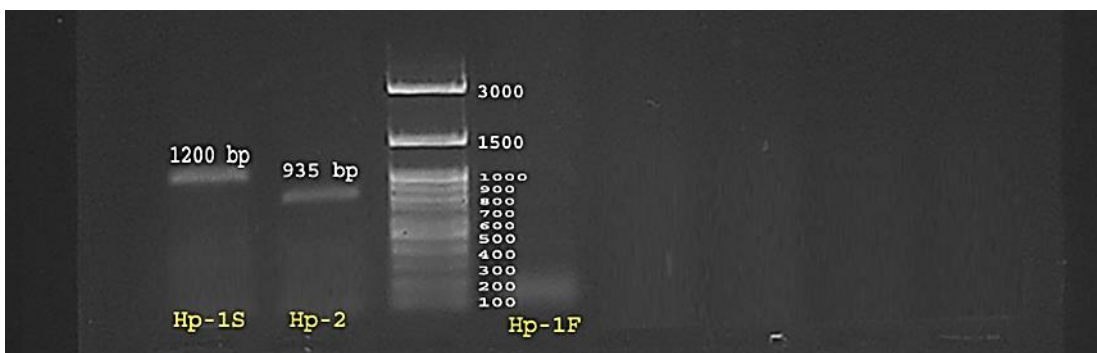


Figure (2): Hp1-1 (Hp 1S – Hp 1F) Agarose gel electrophoresis for amplified HP gene. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (1 h., 80V/cm) and visualized under U.V. light after staining with ethidium bromide staining. (L:100 - 3000bp M: ladder) .



Figure(3):Hp 2-2 (Hp2 – Hp1F) Agarose gel electrophoresis for amplified HP gene. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (1 h., 80V/cm) and visualized under U.V. light after staining with ethidium bromide staining. (L:100 - 3000bp M: ladder) .



Figure(4):HP 2-1 (HP1S – -Hp2) Agarose gel electrophoresis for amplified HP gene. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (1 h., 80V/cm) and visualized under U.V. light after staining with ethidium bromide staining. (L:100 - 3000bp M: ladder) .

Frequency of Hp Types between Different Groupes

Table (3) indicates the distribution of the haptoglobin genotype between the patient groups and the control group, where it was observed that the genotype HP (2-2) is the most prevalent among all groups. It showed a significant increase ($P \leq 0.05$) compared with the rest of the genotypes where the proportions were respectively,

1-in Healthy Control HP1-1(0.30),HP2-2(0.50),HP2-1(0.20)

2-inSickle cell Patient HP1-1(0.13),HP2-2(0.55),HP2-1(0.32)

3-Hepatitis Patient HP1-1(0.13),HP2-2(0.63),HP2-1(0.24)

4-Sickle cell with Hepatitis Patient HP1-1(0.20),HP2-2(0.60),HP2-1(0.20)

the results showed that there was no significant difference between patients and healthy people in the distribution patterns, and the Hp2-2 phenotype was the most prevalent among the cohorts.

Table. (3)the Frequencies of haptoglobin gene among the study groups.

Group		Type	Type Frequency	Chi-Square
Healthy Control		Hp1-1	0.30	P = 0.61
		Hp2-2	0.50	
		Hp2-1	0.20	
Sickle cell Patient		Hp-1-1	0.13	
		Hp2-2	0.55	
		Hp2-1	0.32	
Hepatitis Patient		Hp1-1	0.13	
		Hp2-2	0.63	
		Hp2-1	0.24	
Hepatitis & Sickle cell Patient		Hp1-1	0.20	
		Hp2-2	0.60	
		Hp2-1	0.20	

Determination of Iron concentration and its Association with haptoglobin genotypes:

Type₁: The data of the statistical analysis presented in Table (4) indicate a significant increase ($P \leq 0.05$) in the concentration of iron in all patient groups compared to the control group.

Type₂: The results of the statistical analysis presented in Table (4) indicate a significant increase ($P \leq 0.05$) in iron concentration in all groups of patients compared to the control group.

Type₃: The results of the statistical analysis presented in Table (4) indicate a significant increase ($P \leq 0.05$) in iron concentration in all groups of patients compared to the control group.

There are no statistically significant differences in iron concentration between patient groups **According to types of haptoglobin:**

Statistical analysis in Table (4) showed that there were no statistically significant differences in iron concentration between all types of haptoglobin in the control group and the patients.

Table (4): Comparing serum Fe levels between patients and controls and their relationship with haptoglobin types.

	Type . Hp Groups	HP1-1 (M±SD)	HP2-2 (M±SD)	HP2-1 (M±SD)	LSD
Fe	Control	94.00 ± 17.07 Ba	96.50 ± 12.70 Ba	96.33 ± 13.27 Ba	4.37
	Sickle cell patients	155.80 ± 2.68 Aa	151.50 ± 9.82 Aa	156.30 ± 9.44 Aa	6.51
	Hepatitis patients	154.20 ± 13.01 Aa	152.00 ± 11.94 Aa	155.30 ± 12.30 Aa	7.8
	Sickle cell with Hepatitis	154.00 ± 12.83 Aa	155.70 ± 14.58 Aa	153.00 ± 10.04 Aa	4.32
	LSD	7.40	5.20	6.99	

Values expressed as Mean ±SD*

*Different capital letters denote significant differences ($P \leq 0.05$) between groups.

*Different small letters denote significant differences ($P \leq 0.05$) between types of haptoglobin.

Determination of Zinc concentration and its Association with haptoglobin genotypes:

Type₁: The data of the statistical analysis presented in Table (5) indicate a significant decrease ($P \leq 0.05$) in the concentration of zinc in all patient groups compared to the control group.

Type₂: The results of the statistical analysis presented in Table (5) indicate a significant decrease ($P \leq 0.05$) in zinc concentration in all groups of patients compared to the control group.

Type₃: The data of the statistical analysis presented in Table (5) indicate a significant decrease ($P \leq 0.05$) in the concentration of zinc in all patient groups compared to the control group.

It was found that there were no statistically significant differences in zinc concentration between the patient groups.

According to types of haptoglobin:

The statistical analysis in Table (5) showed a significant decrease ($P \leq 0.05$) in serum zinc concentration in HP1-1 and HP2-1 in sickle cell patients compared with HP2-2, While there was decrease ($P \leq 0.05$) in serum zinc in HP1-1 and HP2-2 in sickle cell with hepatitis compared with HP2-1, while no significant differences were observed in serum zinc between all types of haptoglobin in Control and hepatitis patients groups.

Table (5): Comparing serum Zn levels between patients and controls and their relationship with haptoglobin types.

	Type . Hp Groups	HP1-1 (M±SD)	HP2-2 (M±SD)	HP2-1 (M±SD)	LSD
Zn	Control	118.50 ±9.64 Aa	113.80 ± 15.70 Aa	130.50 ±36.92 Aa	16.9
	Sickle cell patients	58.00 ±12.00 Bb	69.63 ± 12.77 Ba	57.07 ±18.91 Bb	10.65
	Hepatitis patients	69.20 ±12.83 Ba	70.20 ± 16.80 Ba	73.30 ±12.43 Ba	7.00
	Sickle cell with Hepatitis	54.00 ±6.82 Bb	54.50 ± 4.70 Bb	71.00 ±8.53 Ba	6.45
	LSD	13.2	16.57	16.22	

Values expressed as Mean ±SD*

*Different capital letters denote significant differences ($P \leq 0.05$) between groups.

*Different small letters denote significant differences ($P \leq 0.05$) between types of haptoglobin.

Determination of Copper concentration and its Association with haptoglobin genotypes:

Type₁: The statistical analysis presented in Table (6) indicate a significant increase ($P \leq 0.05$) in the concentration of Copper in all patient groups compared to the control group.

Type₂: The results of the statistical analysis presented in Table (6) indicate a significant increase ($P \leq 0.05$) in Copper concentration in all groups of patients compared to the control group.

Type₃: The data of the statistical analysis presented in Table (6) indicate a significant increase ($P \leq 0.05$) in the concentration of Copper in sickle cell, sickle cell with hepatitis groups compared to the control and hepatitis groups,.

while, there was no significant difference in Copper concentration between patients groups.

According to types of haptoglobin:

The statistical analysis in Table (6) showed no significant differences were observed in serum Copper between all types of haptoglobin in Control, and patients groups

Table (6): Comparing serum Cu levels between patients and controls and their relationship with haptoglobin types.

	Type . Hp Groups	HP1-1 (M±SD)	HP2-2 (M±SD)	HP2-1 (M±SD)	LSD
Cu	Control	80.600 ±8.82 Ba	75.18 ±14.09 B a	64.81 ± 18.78 Ba	17.55
	Sickle cell patients	131.33 ±15.78 Aa	130.90 ±10.24 Aa	130.00 ±9.76 Aa	10.34
	Hepatitis patients	129.00±13.88 Aa	123.00 ±12.61 Aa	128.50 ±13.78 Aa	6.5
	Sickle cell with Hepatitis	122.00 ±17.07 Aa	124.50 ±17.67 Aa	129.66 ±6.21 Aa	8.51
	LSD	10.133	11.90	8.65	

Values expressed as Mean ±SD*

*Different capital letters denote significant differences ($P \leq 0.05$) between groups.

*Different small letters denote significant differences ($P \leq 0.05$) between types of haptoglobin.

Determination of Chromium concentration and its Association with haptoglobin genotypes:

Type₁: The statistical analysis presented in Table (7) indicates that there is no significant difference in serum chromium concentration between the patient groups and the control group.

Type₂: The results of the statistical analysis presented in Table (7) indicate that there is no significant difference in serum chromium concentration between the patient and the control group.

Type₃: The data of the statistical analysis presented in Table (7) indicate that there is no significant difference in serum chromium concentration between the patient groups and the control group.

Also, the results showed that there is no significant difference in serum chromium concentration between the groups of patients themselves.

According to types of haptoglobin:

The statistical analysis in Table (7) found no significant difference in serum chromium levels between the control group and patient groups with different haptoglobin genotypes.

Table (7):Comparing serum Cr levels between patients and controls and their relationship with haptoglobin types.

Cr	Type . Hp Groups	HP1-1 (M±SD)	HP2-2 (M±SD)	HP2-1 (M±SD)	LSD
	Control	0.88 ±0.04Aa	0.87 ±0.27Aa	0.83±0.23Aa	0.07
	Sickle cell patients	0.71 ±0.04Aa	0.71 ±0.10Aa	0.74 ±0.22Aa	0.05
	Hepatitis patients	0.85 ±0.15Aa	0.96 ±0.21Aa	0.84 ±0.20Aa	0.31
	Sickle cell with Hepatitis	0.66 ±0.09Aa	0.49 ±0.11Aa	0.62 ±0.15Aa	0.19

Values expressed as Mean ±SD *

*Different capital letters denote significant differences ($P \leq 0.05$) between groups.

*Different small letters denote significant differences ($P \leq 0.05$) between types of haptoglobin.

5. Discussion

The Hp2-2 genotype was found to be overrepresented in patients with sickle cell anemia (55%), hepatitis (63%), and sickle cell anemia and hepatitis (60%), but otherwise showed no significant differences between the three diseases and the healthy control group (table 3).

The findings are consistent with those reported by¹⁹, who observed a higher frequency of the Hp2-2 genotype in sickle cell anemia patients compared to the control group. Their study showed a significant difference ($P < 0.05$) in the Hp2-2 genotype frequency, with sickle cell anemia patients having a higher frequency (54%) compared to sickle cell trait (42%) and healthy individuals (38%).

These findings are also consistent with those obtained by²⁰ in Kuwaiti patients, Contrary to the findings of both¹¹ in Nigeria, who discovered that the distribution of Hp genotypes among the patients and controls were Hp1-1, 43 (42.6 %); Hp2-1, 40 (39.6%); Hp2-2, 18 (17.8%) and Hp1-1, 35 (54.7%); Hp2-1, 24 (37.5%); Hp2-2, 5 (7.8%), respectively, with no difference between the sickle cell anemia patient and control group ($P < 0.05$). and²¹ who found 20.2% had Hp1-1 phenotype, 48.8% had Hp2-1 phenotype and 31.0% had Hp2-2 phenotype in chronic liver disease.

The exact mechanism of sickle cell disease is not fully understood, but it is thought that hemolysis (the breakdown of red blood cells) causes a lack of nutrients that are vital for healthy cells to operate²², including as antioxidants and trace elements²³. In patients with hepatitis C, essential micronutrients (such as iron, zinc, copper, and chromium) can exacerbate liver disease if they are deficient, imbalanced, or toxic²⁴. Across the spectrum of liver damage caused by hepatitis C, individuals have been demonstrated to have impaired metabolism of trace elements in clinical trials.

In the current study it was observed that some trace elements were significantly elevated or depleted ($P < 0.05$) in SCD, HCV and SCD infected with HCV patients compared to healthy controls. These trace elements included iron, zinc, copper, and chromium.

In present study the amount of iron in sickle cell patients blood was significantly elevated when compared with the control ($P < 0.05$) table (4). Our results agree with²⁵. In addition,²⁶ found that among the sickle cell disease study population, 4.5% had iron shortage and 21.3% had iron overload.

Individuals with sickle cell disease who require frequent blood transfusions are at risk of developing iron overload²⁷, Diseases of the liver, heart, and blood sugar are only some of the outcomes of too much iron in the body.

The present results showed an increase level of iron in the blood of patients with hepatitis C when compared with the control ($P < 0.05$) table (4), and the current study is consistent with a study by^{28,29}. Studies suggest, that a high level of iron in the liver plays a crucial role in the progression of liver disease and increasing the risk for liver cancer^{28,30}, Hepatocyte necrosis, which triggers the release of ferritin and the iron absorption by macrophages and Kupffer cells, has been hypothesized to be the origin of mesenchymal hepatic iron excess during HCV infection³¹.

the level of serum zinc in sickle cell patients was significantly depleted when compared with the control ($P < 0.05$) table (5). The significantly low level of zinc is consistent with³², who found a connection between sickle cell disease and symptoms such growth retardation, hypogonadism in males, hyperammonemia, incorrect dark adaption, and cell mediated immunological dysfunction, and zinc insufficiency.

Zinc deficiency is a common problem in sickle cell anemia patients³³. It can be caused by a number of factors, including the increased hemolysis (breakdown of red blood cells) that is characteristic of sickle cell disease, the inflammation that is often associated with the disease, and the use of hydroxyurea, a medication that is used to treat sickle cell anemia¹¹.

Zinc's anti-inflammatory, antioxidant, and radical scavenging properties in hepatitis C are all induced by the presence of this virus antigen³⁴. Zn levels in the serum of HCV infected people were significantly lower than those in the control group, according to the results of the current study, table (5). These findings corroborated those of^{35,36,37}, whose reported that individuals with chronic hepatitis C had significantly lower zinc concentrations than healthy control groups when zinc concentrations were examined at the level of the whole body.

In the present study Serum copper levels were found to be significantly increased in all patient groups compared to the control group ($P < 0.05$), table (6). Copper is known to be essential in the proper functioning of various metal enzymes, including ceruloplasmin involved in iron metabolism³⁸.

In the sickle cell disease group, the mean blood copper level was significantly elevated ($P < 0.05$) than the control group

The current finding agrees with the reports^{39,44} which revealed a significantly elevated level of copper in sickle cell patients. However, it is contrary to previous reports.⁴⁰

Copper, which functions as an antioxidant, is another cofactor found in many enzymes⁴¹. It may however act as a pro-oxidant when present in high concentrations⁴². High serum copper levels in SCD patients may be related to free radical promotion, which has negative consequences⁴³, often observed in sickle cell patients generally.

Our study revealed a significant increase in serum copper levels in patients infected with hepatitis C virus (HCV) compared to the control group table(6), This finding is consistent with previous studies conducted by^{34,45}, which found elevated copper levels in patients with chronic viral hepatitis.

Serum copper increases in response to acute HCV infection; this effect is heightened in CHC and fibrotic liver disease. CHC causes a rise in hepatic copper, most of which is found bound to MTs (Cu-MTs) and contributes to hepatic copper overload^{46,47}.

Although chromium's role in SCD and HCV patients is unclear, it is well established that chromium acts as a cofactor in the first response of insulin-sensitive cell membranes, hence amplifying insulin's effects⁴⁸. The present results indicate that there is no significant difference in serum chromium concentration between all patients groups and the control table(7).

The current results did not show a statistically significant relationship between the polymorphisms of haptoglobin and the levels of iron, copper and chromium in all the participating groups, while the concentrations of zinc differed between the three types of haptoglobin. However, differences in nutrient concentrations may be due to the nature of the disease and the different environmental and dietary factors among the participating groups. Many studies have suggested that genetic variation can affect nutrient metabolism, absorption, and biosynthesis⁴⁹.

An example of a polymorphism with potential influence on chronic disease through effects on nutritional status is the common haptoglobin polymorphism. A study by⁵⁰ found that people with Type 2-2 haptoglobin have a deficiency of vitamin C and iron compared to Type 1-1 and 2-1, and therefore are more susceptible to oxidative stress due to vitamin C deficiency.

6. Conclusion: Hp2-2 was the most prevalent haptoglobin phenotype among patients groups. This suggests that Hp2-2 may play a role in the pathogenesis of sickle cell anemia and hepatitis C, while Haptoglobin polymorphisms were not related to levels of essential minerals in the participating groups. More research is needed to understand this Link better.

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