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The role of *Hepcidin* Gene Polymorphism (rs10421768 A>G and rs1173345431C>T) on β-Thalassemia Major in Iraqi Patients

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Abstract Thalassemia is a genetic blood illness that can cause haemoglobin to develop abnormally and is inherited from a person's parents. Thalassemia has two primary subtypes: alpha and beta. The number of the four alpha globin genes or the two *beta globin* genes that are absent determines how severe alpha and -thalassemia is. The Mediterranean region, Africa, the Middle East, the Indian subcontinent, and South-East Asia all have a high prevalence of thalassemia. This study's goal is to determine whether there is a relationship between serum ferritin levels in Iraqi patients with major -thalassemia and the single nucleotide polymorphisms (SNPs) (rs10421768 A>G and (rs1173345431C>T) in the *Hepcidin* gene. The study is divided into two groups: fifty individuals with -thalassemia (35 females and 15 males) and fifty individuals who appear to be in good health (13 males and 37 females). The participants ranged in age from (14 – 40). All information about the study groups, including age, gender, family history, length of blood transfusions, and severity of the disease, was gathered in questionnaire form during the recruitment period, which lasted from June to October 2022, at the Ibn Albaladi Centre for Thalassemia Disease in Baghdad. The level of ferritin in the blood serum and the whole picture of the blood were determined, and the single nucleotide polymorphisms (SNPs) were investigated using the (real-time PCR-HRM) technology. The results of the survey for the group of patients and persons who appeared to be in good health were as follows: a significant decrease (p0.01) in the level of haemoglobin (Hb) for beta-thalassemia patients (8.57+0.22) compared with the control group (11.15+0.24*):* while the result shows no significant increase in white blood cells of beta thalassemia patients (11.31+2.15)x109/L when compared with the control group (8.81+0.35)x109/L. The red cells distribution width (RDW) results in a significant increase in beta-th The patients with severe symptoms (31(62.00%) and those with moderate symptoms (19(38.00%) had the highest severity percentages, *respectively*. Blood transfusion duration shows a significant difference increase in duration over two weeks (21(42.00%*):* three weeks (18(36.00%*):* and four weeks (11(22.00%) with P-value (0.0073). Patients with -thalassemia had a significantly higher level of serum ferritin (2929.16+1877.54 ng/ml) compared to the control group (21.96+14.21) ng/ml, with a p-value of (0.0001*):* in the laboratory. The genotyping and allele results for the SNP (rs10421768 A>G) did not reveal any appreciable differences between the control group and the sick population. Additionally, there was no discernible difference between the two groups of patients and controls for (rs1173345431C>T). In conclusion, environmental and genetic factors such family history, the severity of the disease, and the presence of the A allele and AA genotype in the *Hep.* gene (rs10421768 A>G). They could be viewed as crucial contributors to the pathophysiology of Iraqi patients with -thalassemia major.

Keywords: Real-time PCR-HRM, polymorphism of the *Hep.* gene.

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Introduction

Hereditary hemolytic anaemia known as thalassemia is accompanied by a lack of or a reduction in one or more synthesises of the -globin chains. It is categorised by aberrant production

or a decrease in the rate of creation of the normal beta or alpha haemoglobin (Hb) subunits. A. Two alpha and two *beta globin* subunits make up haemoglobin A. Haemoglobin subunit beta gene (HBB*):* which codes for globin, is located on the short arm of chromosome 11, while globin genes are located on chromosome 16. The protein known as haemoglobin, which is found in red blood cells (RBCs*):* is in charge of transporting oxygen from the alveolus to tissues. Patients with significant -thalassemia experience severe anaemia and require blood transfusions for the rest of their lives (1). The frequency of thalassemia per 100,000 people by governorate in Iraq is shown in 2015. Dohuk governorate had the second-highest prevalence of thalassemia (54.1/100,000 people*):* followed by Basrah governorate (54.5/100,000 people*):* while Muthanna governorate had the lowest incidence (19.7/100,000 people). A total of 335 newly diagnosed symptomatic thalassemia patients were registered in 2015, resulting in an incidence rate of 34.6/100,000 live births. An imbalance in the ratio of globin chains, which is often under good control, is the primary pathophysiology of thalassemia. Erythroid precursors are destroyed as a result of precipitation of unbound globin chains, such as -globin and -globin in -thalassemia. RBC hemolysis and poor erythropoiesis in the bone marrow are caused by RBC precursor damage (2). Thalassemia major is a more serious variant in which individuals require regular blood transfusions to survive and develop high iron levels as a result. The vascular, endocrine, and hepatic systems become impaired when the iron stores are overloaded because this causes the creation of harmful reactive oxygen species. Several laboratory techniques, including blood smears, prenatal testing (genetic testing of amniotic fluid*):* DNA analysis (genetic testing*):* and total blood counts, can be used to identify and find thalassemia. A natural mechanism for removing the excess iron burden brought on by blood transfusions does not exist in the human body. Transfused packed red blood cells have 200 to 250 mg of elemental iron in each unit. Different clinical problems of iron overload are caused by the accumulation of iron in various organs. The primary regulator of systemic iron homeostasis is the peptide hormone *Hep.*, which is produced in the liver. By preventing intestinal iron absorption, iron recycling by macrophages, and iron mobilisation from hepatic reserves, *Hep.* regulates plasma iron concentration and the distribution of iron in tissues (3).

The *HAMP* gene on 19q13, which has 2637 bp and three exons, encodes the tiny antimicrobial peptide *Hep.*, a crucial component of iron hemostasis. It works by lowering the expression of ferroportin (FPN*):* an iron transporter on the surface of intestinal cells, which is primarily expressed in the liver. Duodenal enterocytes, liver Kupffer cells, periportal hepatocytes, and splenic macrophages are the main cell types that express FPN. One important negative regulator of iron egress from hepatocytes is thought to be the *Hep.*-FPN complex. *Hep.* expression increases in response to iron buildup and inflammation to reduce iron release into the bloodstream. However, *Hep.* is strongly downregulated in anaemic and hypoxic conditions, increasing the availability of iron. Anaemia and iron overload coexist in -thalassemia patients and impose competing signals on *Hep.* expression. In this article, we will investigate the relationship between serum ferritin and the *Hep.* gene *HAMP*

SNPs (rs10421768 A>G and (rs1173345431) C>T) in Iraqi patients with significant -thalassemia.

Material and methods

The samples used in this investigation were received at the Ibn Albaladi Thalassemia Centre in Baghdad between June and October of 2022. The Institute of Genetic Engineering and Biotechnology for Postgraduate Studies at the University of Baghdad gave its approval to the study's design. The study included 50 patients (15 male and 35 female) with exacerbations of -thalassemia major as the first group, and 50 controls (13 male and 37 female) who appeared to be in good health. Each participant's personal information, including age, sex, family history, disease severity, and duration, was gathered using a questioner form. All patients and the ostensibly healthy control group provided written informed consent before being diagnosed with haematological and genetic diseases in accordance with international standards using the clinical details of their medical histories, physical examinations, laboratory tests, and medications.

Genomic DNA extraction

Peripheral venous blood samples in the amount of two millilitres were taken from both -thalassemia patients and seemingly healthy controls. In order to use blood as a source for DNA extraction, EDTA anticoagulant tubes were maintained in a freezer at -20 degrees. Using the gSYAN DNA kit extraction kit (Geneaid. USA Company*):* DNA was retrieved from blood samples of people with -thalassemia and people who appeared to be in good health. Agarose gel electrophoresis was used to verify the existence and integrity of the extracted DNA after genomic DNA was extracted. Then, using a nanodrop spectrophotometer, DNA concentration and purity were evaluated.

Primer design

Hepcidin (rs10421768) and (rs117345431) gene polymorphism preparation HRM qPCR primers were created using the NCBI-Gene Bank database and Primer 3 design online. Lists the primers that were provided by Scientific Research Co. Ltd. Iraq.

High resolution melting (HRM)

High resolution melting (HRM-qPCR) master mix was prepared by using GoTaq® qPCR Master Mix and done according to the company instructions as in the Table (1).

Table (1): High resolution melting (HRM)

qPCR master mix	Volume (µL)
DNA template 5-50ng	5
Hepcidin forward primer (10pmol)	1
Hepcidin reverse primer (10pmol)	1
qPCR Master Mix	10
Nuclease free water	3
Total volume	20

Starting with PCR amplification of the target region in the presence of a dsDNA binding dye, the HRM analysis is conducted. PCR-HRM was carried out on a Rotor gene Q Real-time PCR System (QIAGEN) and then an HRM analysis with 0.2 °C scaling from 55 to 95 °C was conducted. In order to test the duplication of the 2xTransStart® Tip Green qPCR Super Mix Synthetic SNP sequences, EVA-Green was employed in the master mix. Real-time PCR-HRM was utilised on duplicate synthetic controls to detect allelic differences, and the HRM Tool and the programme provided in table (2) were used to create normalised melting curves (NMC) and differential curves (DC).

Table (2): Thermal profile of HRM genotyping *Hep.* gene for two SNPs (rs10421768 A>G and rs1173345431C>T).

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	60 sec	1
Denature	94ºC	5 sec	40
Annealing	ºC58	15 sec	
Extension	72ºC	20 sec	
HRM	55-95	0.2sec for 1 degree	

Statistical analysis

To identify the impact of various factors on study parameters, the Statistical Analysis System SAS (2018) programme was utilised. T-test and the least significant difference (LSD) test (ANOVA) were employed to statistically compare between means. Chi-square test was used to compare percentages (0.05 and 0.01 likelihood) in a significant way. estimate of the correlation between the two variables. Estimated odds ratio and confidence interval in this study (4).

Result and discussion

Evaluation of clinical features of β-thalassemia patients according to severity, blood transfusion, hematological parameters and serum ferritin level

Major issues are brought on by - thalassemia major, which can also cause early death. As the disease progresses, complications could lead to slow growth, bone issues that could change the appearance of the face, issues with the liver and gall bladder, enlarged spleen, enlarged kidneys, diabetes, hypothyroidism, and heart issues. According to the severity of the disease, a recent investigation of the clinical characteristics of - thalassemia patients showed that there were significant differences (P 0.05) between the degree of disease severity and the proportion of patients who experienced severe symptoms as opposed to those who experienced mild symptoms. According to Table (3*):* the maximum percentage of patients with severe symptoms was 31(62.00%*):* while the lowest percentage with mild symptoms was 19(38.00%*):* with a P-value of (0.0287).

Table (3): Distribution of sample study according to severity and blood transfusion duration in B-thalassemia patients groups

Factor		No	%	P-value
Severity	Sever	31	62.00	0.0287 *
	Mild	19	38.00	
Blood Transfusion/ Weeks(W)	2 W	21	42.00	0.0073 **
	3 W	18	36.00	
	4 W	11	22.00	
* (P≤0.05*):* ** (P≤0.01).				

The mutation of two *beta globin* genes, which leads to a flawed synthesis of haemoglobin, causes this type of severe thalassemia. Chronic severe anaemia, inefficient erythropoiesis (IE*):* iron overload, and consequent clinical consequences are its defining features. The excess unpaired and soluble globin chains that accumulate at the membrane of the red cell progenitors in -thalassaemia major catalyse the generation of reactive oxygen species, which causes oxidative cell damage and premature cell death through apoptosis. As a result of this occurring within the erythropoietic tissue, mature red blood cells are hemolyzed and erythropoiesis is rendered inefficient (IE). In addition to causing pathological long bone fractures, distinctive abnormalities of the skull and face, and increased erythropoietin production that causes bone marrow growth, severe anaemia can also result in the development of extramedullary erythropoietic tissue. Iron is absorbed and released from deteriorating red blood cells, which causes an iron overload (5).

The majority of beta-thalassemia major patients require routine red blood cell transfusions to survive. Iron excess is a result of frequent transfusions, and it can have fatal consequences. Patients with beta thalassemia major typically die within the first five years of life without transfusions, and in high-income nations, even with transfusions, only 50–65% of patients reach the age of 35. The mainstay of treatment for people with thalassemia major and many with intermedia is blood transfusion. Transfusions have two goals: to treat anaemia and to decrease inefficient erythropoiesis. Most of the severe growth, skeletal, and neurological problems of thalassemia major are avoided by chronic transfusions. But once they begin, transfusion-related problems turn into a significant cause of morbidity. To provide a safe and logical approach to the use of blood transfusions in the management of these uncommon illnesses, standards must be created and upheld (6). According to blood transfusion length in Table (4*):* there has been a statistically significant difference rise in the duration of blood transfusions over the past two, three, and four weeks, with a P value of (0.0073).

Table (4): Comparison between patients and control groups in Blood parameters.

Group	Mean ± SE		
	WBC (10^9/L)	HGB (g/dL)	RDW-SD (fL)
Patients	11.31 ±2.15	8.57 ±0.22	49.86 ±1.75
Control	8.81 ±0.35	11.15 ±0.24	38.06 ±1.05
T-test	4.331 NS	0.652 **	4.062 **
P-value	0.255	0.0001	0.0001
** (P≤0.01*):* NS: Non-Significant.			

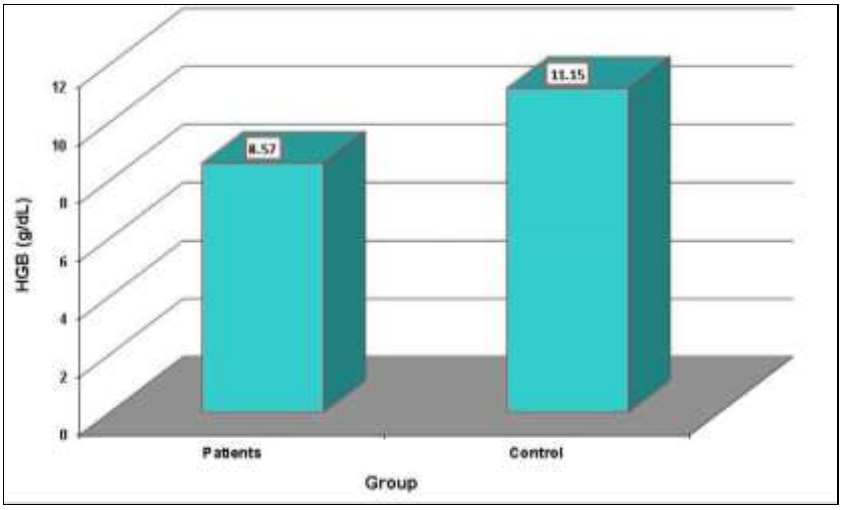

Figure (1-A): Comparison between patients and control for Hb in β-thalassemia patients.

The findings of this study are consistent with a study by (Ayyash and Sirdah, 7) whose hypothesis was that significant changes in blood cell counts have been recorded following splenectomy. These include thrombocytosis, monocytosis, and leukocytosis with a propensity for lymphocytosis.

The significant increases in WBC count in our splenectomized TM patients may be explained by the fact that splenectomized patients were found to have higher levels of susceptibility to infections and a higher risk of septic complications with a high mortality rate than non-splenectomized patients.

More research conducted in Saudi Arabia revealed a drop in Hb levels in patients compared to controls(8).

Patients with severe anaemia due to thalassemyosis need transfusion therapy to survive, but it has drawbacks as well, the most notable of which are complications caused by iron overload. As seen in the study that revealed iron overload cardiomyopathy, increased liver iron, and endocrine organopathy in transfusion-dependent thalassemia patients that were caused by iron deposition in these organs (9*):* not only the volume of blood transfused but also the duration of iron exposure gives the best estimate of organ dysfunction.

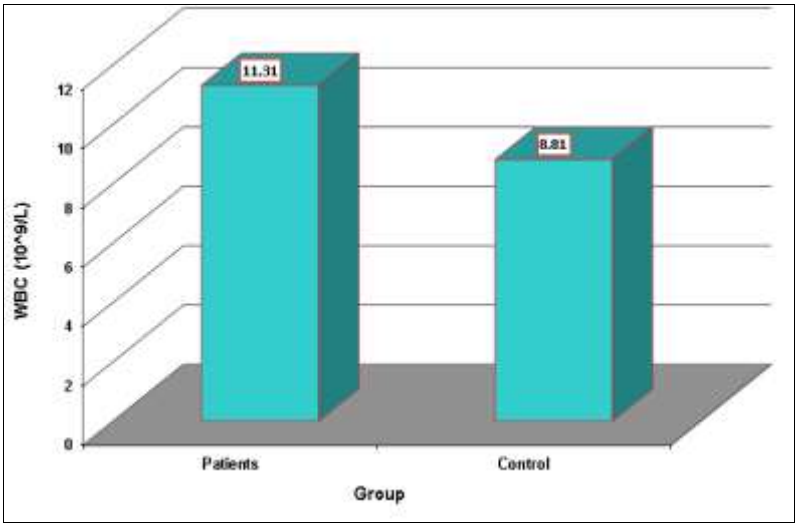

Figure (1-B): Comparison between patients and control for WBC in β-thalassemia patients.

In contrast to the control group (38.061.05*):* the patient with -thalassemia had a significantly higher level of RDW (49.861.75).

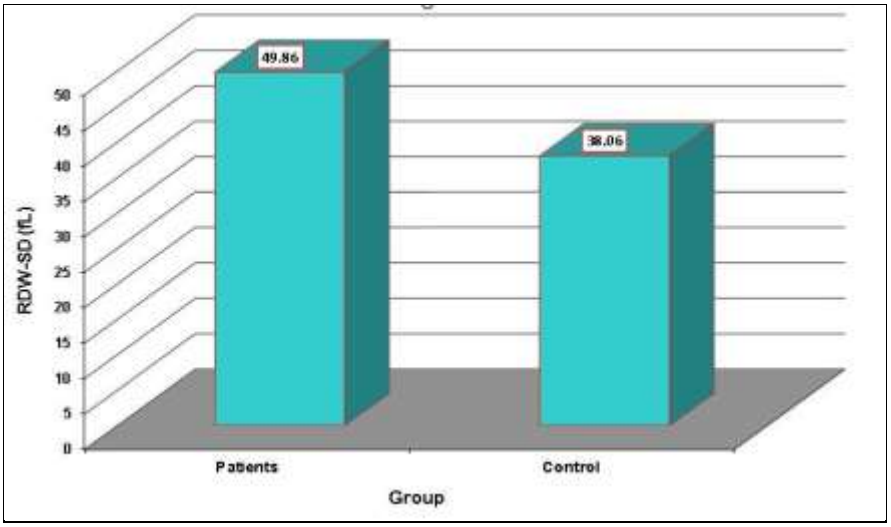

Figure (1-C): Comparison between patients and control for RDW-SD in β-thalassemia patient.

Ferritin is the main form in which iron is stored in the body. Small amounts of ferritin are secreted into the plasma throughout the body. The amount of the body's total iron storage in the absence of inflammation is directly connected with the concentration of this plasma (or serum) ferritin, and patients with -thalassemia major had extremely high ferritin levels(10). With a p-value of 0.0001, demonstrates a substantial rise in ferritin levels for individuals with -thalassemia (2929.161877.54 g/l vs. 21.9614.21 g/l in the control group).

It was discovered that 87.4% of patients with -thalassemia major had extremely high ferritin levels. The mean serum ferritin level, which is consistent with the current investigation, was determined to be 2767.52 ng/ml. These ferritin levels indicate insufficient chelation and susceptibility to consequences from iron overload (11). Children with major -thalassemia showed no significant variation in serum ferritin levels.

Demographic studies

Distribution of β-thalassemia patients and control according to age, sex life style and family history

Patient and control populations for -thalassemia are distributed according to age, sex, and family history. Patients with -thalassemia ranged in age from (14–40) years (mean 18.6–5.1*):* whereas the age of the control group was also between (14–40). The 36 patients with the highest percentage of thalassemia cases (72.0%) were under the age of 20. As shown in Table (5 A*):* 3 patients (6.00%) at age (30) years were followed by 11 (22.00%) for patients (20-29) years.

The results of the -thalassemia patient group showed that the largest percentages in terms of age (20) years compared with the control groups were 36(72.00%*):* 32(64.00%*):* and (20-29) years were 11(22.00%) for patients and 10(20.00%) for controls, respectively. While the means were (18.6 5.1) for patients and (20.2 6.6) for the control group with a P value of (0.279*):* the (30) years were 3 (6.00%) for patients and 8 (16.00%) for the control group.

Table (5 A,B,C): Distribution of β-thalassemia patients and control according to age, sex life style and family history.

A

Demographic characteristics		β-Thalassemia		Control		P value
		No	%	No	%	
Age (years)	<20 years	36	72.0	32	64.0	0.279
	20---29	11	22.0	10	20.0	
	≥30 years	3	6.0	8	16.0	
	Mean±SD (Range)	18.6 ±5.1 (14-35)		20.2±6.6 (14-35)		0.200

B

Demographic characteristics		β-Thalassemia		Control		P value
		No	%	No	%	
Sex	Male	15	30.0	13	26.0	0.656
	Female	35	70.0	37	74.0	

C

Factor		No	%	P-value
Location	Urban	30	60.00	0.048 *
	Rural	20	40.00	
Life style	High	12	24.00	0.0392 *
	Low	17	34.00	
	Medium	21	42.00	
* (P≤0.05) ** (P≤0.01)				

The current study's findings were in line with those of Wasit (12*):* whose research revealed that the prevalence of the condition increased with age (11–18) and was 54(90.00%).

β-thalassemia patients were 35 (70%) percent female overall, 15 (30.00) percent related to male patients, and the control group included 37 (74.00%) percent females and 13 (26.00%) percent males, according to Table 5b, with a P value of (0.656).

The number of males with thalassemia was (111) people, at a rate of 55.5%, which is in line with a study by (13) conducted in Baaquba City, Iraq, which found that the number of males with the disease is larger than the number of infected females. In terms of females, there were (89*):* or 44.5%, who could have a sex-related reason.

Increased knowledge, attitude, and practise awareness among patients and their parents about the condition and its management is the most efficient strategy to lessen the difficulties that thalassemia patients experience. This will also improve their quality of life. This will enhance their ability to manage their chronic illness and its difficulties while it is being managed. Education, time away from school, sports, differences from classmates and siblings, social interactions, and stigmatisation are all significantly impacted by its problems. For the creation of effective therapeutic programmes, social support, and better treatment outcomes, it is essential to have knowledge of the variables that affect quality of life in thalassemia patients (14).

There was a significant difference in the distribution of patients with high life style (H) 12 (24.00%*):* low life style (L) 17 (34.00%*):* and moderate life style (M) 21 (42.00%*):* as shown in Table (5 C): with a P-value of (0.0392).

The table (5 C) shows a significant difference (P0.05) in the distribution of -thalassemia patients between urban (U) 30(60.00%) and rural (R) 20(40.00%) with a P-value (0.048). Other factors that affect -thalassemia patients include education, therapy, and geography.

All patients with -thalassemia have a positive family history, proving that the disease is inherited from parents and grandparents, according to data gathered in a questionnaire form and family history for -thalassemia major (15).

Measuring serum ferritin

The body's iron stores are reflected in the plasma ferritin level. The standard method to assess iron overload and other issues seen in thalassemia subjects, such as growth impairment, is serum ferritin measurement (20).

Molecular Analysis

DNA from a genome extracted

Using a DNA purification kit (DNase I enzyme kit) and following a procedure developed by (Promega company*):* genomic DNA is extracted and purified from whole blood for -thalassemia patients and controls. All samples had bands on the gel electrophoresis that represented genomic DNA. In Figure (2), 18 samples are shown.

The NanoDrop spectrophotometer automatically calculates the nucleic acid concentration and purity ratio, with acceptable ranges of (100- 700 ng/ml) and (1.8-2) correspondingly.

Genetic polymorphism of *Hep. gene* (*rs10421768*) and (*rs1173345431*)

The regulation of iron metabolism relies heavily on *Hep.*. When it binds ferroportin, the primary iron export protein, it causes its internalisation and breakdown, which causes iron to be sequestered within ferroportin-expressing cells. Erythropoiesis with iron restriction and/or systemic iron insufficiency are caused by abnormally high *Hep.*. Additionally, numerous conditions linked to iron overload show inadequately high *Hep.* levels (16). The *Hep.* gene was chosen for this work in order to discover genetic variation utilising high-resolution technique (HRM Real-Time PCR). The SNP (rs10421768) was chosen so that its relationship to the primary form of -thalassemia could be examined.

Hep. gene (rs10421768) genotyping was performed on DNA samples from the two study groups. HRM real-time PCR was employed to detect SNPs. Figure (2 A, B) displays the thermocycler's output for the two genotypes. Additionally, the HRM output for the genotype is shown in Tables (6, 7).

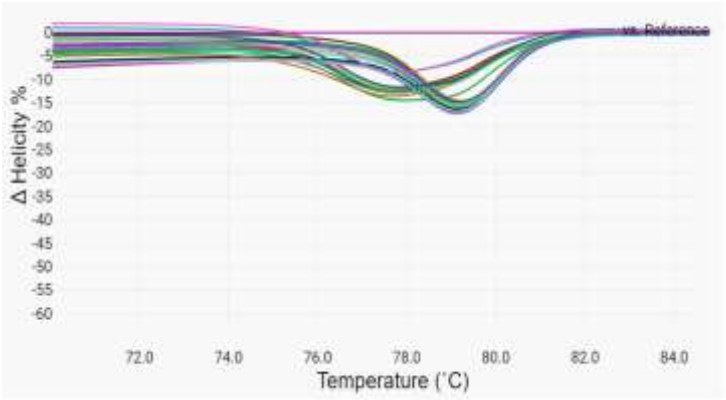

Figure (2 A) : Real time PCR high resolution melting data analysis *Hep. gene* polymorphism that showed homozygote mutant types samples by using uAnalyze [SM] is a web-based application.

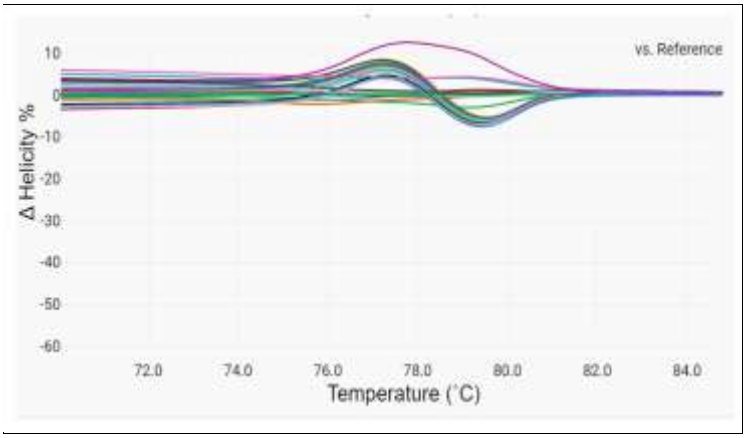

Figure (2 B): Real time PCR high resolution melting data analysis *Hep. gene* polymorphism that showed heterozygote samples by using uAnalyze [SM] is a web-based application.

Genetic polymorphism and allele frequency of *Hep.* (rs10421768) A>G

The results of genotype and allele frequency of the SNPI *Hep.* gene (rs10421768) AG in patients with -thalassemia and the control group are represented in Table (6).

The current study's findings demonstrated that there was no statistically significant difference between the wild type AA for SNP (rs10421768) in the patient group of -thalassemia patients and the control group 26 (52.00%) or the control group 30 (60.00%).

According to the analysis of the GG genotype results, there was no discernible difference between the mutant control group and the thalassemia patients.

Additionally, it was shown that there were no variations in homozygous AG between the control group (heterozygous) 13(26.00%) and thalassemia patients 11(22.00%) (p0.01).

Table (6): Genotype distribution and allele frequency by hardy- of *Hep*. gene polymorphism (rs10421768) in patients and control groups.

Genotype (rs10421768)	Patients (No. = 50)	Control (No. =50)	Chi-Square	*P*-value	OR (C.I.)
AA	26 (52.00%)	30 (60.00%)	0.761 NS	0.438	Ref.
AG	11 (22.00%)	13 (26.00%)	0.894 NS	0.837	0.431 (0.24-0.92)
GG	13 (26.00%)	7 (14.00%)	2.051 NS	0.164	0.557 (0.28-1.16)
Allele frequency					
A	63 (0.73)	73 (0.73)	P-value= 0.077 NS		
G	37 (0.27)	27 (0.27)	P-value= 0.084 NS		
NS: Non-Significant.					

The frequency of the A allele was 63 (0.73%) for -thalassemia patients and 73 (0.73%) for controls, while the frequency of the G allele was 37 (0.27%) for patients and 27 (0.27%) for controls. There was no statistically significant difference in genotype and allele frequency between -thalassemia patients and controls, with P-values for the A allele and the G allele being (0.077) and (0.084*):* respectively.

Genetic polymorphism and allele frequency of *Hep.* (rs1173345431) C>T

The genotype and allele frequency distribution of the SNPII *Hep.* gene (rs1173345431) CT in

patients with -thalassemia and the control group are shown in Table (7).

According to the current study's findings, there was no significant difference between the genotype CC for wild type SNP (rs1173345431) in patients with -thalassemia and the control group 39 (78.00%) or the control group 42 (0.84%).

According to the TT genotype results, there was no discernible difference between the homomutant control group and the patients with -thalassemia.

Additionally, genotype CT results showed no significant differences (p0.01) between the control group (Heterozygous) and the thalassemia patients 8(16.00%).

The frequency of the C allele was 86 (0.86%) for -thalassemia patients and 39 (0.65%) for controls, while the frequency of the T allele was 21 (0.35%) for controls and 14 (0.14%) for patients. The genotype and allele frequency differences between -thalassemia patients and controls were not statistically significant, with P-values for the C allele and T allele, respectively, being (0.583) and (0.411*):* respectively.

Table (7): Genotype distribution and allele frequency by hardy- of *Hep.* gene polymorph (rs1173345431) in patients and control groups.

Genotype rs1173345431	Patients (No. = 50)	Control (No. =50)	Chi-Square	*P*-value	OR (C.I.)
CC	39 (78.00%)	42 (0.84%)	0.592 NS	0.769	Ref.
CT	8 (16.00%)	8 (0.16%)	0.00 NS	1.00	0.055 (0.02-0.37)
TT	3 (6.00%)	0 (0.00%)	0.844 NS	0.307	0.28 (0.13-0.62)
Allele frequency					
C	86 (0.86)	39 (0.65)	P-value= 0.583 NS		
T	14 (0.14)	21 (0.35)	P-value= 0.411 NS		
NS: Non-Significant.					

In accordance with studies (17*):* there were no significant differences in this study because iron overload in the heart and liver and blood ferritin levels are not affected by the presence of the c.-582 A>G polymorphism (rs10421768) located in the *HAMP* promoter (*HAMP*-P) in patients with -thalassemia major. However, *Hep.* and HFE polymorphisms and ferritin level in -thalassemia major (18) showed

Disagree with research (19) *Hep.* gene polymorphisms and iron overload in patients with -thalassemia major unresponsive to iron chelating treatment, as all results were homozygous and there were no significant changes.

Haplotype analysis for *Hep.* gene for rs10421768 A>G and rs1173345431C>T

Table (8) and Figure (3) present the findings of the haplotype frequency of rs10421768 AG and rs1173345431CT SNPs of *Hep.* gene in Iraqi -thalassemia patients and seemingly healthy control. However, results for GC haplotypes frequency were higher in patients 13(26.0%) than apparently healthy control 8(16.0%*):* with no significant differences with p-value 0.220 and OR = 1.845. The study showed that AC haplotypes frequency was higher in the apparently healthy control 42(84.0%) and 34(68.0%) in patients, and gives no significant

differences with p-value 0.061 and OR = 0.405.

This result suggests that these haplotypes reduce the risk of developing the disease, and the presence of the C allele for (rs1173345431) provides protection even if T is substituted with G in the case of rs10421768. The last haplotypes frequency AT was 3(6.0%) in -thalassemia patients and apparently healthy controls, giving no significant differences with p-value 0.307 and OR = 3.128.

This study suggests a substantial correlation between the disease and this haplotype, which raises the likelihood that those who possess it will develop the disease. This outcome was consistent with the genotyping results, which showed a risk effect for both the variant alleles for rs10421768 (G) and rs1173345431 (T). The TT haplotype revealed no correlation.

In agreement with (Gao *et al.,* 21*):* who came to the conclusion that TC haplotype reduce the incidence of Immunoglobulin A nephropathy (IgAN) in the Chinese population, T rs1173345431 and C rs10421768 haplotype are related with decrease the risk. And disagree with (Kondkar *et al*., 22) who discovered that the TC haplotype increases the risk of Saudi Primary Open-angle Glaucoma (POAG). Kondkar also came to the conclusion that the GC haplotype is a protective factor, which was consistent with the findings of the current study.

Table (8): The frequency of haplotype between rs10421768 A>G and rs1173345431 C>T of *Hep.* gene in Iraqi β-thalassemia patients.

Gene characteristics		β-Thalassemia		Control		χ^2	P value	OR	95%CI
		No	%	No	%				
Haplotype	AC	34	68.0	42	84.0	3.809	0.061	0.405	0.155-1.059
	GC	13	26.0	8	16.0	1.507	0.220	1.845	0.689-4.941
	AT	3	6.0	0	0	1.042	0.307	3.128	0.314-31.14
*Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.									

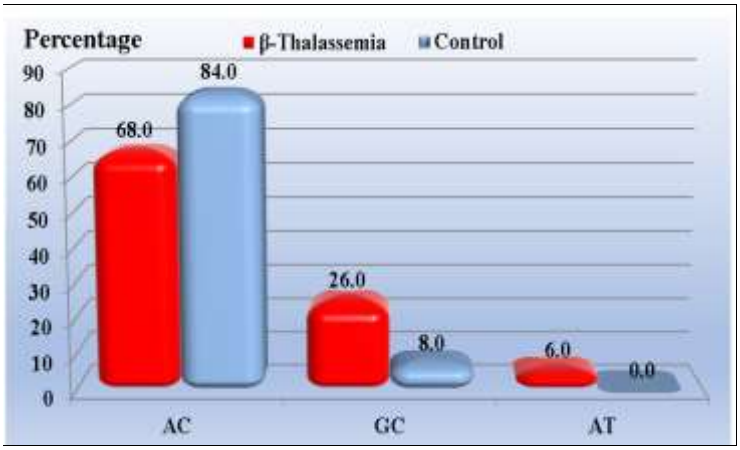

Figure (3): Distribution haplotype frequency for *Hep.* gene rs10421768 and rs1173345431 between patient and apparently health control.

Connection disequilibrium findings based on haplotype revealed a weak connection between the two SNPs rs10421768 and rs1173345431 with D' values of 0.28 and r2 values of 0.07, respectively. Figure (4) displays r 2 and D' values.

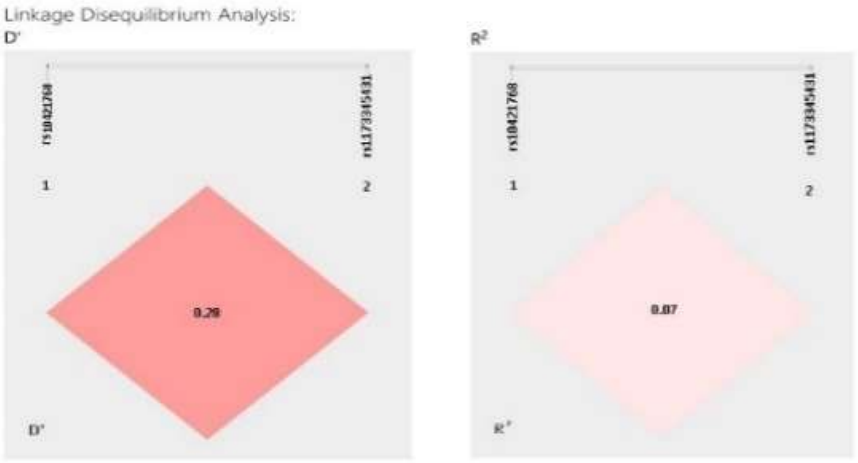

Figure (4): Linkage disequilibrium (LD) plot for rs10421768 and rs1173345431 in Iraqi β-thalassemia patients. D value represent a weak link between the two SNPs.

Correlations between serum iron and *Hep.* gene polymorphism

Studies on iron absorption in beta-thalassemia intermedia patients reveal that the rate of iron loading from the GI tract is roughly three to four times higher than normal. Depending on the degree of erythroid enlargement, improper dietary iron absorption causes non-transfused patients with severe thalassemia to have an elevated body iron burden of 2 to 5 g year (23).

Iron overload is a prevalent complication of HbH illness, according to analysis of nontransfused adult patients. The rate of iron accumulation doubles if frequent transfusions are necessary, as is the case for patients with beta-thalassemia major. Increased iron absorption, whose significance is inversely correlated with Hb levels in beta-thalassemia major, also contributes to the iron overload brought on by transfusions.

Erythropoiesis and iron metabolism are so closely related that changing one of them can have a significant effect on the other. The relative amounts of hypoxia, iron overload, and inefficient erythropoiesis are probably what regulate iron homeostasis in thalassemia. The iron export molecule ferroprotein, which is expressed in enterocytes and macrophages, is inhibited or limited in action by the circulating peptide hormone *Hep.* in response to iron overload (24).However, in conditions of strong erythroid demand, apoptosis, hypoxia, and if levels of erythropoietin (Epo) are elevated, *Hep.* expression can be significantly decreased (25). Therefore, despite the fact that very low levels of *Hep.* are particularly useful in treating acute or temporary blood loss, their prolonged downregulation will lead to iron overload since insufficient erythropoiesis is insufficient in treating anaemia.

The connection between serum iron and the *Hep.* gene polymorphism for SNIPs rs10421768 and rs1173345431 in Iraqi -thalassemia major patients is significantly different, as shown in Table (9) for patients.

Table (9): The correlation between serum iron and *Hep.* gene polymorphism

		Serum iron (µmol/l)		P value
		β-Thalassemia	Control	
***Hep.* gene (rs10421768 genotype)**	AA	10.00±1.63	12.78±3.02	0.0001#
	AG	10.16±1.61	11.50±4.41	0.353
	GG (Ref)	9.32±1.21	13.96±2.74	0.0001#
***Hep.* gene (rs1173345431 genotype)**	CC	9.73±1.41	12.17±3.34	0.0001#
	CT	10.14±1.49	14.91±3.03	0.001#
	TT (Ref)	10.82±3.18	-	-
#Significant difference between two independent means using Students-t-test at 0.05 level.				

Conclusion

In this study, environmental and genetic factors including place, family history of thalassemia, disease severity, length of blood transfusions, elevated serum ferritin levels, and the existence of the A allele and AA genotype in the *Hep.* gene (rs10421768) were examined. They could be viewed as crucial elements in the development of -thalassemia in Iraqi individuals. The SNP (rs1173345431TC) *Hep.* gene has not been studied in relation to -thalassemia in the Iraqi population, and this study is the first of its kind in terms of the Iraqi people.

References

1. Lama, R.; Yusof, W.; Shrestha, T. R.; Hanafi, S.; Bhattarai, M.; Hassan, R. et al. (2021). Prevalence and distribution of major β-thalassemia mutations and HbE/β-thalassemia variant in Nepalese ethnic groups. Hematology/oncology and stem cell therapy. (in press).
2. Nienhuis, A. W. and Nathan, D. G. (2012). Pathophysiology and clinical manifestations of the β-thalassemias. Cold Spring Harbor Perspectives in Medicine, 2(12).
3. Muncie, J. H. L. and Campbell, J. S. (2009). Alpha and Beta thalassemia. American Family Physician, 80(4): 339-344.
4. SAS, S. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9. SAS. Inst. Inc. Cary. NC USA.
5. Kababi, S. E.; Khalfi, B. E.; Maani, K. E. and Soukri, A. (2020). Beta Thalassemia Major: Overview of Molecular Etiology, Pathophysiology, Current and Novel Therapeutic Approaches. International Blood Research & Reviews, 10(2): 1-15.
6. Forget, B. G. (2010). Preface: thalassemia. Hematology/Oncology Clinics, 24(6): 13-15.
7. Ayyash, H. and Sirdah, M. (2018). Hematological and biochemical evaluation of β-thalassemia major (βTM) patients in Gaza Strip: A cross-sectional study. International Journal of Health Sciences, 12(6): 18.
8. Faraj, S. A.; Abd Al Jabar, H. N. and Mahdi, L. S. (2019). Value of Cell Counter-Based Parameters and Formulas in Detection of βThalassemia Minor, the experience of a single Haematological Centre in Iraq. International Journal of Pharmaceutical Research and Allied Sciences.
9. Vichinsky, E. P. (2005). Changing patterns of thalassemia worldwide. Annals of the New York Academy of Sciences, 1054(1): 18-24.
10. Mishra, A. K. and Tiwari, A. (2013). Iron overload in Beta thalassaemia major and intermedia patients. Maedica, 8(4): 328.
11. Koreti, S.; Gaur, B. K.; Das, G. and Gaur, A. (2018). Study of Serum ferritin levels in β-Thalassemia major children. International Journal Pediatric Research, 5(6): 308-313.
12. Khalaf, M. A.; Al-Saadi, B. Q. H. and Mohammed, H. Q. (2022). Evaluation of TLR-3, TLR4, IL-7, and IL37 Immunological Markers in β-Thalassemia Major Iraqi Patients. Iraqi Journal of Biotechnology, 21(1).
13. Sahm, S. F. (2022). Studying Some Factors That Increase the Risk of Thalassemia in The City of Baquba. Journal of Techniques, 4(33): 181-184.
14. Cappellini, M. D.; Cohen, A.; Porter, J.; Taher, A. and Viprakasit, V. (Eds.). (2014). Guidelines for the management of transfusion dependent thalassaemia (TDT).
15. Shafique, F.; Ali, S.; Almansouri, T.; Van Eeden, F.; Shafi, N.; Khalid, M., *et al*. (2021). Thalassemia, a human blood disorder. Brazilian Journal of Biology, 83.
16. Ginzburg, Y. Z. (2019). *Hep.*-ferroportin axis in health and disease. Vitamins and hormones, 110, 17-45.
17. Fekri, K.; Asle Rasouli, N.; Tavallai Zavareh, S. A.; Jalil, M.; Moradi, F.;

Hosseinpour, M., *et al*. (2019). *Hep.* and HFE Polymorphisms and Ferritin Level in β-Thalassemia Major. International Journal of Hematology-Oncology and Stem Cell Research, 13(1): 42–48.

18. Jabbar, Esraa E. and Wejdan T. Mahdi. "The *Hep.* Gene Polymorphism in Patients with B -Thalassemia." International Journal of Health Sciences, I,. 2022, 11594-11601.
19. Zarghamian, P.; Azarkeivan, A.; Arabkhazaeli, A.; Mardani, A. and Shahabi, M. (2020). *Hep.* gene polymorphisms and iron overload in β-thalassemia major patients refractory to iron chelating therapy. BMC Medical Genetics, 21(1): 1-5.
20. Singh, P. and Seth, A. (2017). Growth and endocrine issues in children with thalassemia. Pediatric Hematology Oncology Journal, 2(4): 98-106.
21. Gao, J.; Wang, M.; Wei, L.; Niu, D.; Wei, J.; Ou, Y.; Wang, L., *et al.* (2017). The endothelial nitric oxide synthase gene polymorphism is associated with the susceptibility to immunoglobulin a nephropathy in Chinese population. Kidney and Blood Pressure Research, 42(3): 608-616.
22. Kondkar, A. A.; Azad, T. A.; Sultan, T.; Osman, E. A.; Almobarak, F. A. and Al-Obeidan, S. A. (2020). Association of endothelial nitric oxide synthase (NOS3) gene polymorphisms with primary open-angle glaucoma in a Saudi cohort. PLoS One, 15(1): e0227417.
23. Gardenghi, S.; Marongiu, M. F.; Ramos, P.; Guy, E.; Breda, L.; Chadburn, A., *et al*. (2007). Ineffective erythropoiesis in β-thalassemia is characterized by increased iron absorption mediated by down-regulation of *Hep*. and up-regulation of ferroportin. Blood, The Journal of the American Society of Hematology, 109(11): 5027-5035.
24. Nemeth, E.; Tuttle, M. S.; Powelson, J.; Vaughn, M. B.; Donovan, A.; Ward, D. M., *et al*. (2004). *Hep.* regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science, 306(5704): 2090-2093.
25. Pinto, J. P.; Ribeiro, S.; Pontes, H.; Thowfeequ, S.; Tosh, D.; Carvalho, F., *et al*. (2008). Erythropoietin mediates *Hep*. expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. Blood, 111(12): 5727–5733.