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ORIGINAL RESEARCH ARTICLE

Role of blood hepcidin alteration as a biomarker in β-thalassemia patients' diagnosis

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ABSTRACT

The objective of this research was to determine the change in hepcidin levels and other biochemical markers, including total iron binding capacity (TIBC), serum iron, testosterone, and specific vitamins in the blood of individuals with β-Thalassemia. Here, 140 participants were involved in the study, of whom 110 were affected by β-thalassemia and 30 were healthy. The samples were obtained from Baqubah Teaching Hospital, the blood bank and blood donation center. The study was carried out from May 2022 to August 2022, and the patients were housed in Diyala Provence, Baquba City and its suburbs. The participants in this investigation were split into three groups: A: 55 male patients with β-thalassemia; B: 55 female patients with β-thalassemia; and C: 30 healthy individuals that were set as a control group. The findings of the study showed that the levels of HbA1 in males and females were 93.44 ± 0.71 and 93.53 ± 0.91, respectively, while the levels of HbA2 in males and females were 4.99 ± 0.59 and 5.29 ± 0.72, respectively. In contrast, the control group had HbA1 levels of 97.33 ± 0.40 in males and 97.66 ± 0.46 in females, but HbA2 levels were 2.32 ± 0.33 in males and 2.24 ± 0.24 in females. The study revealed remarkable differences ($P < 0.05$) between these variables. Hematological measures, such as hemoglobin concentration and percentages of mean corpuscular volume (MCV), packed cell volume (PCV), and mean corpuscular hemoglobin (MCH) were substantially reduced ($P < 0.05$) in β-thalassemia patients when compared to the controls. Serum iron and TIBC were significantly increased ($P < 0.05$), while Hepcidin levels were markedly decreased ($P < 0.05$) in the serum of β-thalassemia patients compared to controls. Therefore, as a conclusion the reduction of hepcidin levels and increase in iron levels are correlated with β-thalassemia and can be used as ~~a~~ biomarkers in monitoring β-thalassemia disease.

Keywords: hepcidin; β-Thalassemia; serum iron; TIBC; testosterone; vitamins

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1. Introduction

Thalassemia is a type of inherited anemia that results from mutations that occur during the production of globin, the protein component of hemoglobin. Globally, thalassemia causes several public health problems[1,2]. Most individuals affected by thalassemia live in Mediterranean countries, particularly those located near the equator in Africa and Asia[3,4]. Thalassemia is classified into different types based on which globin chain(s) are significantly deficient. An unequal production of globin chains can lead to hemolysis, ineffective erythropoiesis, and varying degrees of anemia[2,5].

Thalassemia can be classified into three different clinical categories based on its severity: major, which requires lifelong blood transfusions; intermedia, which causes non-fatal anemia on its own; and mild thalassemia, also known as thalassemia trait, which typically

presents with no observable symptoms[6–8]. The type of gene mutation responsible for thalassemia is linked to its phenotype. However, in β-Thalassemia, only one gene is damaged. Beta thalassemia is classified into triple primary subgroups depending on the seriousness of α and β-globin chains' imbalance. Beta thalassemia major (named Mediterranean anemia or Cooley's anemia) is the severest form and results in dangerous anemia that requires regular medical care, including blood transfusions[5]. Beta thalassemia intermedia is a moderately severe form of the condition, and some individuals with this form may require blood transfusions and other medical treatment[9]. Beta thalassemia minor (also known as trait) results in mild anemia, and most individuals with this form do not require any medical intervention[6]. Beta thalassemia is characterized by a deficient or absent β-globin chain[10]. Individuals with thalassemia who receive blood transfusions may experience symptoms of iron overload, which can cause a delay or absence of puberty in children. While iron is a crucial component that enters all life forms and is important for humans, the recommended daily intake varies from children to adults with amounts of 8 mg to 11–18 mg, respectively. In developed countries, the average healthy adult is believed to have iron of around 4–5 g, hemoglobin of approximately 2.5 g, and ferritin making up most of the remaining amount[11].

Iron is predominantly absorbed by the intestinal cells lining the duodenum after ingestion of supplements or digestion of food. The absorbed iron is then transformed into transferrin by binding to apotransferrin. Transferrin-bound iron is loosely bound, enabling it to be transported to various cells in the tissues throughout the body. Ferritin, a protein complex, is the primary storage form of iron that presents in reticuloendothelial cells, liver cells, and erythroid precursors inside bones marrow[12,13]. Iron is crucial for vital metabolic processes, including DNA synthesis, energy metabolism, and oxygen transport, with its biological functions reliant on its ability to interchange the oxidation states between ferrous (Fe^{+2}) and ferric (Fe^{+3}) and its interactions with proteins[14]. Mammals have evolved highly effective mechanisms to absorb iron from their diet, store any surplus, and only release the necessary amount for regular bodily functions. Heme, which is involved in the fabrication of hemoglobin, accounts for around 3–5 g of total body iron in adults[15].

Hepcidin is a peptide hormone with a 25-amino acid sequence. Its principal role is to prevent iron from being absorbed in the duodenum and from being secreted by macrophages and hepatocytes into the circulation[16]. The importance of keeping iron levels steady in the body cannot be overstated. The production of hepcidin, which is produced mostly by the liver, is controlled by the body's iron levels, erythropoietic activity, and immune response[16,17]. Hepatocytes escalate hepcidin production in response to excessive iron levels in the body[18]. Elevated hepcidin concentrations impede further iron absorption or secretion. However, a shortage of iron decreases hepcidin production, which increases iron absorption[18]. Ferric plasma transferrin and iron in hepatocytes promote hepcidin synthesis[19]. High erythropoiesis reduces hepcidin levels, increasing iron availability for hemoglobin production[20,21]. Hepatocytes produce most hepcidin, while macrophages and adipocytes also produce some. β-Thalassemia is characterized by low hemoglobin (7 g/dL), low MCH (12–20 pg), and high MCV (50–70 FL). The disease is connected to hepcidin control[5,22]. The study assessed hematological parameters (Hb, PCV, MCV, and MCH) in β-thalassemia patients by measuring blood iron and TIBC levels. Additionally, β-thalassemia patients' blood levels of hepicidin, testosterone, and vitamins B9 and B12 were compared to healthy persons.

After estimating serum iron and total iron binding capacity (TIBC) levels of β-thalassemia patients, the study aimed to determine the values of several hematological parameters (hemoglobin, packed cell volume, mean corpuscular volume, and mean corpuscular hemoglobin). Furthermore, levels of hepcidin, testosterone, and vitamins B9 and B12 in the serum of β-thalassemia patients were compared with those of healthy controls.

2. Materials and methods

2.1. Patients and controls

In this research, a total of 140 individuals participated, with 110 of them diagnosed with β-thalassemia (55 males and 55 females) at Baquba Teaching Hospital, based on information from the blood bank and blood donation center. The age range of the β-thalassemia patients was 15 to 60 years, with a mean age of 35.81 ± 8.16 years. In addition, 30 healthy individuals (15 males and 15 females) without thalassemia participated as a control group, who were matched with the patients in terms of demographics. The age range of the control group was 20 to 50 years, with a mean age of 30.18 ± 6.81 years. The study was conducted from May-August 2022 in Diyala Provence/Baqubah City and its surrounding areas. Private labs were utilized to conduct the necessary analyses for the study.

2.2. Blood samples: Collection and analysis

Competent nursing professionals obtained blood samples from all participants who were part of the research. Once a 5 mL venous blood sample was collected from each person, it was divided into two tubes. One tube, which contained 1 mL of ethylenediaminetetraacetic acid (EDTA), was used to assess HbA1c within three hours. The other tube, which held 4 mL and was a vacutainer plain tube, was used to produce serum samples. The blood in the vacutainer plain tube was allowed to clot for a short time and then centrifuged for 10 min at 4000 rpm to detach the serum. Resulted serum was then split into five standard Eppendorf tubes and stored at a temperature of −20 °C until the time of analysis. When the serum samples were needed for analysis, they were removed from the freezer and allowed to reach a temperature of 4–8 °C. Finally, the specimens were obligingly agitated at room temperature (RT) to guarantee well-mixing.

2.3. Analytical methods for hematological parameters of groups

Hemoglobin: A testing system for Hemoglobin, called Bio-Variant Rad's, utilized a brief program cation-exchange high-performance liquid chromatography (HPLC) was used to automatically examine both normal and abnormal hemoglobin present in blood samples[23]. The determination of Glycohemoglobin (HbA1 and HbA2) in whole blood was carried out by using electrophoresis and colorimetric methods[24].

Complete Blood Counts (CBC): It is a set of tests used to examine the cells present in a person's blood. This test may help diagnose a range of ailments including anemia, infections, and even leukemia. To perform hematological tests such as HB, RBC, HCT, and MCV, blood (1 mL) in an EDTA test tube was analyzed employing a Sysmax device. In addition, serum iron and total iron-binding capacity (TIBC) concentration were measured. Transferrin, a protein in human blood which aids in the transportation of iron throughout the body, binds to the iron in the blood. TIBC measurements could be employed in detecting and monitoring iron deficiency anemia, chronic inflammatory disorders, and Transferrin saturation.

Hepcidin: An ELISA kit obtained from Shanghai Company. China was used to quantify hepcidin in serum. The competitive inhibition enzyme immunoassay technique provided by this kit can be employed to identify and measure the concentration of hepcidin in various human bodily fluids such as plasma, blood, cell lysates, tissue homogenates, and cell culture supernatants.

Testosterone: It was determined by the automated hormones analyzer COBASe411 (Roche, Germany)**.** Vitamin B12: B12 and B9 concentrations were assessed by enzyme linked immuno-sorbent assay (ELISA) kits from Cusabio Biotech Co., LTD, China.

2.4. Ethical approval

The study received ethical approval from the Iraqi Health's National Centre for Training and Human Development/ Baquba Teaching Hospital Ethics Committee, and the University of Diyala Ethical Committee. Before participating, all individuals provided written detailed consent.

2.5. Statistical analysis

The statistical analysis of the information was carried out using Excel and Minitab, a tool for statistical

analysis. The results were presented as mean and the standard deviation was calculated. The Dunkin' multiple and the analysis of variance (ANOVA) tests were utilized to compare the geometric means of the experimental groups and to determine the statistical differences between them. Significant differences were reported when the probability level was smaller than 0.5, and the absence of noticeable variations was reported once it was larger than 0.5. The use of letters was employed to identify major distinctions (similarity and difference) between two things.

3. Results

3.1. Quantitative and qualitative assessment of Hemoglobin and comparative in studied groups

The aim of this study was to figure out the proportions of different kinds of hemoglobin (HbA1 and HbA2) in β thalassemia anemia patients, as well as in healthy individuals. Hemoglobin concentration was measured, and qualitative and quantitative estimations of hemoglobin were used to obtain these percentages. The results indicate that HbA1 levels in male and female patients were 93.44 ± 0.71 and 93.53 ± 0.91, respectively, while HbA2 levels were 4.99 ± 0.59 and 5.29 ± 0.72, respectively. In contrast, the control group showed HbA1 levels of 97.33 ± 0.40 in males and 97.66±0.46 in females, and HbA2 levels of 2.32 ± 0.33 in males and 2.24 ± 0.24 in females. The goal of this analysis was comparing the hemoglobin proportions of patients with β thalassemia anemia with those of healthy individuals. **Table 1** summarizes the results.

Table 1. Qualitative and quantitative estimation of hemoglobin in studied groups.

Parameters	**Controls (30)**		**Patients (110)**		***P*-Value**
	Males (15)	**Females (15)**	**Males (55)**	**Females (55)**	
HbA1%	97.33 ± 0.40	97.66 ± 0.46	93.44 ± 0.71*	93.53 ± 0.91*	10^{-4}
HbA2%	2.32 ± 0.33	2.24 ± 0.24	4.99 ± 0.59*	5.29 ± 0.72*	10^{-4}

* Significant.

3.2. Comparison of hematological parameters in studied groups

Table 2 presents the results of the comparison between patients with β-thalassemia and the controls in terms of hematological parameters. The data indicate that the patients had obviously ($P < 0.05$) minimal hemoglobin concentration, pulmonary capillary wedge pressure, mean corpuscular volume, and mean corpuscular hemoglobin concentration than the control group.

Table 2. Comparative hematological parameters in studied groups.

Parameters	**Controls (30)**		**Patients (110)**		***P*-Value**
	Males (15)	**Females (15)**	**Males (55)**	**Females (55)**	
Hb (g/dL)	14.97 ± 2.12	14.29 ± 1.29	8.15 ± 0.81*	7.87 ± 1.13*	0.001
PCV%	43.98 ± 5.85	41.16 ± 2.76	30.18 ± 2.08*	23.55 ± 1.42*	0.001
MCV (FL)	78.82 ± 4.79	73.91 ± 4.94	68.73 ± 5.63*	62.91 ± 4.89*	0.003
MCH (Pg)	30.04 ± 1.38	27.19 ± 1.27	18.65 ± 1.27*	15.98 ± 1.36*	0.002

* Significant.

3.3. Comparison of Iron and TIBC in studied groups

Upon analyzing the data in **Table 3**, it is evident that patients with β-thalassemia had a remarkably higher ($P > 0.05$) iron concentration compared to the control group. The results also showed that the total iron-binding capacity (TIBC) was notably ($P > 0.05$) larger in the patients as compared to the controls.

Table 3. Iron and TIBC concentrations in studied groups.

Parameters	Controls (30)		Patients (110)		*P*-Value
	Males (15)	Females (15)	Males (55)	Females (55)	
Iron (mg/dL)	67.91 ± 12.68	73.95 ± 9.43	119.4 ± 20.93*	108.93 ± 15.8*	0.039
TIBC (mg/dL)	162.33 ± 25.22	150.97 ± 18.94	282.9 ± 42.88*	275.2 ± 34.9*	0.026

* Significant.

3.4. Comparation of Hepcidin, Testosterone, B9, and B12 levels in studied groups

Table 4 presents the findings of hepcidin, testosterone, vitamin B12, and vitamin B9 levels analysis in patients and the control group. The data indicate that the patients had noticeably lower ($P < 0.05$) levels of hepcidin and testosterone compared to the control group. Additionally, the concentrations of vitamin B12 and B9 were significantly lower ($P < 0.05$) in the patients compared with the controls.

Table 4. Hepcidin, Testosterone, B9, and B12 levels in studied groups.

Parameters	Control (30)		Patients (110)		*P*-value
	Male (15)	Female (15)	Male (55)	Female (55)	
Hepcidin (ng/dL)	3.83 ± 0.76	3.56 ± 1.35	0.45 ± 0.27*	0.37 ± 0.33*	0.027
Testosterone (ng/mL)	5.88 ± 0.81	0.34 ± 0.08	1.22 ± 0.34*	0.18 ± 0.04*	0.0001
B12 (Pg/mL)	481.6 ± 32.3	490.43 ± 74.9	145.8 ± 43.6*	139.2 ± 43.5*	0.043
B9 (Pg/mL)	13.95 ± 2.94	12.88 ± 1.76	4.87 ± 0.66*	5.75 ± 1.13*	0.001

* Significant.

4. Discussion

Hemoglobin A (HbA), could be referred to as HbA1, is the predominant type of hemoglobin in human adults, comprising over 97% of the overall hemoglobin in red blood cells. It exists in the form of a tetramer that contains two alpha and two beta subunits ($\alpha 2\beta 2$)[25]. On the other hand, Hemoglobin A2 (HbA2) is an infrequent type of adult hemoglobin structured of two alpha and two delta-globin subunits. It makes up only 3% of the total hemoglobin in adults[26]. **Table 1** demonstrated a remarkable decrease in the mean values of hemoglobin A1 in the studied groups of β-thalassemia patients compared to the control group. On the other hand, there was a significant increase in the mean values of hemoglobin A2 in β-thalassemia patients compared to the control group. These values can be used as reliable indicators for the diagnosis of β-thalassemia. Galanello and Origa in 2010[5] noted a marked variation in the qualitative and quantitative estimation of hemoglobin in thalassemia patients thanks to genetic molecular-level differences. This involves the kind of mutations causing the illness and their effects on the hemoglobin types (HbA1 and HbA2) ratios and the chromosomal abnormalities (homozygous, heterozygous, and compound)[27,28].

In the current work, it was observed that thalassemic patients had minimal levels of both PCV and Hb compared to the healthy individuals. The decrease in these values can be attributed to ongoing anemia and poor perfusion, leading to hypoxia. The thalassemic patients evaluated in this study showed remarkable microcytosis out of hypochromia, in addition to other appreciable changes in RBC mass and related indices such as RBC count, HCT, and MCV. The results are consistent with the usual clinical manifestations reported in persons with thalassemia, who frequently require blood transfusions[29]. Patients diagnosed with β-thalassemia in Sicily had notable alterations in their hematological parameters, such as profound anemia, heightened platelet count, and high white blood cell count[30]. A possible link may exist between the kind and degree of poikilocytosis observed in a patient with the seriousness of their clinical state[29]. The study documented a reduction in the quantity of red blood cells (RBCs) in persons with β-thalassemia, perhaps as a result of macrophages phagocytosing RBCs inside the reticuloendothelial system. Disorders such as

anisocytosis, poikilocytosis, and the presence of target cells, which are caused by the buildup of α chains on the membrane of red blood cells, might result in the demise of these cells[31,32]. As per prior research, the study showed a notable reduction in hemoglobin levels among persons with β-thalassemia as compared to the control group[33–35]. The decline in the number of red blood cells can be linked to insufficient generation of heme and a reduction or absence of synthesis of β globin chains[36,37]. The decrease in packed cell volume (PCV) in persons with β-thalassemia, as compared to the control group, aligns with previous research findings[38]. The observed drop can be attributed to a reduction in the quantity of red blood cells, the occurrence of microcytosis, and a loss in hemoglobin concentration. The findings of this investigation are consistent with those reported in other studies[39,40].

The TIBC test measures blood iron binding and transport. The liver's transferrin protein, which regulates blood iron absorption, is measured in the transferrin test. TIBC depends on blood transferrin for iron binding. Thalassemia, a hereditary blood condition, can cause organ and tissue iron buildup. In thalassemia patients, TIBC and transferrin levels, among other indicators, are high, suggesting these tests may be diagnostic[41,42]. Thalassemia patients experience higher plasma iron turnover due to an increased intake of iron resulting from ineffective erythropoiesis and regular blood transfusions[43]. On the other hand, iron deficiency anemia is associated with low iron stores in the body, while hemolytic anemias, such as thalassemia, are characterized by elevated serum iron levels. A study reported a significant difference in TIBC levels between β-thalassemia patients and those with other types of thalassemia, as well as a control group. The same study found that TIBC and serum iron levels are better indexes than transferrin levels in iron overload tracking in children with thalassemia. Diagnosis of β-thalassemia in anemic children involved an enhancement in serum iron levels but a decrease in TIBC ones[44].

Hepcidin is a hormone responsible for regulating iron levels in the body. Its primary function is to prevent the release of iron into the plasma by inhibiting the absorption of iron from food in the duodenum and blocking the release of iron by macrophages and hepatocytes[17]. In response to high iron levels, the body produces more hepcidin to limit further absorption. Conversely, during iron deficiency, hepcidin secretion is reduced to allow for more efficient iron absorption. Hepcidin levels increase during inflammation and decrease with hypoxia or iron overload[45,46]. Despite having excess iron, patients with β-thalassemia often exhibit low levels of hepcidin, likely due to tissue hypoxia and the high iron demand for erythropoiesis.

The study revealed a noteworthy reduction in testosterone levels, which is consistent with earlier research investigating the link between hypogonadism and sexual dysfunction, delayed or absent sexual development, amenorrhea, and infertility in β-thalassemia patients[47]. The findings of the current study, especially the marked increase in ferritin, align with previous research on the pituitary-gonadal axis and the endocrine system, which suggested that the primary cause of gonadal failure is iron accumulation in the gonads.

Vitamin B9 (Folate) and B12 are necessary coenzymes in the human body. Folate and B12 decreased in the patients' blood compared to healthy individuals. Folate and B12 are vital B vitamins that play a critical role in the synthesis of white and red blood cells inside the bone marrow, as well as in the synthesis of DNA and RNA, and the conversion of carbohydrates into energy. A deficiency of the coenzyme needed for DNA synthesis can result in megaloblastic anemia, a condition characterized by enlarged, immature red blood cells. Megaloblastic anemia often results from a lack of vitamin B12 due to its critical role in folate metabolism[48,49]. When there is not enough B12, folate becomes trapped in an inactive form, preventing its release biochemically and ultimately leading to a limited production of DNA[50].

5. Conclusions

Patients diagnosed with β-thalassemia exhibited statistically significant decreases in hemoglobin concentration, MCV, PCV, and MCH ratios compared to the healthy control group ($P < 0.05$). Conversely, the

patient group demonstrated higher levels of serum iron concentrations and TIBC than the healthy control group, although these increases did not reach statistical significance ($P > 0.05$). Furthermore, patients showed statistically significant reductions ($P < 0.05$) in testosterone levels, as well as decreased levels of vitamin B9 (folate) and vitamin B12 compared to the healthy group. Notably, serum iron and TIBC were significantly increased ($P < 0.05$), while hepcidin levels were markedly decreased ($P < 0.05$) in the serum of β-thalassemia patients compared to controls. In conclusion, the observed decrease in hepcidin levels and increase in iron levels are strongly associated with β-thalassemia and can serve as biomarkers for monitoring the disease.

Author contributions

Conceptualization, WYMA and KSS; methodology, WYMA; software, ESM; validation, WYMA, KSS and ESM; formal analysis, KSS; investigation, ESM; resources, MK; data curation, MK; writing—original draft preparation, WYMA; writing—review and editing, MK; visualization, ESM; supervision, WYMA; project administration, ESM. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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