DETERMINAÇÃO DO EFEITO DA SOBRECARGA DE FERRO NA VITAMINA D E DA PERCENTAGEM DE CADA GRUPO SANGUÍNEO EM PACIENTES COM TALASSEMIA

DETERMINATION THE EFFECT OF IRON OVERLOAD ON VITAMIN D AND THE PERCENTAGE OF EACH BLOOD GROUPS IN PATIENTS WITH THALASSEMIA

تحديد تأثير الحديد الزائد على فيتامين د ونسبة كل فصيلة دم لدى مرضى الثلاسيميا

AL-MASAOODI, Rusul Ali^{1*}; ALWAID, Shumoos H¹; Al-SHEMERY, Maryam Kadhim¹

¹ Department of Medical Laboratory Technique, College of Medical and Technique, Islamic University, 54001 Najaf, Iraq.

* Corresponding author
e-mail: rusulali.k@gmail.com

Received 12 June 2020; received in revised form 22 August 2020; accepted 09 September 2020

RESUMO

A talassemia pode ser caracterizada como anormalidades eritrocitárias hereditárias resultantes do defeito na síntese da cadeia de globina da hemoglobina que leva à anemia hipocrômica microcítica. Este estudo teve como objetivo encontrar alguma relação entre o sistema ABO e a doença talassêmica e determinar a relação entre ferritina e vitamina D em pacientes com talassemia. Este estudo estimou o nível de Hb, ferritina e vitamina D e também calculou a percentagem dos tipos sanguíneos ABO em pacientes talassêmicos e não-talassêmicos (grupo controle). Este estudo foi conduzido com 60 amostras sendo 20 para grupo controle e 40 de pacientes com talassemia. A idade dos pacientes com talassemia e controles variou de 2 a 29 anos. Este estudo encontrou a correlação entre ferritina e vitamina D. Os resultados demonstraram uma redução significativa ($p < 0,05$) de Hb e vitamina D, mas um aumento significativo ($p < 0,05$) na concentração de ferritina. O estudo encontrou ainda uma relação negativa não-significativa ($p < 0,05$) entre o nível de ferritina e vitamina D. Foi demonstrado que o grupo sanguíneo O está mais presente em pacientes talassêmicos (40%) ao passo que os grupos A, B e AB, estão, respectivamente, presentes em 23%, 28%, 10% dos pacientes. Pode-se concluir que os pacientes com talassemia são mais frequentes com o sangue tipo O seguido do tipo B e, menos frequente, no tipo sanguíneo AB.

Palavras-chave: *Talassemia; vitamina D; Ferritina; Sistema de grupo sanguíneo ABO.*

ABSTRACT

Thalassemia can be characterized as hereditary erythrocyte abnormalities resulting from the defect in the synthesis of the hemoglobin globin chain that leads to microcytic hypochromic anemia. This study aimed to find some relationship between the ABO system and thalassemia disease and to determine the relationship between ferritin and vitamin D in patients with thalassemia. This study estimated the level of Hb, ferritin, and vitamin D and also calculated the percentage of ABO blood types in thalassemia and non-thalassemia patients (control group). This study was conducted with 60 samples, 20 for the control group, and 40 for patients with thalassemia. The age of patients with thalassemia and controls ranged from 2 to 29 years. This study found a correlation between ferritin and vitamin D. The results demonstrated a significant reduction ($p < 0.05$) of Hb and vitamin D, but a significant increase ($p < 0.05$) in the concentration of ferritin. The study also found a non-significant negative relationship ($p < 0.05$) between the level of ferritin and vitamin D. It was demonstrated that blood group O is more present in thalassemia patients (40%) whereas groups A, B, and AB, respectively, are present in 23%, 28%, 10% of patients. It can be concluded that patients with thalassemia are more frequent with blood type O followed by type B and, less frequently, blood type AB.

Keywords: *Thalassemia; vitamin D; Ferritin; ABO blood group system.*

المخلص

مرض الثلاسيميا يمكن أن يُعرف على أنه تشوهات وراثية في كريات الدم الحمراء ناتجة عن خلل في تصنيع سلسلة غلوبين الهيموجلوبين يؤدي إلى فقر الدم الناقص الصبغ. هدفت هذه الدراسة إلى إيجاد علاقة بين نظام زمر الدم ABO ومرض الثلاسيميا وتحديد العلاقة بين الفيريتين وفيتامين د في مرضى الثلاسيميا. قدرت هذه الدراسة مستوى الهيموجلوبين والفيريتين وفيتامين د أيضًا حسب النسبة المئوية لأنواع ABO في مرضى الثلاسيميا وير المصابين

بالتلاسيميا (المجموعة الضابطة). أجريت هذه الدراسة على (60) عينة، (20) عينة للمجموعة الضابطة، 40 عينة لمرضى التلاسيميا. تراوحت اعمار مرضى التلاسيميا والمجموعة الضابطة بين (2-29) سنة. وجدت هذه الدراسة علاقة بين الفيريتين وفيتامين د. وأظهرت النتائج انخفاضاً معنوياً ($p < 0.05$) في الهيموغلوبين وفيتامين د، ولكن زيادة معنوية ($p < 0.05$) في تركيز الفيريتين. كما وجدت الدراسة وجود علاقة سلبية غير معنوية ($p < 0.05$) بين مستوى الفيريتين وفيتامين د. من النتائج تبين أن فصيلة الدم O هي الأكثر وجوداً في مرضى التلاسيميا (40%) بينما مجموعات الدم الأخرى من النوع A ، B ، و AB موجودة بنسبة (23%، 28%، 10%) على التوالي من المرضى. يمكن الاستنتاج أن مرضى التلاسيميا يكونون أكثر تكراراً مع فصيلة الدم O يليها فصيلة B ، والأقل تكراراً ، فصيلة الدم AB.

الكلمات المفتاحية: التلاسيميا، فيتامين د، الفيريتين، نظام زمر الدم ABO.

1. INTRODUCTION:

The thalassemia is an inherited disease of the blood that causes your body to have less hemoglobin than normal. Hemoglobin allows red blood cells to carry oxygen. Thalassemia accounts for about 3% of the world population, which has β -thalassemia, being the most common of congenital diseases in the world (Gregg *et al.*, 2019). Thalassemia results from a reduction in the synthesis of one or more of the polypeptide chains to hemoglobin (Hb), leading to interference in erythroid maturation, function, and ineffective erythropoiesis (Chaudhari, 2011). Two major factors related to the squeal of thalassemia: decrease the synthesis of the beta-globin chain leading to a reduced level of HbA, unbalanced alpha, and beta-globin chain synthesis (Be-Baun *et al.*, 2011).

Thalassemia was found with high commonness in Mediterranean countries, the Middle East, and Southeast Asia (Wong *et al.*, 2016), data from the World Health Organization (WHO) in 201. There are two major types of thalassemia (Galanello and Origa, 2010): i) α -thalassemia (α -T) – persons with alpha thalassemia, which inadequate synthesis of alpha-globin and ii) β -thalassemia (β -T) – persons with β -T, which inadequate synthesis of a beta-globin chain.

Alpha-Thalassemia is a congenital autosomal recessive disorder produced from mutations, includes deletion within the α -globin gene cluster, including two of alpha 1 ($\alpha 1$) and alpha 2 ($\alpha 2$) globin genes that are found on chromosome 16p13. More than 750 different variants in α -globin genes have been identified, leading to α -thalassemia worldwide (Giardine *et al.*, 2014). The α -thalassemia is most commonly found in the Mediterranean regions, sub-Saharan Africa, Middle East, Indian Subcontinent, East, Southeast Asia, and immigrants to these areas (Weatherall, 2011). The intensity of α -thalassemia relies on the type of mutation (deletional or non-deletional), and copies numbers of affected α - gene (Miri-Moghaddam *et al.*, 2015). The Hb Bart's hydrops fetalis (four defective α -globin genes) or Hb H disease (three

defective α -globin genes) can be diagnosed during prenatal (Zamani *et al.*, 2017).

Beta-thalassemia is considered a prevalent congenital disorder in the world, the cause of this disease is due to reducing or the absence of β -polypeptide chain synthesis. There are 200 mutations linked with a β -thalassemia phenotype that affect the expression phases of the β -globin gene, leading to decrease (β^+) or complete absence (β^0) of β -globin chain production (Bilgen *et al.*, 2013). β -thalassemia includes three main forms (Al-Mosawy, 2017):

i) β -thalassemia minor (trait) – is a heterozygous disorder that single mutation in the β -globin chain, leading to a reduction in synthesis of the β -polypeptide chain, and resulting in mild to moderate microcytic anemia called β -thalassemia trait (Sinha *et al.*, 2017). Thus, the patient with a β -thalassemia trait is clinically asymptomatic, without hepatosplenomegaly or jaundice present, with normal or close to normal hemoglobin levels (Al Raeesi *et al.*, 2017);

ii) β -thalassemia intermedia – can be defined as a heterogenetic disorder. The β -globin chain production is often mild when two genes are defective (Al Raeesi *et al.*, 2017). Beta-thalassemia intermedia is a less severe anemia in adolescence or early adulthood (Taher *et al.*, 2011). In this type of thalassemia, blood transfusions are not necessary or required frequently, but the patient continues to suffer the effects of the anomaly (Galanello and Origa, 2010).

iii) β -thalassemia major – is homozygous or compound heterozygous for more severe beta chain mutations (Jha and Jha, 2014). Beta-thalassemia is a major referred to sever anemia and medical attention, usually demanding in the first two years of life and depending on blood transfusions (Galanello and Origa, 2010). In β -thalassemia, major patients required iron chelation therapy to regulate iron overload. Blood transfusions are recommended, as they are caused by inefficiency in the production of β protein. Excessive α protein causes hemolytic anemia, reduced red blood cell survival, and

bone marrow over-stimulation (Rund and Rachmilewitz, 2005).

The unbalanced in α -globin and β -globin protein synthesis were to appear excess in alpha-globin chain production, which is the major pathophysiology of β -thalassemia (Leecharoenkiat *et al.*, 2016). Alpha-globin chains are different from β -globin chains when incapable of forming stable tetramers. So, the free excess of α -globin chains causes insoluble aggregates, accumulating in the erythroid cell (Mathias *et al.*, 2000). The free α -globin chains in β -thalassemia interfere with red cell maturation, and erythrocytes are ineffective and cause marrow expansion (Taher *et al.*, 2010). In the small percentage of erythroid cells that can be developed to mature cells, the precipitate of free α -globin chains produced reactive oxygen species and oxidative stress. Leading to red blood cell membrane destruction and hemolytic increase (Schrier, 2002).

Therapy of thalassemia is a chronic blood transfusion to improve the quality of life. Transfusion causes complications to include hemosiderosis and hemosiderosis. Induces morbidity can be prevented by adequate iron chelation therapy (Be-Baun *et al.*, 2011). Repeated blood transfusion causes progressive iron overload, a major complication of treatment (Suman *et al.*, 2016). The major complication of iron toxicity is the damage of organs and stunted growth. Iron is primarily deposited in the liver, then in the endocrine system, and then in the cardiac muscle. This leads to iron-induced liver injury, hypothyroidism (Be-Baun *et al.*, 2011).

The mortality in patients with thalassemia that are dependent on blood transfusion is iron overload-related to cardiomyopathy (Aboul-Enein *et al.*, 2015). Free iron is toxic to cells because free iron is the catalysis of free radicals' formation from Reactive Oxygen Species (ROS) through the Fenton reaction (Ezzat *et al.*, 2015). When erythrocytes die, phagocytosis occurs where hemoglobin is digested, and iron is eliminated (Al-Mosawy, 2017). Diagnosis of β -thalassemia can be performed by laboratory tests such as complete blood count (CBC), Hb electrophoresis, genetic testing (Tahir *et al.*, 2011).

The possible diagnoses for this anomaly are:

i) Clinical diagnosis: Usually, TM is diagnosed in an infant younger than two years of age with jaundice, severe microcytic anemia, and hepatosplenomegaly (Jha and Jha, 2014);

ii) Hematologic diagnosis: Is characterized by decrease in Hb concentration (< 7 g/dl), mean cell volume (MCV) $> 50 < 70$ fl (femtoliters) and mean cell Hb (MCH) $> 12 < 20$ pg (Al-Mosawy, 2017). Peripheral blood smear: The blood film of a patient with beta-thalassemia shows RBC microcytosis, hypochromic, anisocytosis, poikilocytosis (elongated cells and tear-drop), and nucleated RBC (erythroblasts) (Cao and Galanello, 2010).

iii) HPLC/electrophoresis: Hemoglobin electrophoresis is next step for patients with MCV < 80 fL or MCH < 27 pg, (Old, 2003). Separation of hemoglobin by different electrophoretic techniques is a mainstay of diagnosing hemoglobin disorders (Borbely *et al.*, 2013). However, several methods of HPLC high-performance liquid chromatography (HPLC) analysis are observed from different manufacturers, that are separated from hemoglobin dependent on cation exchange chromatography (Rhea and Molinaro, 2014). Also, an increase in hemoglobin A2 associated with β -T and an increase in HBB protein associated with HbH disorder are diagnosed by isoelectric focusing (Sabath, 2017).

iv) Molecular Genetic Analysis: Until recently, allele-specific oligonucleotide hybridization was a primary test for the detection of β -T mutations in the laboratory, which performed by using several mutation-specific probes (Vrettou *et al.*, 2003). Nowadays, the polymerase chain reaction (PCR) is a well-known technique for mutation detection, and the commonly used for detection of thalassemia mutation is Amplification Refractory Mutation (ARMS) (Rosatelli *et al.*, 2009).

The treatments of thalassemia are regular blood transfusions, iron chelating therapy, treatment of complications such as endocrine disorder, osteoporosis, and heart failure (Petrou *et al.*, 2010). The regular blood transfusions in TM were important to correct the anemia, inhibit erythropoiesis, and decrease in iron absorption (Cao and Galanello, 2010). The blood transfusions are based on several factors, such as weight, hemoglobin concentration, and PCV of blood (Rachmilewitz and Giardina, 2015). Chelation therapy iron overload is one of the serious complications of blood transfusion. (Zafeiriou *et al.*, 2006). The only approach to eliminate excess iron is the usage of iron chelators because the body has notability for removing iron (Glickstein *et al.*, 2006).

Therefore, there are three main iron-

chelating therapy: i) desferrioxamine (DFO); ii) deferiprone (DFP) and iii) deferasirox (DFX), that reducing the toxicity in cardiac, endocrine and hepatic which caused by excess iron in the body (Prus and Fibach, 2010). Bone marrow transplantation: In patients with thalassemia, the transplantation of bone marrow is a good therapy during childhood (Lawson *et al.*, 2003). Gene therapy is a genetic treatment in thalassemia, includes the replacement of nonfunctional globin genes with cloned beta-globin genes, while numerous factors of problems have hurdled this therapy due to the vectors, to the complex controlled of globin gene expression, as well as the very high level an expression which was required for the correction of genetic defects (Genovese *et al.*, 2014). Ferritin is an intracellular protein that storage and liberation of iron in a controlled fashion.

The protein is synthesis by nearly all organisms (Casiday and Frey, 2000). Ferritin is present in almost tissues as a cytosolic protein, but it excreted minor quantity into the bloodstream, and it acts as an iron carrier. The ferritin present in the plasma can act as indirect markers of the total iron storage quantity in the body. Thus, serum ferritin is used as a diagnostic test for iron-deficiency anemia (Wang and Knovich, 2010). Ferritin is a globular protein complex consisting of 24 protein subunits, forming a nanocage with multiple metal-protein interactions (Theil, 2012). The entry of ferritin in lysosomes is high when the cell needs iron for cellular use. On the other hand, when iron levels are high, the entry of ferritin into the lysosome decreases (Mancias *et al.*, 2015).

The ABO blood group system has an important role in human blood transfusions. The ABO system contains A antigens, B antigens, and antibodies against these antigens. The ABO system was exposed by Landsteiner in 1900. Additionally, in the ABO system Rh system was found, and this system has "naturally occurring" antibodies against A and B antigens. Those who do not express these antigens (Landsteiner's Law) it causes negative and sometimes fatal results in the first mismatched transfusion (Koeppen, 2010).

The presence of ABO and Rhesus (Rh) antigens is clinically important, as it plays a fundamental role in blood transfusions and organ transplants. Although all humans have these systems and blood groups, the frequency of ABO and Rh antigens varies between populations (Avent, 2000).

The vitamin is a steroid hormone, well known for its important role in regulating body levels of calcium and phosphorus in bone mineralization (Morris, 2014). This vitamin is synthesized in the skin after exposure to sunlight (Vanlint *et al.*, 2011). Vitamin D is found in two forms: D3 (cholecalciferol) and D2 (ergocalciferol). Vitamin D3 is synthesized in human skin from 7-dehydrocholesterol after exposure to sunlight and through nutritional sources (Chen *et al.*, 2009). The first hydroxylation site of vitamin D occurs in the liver and is converted to 25-hydroxyvitamin D[25(OH)D], and this structure is characterized by low biological activity. The second site that produces biologically active vitamin D is in the kidneys (Man *et al.*, 2016; Aloia *et al.*, 2006). Hydroxylation in the kidneys is stimulated by parathyroid hormone (PTH) and suppressed by phosphate (Weaver and Fleet, 2004).

The key function of vitamin D is regulated of phosphorus and calcium by that VD increases intestinal and tubular absorption of calcium. Additionally, vitamin D reduces the physiological activity of parathyroid hormone (PTH) in two ways: i) directly (by influenced parathyroid glands) and ii) indirectly by hyperkalemia (Aloia *et al.*, 2006). Vitamin D plays an important role in bone formation and resorption (Morris, 2014).

The role of vitamin D on the bone formation is by the affecting of vitamin D on osteoblasts via vitamin D receptors (VDR), increases the synthesis of osteocalcin, alkaline phosphatase (ALP), and the collagen type, whereas the vitamin D has an effect on osteoclasts it is doubled; indirect via osteoblasts (RANKL/RANK/osteoprotegerin system), and directly – by inhibition of differentiation from promyelocytes to monocytes, that are the precursors of osteoclasts (Boyle *et al.*, 2003).

Vitamin D is essential for maintaining calcium balance in the skeleton. Absorbed in the intestines, calcium promotes an increase in the number of osteoclasts, allowing the parathyroid hormone (Boyle *et al.*, 2003).

The influence of vitamin D on muscle cells is by regulating calcium metabolism in muscle cells that are vital for the process of contraction and relaxation of muscle fibers (Janssen *et al.*, 2002). The active form of vitamin D (1,25-(OH)₂) acts through the vitamin D receptor (VDR) that is found in the kidney, bone, intestine, and parathyroid gland, also found in tissues and organs that are not directly involved in the maintenance of calcium homeostasis, such as

the brain, breast, immune cells, muscle tissue (Holick, 2009). The biologically active form of vitamin D is 1,25-dihydroxy vitamin D ($1,25(\text{OH})_2\text{D}$). This molecule acts by means of VDR found in the kidneys, bone, intestine, and parathyroid gland. It is also found in tissues and organs that are not directly involved in maintaining calcium homeostasis, such as the brain, breast, immune cells, and muscle tissue (Holick, 2009; Holick, 2004).

2. MATERIALS AND METHODS:

This study was conducted at the Thalassemia Center in Al-Zahraa Teaching Hospital in Al-Najaf governorate, Iraq. A serum sample was collected from thalassemia transfusion patients in addition to a control group (healthy). The samples tested were (60) samples that were divided the control group, (20) samples were beginning from the (2-29) years, (40) samples are from thalassemia patients. The age of thalassemia patients was a range from the (2) year to (29) year. The study cases can be divided according to blood transfusion duration to < 1 year, 1-5 years, and > 5 years.

This study was conducted at the Thalassemia Center at Al - Zahraa University Hospital under the governorship of Al - Najaf, Iraq. A serum sample was collected from patients transfused with thalassemia, in addition to a control group (healthy). The samples were tested on 60 patients and divided into groups: i) 20 control patients aged 2 to 29 years and ii) 40 patients aged 2 to 29 years with thalassemia. The study is still divided according to the blood transfusion period (ranging from < 1 year, 1-5 years, and > 5 years).

2.1. Collection of samples

A blood sample was obtained from the elbow vein by sterilized synergies. The sample was placed in two tubes. The first tube contains EDTA as anticoagulants to prevent blood clotting for used in physiological studies. The second gel tube was used to allow clotting the blood at room temperature to prepare a serum after using the serum for biochemical biomarkers studies.

2.2. Hemoglobin Estimation

Hematological parameters have been used in the Hematology Laboratory at al-Zahraa Teaching Hospital with Mythic 18 (RINGELISA N CO., Turkey). This equipment provides an EDTA

anticoagulated blood count (Wasmuth, 2010) through a fully automated analyzer. Hemoglobin analysis is performed directly by 555 nm spectrophotometry. The formation of chromogenic cyanmethemoglobin detects hemoglobin (cyanide lytic solution) and oxyhemoglobin (cyanide-free lytic solution). Blank hemoglobin is measured for each analytical process and during the rinse phase.

2.3. Determination of Ferritin

The level of ferritin in serum was examined by using enzyme-linked immunosorbent assay (ELISA), according to a prepared processed from the Manufacturing company (E-EL-H0168).

2.3.1 Preparation of the reagent

i) Hold all reagents until they are used at room temperature ($18 \sim 25^\circ\text{C}$). Follow the Microplate Setup Reader Manual and preheat it to OD for 15 minutes.

ii) Wash buffers: dilute 30 mL of concentrated wash buffer with 720 mL of deionized or distilled water to make 750 mL of wash buffer. Note: if the crystals are concentrated, heat in a 40°C water bath and blend gently until the crystals are completely dissolved.

iii) Standard working solution: a standard centrifuge of 10,000 kilograms every 1 minute. Add 1.0 mL of regular reference and sample thinner, allow 10 minutes to stand, and reverse several times. All in all, combine it with a pipette until it is dissolved. This reconstitution results in a working solution of 10 ng/mL. Make serial dilutions as needed. The dilution gradient suggested is as follows: 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0 ng/mL. Take 7 EP tubes and add 500 μL of normal reference and diluent to each tube. 10 ng/mL working solution pipette 500 μL on the first tube and blend to produce a working solution of 5 ng/mL. The pipette 500 μL of the solution in the latter according to these steps of the former pipeline. The following example is for reference purposes. Note: the last tube is empty. Don't pipe the water in the old tube.

iv) Biotinylated Detection Working solution: calculate the quantity required before the experiment (100 μL /well). In preparation, a little more than estimated should be prepared. Until use, center the stock tube, dilute 100% bis, Concentrating Detection Biotinylated Ab to 1

using Biotinylated Detection Ab Diluent Working Solution.

v) Concentrated HRP Working solution: Measure the quantity required before the experiment (100 μ L/well). In preparation, a little more than estimated should be prepared. Dilute 100, Concentrate HRP, mix with 1 concentrated HRP, and conjugate dilute working solution.

2.3.2 Procedure

- i) Prepare the sample and the reagent:
- ii) Add 100 μ L standards, blanks, and samples to specific wells;
- iii) Cover the plate by a sealer that we have supplied. Incubate for 90 minutes at 37 minutes;
- iv) Remove the liquid well, do not wash it. Immediately apply 100 μ L of biotinylated detection;
- v) Incubate at 37 °C for 1 hour, then vacuum and wash each well;
- vi) Add 100 μ L of HRP. Join the work solution for each well;
- vii) Incubate at 37 °C for 30 minutes and then repeat the washing cycle five times.
- viii) Add 90 μ L of substrate solution to each well. Incubate at 37 °C for approximately 15 minutes;
- ix) Apply 50 μ L of stop solution to each well of water and read at 450 nm within 10 minutes.

2.4. Estimation of human vitamin D

25(OH) Vitamin D concentrations in the serum were examined by using enzyme-linked. Immunosorbent assay (ELISA), according to prepare processed from CALBIOTECH, China. All reagents and specimens must be approved for use at room temperature, and both reagents must be commonly combined without spraying. All procedures should be completed immediately after the start of the process.

2.4.1 Procedure

- i) Deposit 10 μ L of 25-OH Vitamin D levels, controls, and samples in each well, if necessary.
- ii) Distribute 200 μ L of biotinylated 25(OH)D reagent solution into each well.

iii) Mix the contents carefully in the wells for 20 seconds using a 200 to 400 RPM shaker (or similar movements). Remove the shaker and cover the plate with the adhesive plate seal to ensure that each well is completely screened.

iv) Incubate the sealed plate for 90 minutes at room temperature.

v) Carefully remove the plate seal.

vi) Shake the contents of the wells quickly into a waste reservoir.

vii) Dispense 300 μ L of 1X wash buffer in each well and shake it quickly into a waste storage tank. Shake the wells on the paper towel vigorously to extract the remaining droplets. Repeat two more times in all for three washes.

viii) Dispense 200 μ L (Streptavidin-HRP) of the conjugate enzyme in each well.

ix) Incubate at room temperature for 30 minutes.

x) Shake the contents of the wells in the waste container quickly.

xi) Dispense a 300 μ L 1X wash buffer in each well and shake it quickly into a reservoir. Shake the wells on the paper towel vigorously to extract the remaining droplets. Repeat two more times in all for three washes.

xii) Use a multichannel pipette to dispense 200 μ L of TMB Substrate into each well.

xiii) Incubate at room temperature, preferably in the dark, for 30 minutes.

xiv) Dispense 50 μ L of Stop Solution in each well to stop the enzyme response. Mix the contents of the plate carefully for 20-30 seconds.

xv) Write the ELISA Reader for absorption at 450 nm within 10 minutes of the stop solution applied.

xvi) Standard Curve: Seven standard rates are included for each run.

2.5. Statistical analysis

The results were analyzed using the GraphPad Prism software, which allows non-linear regression, understandable statistics, and data organization. The data is displayed as mean $\pm$ standard error (SE). The T-test analyzed the comparison between patients and control groups. The value of $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION:

3.1. Hematological characteristic

The result showed a significant ($p < 0.05$) decrease in the level of Hb in patients with thalassemia compared with the control group. The Hb value in patients with thalassemia (7.458 ± 0.2406) and the Hb value in control patients (12.60 ± 0.1748) are shown in Figure 1.

The results are following the studies by Shanthi *et al.* (2013), who observed a decrease in the level of Hb due to the early and continuous degradation of red blood cells. This anomaly leads to the destruction of red blood cells before maturation. Bhuiyan *et al.* (2020) studied 40 patients, and their results showed that 97.5% had a Hb level below 10 gm/dL, and only 2.5% of the patients reached high Hb levels, allowing to conclude that it is an adequate and accurate implementation in the regimen transfusion. The correct transfusion practice can guarantee normal growth without increasing the expansion of erythropoiesis and effective iron overload prevention.

The result in Figure 2 shows the percentage of ABO types in thalassemic patients. This result demonstrates the O blood group is most in thalassemic patients (40%), but other blood groups type A, B, and AB have a percentage (23%, 28%, 10%), respectively.

In Figure 3 revealed the patients with Rh-positive more than patients with Rh-negative. This result agrees with the study of Marbut *et al.* (2018). Another study shown the blood group O+ is most blood group in thalassemic patients, also shown the people with blood group O is most chance of family history diseases (Abbas, 2013; Sinha *et al.*, 2017).

According to the above studies, which demonstrations with the ABO types have related to thalassemia disorder, because thalassemia is one of the genetic diseases, and O blood groups were related to a family history disease.

3.2. Biomarker

Figure 4 shows a significant increase ($p < 0.05$) in serum levels of ferritin in a patient with thalassemia ($2,984 \pm 321.3$) compared with a control group (97.50 ± 0.7159), this study agrees with the study of Eissa and El-Gamal (2014). The increase in the concentration of ferritin in thalassemia patients is related to frequent blood transfusions and an ineffective red blood cell production process (Pasricha *et al.*, 2013).

The early organs affected by iron overload

are the liver, leading to damage in the tissue of the liver from direct toxic of iron on liver cells. This study found the relationship between serum ferritin and liver enzymes (Suman *et al.*, 2016). In thalassemia, the patients suffer from chronic anemia. Hypoxia causes an increase in iron absorption from the gastrointestinal tract and blood transfusions. This leads to a gradual accumulation of iron due to insufficient excretory pathways, when serum transferrin exceeds saturation 70% and high of ferritin, free iron species, that plays a role in generating reactive oxygen species with ultimate damage of tissue, dysfunction of organs, and death (Rachmilewitz and Giardina, 2011).

Figure 5 shows a decrease ($p < 0.05$) in serum vitamin D levels in patients with thalassemia ($8,390 \pm 1,081$). On the other hand, control groups have $29.44 \pm 2,771$ of vitamin D. These results are in agreement with the studies by Ezzat *et al.* (2015), where they concluded that high levels of iron in the liver interrupts the production of 25-hydroxyvitamin D. Soliman *et al.* (2013) identified a decrease in vitamin D levels in children with thalassemia. This behavior may be related to low mineral absorption in the bone matrix (Shaykhbaygloo *et al.*, 2020).

The decrease in vitamin D in patients with thalassemia causes an accumulation of iron in the liver and skin. A serious achievement of this anomaly is related to the synthesis of hydroxyl, a fundamental molecule in the structure of vitamin D (Pirinçcioğlu *et al.*, 2011). Al-Shemery and Al-Dujaili, (2019), in this study, indicated a high level of Procollagen type III peptide in patients with thalassemia that showed liver damage.

Figure 6 reveals discrepancies in serum ferritin levels according to the time of blood transfusion (<1 year, 1-5 years, and > 5 years). Significant amounts are evident between transfusions in the <1 year and > 5 year period. On the other hand, the duration of 1-5 years is of little significance, which is in accordance with the study by Bhuiyan *et al.* (2020). The researchers say that high levels of iron in the blood are a direct result of blood transfusions for patients with thalassemia. High levels of iron are due to the absence of a biological mechanism capable of eliminating excess iron in the body (Pasricha *et al.*, 2013).

Figure 7 again shows the discrepancies in the serum vitamin D concentration according to the duration of the blood transfusion (<1 year, 1-5 years, and > 5 years).

3.3. Relationship between biomarkers

The results of the linear regression (Figure 8) between the concentrations of ferritin and vitamin D in patients with thalassemia revealed a non-significant negative relationship ($r = -0.09$).

Gombar *et al.* (2018) demonstrated that the increase in serum ferritin in patients with thalassemia, resulting from multiple blood transfusions, led to the accumulation of iron in the liver. Thus, low levels of vitamin D decrease the synthesis of hydroxyl and, consequently, decrease serum levels. Shaykhbaygloo *et al.* (2020) report the same trend in the behavior of patients with thalassemia in diseases of the endocrine system due to the accumulation of iron.

4. CONCLUSIONS:

In view of the results obtained in this study, we can conclude that patients with thalassemia are more susceptible to blood type O+. The increase in ferritin in the liver and skin due to the accumulation of iron has the direct effect of decreasing vitamin D levels. Another factor that induces the increase in ferritin is the frequent blood transfusions in patients with thalassemia. Thus, the research carried out shows us a significant contribution to the scientific community, leading to conclusions on the concern and care for patients with thalassemia in the search for understanding and effective treatments. The data discussed in the Results and Discussion showing the relevance of the work and how different it is from other researches. Also, point out the benefits and improvements that can be observed to develop new science standards that can change something in the related.

5. ACKNOWLEDGMENTS:

The authors offer their thanks and great appreciation to the central thalassemia for their facilities to accomplish this research.

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Figure 1. Level of hemoglobin in thalassemic patients and control group.

Figure 2. Percentage of ABO Types in patients with thalassemia.

Figure 3. Percentage of Rh of ABO types in patients with thalassemia.

Figure 4. Concentration of the ferritin in the thalassemic and control group.

Figure 5. Concentration of the vitamin D in the thalassemic patients and control group.

Figure 6. Concentration of ferritin according to the duration of blood transfusion in thalassemic patients.

Figure 7. Concentration of vitamin D according to the duration of blood transfusion in patients with thalassemia.

Figure 8. Relationship between ferritin and vitamin D.