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Relationships Of Vitamin D And Vitamin B12 With Malonaldehyde In Patients With Beta Thalassemia Major

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Abstract

Background: Beta thalassemia major (Cooley's anemia) is a congenital genetic blood disorder resulting from a deficiency of β-globin chains due to severe anemia that depends on continuous blood transfusions for survival. Patients suffer from hemoglobinopathy, infection, diabetes mellitus, growth retardation, enlargement and damage to the liver and spleen, bone marrow aplasia and delayed sexual maturity. This complication arises due to the accumulation of iron, the impairment of antioxidants from vitamins, and an increase in lipid peroxides (MDA).

Objectives: The study aimed to evaluate vitamin D and B12 levels and their relationship with MDA, liver enzymes (AST, ALT, ALP), iron and (Total Billirubin, Direct Billirubin, Indirect Billirubin).

Materials and methods: The samples of BTM patients under study were (100 samples), (50 samples) for both males and females. Patients' ages ranged from (2-30 years). While the control sample was (40), with (20 samples) for each of the two sexes. Their ages ranged from (2-30 years). Their disease was confirmed by blood tests, hemoglobin electrophoresis, DNA analysis, and elevated ferritin levels. Vitamin D and B12 levels were assessed by immunoassay. While (MDA, Fe, AST, ALT, ALP) and (Total Billirubin, Direct Billirubin, Indirect Billirubin) were evaluated by colorimetric methods.

Results: Vitamin D and B12 levels decreased significantly ($p \leq 0.05$). While the levels of (MDA, Fe, AST, ALT, ALP) and (Total Billirubin, Direct Billirubin, Indirect Billirubin) increased significantly at ($p \leq 0.05$) in BTM patients compared with the control group. Where the effect of the disease according to the results was clear in patients with age.

conclusion: Our results indicate that the weakness of the antioxidant systems (vitamin D and B12) with the increase in oxidative factors resulting from the complications of the disease led to the exacerbation of the disease with iron overload, causing an increase in the levels of liver enzymes in the blood, damaging the liver and spleen.

Keywords: Beta thalassemia major, Vitamin D, Vitamin B12, MDA, Liver enzymes, Iron.

Introduction

Thalassemia is a congenital genetic disorder of the blood, in which the body produces abnormal forms of hemoglobin.[1] It occurs due to mutations or deletions in hemoglobin genes, leading to a lack of production or the absence of alpha or beta chains.[2] The two main categories of thalassemia are alpha and beta. Then the formation of ineffective red blood cells, a varying degree of anemia, and then hemolysis.[3] Beta thalassemia (BT) is one of the most common inherited hemoglobin disorders.[4] A severe decrease in the production of beta-clobin chains causes hemolysis. BT patients require chronic and frequent blood transfusions since childhood in order to survive.[5] BT spreads all over the world. Where patients suffer from iron accumulation in the skin, liver and kidneys.[6] As well as bone deformities such as osteoporosis.[7] BT is classified on the basis of clinical severity into thalassemia major, thalassemia intermedia and thalassemia minor.[8] Beta thalassemia major (BTM) also called Cooley's anemia, in which hemoglobin (Hb) levels (Hb < 7 g/dL).[9] Clinical symptoms of BTM often appear between (3-6) months after birth with other initial symptoms of growth retardation and pallor.[10] Hypersplenism, hepatosplenomegaly and bone marrow aplasia. Bone changes are evident in the facial features in terms of enlargement of the frontal front and enlargement of the upper jaw.[11] BTM is diagnosed by microcytosis by blood tests, hemoglobin electrophoresis, deoxyribonucleic acid analysis and elevated ferritin levels.[12] In order to prevent the disease, the condition of the parents must be known before the birth of the child. If the test result for both parents is positive for the carrier condition, the couple is advised to have the fetus medically terminated.[13] Infection and hemoglobinopathy are frequent complications in BTM.[14] In addition to growth disorders, diabetes mellitus, delayed sexual maturity.[6] Bone marrow expansion, blood formation outside the bone marrow,[12] Expansion of the bones of the face and skull, which increases the risk of bone fractures.[7] Liver damage, hepatitis C (HCV) and hepatitis B (HBV) acquired through repeated blood transfusions.[15] The appropriate treatment for BTM is through lifelong red blood cell transfusions to maintain the hemoglobin level at (9-11gm/dL).[16] Removal of heavy metals such as iron by iron chelation therapy.[17] Taking folic acid supplements, umbilical cord

blood transplantation, splenectomy [12,18] Transplantation of blood-forming stem cells or bone marrow because it is the only way that may lead to a definitive cure for BTM.[13] Oxidative stress has a major role in the pathophysiology of BTM patients. High levels of reactive oxygen species (ROS) cause cytotoxicity.[19] In addition, iron overload in red blood cells is absorbed in the digestive system, causing its deposition mainly in the heart, liver, and endocrine glands.[20] Blood transfusions in BTM have risks and complications arising from iron overload, from physical, psychological, social and economic effects, [21] as well as neurological problems, failure to thrive, cardiovascular problems, liver disease, bacterial infection problems, as well as abnormalities. Different immunoglobulins, such as spleen removal and liver damage.[22]

Vitamin D has an important role in maintaining bones, balancing minerals such as calcium and phosphorus.[23] Vitamin D is a hormone in nuclear receptors, modulating the inflammatory system by regulating the formation of inflammatory cytokines and immune cells. It also significantly reduces the level of ferritin in the blood.[24] The risk of vitamin D deficiency in BTM is associated with bone diseases, including osteoporosis, rickets, spinal deformities and fractures as well as heart failure. Vitamin D is transported to the liver and converted to 25-hydroxyvitamin D, then additional hydroxylation is added to convert it to 1,25-dihydroxyvitamin D3 in the kidneys.[25] The important factors that help increase vitamin D levels in the body are exposure to sunlight, nutritional status, nutrient intake of nutrients, especially energy and proteins such as fish, fortified milk, meat and fats to help absorb vitamin D.[7] Vitamin B12 (Vit.B12) known as cobalamin has important roles in blood formation, maintaining the function of peripheral nerves and the integrity of the nervous system, and any deficiency of it leads to the risk of developing peripheral neuropathy.[26] Vit.B12 is a micronutrient required for the production of red blood cells. Because of the acceleration of erythropoietic turnover in the BTM, this leads to an increased demand for nutrients required for erythropoietic production.[27] Vit.B12 deficiency causes ineffective erythropoiesis, megaloblastic anemia, and neuropsychiatric manifestations such as neuropathy, myelopathy, depression, and dementia.[28] Vit.B12 cannot be synthesized in the body but must be ingested from external sources to be absorbed mainly in the terminal ileum. It is synthesized by gastric parietal cells.[29] Vit.B12 is a water-soluble vitamin with an essential role in cellular metabolism, DNA synthesis and erythropoiesis.[28] Malondialdehyde (MDA) is a compound formed as a by-product of lipid peroxides and is often used as a biomarker of oxidative stress.[30] MDA is an important measure of increased lipid oxidation and a sensitive biomarker indicating tissue injury.[8] MDA levels increase in the BTM due to iron overload through frequent blood transfusions, oxidative stress from ROS, and high levels of ferritin.[31] As well as increased lipid peroxidation in the membranes of red blood cells, the destruction of

proteins, fats and nucleic acids.[19] Iron is one of the basic elements found in abundance in nature and is not easily absorbed because it oxidizes when exposed to oxygen, which makes it insoluble in a large way. The majority of iron is found in red blood cells in the body and produces hemoglobin during the formation of red blood cells.[32] Iron is important in DNA synthesis and oxygen and electron transport.[10] About 1-2 mg of iron per day is absorbed from the diet, with an equivalent amount lost through the digestive tract.[31] Iron overload is called hemochromatosis, which is characterized by improper accumulation of iron in the functional parts of the vital organs in the body, which leads to organ damage and failure.[33] The liver is involved in one of the most complex physiological processes in the human body and maintains a balanced metabolism. The liver is exposed to the risks of oxidative stress due to an imbalance between the natural antioxidant defense mechanism and the production of excess free radicals in the body.[34] The method of estimation of liver enzymes is one method of determining liver damage, caused by oxidative injury and the direct toxic effect of iron on liver cells in BTM.[35] Aspartate aminotransferase (AST) (EC 2.6.1.1) is called Glutamate Oxaloacetate Transaminase (GOT). It is found in the liver, heart muscle, skeletal muscles, kidneys, brain, pancreas, lungs, leukocytes, and erythrocytes.[36] Serum AST levels are somewhat elevated in almost all liver diseases.[37] Alanine aminotransferase (ALT) (EC 2.6.1.2) is called Pyruvate Transaminase Glutamate (GPT). It is present in high concentration in the liver and to a lesser extent in the skeletal muscles, kidneys and heart. Serum ALT activity is an indicator of liver cell damage. The serum ALT level is considered an indicator of necroinflammatory.[15] ALT levels in the blood rise in liver diseases, non-alcoholic steatohepatitis, chronic viral hepatitis, acute viral hepatitis, toxins and ischemic hepatitis.[37] Alkaline phosphatase (ALP) (EC 3.1.3.1) is found in the liver and bone marrow. It can be considered a diagnostic sign for many diseases such as: liver diseases, bones, thalassemia, hemolytic anemia and cancer.[38] ALP enzymes were found in the hepatic cell membranes, specifically in the hepatic sinuses and bile ducts. ALP activity levels rise in BTM patients in cases of obstruction of the bile ducts inside and outside the liver, sinus obstruction, damage to the liver cells,[39] bone tissue dissolution.[40] In BTM patients dependent on blood transfusions, the levels of AST and ALT enzymes increase. Liver biopsy is the preferred standard test to find out the status of excess iron in the liver, because it is deposited in the liver first, then in the endocrine organs, then the heart, leading to liver injury, hypothyroidism, Hypoparathyroidism, growth hormone deficiency, diabetes mellitus and heart failure.[41] Bilirubin is the end product of heme catabolism. Bilirubin is often portrayed as a toxic compound to tissues. Several promising scientific studies have been able to demonstrate its effectiveness as an endogenous antioxidant. It has been shown to scavenge free radicals, provide cardiovascular protection as well as boost the immune system. The normal range of bilirubin is

about (0.2-1.2 mg/dL), so any increase beyond this level is harmful to the body.[42] Serum bilirubin is a sensitive indicator for evaluating liver function to detect extended hepatocyte damage. In BTM patients, hyperbilirubinemia could be due to cirrhosis of the liver cells.[15] Nearly two-thirds of BTM patients have several calcified bilirubin stones by the age of 15 years. Which leads to the formation of gallstones.[43] Bilirubin can be measured as: direct (conjugated), or the amount of total bilirubin in the blood.[44]

Materials &Methods

Patients and control group

The samples of BTM patients under study were (100 samples), (50 samples) for each of the males and females. Patients' ages ranged from (2-30 years). While the control samples were (40), by (20 samples) for each of the two sexes. Their ages also ranged from (2-30 years).

Collection of patients and control blood samples

Blood samples were collected from patients with BTM from the Thalassemia Center in Azadi Teaching Hospital in Kirkuk Governorate in Iraq, when they visited the center for frequent and continuous blood transfusion. It was confirmed that they had the disease through blood tests, hemoglobin electrophoresis, DNA analysis and elevated ferritin levels.

Biochemical parameters measurements

Vitamin D and vitamin B12 levels were measured according to the competition principle. The total duration of the assay was (27 minutes) using the Cobas device from the German company Roche. Using a (ruthenium) protein to bind vitamin D (vitamin D binding protein) with both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2. Then the total vitamin D concentrations are calculated in (ng/mL) units. Vitamin B12 present in the sample competes with vitamin B12 added with ruthenium. Vitamin B12 concentrations are calculated for each sample in units (pg/mL).[45] The measurement of MDA depends on the principle of action when, by reacting Thiobarbituric acid (TBA) with MDA under specific thermal conditions and a decrease in pH, the complex [TBA] 2-malondialdehyde is pink.[46] Iron concentrations were measured, as a result of the dissociation of iron-transferrin in an acidic medium, which leads to the reduction of iron (III) ions to iron (II) ions by ascorbic acid. Fe(II) ions form a colored complex with ferene.[47] The principle of measuring AST activity depends on the interaction of α-oxoglutarate with L-aspartate in the presence of AST to produce L-glutamate and oxaloacetate. AST levels are measured by oxaloacetate hydrazone concentrations of 2,4-dinitrophenylhydrazine.[48] The activity of ALT was measured based on the interaction of α-oxoglutarate with L-alanine

in the presence of ALT to produce L-glutamate and pyruvate. ALT concentrations are measured by pyruvate hydrazone concentrations consisting of 2,4- dinitrophenylhydrazine.[48] While the effectiveness of ALP was measured by the interaction of phenyl phosphate with ALP to form phenol and phosphate. Where the liberated phenol reacts with 4-amino antipyrine in the presence of K3Fe(CN)6 to form a red complex.[49] Total and direct bilirubin levels were estimated by the Diazo method by reacting bilirubin with diazotized sulfanilic acid forming the colored azobilirubin complex in highly acidic or alkaline media.[50]

Statistical Analysis

Statistical analysis was carried out using Minitab program to find the mean and standard deviation. Using the T-test, the Anova test and the Duncan test at the probability level (P value), which give significant differences at ($P \leq 0.05$) and non-significant differences when ($P > 0.05$).[51]

Results

The results in Table (1) showed a significant decrease in the levels of vitamins (Total Vit.D, Vit.B12) at the probability level ($p \leq 0.05$), as the results in patients were (12.25 ± 4.58, 252.9 ± 65.8) compared to the control group (42.8 ± 18.4, 564 ± 157) respectively. While the levels of (MDA, Fe, AST, ALT, ALP) increased significantly at ($p \leq 0.05$). Where the results were when in patients (45.96 ± 8.84, 236.0 ± 47.1, 36.2 ± 16.5, 41 .4 ± 20.0, 202.1 ± 45.5) compared with control (12.97 ± 3.78, 110.07 ± 32.2, 9.63 ± 1.46, 12.68 ± 2.89, 68.3 ± 19.0) respectively. The levels of (Total Billirubin, Direct Billirubin, Indirect Billirubin) increased significantly at ($p \leq 0.05$). Where the results for patients (3.611 ± 0.753, 0.740 ± 0.277, 2.871 ± 0.476) compared to control (0.837 ± 0.262, 0.121 ± 0.048, 0.716 ± 0.214) respectively.

Table (1): Levels of biochemical variables in the blood serum of patients with beta thalassemia major and the control group.

Parameters	Controls (n=40)	Patients (n=100)
	mean ± SD	mean ± SD
Total Vit.D (ng/mL)	42.8 ± 18.4	12.25 ± 4.58
Vit.B_{12} (pg/mL)	564 ± 157	252.9 ± 65.8
MDA (nmol/mL)	12.97 ± 3.78	45.96 ± 8.84
Iron Fe (µg/dL)	110.07 ± 32.2	236.0 ± 47.1
AST (GOT) (U/I)	9.63 ± 1.46	36.2 ± 16.5
ALT (GPT) (U/I)	12.68 ± 2.89	41.4 ± 20.0
ALP (IU/L)	68.3 ± 19.0	202.1 ± 45.5
Total Billirubin (mg/dL)	0.837 ± 0.262	3.611 ± 0.753
Direct Billirubin (mg/dL)	0.121 ± 0.048	0.740 ± 0.277

Indirect Billirubin (mg/dL)	**0.716 ± 0.214**	**2.871 ± 0.476**

The results are shown in Table (2) and according to gender, there are statistically significant differences at (p≤0.05) when comparing groups of male and female BTM patients versus control groups for all parameters. The patients' results for Total Vit.D levels in males and females were (12.066 ± 4.743, 12.438 ± 4.461) compared to control (46.310 ± 9.580, 39.280 ± 6.830) respectively. Vit.B12 levels in male and female patients (256.87 ± 30.07, 252.85 ± 30.55) compared to control (604.60 ± 59.50, 522.60 ± 47.60), respectively. When comparing between the groups of patients only and of both sexes for (Total Vit.D, Vit.B12) levels, there were no statistically significant differences (P>0.05). However, Total Vit.D levels for female patients (12.438 ± 4.461) were higher than for male patients (12.066 ± 4.743). The levels of Vit.B12 for male patients (256.87 ± 30.07) were higher than those for females (252.85 ± 30.55). The MDA levels in male and female patients were (45,600 ± 4,130, 46,330 ± 4,620) compared to control (12,904 ± 2,979, 13,036 ± 2,665), respectively. However, there were no significant differences (P>0.05) when comparing between groups of patients only and according to gender. MDA levels in female patients (46,330 ± 4,620) were higher than in males (45,600 ± 4,130). Iron (Fe) levels increased significantly at (p≤0.05) for groups of male and female patients (224.64 ± 30.00 and 247.45 ± 31.12) compared to control (118.03 ± 17.55 and 102.11 ± 18.67), respectively. While the comparison between groups of patients only and for both sexes, the levels of iron (Fe) for female patients (247.45 ± 31.12) were higher than that of males (224.64 ± 30.00), but not significant (P>0.05). The levels of liver enzymes (AST, ALT, ALP) were significantly increased at the probability level (p≤0.05) in the serum of BTM patients according to gender compared to control. The results for male patients were (35.70 ± 6.650, 40.50 ± 7.100, 195.10 ± 23.21) and for male control (10.00 ± 1.376, 13.15 ± 3.150, 69.37 ± 10.07), respectively. While the results for female patients were (36.62 ± 6.450, 42.34 ± 8.600, 209.04 ± 27.17) and for female controls (9.250 ± 1.482, 12.20 ± 2.587, 77.18 ± 11.82), respectively. When making a comparison between groups of patients only and according to sex, the levels of (AST, ALT, ALP) did not give significant differences (P>0.05). The levels of (AST, ALT, ALP) in female patients were higher than in males. Total Billirubin, Direct Billirubin, Indirect Billirubin levels were increased in BTM patients compared to control. The total bilirubin levels for male and female patients were (3.597 ± 0.878, 3.625 ± 0.611) compared to control (0.842 ± 0.234, 0.832 ± 0.293), respectively. While the results of Direct Billirubin levels for male and female patients were (0.677 ± 0.240, 0.803 ± 0.299) compared to control males and females (0.110 ± 0.044, 0.133 ± 0.051), respectively. Indirect bilirubin levels for male and female patients were (2.920 ± 0.908, 2.822 ± 0.631) compared with (0.732 ± 0.238, 0.699 ± 0.311), respectively. When comparing between groups of patients only and according to sex, it did not give significant differences at (P>0.05) when comparing between groups of patients only and according to sex.

While the levels of (Total Billirubin, Direct Billirubin) in females were higher than in males, the levels of Indirect Billirubin in males were higher than in females.

Table (2): Levels of biochemical variables in the blood serum of patients with beta thalassemia major and the control group according to sex.

Parameters	Sex	Controls Male (n=20) Female (n=20)	Patients Male (n=50) Female (n=50)
		mean ± SD	mean ± SD
Total Vit.D (ng/mL)	Male	46.310 ± 9.580	12.066 ± 4.743
	Female	39.280 ± 6.830	12.438 ± 4.461
Vit.B_{12} (pg/mL)	Male	604.60 ± 59.50	256.87 ± 30.07
	Female	522.60 ± 47.60	252.85 ± 30.55
MDA (nmol/mL)	Male	12.904 ± 2.979	45.600 ± 4.130**
	Female	13.036 ± 2.665	46.330 ± 4.620**
Iron Fe (µg/dL)	Male	118.03 ± 17.55	224.64 ± 30.00
	Female	102.11 ± 18.67	247.45 ± 31.12
AST (GOT) (U/I)	Male	10.00 ± 1.376	35.70 ± 6.650
	Female	9.250 ± 1.482	36.62 ± 6.450
ALT (GPT) (U/I)	Male	13.15 ± 3.150	40.50 ± 7.100
	Female	12.20 ± 2.587	42.34 ± 8.600
ALP (IU/L)	Male	69.37 ± 10.07	195.10 ± 23.21
	Female	77.18 ± 11.82	209.04 ± 27.17
Total Billirubin (mg/dL)	Male	0.842 ± 0.234	3.597 ± 0.878
	Female	0.832 ± 0.293	3.625 ± 0.611
Direct Billirubin (mg/dL)	Male	0.110 ± 0.044	0.677 ± 0.240
	Female	0.133 ± 0.051	0.803 ± 0.299
Indirect Billirubin (mg/dL)	Male	0.732 ± 0.238	2.920 ± 0.908
	Female	0.699 ± 0.311	2.822 ± 0.631

The results showed in Table (3) that there were significant differences at ($p \leq 0.05$) in all age groups of patients when compared with the corresponding age groups in the control group. The effect of the disease was clear on BTM patients with age. Where the levels of (Total Vit.D, Vit.B12) vitamins decreased for the age groups in the patients compared to the control. The levels of (MDA, Fe), liver enzymes (AST, ALT, ALP) and (Total Billirubin, Direct Billirubin, Indirect Billirubin) increased for all age groups of BTM patients compared with the age groups of the control group. However, when comparing the groups of patients only for the three age groups, there were no significant differences ($P > 0.05$).

Table (3): Levels of biochemical variables in the serum of patients with beta thalassemia major and the control group, according to age.

Parameters	Ages (years)	Controls (n=40) (2-10) years (n=12) (11-20) years (n=18) (21-30) years (n=10)	Patients (n=100) (2-10) years (n=40) (11-20) years (n=40) (21-30) years (n=20)
		mean ± SD	mean ± SD
Total Vit.D (ng/mL)	(2-10) years	37.340 ± 4.000	14.125 ± 3.284
	(11-20) years	44.660 ± 8.410	11.104 ± 3.299
	(21-30) years	45.990 ± 7.430	10.802 ± 2.182
Vit.B_{12} (pg/mL)	(2-10) years	541.2 ± 46.4	257.8 ± 44.5
	(11-20) years	576.8 ± 66.4	253.1 ± 47.7
	(21-30) years	566.7 ± 66.3	242.6 ± 46.4
MDA (nmol/mL)	(2-10) years	10.790 ± 2.840	46.040 ± 3.460**
	(11-20) years	13.019 ± 2.457	44.380 ± 3.250**
	(21-30) years	15.497 ± 2.784	48.980 ± 3.310**
Iron Fe (µg/dL)	(2-10) years	131.80 ± 23.39	221.26 ± 25.28
	(11-20) years	105.41 ± 25.37	230.95 ± 28.56
	(21-30) years	92.37 ± 21.44	275.80 ± 21.40
AST (GOT) (U/I)	(2-10) years	8.583 ± 0.900	32.050 ± 7.010
	(11-20) years	9.722 ± 1.227	33.150 ± 4.400
	(21-30) years	10.700 ± 1.636	50.400 ± 5.050
ALT (GPT) (U/I)	(2-10) years	10.667 ± 1.77	34.630 ± 3.65
	(11-20) years	12.889 ± 2.54	41.730 ± 7.79
	(21-30) years	14.700 ± 3.16	54.400 ± 7.71
ALP (IU/L)	(2-10) years	65.01 ± 15.44	190.25 ± 35.91
	(11-20) years	69.72 ± 13.55	195.81 ± 44.23
	(21-30) years	71.36 ± 13.37	238.30 ± 48.9
Total Billirubin (mg/dL)	(2-10) years	0.677 ± 0.288	3.464 ± 0.767
	(11-20) years	0.868 ± 0.219	3.481 ± 0.688
	(21-30) years	0.972 ± 0.221	4.162 ± 0.610
Direct Billirubin (mg/dL)	(2-10) years	0.112 ± 0.049	0.689 ± 0.265
	(11-20) years	0.124 ± 0.048	0.646 ± 0.227
	(21-30) years	0.127 ± 0.052	1.030 ± 0.189
Indirect Billirubin (mg/dL)	(2-10) years	0.565 ± 0.297	2.775 ± 0.825
	(11-20) years	0.744 ± 0.239	2.835 ± 0.732
	(21-30) years	0.845 ± 0.238	3.132 ± 0.758

Discussion

Total Vit.D

Several studies were consistent with the current study in terms of lower vitamin D levels in BTM patients compared to the control group. Including (Dhale, S., et al.)[52], (Herawati, Y., et al.)[53], (Caroline, P. O. L., et al.)[7], (Thiagarajan, N. R., et al.)[54], (Pala, M., et al.)[55] and (Manolopoulos, P. P., et al.)[56]. Observed in BTM patients who undergo

regular blood transfusions have decreased levels of vitamin D associated with the accumulation of excess iron in the liver, leads to inactivation of vitamin D hydroxylation. Also, its accumulation under the skin reduces the process of converting 7-Dehydrocholesterol into vitamin D, thus causing an imbalance in its composition. In BTM, ROS is also formed as a result of oxidative stress caused by iron overload, which causes increased levels of lipid peroxides represented by MDA and thus lowers vitamin D levels.[7] (Herawati, Y., and others) stated that exposure for one hour to sunlight is one of the most important factors affecting 25-hydroxyvitamin D. Genetic and ethnic factors of skin color and dark complexion, which prevent adequate exposure to sunlight, cause low levels of 25-hydroxyvitamin D. Lack of physical activity in BTM and use of sunscreens are associated with inhibition of the formation of vitamin D through the skin by sunlight.[57] The researchers (Manolopoulos, P. P., et al.) confirmed that the deficiency of vitamin D levels in BTM patients is associated with bone diseases including osteoporosis,[56] Progressive expansion of the marrow, spinal deformities, decreased bone mass, fractures, chronic hypoxia, direct iron toxicity, nutritional deficiencies, rickets and osteomalacia. Vitamin D helps homeostasis of calcium and mineralization of the skeleton, by increasing calcium concentrations inside and outside the cell through several important mechanisms: mobilizes calcium from the bones, reduces renal calcium excretion, absorbs calcium in the intestines and is associated with parathyroid hormone. Impaired calcium homeostasis is thought to result from iron overload in patients with BTM.[54] Whereas (Yu, U., et al.) noted that vitamin D levels are lower in older children compared to younger children, despite the high levels of ferritin and excess hepatic iron, which gave an indication of vitamin D deficiency and altered bone metabolism.[58] (Sadri, S., et al.) confirmed in a recent study that one of the important reasons for the low levels of vitamin D in BTM is the process of iron chelation, and taking high doses of desferrioxamine treatment that ultimately leads to deterioration of bone health and its fragility.[59] The dietary intake of nutrients, especially proteins, fats and energy, affects the decrease in vitamin D levels in the BTM, as a result of the formation of ineffective red blood cells, causing an increase in the patient's use of energy and protein necessary for the formation of enzymes and proteins that bind vitamin D.[6] A recent study confirmed the administration of BTM vitamin D supplements with a health-recommended dose of (50,000 IU) for those with vitamin D levels less than (20 ng/dL) once a week and given until the child reaches normal levels.[60]

Vit.B12

Several studies agreed with our study in terms of a decrease in Vit.B12 levels in BTM, including (Gamayani, U., et al.), [26] (Sharma, N., et al), [61] (Sharma, A. , et al.), [27] and (Houdhary, R., and his colleague). [62] Vit.B12 deficiency affects growth retardation in a common way and is

more evident at puberty, including chronic anemia, iron toxicity, and endocrine abnormalities.[63] (Mohammed, W.R., et al.) showed that Vit.B12 deficiency causes megaloblastic anemia for its role in folate metabolism. And they confirmed taking Vit.B12 supplements daily, at least (2-3) mg.[44] Being a vitamin that cannot be synthesized in the body, it is found in many sources, including meat, fish, eggs, and dairy products, and it is rarely found in products of plant origin.[64] And (Sharma, A., et al.) mentioned that megaloblastic in BTM is caused by microcytosis and hypochromia.[27] (Sharma, N., et al.) conducted a study on BTM patients to see differences in food and nutrient intake, where they noticed that BTM patients had lower intakes of most vitamins, fruits, vegetables, and mineral supplements, which helps exacerbate anemia problems. Inadequate health care is one of the main obstacles to effective treatment of iron overload patients, whose complications are the main cause of death in BTM.[61] (Berz, D., and others) showed that transfusions of packed red blood cells do not lead to a significant change in the levels of Vit.B12 in the blood, although the blood transfused to patients is likely to contribute to an increase, even a small amount, of Vit.B12.[65] (Usman, S. Y., and others) showed that vitamin B12 (cobalamin) in BTM patients has an essential role in the formation of red blood cells, and in maintaining the function of peripheral nerves. Therefore, its deficiency causes the risk of peripheral neuropathy.[66] Peripheral neuropathy occurs in BTM for various reasons: including high levels of ferritin in the blood, anemia, and a lack of B vitamins, including Vit.B12.[26] (Lardhi, A., and his group) confirmed that Vit.B12 is one of the essential vitamins for the formation of DNA, red blood cells, and the proper functioning of the nervous system. And any deficiency in its levels causes a group of neurological, bloody, and psychological manifestations, as well as chronic atrophic gastritis, which results in the loss of parietal cells in the stomach due to the presence of certain autoantibodies.[28] A study was conducted by (Shaikh, A., and others) on BTM, where they noted the deficiency of Vit.B12 in the younger age groups. It was more in females at childbearing age, and they attributed the reason to the increased need of the body for these vitamins or the inadequacy of the diet or the loss of micronutrients in the blood through the regular menstrual cycle in women. Its levels certainly decrease physiologically during pregnancy. A contributing cause is low birth weight, growth retardation, or neural tube defects. The study also indicated that the mother who suffers from a deficiency of Vit.B12 and has a nutritional deficiency while she is breastfeeding her child at the age of 4 to 8 months, which shows that they have a deficiency of Vit.B12.[67]

MDA

MDA levels increased in BTM patients, as this study was consistent with the studies of (Awadallah, S.M., et al.),[68] (Caroline, P.O.L., et al.),[7] (Jabbar, E.E., et al.),[39] and (Najia). , N.A., et al.).[69] Frequent BTM

transfusions lead to oxidative tissue injury due to iron overload as a result of oxidative stress leading to increased levels of MDA lipid peroxides and free radicals.[70] The researchers confirmed (Caroline, P. O. L., and others) that patients with elevated levels of MDA result from congenital disorders resulting from reduced formation of beta-clobin chains, patients with elevated levels of MDA result from congenital disorders resulting from reduced formation of beta-clobin chains, Which causes the production of ineffective red blood cells due to the occurrence of oxidative stress to increase the accumulation of unstable free α-globin chains, leading to the destruction of red blood cells. In addition to the release of iron from heme, causing its accumulation inside the cells and in the blood plasma to produce ROS represented by free radicals such as hydroxyl radicals and hydrogen peroxides that disrupt the integrity of the cellular membranes of red blood cells. Hydroxyl radicals break chains of polyunsaturated fatty acids present in the structure of cellular membranes, forming lipid peroxides, and MDA is one of these harmful products.[7] MDA lipid peroxides and protein oxidation lead to disturbances in the regulation of the lipid membrane of cells and lead to deformation leading to engulfment of red blood cells and their decomposition when exposed to phosphatidylserine. This phenomenon appears in patients with BTM and is called (Eryptosis). It is the process of decomposition of red blood cells after disease and before aging, leading to shrinkage of cells with the loss of their ability to organize membranes.[71]

Iron (Fe)

Significantly higher levels of iron in BTM patients compared to control were consistent with several studies, including (Jabbar, E.E., and others), [39] (Salah Noori, R., and others), [72] (Sposi, N. M.), [31] (Chaudhry, A. F., and others), [73] (Yadav, P. K., and his colleague), [33] and (Abdulla, A. A.).[74] Iron accumulation and increased levels induce oxidative stress that depletes antioxidants in the cell. And when the oxidants increase, they cause significant damage to the organelles and membranes of red blood cells in the stages of their formation, as a result of the inability or efficiency of the drugs used to get rid of iron. Iron also rises through absorption in the digestive tract, and its buildup may exceed the body's ability to detoxify ferritin. Blood transfusions generate about (1 mg) of iron per (1 ml) of blood, which makes it difficult to get rid of iron in the body in return for increasing the absorption of iron in the small intestine.[39] To result in primary and secondary hemochromatosis, whose symptoms lead to genetic diseases.[72] The study (Ram, G., et al.) indicated that BTM patients with age often had higher iron stores after chelation therapy. It has been proven that iron deposition from repeated blood transfusions causes thyroid dysfunction.[75] It also causes early osteoporosis.[59] (Pilo, F., et al.) stated that the iron in BTM is highly toxic due to its high ability to gain and lose electrons and interact with oxygen leading to the formation of ROS, which destroy the

physiological cellular antioxidant system. It causes damage to the pituitary gland even in the absence of cirrhosis.[76] (Salah Noori, R., et al.) showed that free radicals act on the deposited iron to overcome the cellular antioxidant mechanisms, leading to peroxide damage that causes many diseases in tissues, including the heart and pancreas.[77] and developmental delay, cirrhosis, failure of sexual maturation, diabetes and subsequent death.[9]

Liver enzyme levels (AST, ALT, ALP)

Levels of liver enzymes (AST, ALT, ALP) were high in BTM patients compared to control, especially with age. The study came in agreement with several studies conducted by (Jabbar, E. E., and others),[39] (Najia, N. A., and others),[69] (Hamed, O. M., and others),[38] (Abdulla, A. A.),[74] (Harish, G., and his colleague),[41] (Saleh, K. K., and others),[78] (Suman, R. L., and others),[35] and (Goyal, P., and his colleague).[79] The liver in BTM is affected by iron overload due to its direct toxic effect on liver cells, leading to elevated levels of liver enzymes (AST, ALT, ALP).[41] (Hamed, O.M., et al.) showed that the high levels of liver enzymes in BTM are attributed to damage and not necessarily to death of liver cells. Which leads to an increase in cell permeability due to the change in the chemical composition of the protein, causing an increase in the activity of these enzymes and their release into the blood circulation.[38] Several studies, including (Harish, G., and his colleague) confirmed that the reason for the high levels of liver enzymes (AST, ALT, ALP) in patients with BTM is related to high ferritin levels, when its levels exceed (1000 ng/L) and consider it as an indicator with a clear effect for evaluating the condition liver.[41] It was also observed that repeated blood transfusions lead to an increase in ferritin levels as well, and its crossing (2000 ng/L), especially after (30) blood transfusions. The liver is the first organ to be affected by iron overload as a result of peroxide damage and the direct toxic effect of iron on liver cells.[35] The study conducted by (Jabbar, E.E., and his colleague) showed that ALP levels increase in BTM with age, specifically after the age of ten, since the bulk of ALP is secreted from the two tissues (bone marrow - liver) into the blood circulation due to the pathological conditions of these two tissues. ALP levels increase in the liver due to the accumulation of iron and its high levels. While in the bone tissue caused by osteoporosis. Since ALP is secreted from cell membranes in the hepatic sinuses and bile ducts, any blockage in the bile ducts, whether inside or outside the liver, leads to high levels of ALP in the blood due to damage to the liver cells.[39] ALP is found in various tissues in the body, such as the liver and bone marrow. Its evaluation is a diagnostic marker for liver diseases, hemolytic anemia, bones, thalassemia and cancer.[38] In addition to the liver being infected with metastatic liver cancer and lymphoma.[39] The high levels of (AST, ALT) in the BTM leads to an enlarged spleen and affects liver functions.[74]

Total, direct and indirect bilirubin levels

The results of the current study agree with the studies of (Faiq, A. B., et al),[80] (Saleh, K. K., and his colleague),[78] (Nafady, A., et al),[81] and (Mohammed, W.R., et al).[44] Non-enzymatic liver variables such as total, direct and indirect bilirubin levels are among the most important variables experienced by BTM patients who undergo frequent blood transfusions due to an increase in the amount of total bilirubin in their bloodstream. The main cause of hyperbilirubinemia is due to increased hemolysis resulting from the destruction of erythrocytes by the spleen and a decreased ability of the liver to conjugate bilirubin.[81] On the other hand, damage to liver cells resulting from iron overload can lead to hepatotoxicity, thus increasing bilirubin concentrations and its release into the bloodstream.[78] Increased bilirubin production leads to other complications in BTM patients by hemolysis and biliary obstruction. The deposition of high concentrations of indirect bilirubin and calcium bilirubinate inside the cavity of the gallbladder leads to the formation of gallstones. Serum bilirubin and ALP can be good markers for follow-up and control of bile duct obstruction due to repeated transfusion and hemolysis.[80] The liver works to discharge and excrete more than (3000 g) of bilirubin per day, while the natural production increases in the conjugation and secretion of Bilirubin Digluconide. Due to the massive decomposition of red blood cells in BTM patients, it leads to the excretion of bilirubin in the bile, with an increase in the quantities of urobilinogen that enter the enterohepatic circulation and urinary urobilinogen, and this makes the levels of indirect bilirubin in a continuous rise in the blood, causing jaundice.[44] And (Mahomoodally, M. F., and his colleague) proved that bilirubin acts as an antioxidant of endogenous origin, by proving its ability to get rid of harmful free radicals in the body, strengthen the immune system, and provide protection for the heart and blood vessels, but within the normal range specified for it in the body (0.2-1.2mg). /dL). Bilirubin is the final product of heme degradation, and it is a toxic compound for tissues. If its level exceeds the normal limit, it is harmful to the body.[42]

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