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Ministry of Higher Education and Scientific Research
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Assessment of Urinary N-acetyl-beta-Glucosaminidase level and Renal Function in Patients with Transfusion Dependent beta – Thalassemia Major in Babylon Center of Hereditary Blood Disorders

A Thesis

Submitted to the Council of the College of Medicine/ University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master of Science / Clinical Biochemistry

By

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بسم الله الرحمن الرحيم

قَالَ رَبِّ ٱشۡرَحۡ لِي صَدۡرِي ٢٥ وَيَسِّرۡ لِيٓ أَمۡرِي ٢٦ وَٱحۡلُلۡ عُقۡدَةٗ مِّن لِّسَانِي ٢٧ يَفۡقَهُواْ قَوۡلِي ٢٨

صدق الله العلي العظيم

سورة طه (٢٥-٢٨)

Dedication

To my lovely father and mother.

To soul of my dear brother" Ahmed "

To my wife, to my roses, Ridha& Abbas

To everyone I love.

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I would like to express my extreme thanks to Allah, Most Gracious, and Most Merciful.

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Also, I'd want to express my thanks to everyone who has assisted me.

AMJAD

Supervisor Certification

We certify that this thesis entitled (Assessment of N-acetyl-beta-Glucosaminidase (NAG) and Renal Function in Patients with beta – Thalassemia Major with Repeated Blood Transfusions in Babylon Center of Hereditary Blood Disorders) has been prepared under our supervision at the Department of Biochemistry, College of Medicine, University of Babylon, in partial requirements for the degree of master in Clinical Biochemistry.

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In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

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COMMITTEE CERTIFICATION

We, the examination committee, certify that we have read the thesis entitled "**Assessment of Urinary N-acetyl-beta-Glucosaminidase level and Renal Function in Patients with Transfusion Dependent beta – Thalassemia Major with in Babylon Center of Hereditary Blood Disorders "** and have examined the student Amjad Hussein Jawad in its contents and that in our opinion it is accepted as a thesis for degree of Master of Science in clinical biochemistry with excellent estimation.

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List of Abbreviations

Abbreviation	Details
A/C Ratio	Albumin/creatinine ratio
AUC	Area under curve
B2M	Beta2-microglobulin
BMD	Bone mineral density
CE	Capillary electrophoresis
CKD	Chronic renal disease
DFO	Desferrioxamine
DFP	Deferiprone
DFX	Deferasirox
DXA	Dexa scan
fl	Femtoliter
G6PD	Glucose-6-phosphate dehydrogenase
GFR	Glomerular filtration rate
HbA	Adult hemoglobin
HbA2	Minor Adult hemoglobin
HbF	Fetal hemoglobin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography

HRP	Horseradish Peroxidase
HSCT	Hematopoietic stem cell transplantation
IE	Ineffective erythropoiesis
IL-18	Interleukin-18
KDa	Kilo Dalton
LCR	Locus control region
LDH	Lactate dehydrogenase
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
NAG	N-acetyl-beta-D-glucosaminidase
NGAL	Neutrophil gelatinase–associated lipocalin
NPV	Negative predictive value
NTBI	Non-transferrin bound iron
NTDT	Non-transferrin dependent thalassemia
OD	Optical density
PCR	Polymerase chain reaction
pg	Pico gram
PPV	Positive predictive value
RBP	Retinol binding protein
RDW	Red cell distribution width

RNA	Ribonucleic acid
ROC	Receiver operator characteristic
SD	Standard deviation
SPR	Solid Phase Receptacle
STP	Strip
TDT	Transfusion-dependent thalassemia
TIMP-2	Tissue inhibitor of metalloproteinases-2
VIDAS	Vitek Immuno Diagnostic Assay System
WHO	World Health Organization
α	Alpha
β	Beta
βTM	Beta thalassemia major
γ	Gama
δ	Sigma
ζ	Zeta
ε	Epsilon

Summary

Beta-thalassemia is most prevalent genetic hemoglobinopathy in the world. It is caused by a reduction or absence of beta globin chain production, which is typically a portion of adult hemoglobin (HbA, which is $\alpha 2\beta 2$). A compensatory ineffective erythropoiesis, severe anemia, and accelerated erythrocyte turnover will result from this genetic abnormality. Thalassemia syndrome is frequently accompanied with a wide range of problems resulting from both the disease and the treatments used to treat it.

This study was designed as a case-control study and was constructed to study the renal complications in beta-thalassemia major patients with repeated blood transfusion. To achieve this aim, 60 patients with beta-thalassemia major, 33 of them male and 27 of them female, and with 60 apparently healthy as control group, 33 of them male and 27 of them female.

These patients divided into two subgroups according to age, group I were less than 18 year, 38 patient with mean ± standard deviation (14.00 ± 2.75) year, group II were equal or more than 18 years, 22 patient with mean ± standard deviation (23.13 ± 4.45) year. In this study excluded any patient with: nephropathy, diabetes mellitus, chronic liver disease, Pregnant, and other hemoglobinopathies. In addition, Patients divided into two groups based on the treatment use, 14 patients use desferrioxamine and 44 patients use deferasirox treatment.

All samples were collected from Babylon center of hereditary blood disorder from November 2021 to January 2022 and this study was performed at the laboratory of Department of Chemistry and Biochemistry in College of Medicine- University of Babylon.

The serum and urine samples were used to measure biochemical parameters, N-acetyl D- glucosaminidase, β-2 microglobulin, albumin creatinine ratio, calcium creatinine ratio, serum ferritin, serum creatinine, and blood urea.

Results of the present study revealed that there was a significant differences in the levels of Albumin creatinine ratio, N-Acetyl beta-D-glucosaminidase, Beta2-microglobulin, calcium creatinine ratio, serum creatinine, and blood urea between patients with beta-thalassemia major and its control group ($p < 0.05$).

In this study significant positive correlation between age with ACR and β2M, and there was significant positive correlation between ACR and β2M, UCa/Cr level. There is a statistically significant positive correlation between the ferritin level and the number of previous blood transfusions. positive significant correlation between ferritin with NAG and β2M.

In conclusion, renal hemosiderosis and asymptomatic renal dysfunction are prevalent among β-thalassemia major patients with repeated blood transfusion, which are not found in routine renal investigations, which required regular screening with early markers of glomerular and tubular dysfunction.

1. Introduction

1.1. Thalassemia Syndromes

The thalassemia syndromes are a group of inherited autosomal recessive conditions associated with hemolytic disorders that result from defect in production of hemoglobin and ineffective erythropoiesis(IE) [1, 2].

The word of thalassemia is derived from two Greek words: “Thalassa” which means “the sea” and “Haima” which means “blood” [3]. Dr. Cooley first described thalassemia in 1925 [4]. According to polypeptide chain defective of hemoglobin in red blood cells, there are two types of thalassemia, alpha thalassemia that is caused by a defect in the rate of synthesis of α chains, and beta thalassemia that is caused by a defect in the rate of synthesis of β chains [3].

Ineffective erythropoiesis and RBCs hemolysis are the reason for the anemia, therefore transfusions process of packed red blood cells are a common treatment for most thalassaemic patients [1]. Because each unit of blood contains 200 to 250 mg of iron, repeated transfusions generate excessive iron loading, which can lead to transfusional siderosis with accumulation of iron in different organs [5]. Iron may accumulate in a variety of tissues, including reticuloendothelial cells in organs like the spleen, liver, and bone marrow, as well as parenchymal cells in the heart, liver, kidneys, pancreas, and endocrine glands including the Langerhans islets, pituitary gland, testicles, and ovaries [6] .

1.1.1. Hemoglobin

Hemoglobin (Hb) is a hemoprotein of the red blood cells that mainly function to carry oxygen from the lungs to the tissues. Other functions include the transport of carbon dioxide (CO_2) and a buffering action. The molecular weight of hemoglobin is 64–64.5 kDa. Heme is required for oxygen transport, while globin protects heme from oxidation, makes it soluble, and allows for variation in oxygen affinity [7].

Hemoglobin is made up of four different globin subunits (two α- and two non–α-, β-, γ-, or δ-chains). All hemoglobins carry the same prosthetic heme group iron protoporphyrin IX associated with a polypeptide chain (alpha) and (beta), each subunit was folded in half to form a pocket or cleft in which the heme group could nestle.. The ferrous ion of the heme is linked to the N of a histidine[8], as seen in Figure 1.1.

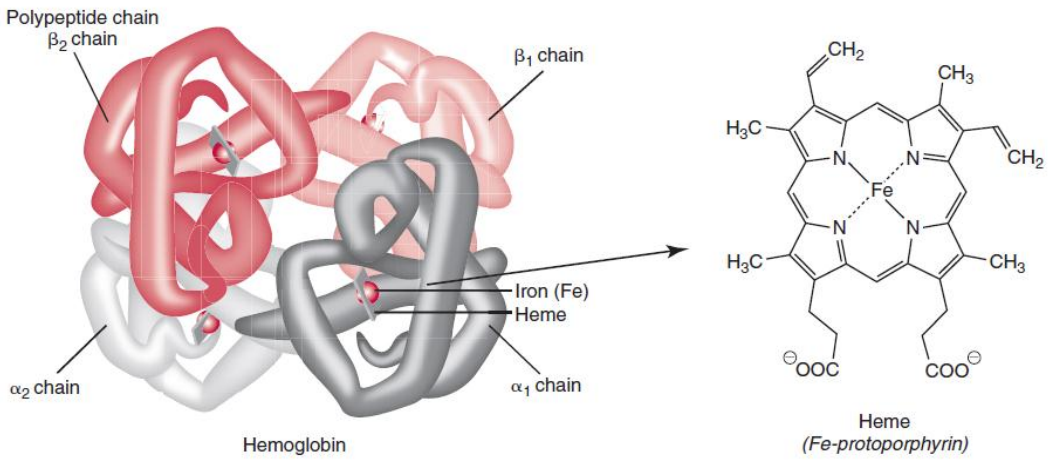

Figure.1.1 Structure of the hemoglobin tetramer [9]

1.1.1.1. Types of Hemoglobin

The globin chains, which make up each molecule of hemoglobin, are made up of two pairs of identical subunits. There are two types of chains in this collection : the α -globin cluster, which includes the globin chains ζ - and -α globin chains, and the β-globin cluster, which includes the globin chains ε,γ, β and -δ globin chains [10].

There are four major types of hemoglobin, these types are formed according to pairing of these globin chains during ontogensis, these types as the following:

1. "Embryonic" hemoglobins, represent ζ2ε2 (Hb Gower 1), α2ε2 (Hb Gower 2), ζ2γ2 (Hb Portland 1); and ζ2β2 (Hb Portland 2); these types of hemoglobin can be detected from the third through the tenth week of pregnancy,
2. "Fetal" hemoglobin (HbF α2γ2), which constitutes the predominant oxygen carrier to the fetus during pregnancy.
3. "Adult" hemoglobin (HbA α2β2), this type of hemoglobin replaces HbF shortly after birth.
4. A minor adult component, HbA2 (α2δ2) [11].

The process of manufacturing and halting different types of hemoglobin at different phases of human development is referred as "haemoglobin switching". Distinct hemoglobin, are generated in the embryo, fetal, and adult as an adaptation to differing oxygen requirements. The increase in the synthesis of fetal hemoglobin (α2γ2) during the first year of life is frequently the cause of severe B –thalassemia [12].

Adult human red cells contain around 97-98 % of HbA, 2-3 % of HbA2, and traces of HbF under normal circumstances [11].

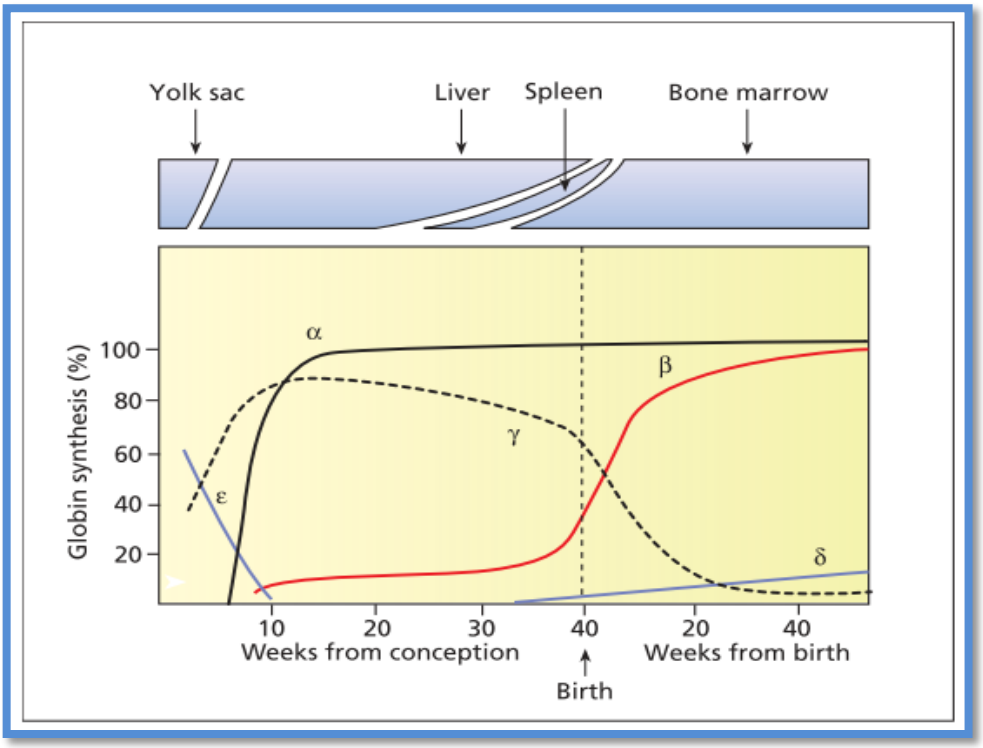

Figure 1.2 Globin synthesis during the embryonic, fetal, and adult phases of erythroid development [13].

1.2. Beta- Thalassemia

The β -thalassemia is the most common type of thalassemia, caused by a reduced rate of β- globin production. There are more than 200 β-thalassemia mutations known. The defect's severity is very varied. Beta- thalassemia can be heterozygous or homozygous because normal individual have two allelic β- globin genes. Because there are so many β-thalassemia mutations, compound heterozygosity (having two mutant genes but no normal β gene) is a possibility [14]. Therefore, the beta globin chains of the hemoglobin (Hb), which is made up of two alpha globin and two beta globin chains, are decreased ($beta^{+}$) or absent ($beta^{0}$) in beta-thalassemia [15].

1.2.1. Classification of Beta Thalassemia.

βeta-thalassemia can be broadly classified into three categories:

- **β-thalassemia minor or β-thalassemia trait:**

Characterized by one defective gene and one normal gene. Individuals may experience mild anemia but do not require transfusion. The hallmark of β-thalassemia minor is an Hb electrophoresis with an elevated HbA2. As with the α-thalassemias, it is important to not inappropriately diagnose or treat these patients as iron-deficient [16].

- **β-Thalassemia intermedia:**

Characterized by two defective genes, but some β-globin production is still observed in these individuals. Symptoms range between that of β-thalassemia minor and β-thalassemia major. However, some individuals may have significant health problems requiring intermittent transfusion [16].

- **β-Thalassemia major (Cooley's anemia):**

Characterized by two defective genes but almost no function of either gene, leading to no synthesis of β-globin. These individuals have a severe form of disease requiring lifelong transfusion and may have shortened life span [16].

1.2.2. Epidemiology

Inherited hemoglobin diseases are mostly seen in low- and middle-income countries in the tropical belt, which stretches from Sub-Saharan Africa through the Mediterranean and Middle East, and then to South and Southeast Asia [17]. This is due to the high prevalence of consanguineous marriages in these areas, as well as carriers' acquired resistance to severe types of malaria in areas where the disease has been or is still widespread [18]. Other causes for the high prevalence of thalassemia in these countries include gene drift and founder effects. As a result of increased movement of individuals from locations where the illness is growing, thalassemia is now present in most regions, including Australia, the United States, Canada, South America, and North Europe [19].

According to the World Health Organization (WHO)in 2018, at least 5.2 % of people worldwide have thalassemia, and around 1.1 % of couples are at risk of producing children with a hemoglobin abnormality[20]. In Iraq, thalassemia has a prevalence of 35.7/100000 and an incidence of 4.5 per 100000 [21].

1.2.3. Etiology

Two multigene clusters on chromosomes 16 and 11 regulate hemoglobin production. Three homologous genes are found on chromosome 16, the zeta (ζ) gene, alpha 1 and alpha 2 genes and five functional genes are located on chromosome 11 (ε , Gγ , Aγ , δ , and β) they are placed on the chromosome in the order of their developmental expression to produce

different Hb tetramers at various stages of embryonic and fetal development [22], as show in Figure 1.3.

The locus control region (LCR) is located upstream of this globin complex, the LCR is made up of numerous DNase I hypersensitive sites that include transcription activator binding sites, and it acts as an enhancer to control the spatiotemporal transcription of globin genes, the expression of the globin gene is low when it isn't present [9].

On the β-gene cluster, more than 200 β-thalassemia mutations have been identified, influencing any stage of B-globin mRNA production, from transcription to RNA processing to translation. The majority of mutations are frame-shift mutations, which are caused by substitutions, deletions, or insert oligonucleotides. Gross gene deletions are rarely the cause of β-thalassemia [20,21].

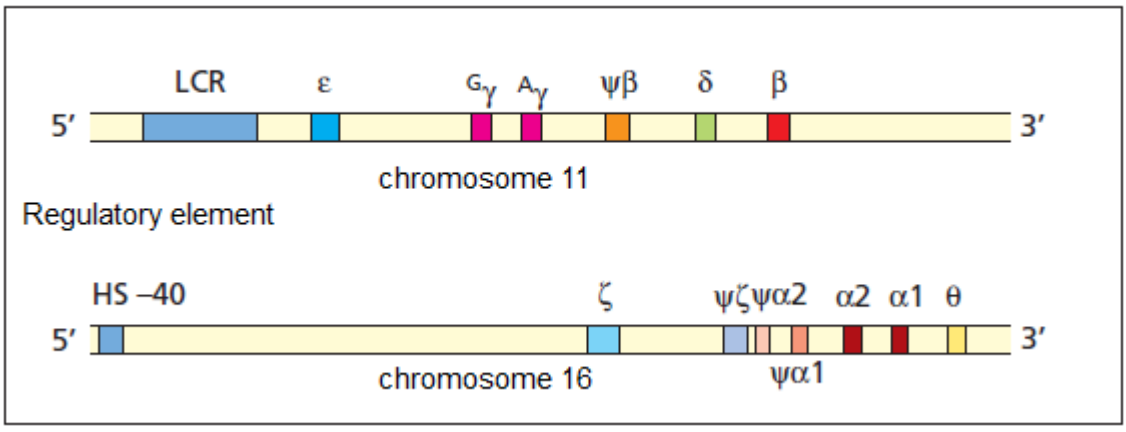

Figure 1.3. Globin α- and β-Gene Clusters[24].

1.2.4. Pathophysiology

When β-globin is decreased or absent, excess unpaired and insoluble α-globin chains precipitate at the membrane of red cell precursors. Free iron catalyzes the generation of reactive oxygen species(ROS), which cause oxidative membrane damage and apoptosis of red blood cell precursors in the bone marrow, resulting in widespread premature destruction (ineffective erythropoiesis) [23,24], as shown in Figure 1.4.

Erythroid marrow hypertrophy in medullary and extramedullary locations causes abnormalities or cranial and facial malformations, as well as extramedullary erythropoietic tissue masses, cortical thinning, and pathological fractures of long bones. The abnormal RBCs' lipid membrane composition might cause thrombotic complications, especially in splenectomized patients[27]. Erythropoiesis, anemia, and hypoxia all suppress hepcidin, which plays a key role in iron homeostasis in individuals who do not get blood transfusions [28].

Increased duodenal iron absorption and systemic iron overload result from hepcidin deficiency, whereas in patients who receive regular blood transfusions, most of the excess iron is due to the breakdown of red blood cells. When transferrin's iron-binding capacity is reached, iron can appear in the serum as non-transferrin bound iron, which is a major catalyst for the generation of free radicals that cause oxidative stress and damage to mitochondria, lysosomes, lipid membranes, protein, and DNA, resulting in organ damage in thalassemia patients [25].

The red cells that have reached the circulation are small, deformed, and full with abnormalities. They also contain a trace amount of Hb, which gives

RBCs their characteristic microcytic hypochromic appearance. Because many of these cells are destroyed by the spleen or hemolyzed directly in the circulation owing to Hb precipitants, thus the destruction of erythrocytes in the bone marrow, spleen and periphery causes anemia [29].

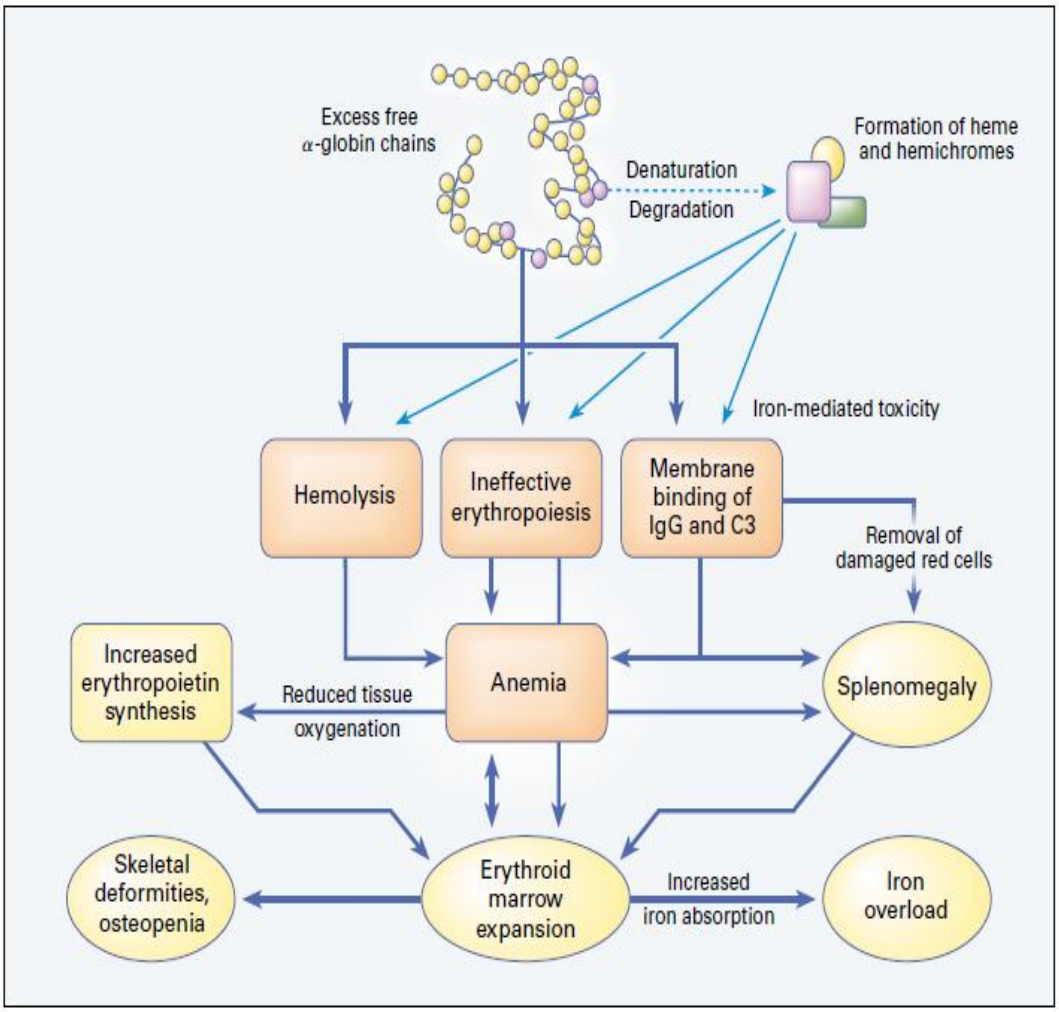

Figure 1.4. Effects of Excessive Free-Globin Chain Production [22].

1.2.5. Clinical presentation

Abdominal girth expansion, small size for age, and failure to develop are common clinical features in children younger than one year of age. The patient's physical examination may indicate frontal bossing, which is produced by thickness of the cranial bones, pallor, and prominence of the cheek bones cause the base of the nose to be obscured and the teeth to be exposed. These characteristics are the result of ineffective erythropoiesis, which causes bone marrow expansion (up to a 30-fold increase), which results in the production of highly unstable α-globin tetramers, triggering a chain of events that includes anemia, hemolysis, splenomegaly, and increased iron absorption [9].

In some developing countries when patients are untreated or inadequately transfused as a result of a limitation of resources, the clinical picture of thalassemia major is marked by retarded growth, jaundice, pallor, weak musculature, hepatosplenomegaly and ulcers on the legs [30].

1.2.6. Diagnosis of β-thalassemia major

Thalassemia may be suspected if an individual shows signs that are suggestive of the disease. However, laboratory diagnosis is required to establish the exact diagnosis and to provide accurate genetic counseling to parents and affected individuals regarding recurrence risks and testing choices [31].

1.2.6.1. Hematological Diagnosis

Hematological diagnosis depends on the blood count and blood smear, patient with β-thalassemia major is characterized by decrease haemoglobin

level less than 7 g/dl), mean corpuscular haemoglobin (MCH) >12 and <20 pg, mean corpuscular volume (MCV) > 50 and <70 fl. The blood smear shows microcytosis, hypochromia, anisocytosis, poikilocytosis (elongated cells with spiculated tear-drops), target cells and erythroblasts. The number of erythroblasts rises after splenectomy and is related to the degree of anemia [30].

It is worth noting that the level of MCH and MCV decreases in other types of beta thalassemia and some types of anemia, but at different levels, and the diagnosis is supported by other types of blood picture indicators such as red blood cell (RBC) count and red cell distribution width (RDW) [32].

1.2.6.2. Haemoglobin analysis

Cellulose acetate electrophoresis or capillary electrophoresis (CE), DE-52 microchromatography, or high-pressure liquid chromatography (HPLC) are used to identify qualitative and quantitative haemoglobin [13]. Electrophoresis or HPLC measurement of hemoglobin is the gold standard for diagnosing β-thalassemia carriers [32].

In β^0 thalassemia homozygotes, HbA is undetectable, whereas HbF accounts for 92-95 % of total hemoglobin. HbA levels in β^+ thalassemia homozygotes range from 10% to 30%, while HbF levels range from 70% to 90%. HbA2 level is varied in β thalassemia homozygotes , although it is increased in thalassemia minor [23].

1.2.6.3. Molecular analysis

Because each group has a small number of mutations, molecular genetic testing has become easier. Methods based on PCR are used to detect

commonly occurring globin gene mutations. Screening for single base mutations has become much easier with the invention of the PCR.

The majority of thalassemia mutations are point mutations. Point mutations are Single-base replacements, minor insertions, and deletions [33].

The most common technique is primer-specific amplification, which employs a set of probes or primers that are complementary to the most prevalent mutations in the group from which the affected individual originated. If focused mutation analysis fails to find the mutation, sequence analysis of the β-globin gene may be used [22].

1.2.7. Complications of β-thalassemia major

A- Iron overload.

There is no physiological mechanism for the body to eliminate excess iron. When iron consumption is increased over time, either as a consequence of red blood cell transfusions or as a result of increased iron absorption through the gastrointestinal (GI) tract, iron overload (haemosiderosis) develops. Both of these occur in patients with β-thalassemia major [34]. In individuals with βTM , iron overload is a major problem with several structural and functional consequences [35].

Another mechanism that accounts for iron overload is dyserythropoiesis. It's been proven that it's caused by the liver producing less hepcidin, an iron-regulating hormone. Hepcidin deficiency causes the cellular iron exporter ferroportin to become activated; this causes an increase in the amount of iron that enters the plasma [36].

The contribution of dyserythropoiesis as a source of iron excess in thalassemia major is low relative to that caused by blood transfusions, but it

may explain why these individuals might acquire considerable iron overload even before receiving blood transfusions [37].

B- Cardiac Complications.

Iron overload is the most important a potential cause of heart failure in patient with βTM. When the heart is exposed to a high quantity of circulating non-transferrin bound iron over an extended period, this is known as cardiac iron loading. Despite advancements in therapeutic care of thalassemia major and the resultant significant increase in patient survival, heart disease has always been and continues to be the primary cause of death and illness [36,37]. In thalassemia major patients, heart disease accounts for more than half of all deaths [38,39]. Heart failure, cardiomyopathy, pulmonary hypertension, arrhythmias, pericarditis, and myocarditis are some of the symptoms that might appear[39,40].

C- Enlargement of the Liver and Liver Disease.

Among several organs damaged in patient with β-thalassemia, the liver is a major target. The major cause is an excess of iron in the body [43]. Hepatitis viruses, particularly the hepatitis B virus (HBV) and hepatitis C virus (HCV), continue to be a major cause of worry [44].

The most prevalent complication of chronic liver disease is the development of liver cirrhosis, which increases the risk of hepatocellular carcinoma (HCC), which is becoming more common as β-thalassemia outcomes improve [45]. Iron overload will affect the hepatic macrophages that are substantially less frequent than hepatocytes. Following that, the iron stored in macrophages will be released into the circulation over time, eventually when it reaches the bone marrow it triggers the formation of new red blood cells [46].

The iron saturation of plasma transferrin occurs during this release phase, As a result, non-transferrin-bound iron appears in the plasma. Hepatocytes, heart cells, and pancreatic cells readily absorb this iron species, causing these organs to become overloaded.

D- Enlargement of the Spleen.

Increased red blood cell breakdown by the reticuloendothelial system, particularly in the spleen, resulting in spleen enlargement (splenomegaly). Modern transfusion regimens, on the other hand, have significantly decreased the incidence of splenomegaly and splenectomy in BTM patients by establishing more appropriate pretransfusional haemoglobin levels and transfusion intervals [45,46].

E- Infections.

Infections and associated consequences were the second leading cause of mortality among transfusion-dependent thalassemia patients (TDT).In some countries infections are becoming the biggest cause of death since the frequency of deaths due to iron-related heart problems has decreased significantly [49].

F- Endocrine Complications.

Endocrine abnormalities such as hypogonadism, hypoparathyroidism, hypothyroidism, and pancreatic and adrenal insufficiency, are among the most prevalent β-thalassaemia major complications [50]. The most common manifestations of endocrinopathies are delayed puberty, growth retardation, diabetes, hypothyroidism, and hypogonadism [49,50].

The most common cause of endocrinopathies in beta thalassemia patients is presence of significant amount of iron toxicity in the endocrine gland[50]. Therefore, most endocrine problems are discovered as iron deposition progresses throughout the second decade of life. Other variables, such as chronic anemia, zinc insufficiency, and the toxicity of iron chelating drugs, contribute to the development of endocrinopathies [52].

G- Bone Abnormalities and Osteoporosis.

In patients with β-thalassemia, bone abnormalities have also been described such as spinal deformities, scoliosis, nerve compression, spontaneous fractures, osteopenia and osteoporosis.

Transfusion programs and chelation treatment have greatly increased survival rates. As a result, osteopenia and osteoporosis are common causes of morbidity in young adults with BTM, and the incidence of osteopenia or osteoporosis in well-treated BTM patients has been reported to be around 40–50 % [53].

Bone mineral density (BMD) is a well-known and commonly applied bone health indicator. The gold standard for determining BMD is DXA. In thalassemia, marrow expansion disrupts bone formation mechanically, leading to cortical thinning; it is still regarded to be a main factor of thalassemia patients' bone deformation and fragility. In addition to the ineffective erythropoiesis and increasing bone marrow expansion, several inherited and acquired variables such as endocrine complications, are implicated in bone destruction in TM [54].

1.2.8. Management of β-Thalassemia Major

A. Prevention Strategies.

To avoid beta-thalassemia, community awareness programs, prenatal screening, carrier detection, and genetic counseling are all employed.

B. Transfusion Programs.

The goals of transfusion treatment are to treat anemia, decrease erythropoiesis, and inhibit iron absorption in the gastrointestinal tract, which happens in untransfused patients due to increased ineffective erythropoiesis [55].

One of the treatments recommended for patients with βTM is blood transfusions on a regular basis for the rest of one's life, generally every two to five weeks to keep the pre-transfusion haemoglobin level above 9-10.5 g/dl. In most patients, this transfusion regimen supports normal development, enables normal physical activities, effectively reduces bone marrow activity, and minimizes transfusional iron deposition [12,54].

Patients diagnosed with beta-thalassemia major should begin transfusion treatment if they have severe anemia with level of hemoglobin less than 7 g/dL on two separate days separated by more than two weeks.

Other causes of anemia should be excluded, such as infections, blood loss, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and folic acid deficiency [55]. Transfusions may be required as early as six months of age [12].

When possible, frequent transfusions should begin before the second or third year of life to avoid the development of numerous anti-red blood cell antibodies and the consequent difficulty in obtaining compatible transfusion units [30].

A pre-transfusion hemoglobin level of 11 to 12 g/dL may be adequate for heart disease patients, clinically relevant extramedullary hematopoiesis, and a patient with a spleen enlargement[56]. The mean target hemoglobin level should be 12 g/dL to avoid the risk of a stroke that arises from hyperviscosity as a result of an increase in hemoglobin level after the transfusion, so the post-transfusion Hb level should be kept between 14 and 15 g/dL [55].

C. Iron Chelating Therapy.

Iron overload must be treated when serum ferritin levels exceed 1000 mg/L, which occurs after 10 to 20 blood transfusions [57]. Chelation treatment aims to regulate the iron deposition after RBC transfusions through enhancing iron excretion in urine and/or utilizing chelators to increase iron excretion in the faces. Because iron is necessary for essential metabolic processes, balancing the benefits of chelation therapy with the risks of over-chelation is one of the most difficult challenges of chelation therapy. The second challenge in the use of these treatments is the commitment to take the treatment over time, even a simple defect in the organization of treatment times and its interruption can negatively affect the patient [58].

There are now three iron chelators approved for clinical use, each with unique iron binding characteristics, absorption, elimination, and metabolism pathways. These is summarized in Table 1.1.

Table: 1.1 –Chemical and pharmacological properties of licensed chelators [59].

COMPOUND	Desferrioxamine (DFO)	Deferasirox (DFX)	Deferiprone (DFP)
Molecular weight (daltons)	560	373	139
Log Iron binding affinity (pM)	26.6	22.5	19.9
Delivery	intravenous or subcutaneous 8-12 hours 5 days/week	Oral, once daily	Oral, 3 times daily
Half-life of iron free drug	20-30 minutes	12-16 hours	3-4 hours
Route of iron excretion	Urinary and fecal	Fecal	Urinary
Elimination of iron complex	Urine + feces Iron complex removed more slowly than free drug	Feces	Urine
Metabolism	Intrahepatic to metabolite B which binds iron	>90% of it is excreted in the feces, and 60% of it is unmetabolized. Iron is bound by the majority of metabolites.	Glucuronide formed in liver does not bind iron
Recommended dose mg/kg/d	30-60 5-7 x/week	20-40 One/day	75-100 in 3 divided doses
Chelation efficiency (% of drug excreted iron	13	27	7

Main Adverse effects	Ocular, auditory, bone growth retardation local reactions, allergy	Gastrointestinal, increased creatinine, increased hepatic enzymes	Gastrointestinal, arthralgia, agranulocytosis/ neutropenia

D. Splenectomy.

Thalassemia major is characterized by splenomegaly and hypersplenism. Splenectomy is the procedure of choice for minimizing excessive consumption of blood and thus, reducing iron overload.

However, the lack of a spleen is linked to many of the complications. Infection risk, most often sepsis from encapsulated organisms (Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitides), varies between researches, ranging from more than 30-fold in contrast to the general population [47].

Other side effects of splenectomy include an increased risk of thrombotic complications and pulmonary hypertension, which may be caused by an increase in circulating platelets and immature red blood cells, as well as altered endothelial function, increased platelet activation, and lower levels of proteins C and S [60].

E. Stem Cell Transplantation.

Bone marrow transplantation is the only curative treatment for beta-thalassemia major [61]. The principle of hematopoietic stem cell transplantation (HSCT) is to replace the thalassemic HSC with ineffective erythropoiesis with an allogeneic HSC with functional erythropoiesis.

The majority of transplantation with stem cells is performed by using human leukocyte antigen (HLA) compatible sibling donor bone marrow, peripheral blood stem cells(PBSCs) and cord blood stem cells [62].

F. Gene Therapy.

The standard curative therapy is bone marrow transplantation from a matched related donor. Nonetheless, it has substantial downsides, including a paucity of major histocompatibility complex MHC-matched donors, the requirement for long-term immunosuppression, restricted use to the youngest patients and an increased risk of immunological problems, as well as non-rejection death in older patients with organ damage [63].

This is because hematopoietic stem cell compartment engraftment can be affected by age-related extramedullary hematopoiesis, chronic inefficient erythropoiesis, and iron overload. Furthermore, alternate donor transplants, such as matched unrelated or identical donors, may not constitute a risk-free procedure [64].

In order to correct ineffective erythropoiesis and hemolytic anemia, gene therapy for beta-thalassemia aims to ensure stable introduction of functioning globin genes into the patient's own hematopoietic stem cells (HSCs). This was considered to offer curative potential for patients who couldn't have transplants or didn't have an MHC-compatible donor, as it avoided the immunological risks of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and the necessity for immune suppression [65].

1.3. Renal Dysfunction in BTM

Patients with Beta-thalassemia have a partial or full loss of production of beta-chain hemoglobin. The excess of α-chain is unstable, and it ultimately precipitates and disintegrates, causing damage to the red blood cell membrane. Premature hemolysis of damaged RBCs in the bone marrow and spleen causes accelerated RBC turnover, ineffective erythropoiesis, and severe anemia. Regular blood transfusions are the only way to correct this. Iron overload, caused by inefficient erythropoiesis and early hemolysis in the plasma and major organs for example heart, liver, and endocrine glands, is one of the principal consequences of this hereditary condition [66]. As a result, iron chelators medicines such as deferoxamine, deferiprone, and deferasirox are required to keep these people alive. The survival of BTM patients with repeated blood transfusions is reduced without proper and suitable chelation treatment.

The kidney's putative role in β-thalassemia patients has received little consideration. Some studies, however, have revealed the presence of various tubular and glomerular abnormalities [67]. Renal failure can develop in BTM patients without clinical manifestations before the onset of any additional consequences caused by chronic hypoxia, anemia, severe iron overload (haemosiderosis) , and excessive chelation use [68]. It is essential to look into ways to effectively assess renal function in order to detect kidney disease early.

Previously, serum creatinine and other traditional tests were employed as a measure since they are easy to quantify from a blood sample. However, despite a normal measured serum creatinine on a routine blood test, 50% of persons have evidence of impaired kidney function, which correlates poorly with the gold standard assessment of renal function [67,68]. So, early

prognostic indicators of renal impairment in Beta-thalassemia major are urgently needed, as early management can considerably improve prognosis [71].

1.3.1. Mechanism of Renal Injury in β-Thalassemia

The main etiologic factors in β-thalassemia renal dysfunction include iron overload from recurrent transfusions, chronic anemia, and particular iron chelation therapy. Furthermore, viral agents such as HBV or HCV, as well as HIV infection, which lead to decrease glomerular filtration rate (GFR) in thalassemia patients. Renal dysfunction can be caused by iron-induced hepatic and cardiac dysfunction [72].

- **Chronic Anemia:**

Chronic anemia and resulting hypoxia can cause oxidative stress and lipid peroxidation, both of which are linked to tubular cell damage. Increased metabolic demand in tubular cells in combination with prolonged hypoxia may cause apoptosis, followed by tubulo-interstitial damage, glomerulosclerosis, and kidney fibrosis [73].

Furthermore, hyperfiltration has been reported in β-thalassemia in several studies. This hyperdynamic circulation initially increases plasma flow and GFR, but glomerular capillary wall expansion and consequent endothelial and epithelial damage can lead to glomerular dysfunction and a steady reduction in GFR [74].

- **Excess Iron.**

Excess free iron is a recognized trigger for lipid peroxidation, which damages cell membranes [75]. Non-transferrin bound iron (NTBI) and heme are both dumped into kidney by blood transfusions. Tubular necrosis, cortical atrophy, and interstitial fibrosis can all be caused by hemosiderin accumulation in the proximal and distal tubules of kidney.

In thalassemia patients, a significant role for these factors in the pathophysiology of acute and chronic kidney injury. Tubular cells that have been injured can produce growth factors and cytokines, resulting in tubulo-interstitial fibrosis and glomerular sclerosis [76].

- **Iron Chelation Therapy.**

The use of iron chelation therapy such as deferoxamine, deferiprone, and deferasirox may cause glomerular damage or reduce their function. The severity of this glomerulopathy varies from a little rise in serum creatinine to severe kidney injury [73].

In thalassemia patients, desferrioxamine and deferasirox might cause more renal harm than deferiprone, especially if proper dose monitoring is not performed. Iron depletion caused by larger dosages of iron chelators may play a role in the etiology of thalassemia renal damage [77]. GFR was reduced in the iron deprived nephrons via impaired mitochondrial function and consequent production of adenosine and adenosine triphosphate that lead to activation of the tubulo-glomerular feedback, vasoconstriction of the afferent pre-glomerular arterioles [73].

1.3.2. Types of Renal Dysfunction

Renal damage in thalassemia patients rises with age and the number of blood transfusions received [78]. Both tubular and glomerular dysfunction can occur in these individuals [79].

- **Tubular dysfunction**.

The renal tubule's integrity can be determined indirectly by measuring functional change and detecting tissue damage. The most prevalent method has been to use immunoassay technology to assess urine quantities of low molecular weight proteins. These are reabsorbed and catabolized within the proximal tubule after being freely filtered at the glomerulus. As a result, the presence of significant amounts of these proteins in the urine indicates that tubular reabsorptive processes have failed [9].Several marker can used to identification of tubular damage such as urinary β2-microglobulin , retinol binding protein(RBP) and α1-microglobulin. Cystatin C, which is usually tested in serum as a marker of GFR, may also be assessed in urine as a sign of proximal tubular injury [80].

Tubular injury causes the release of intracellular components into the urinary tract, which may be measured to determine the tubule's functional integrity. In urine, a vast number of enzymes have been detected such as N-acetyl-B-D-glucosaminidase (NAG) [81]. Many novel biomarkers have emerged as a result of proteome discovery investigations in the previous few years most notably plasma and urinary neutrophil gelatinase–associated lipocalin (NGAL) [82], and kidney injury molecule-1 (KIM-1) [83], interleukin-18 (IL-18) and tissue inhibitor of metalloproteinases-2 (TIMP-2) [84].

- **Glomerular dysfunction.**

Twenty to forty percent of thalassemic patients had glomerular hyperfiltration. Regular blood transfusions can have an effect on this, but they may be linked to an increase in hypercalciuria. Although GFR decline in pediatric thalassemia major patients is uncommon, a steady drop in GFR may develop with advancing age and gradual renal impairment [77].

Chronic anemia is hypothesized to cause hyperdynamic circulation and increased renal plasma flow and glomerular filtration rate (GFR) via lowering systemic vascular resistance [85].

Increase glomerular filtration rate appears to be harmful to the mesangial compartment, causing it to expand in volume and cellular activity, resulting in sclerosis [86].

1.3.3. Biomarker of Renal Dysfunction in Beta Thalassemia Major.

1.3.3.1. Albumin Creatinine Ratio.

The glomerular filter keeps higher molecular weight proteins in the circulation, smaller molecular weight proteins are readily filtered, reabsorbed, and catabolized within tubular cells. As a result, the presence of significant levels of protein in the urine indicates renal dysfunction. The relationship between kidney illness and proteinuria may be traced back to the early nineteenth century, when Bright reported albuminous nephritis [87]. Depending on the type of proteinuria observed, proteinuria can be classified as tubular or glomerular proteinuria. Overflow proteinuria is a third type in which the tubular capacity for reabsorption is exceeded by filtration of large quantities of low molecular weight protein. Examples of the latter, Bence

Jones proteinuria and myoglobinuria, that show in table (1.2). Proteinuria is a strong predictor of renal disease progression, and lowering protein excretion is a treatment goal [9].

Table: 1.2- Characterization of Proteinuria[88]**.**

Type of Proteinuria	Causes
Glomerular	Increased glomerular permeability
Tubular	Proximal tubular damage: decreased tubular reabsorptive capacity and/or release of intracellular components (e.g. due to nephrotoxic drugs)
Overflow	Increased plasma concentration of relatively freely filtered protein

In most forms of proteinuric kidney disease, albumin is the most abundant protein in the urine. As a result, determining albuminuria is the gold standard for determining urine protein concentrations. Increased levels of albuminuria are included in the diagnosis and stage of chronic renal disease (CKD), according to clinical practice standards [89].

1.3.3.2. N-acetyl-beta-D-glucosaminidase.

The urine contains around 40 different enzymes from various sources. They are made up of plasma and blood cells, kidneys, urinary tract epithelium, and urinary tract glands. These enzymes take place the glomerular membrane (such as alanine amino peptidase and γ-glutamyl transferase), mitochondrion (such as malate dehydrogenase), cytoplasm (such as LDH) and lysosome (such as N-acetyl-β-(D)-glucosaminidase) [90]. The lysosomal enzyme N-

acetyl-beta-D-glucosaminidase (NAG) is found in the proximal convoluted tubules of the kidneys and can be useful as a marker of proximal tubular damage and nephrotoxicity. The NAG molecule has a relatively greater molecular weight of around 130000-140000 Da, which prevents it from passing through the kidney's glomerular basement membrane. Finally, the liver quickly eliminates NAG from circulation [91]. Urinary NAG is a simple, quick, and noninvasive approach for detecting and monitoring renal tubular function in a variety of situations. Urinary NAG excretion is abnormal in a variety of renal disorders, including urinary tract infection, vesicoureteral reflux, diabetes mellitus, nephrotic syndrome, glomerulonephritis, nephrocalcinosis, hypercalciuria, urolithiasis, hypertension, perinatal asphyxia, hypoxia, heavy metal poisoning, nephrotoxic drugs as well as cardiac failure [92].

There are two main isoenzyme of NAG, isoenzyme A (acid form) and isoenzyme B (basic form). In normal urine, the A isoenzyme of NAG (NAG-A) is the dominant form. It is classified as functional enzymuria because its excretion is linked to cell maturation and cellular exfoliative turnover. NAG's B isoenzyme is tightly linked to the basal membrane, where it is found. The overall activity of urine NAG, specifically its B form, is increased in individuals with tubular and interstitial renal diseases [93].

1.3.3.3. Beta2-microglobulin.

Beta2-microglobulin is a single-chain polypeptide, with low molecular weight (11.8 kDa) released by all nucleated human cells, with daily synthesis rates ranging from 2–4 mg/kg in healthy individuals. Serum β2M is nearly completely metabolized (99%) by the kidneys and remains steady around 1–

3 mg/mL, the glomeruli filter this molecule, which is then reabsorbed and catabolized in the proximal tubular cells [94].

In a wide range of hematologic and non-hematologic disorders, serum β2M has now been recognized as a significant prognostic predictor. Despite normal plasma levels, urine β2M levels are high in renal tubular diseases, reflecting a problem with proximal tubule reabsorption [95].

The serum β2M level is substantially inversely associated with the glomerular filtration rate, and is one of the traditional low-molecular-weight indicators of kidney function. At the same time, serum β2M concentration is often affected by nonrenal variables including systolic blood pressure, gender, total cholesterol, inflammation [96].

1.3.3.4. Urine Calcium Creatinine Ratio.

Calcium is one of the most abundant elements in the human body. It is a critical component for forming electrical gradients across membranes, a cofactor required by numerous enzymes, and the primary component of bone. Calcium concentrations in blood and cells are closely regulated under normal physiologic circumstances. Calcium is eliminated in the urine as well as in the feces. Approximately 20% to 25% of dietary calcium is absorbed, and 98 percent of filtered calcium is reabsorbed in the kidneys. A complex regulatory system that includes vitamin D and parathyroid hormone carefully regulates calcium traffic between the gastrointestinal tract, bone, and kidney. Bone health requires a sufficient amount of bioavailable calcium [97].

The presence of calcium in the urine above the normal range is evidence of injury to the renal tubules [85].

Although measuring urine calcium excretion using a 24-hour urinary analysis is critical for diagnosing hypercalciuria, it can be challenging to perform in

patients. To get around these problems, researchers have employed the random urine calcium/creatinine ratio (UCa/Cr) [98].

1.3.3.5. Blood urea

Urea ($CO(NH_2)_2$, Mr 60 Da) is the most important nitrogen-containing metabolic product of protein catabolism, more than 75% of nonprotein nitrogen excreted comes from this source. Nitrogen produced from amino acids enters the urea cycle through intermediates such as aspartate and ammonia during protein catabolism. The urea cycle enzymes are responsible for urea production only in the liver. The rate at which urea is produced is determined by the rate at which protein is catabolized from both dietary and endogenous sources, which is mostly sourced from muscle tissue.

Because of its capacity to diffuse across most cell membranes with the help of urea transporter proteins, urea is dispersed equally throughout the whole body fluids after production [99].

Renal excretion accounts for more than 90% of urea elimination from the body, with gastrointestinal and skin losses accounting for the majority of the rest. The glomerulus filters urea readily and tubules do not actively resorb or secrete this substance. However, as part of normal kidney function, (40 % -70 %) of urea passes passively out of the tubule and into the renal interstitial space, eventually returning to plasma. Urea back-diffusion is also affected by urine flow rate, during high flow; the entry of urea into the interstitium is reduced and more in low-flow situations.

For many years, blood and serum urea measurements have been used to assess kidney function. Although serum creatinine is widely considered as providing superior information in this regard. In some cases, serum and urine urea measurements might still give relevant clinical information [100].

1.3.3.6. Serum Creatinine

Two enzymatically mediated processes produce creatine, the immediate precursor of creatinine, in the kidneys, liver, and pancreas. Creatine is carried through the bloodstream to various organs, including muscle and the brain, where it is phosphorylated to phosphocreatine, a high-energy molecule. The metabolic mechanisms of muscular contraction include the interconversion of phosphocreatine and creatine. A small percentage of free creatine in muscle tissue (1% - 2% each day) converted to creatinine, an anhydride waste product, spontaneously and irreversibly. As a result, the quantity of creatinine generated each day in an individual remains relatively constant and is proportional to muscle mass [101].

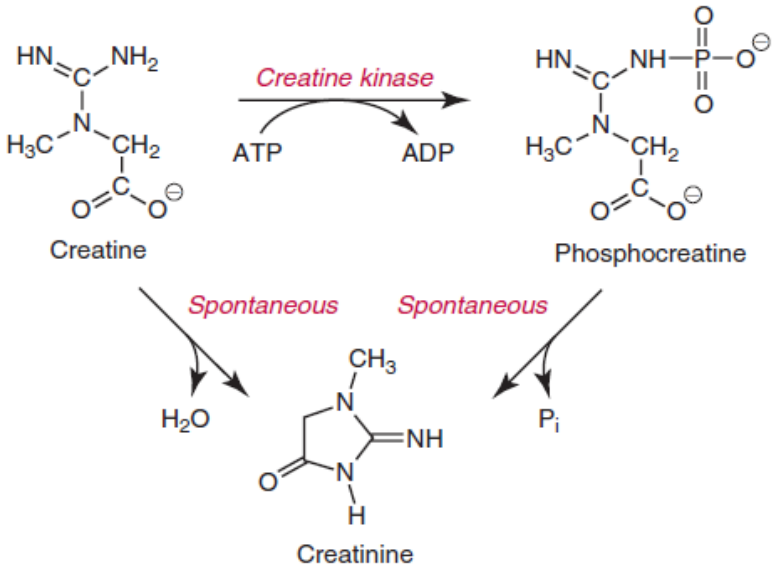

Figure 1.5. Synthesis of Creatinine [101].

Creatinine (Mr 113 Da) is found in all body fluids and secretions, and it is filtered freely by the glomerulus. Although the renal tubules do not resorb it in large amounts, it does produce a small but noticeable tubular secretion, as well as concentration-related losses in the gut. As the circulating concentration of creatinine rises, so does creatinine production; numerous mechanisms have been proposed to explain this, including feedback inhibition of creatine synthesis, reconversion of creatinine to creatine, and conversion to other metabolites [102].

Serum creatinine is the most frequent way to determine GFR. Serum creatinine concentration is a product of the rate of release into the circulation and the rate of clearance. Age, gender, ethnicity, muscle mass, nutritional state, and a variety of other preanalytical and analytical factors all impact its production of creatinine [89].

Aims of the study

- The aim of study is to assess N-acetyl-beta-glucosaminidase and renal function in beta-thalassemia major patients receiving repeated blood transfusion and to look for renal complications in those patients if any.

- The assessment of early biochemical markers of renal function may aid in the prevention of kidney damage prior to the appearance of any clinical symptoms.

2. Material and methods.

2.1. Subjects.

In this case-control study, there are two groups: the first includes patients with beta thalassemia major, and the second includes those who appear to be healthy. The sample size was determined according to the Fisher formula for calculate sample size[103].This formula is :

$$n= \frac{Z^2 P(1-P)}{d^2}$$

Where n= sample size

Z= Z statistic for the level of confidence interval 95% which = 1.96.

P= Prevalence of β-thalassemia which is 3.7 % in the Iraq [104].

d= precision (in proportion of one; if 5%, d = 0.05).

This research was carried out at the laboratory of the College of Medicine at the University of Babylon. The collecting of samples carried out from November 2021 to January 2022. Questionnaires were created to collect data from the control and patients group.

2.1.1. Patients group

The patients group that consisted of 60 patients with β-thalassemia major with repeated blood transfusion. This group was separated into two subgroups, 33 males and 27 females. These patients included in this study whose age ranged (6-35) years. In addition, Patients divided into two groups based on the treatment use, 14 patients use desferrioxamine and 44 patients use deferasirox treatment.

They were obtained at Babylon center of hereditary blood disorder in Babylon teaching hospital for maternity and children. All patients were pre-diagnosed by the physicians at the thalassemia center.

2.1.2. Control Group

The control group consists of 60 individuals who seemed to be healthy and were separated into two subgroups, 33 males and 27 females. These patients included in this study whose age ranged (6-32) years.

They were obtained at Babylon teaching hospital for maternity and children and private clinics in Hilla city.

2.1.3. Criteria for Exclusion

1. Any patient with nephropathy.

2. Any patient with diabetes mellitus.

3. Pregnant.

4. Any patient with chronic liver disease.

5. Patients with other hemoglobinopathies.

2.1.4. Ethical Approval and Consent

All participants in this study were informed before to collecting samples, and verbal agreement was obtained from each of them.

2.2. Chemicals.

All the kits used in this study were shown in Table (2.1).

Table: (2.1)-Kits used in this study

No	Kit	Origin
1	Albumin Creatinine Ratio Kit	Siemens (Germany)
2	Calcium(Colorimetric) Kit	Biolabo (France)
3	Creatinine (Colorimetric) Kit	Biolabo (France)
4	Ferritin Kit	BioMerieux (France)
5	Human Beta2-microglobulin	BioMerieux (France)
6	Human NAGase(N-Acetyl Beta-D-Glucosaminidase) ELISA Kit	Elabscience (China)
7	Urea (Colorimetric) Kit	Biolabo (France)

2.3. Instruments and Equipment.

The instruments and equipment used in this study were shown in Table (2.2).

Table: (2.2) - Instruments and equipment

No.	Instruments and equipment	Company, Origin
1.	Centrifuge	Hettich, Germany
2.	DCA Systems	Siemens, Germany
3.	Deep freeze	GFL, Germany
4.	Disposable syringe (5 mL)	Alrawabi, Jordan
5.	Disposable test tube (10 mL)	Meheco, China
6.	Distiller	GFL, Germany
7.	Elisa reader and washer	Biotek, USA
8.	Eppendorf tube (1.5 µL)	BC, China
9.	Incubator	Memmert, Germany
10.	Micropipettes (5-50 µL), (2-20 µL), (20-200 µL), (100-1000 µL)	Slamed, Germany
11.	Pipette tips 0.2 mL	BC, China
12.	Pipette tips 1 mL	BC, China
13.	Spectrophotometer	C-cell, England
14.	Test tube with separation gel	AFCO, Jordan
15.	Vidas	BioMrieux, France
16.	Water bath	Memmert, Germany

2.4. Methodologies

2.4.1. Collection of Samples

Using a disposable syringe (5 mL), venous blood samples were obtained from control and patients. Subjects were asked to come in for a blood sample. Five milliliters of blood were extracted through vein puncture and progressively pumped into disposable tubes containing separating gel. The blood in the gel-containing tubes was allowed to clot for 10 minutes at room temperature before being centrifuged for 10 minutes at 2000 xg, then separated into small volumes and kept in a deep freezer (-20^{o} C) to carry out the assay. The blood samples obtained from the groups were used to estimate serum ferritin, serum creatinine, and blood urea.

Early morning urine sample collected from patients and control, and putted into disposable container, than centrifuged for 20 minutes at 1000xg, then the supernatant was collected and separated into small volumes and kept in a deep freezer (-20^{o} C) to carry out the assay.

2.4.2. Determination of Albumin Creatinine Ratio

Albumin Creatinine Ratio was measured by DCA Vantage device.

Principle

In the presence of polyethylene glycol, a specific antibody binds to albumin for albumin measurement. The albumin-antibody complexes that are produced causes increased turbidity, which is quantified as absorbance at 531 nm. The albumin is measured using an absorbance versus albumin concentration calibration curve.

The Benedict/Behre reagent is used in the creatinine test, which involves creatinine forming a colored complex with 3,5- dinitrobenzoic acid at a high pH and measuring it at 531 nm. The creatinine is then measured using an absorbance versus creatinine concentration calibration curve. After that, the albumin to creatinine ratio (ACR) is obtained.

The DCA Analyzer performs all measurements and calculations automatically. At the end of the assay, the instrument's screen displays albumin concentration (mg/L), creatinine concentration (mg/dL or mmol/L), and albumin to creatinine ratio(mg/g) [105].

Procedure

The reagent cartridges were allowed 15 minutes to warm up at room temperature after removing it from the refrigerator (in the unopened foil pouch). The reagent cartridges were used within less than 10 minutes.

The cartridge cover was opened by tearing off the case from the side (until the entire long side of the pouch is open).

Unused urine capillary holders was sink in the urine to suction the sample and then the plunger was pressed, then the cartridge put in the instrument. The result was read.

Reference Range.

Normoalbumineuria. < 30 mg/g

Microalbumineuria. 30 – 300 mg/g

Albuminuria. > 300 mg/g

2.4.3. Determination of Urinary N-acetyl-beta-D-glucosaminidase.

N-acetyl-beta-D-glucosaminidase (NAG) level was measured by enzyme linked immunosorbent assay kit.

Principle

The sandwich-ELISA technique is used in this ELISA kit. This kit includes a micro ELISA strip plate that has been pre-coated with a human NAGase specific antibody. In the micro ELISA plate wells, standards or samples are mixed with the specific antibody. After that, a Biotinylated detection antibody specific for human NAGase and an Avidin-Horseradish Peroxidase (HRP) conjugate are incubated in each microplate well. The components that are not needed washed away. Each well is filled with substrate solution. Only those wells that contain human NAGase, the color of Biotinylated detection antibody and the Avidin- HRP conjugate will be blue. The enzyme-substrate reaction is stopped when stop solution is added, and the color turns to yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The optical density value is proportional to the amount of human NAGase in the sample. By comparing the absorbance of the samples to the standard curve, the concentration of NAGase in the samples were determined [106].

Reagent Preparation

1- All materials were placed at room temperature (18~25℃) before used.
2- A volume of 30 mL of concentrated wash buffer was added to 720 mL of deionized or distilled water to prepared 750 mL of wash buffer.

3- A standard solution of 50 ng/mL was produced by addition the original 120µl (100 ng/mL) with 120µl of standard diluent. The working solution was let stand for 10 min. Then, as needed, the serial dilutions were produced, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 ng/mL.

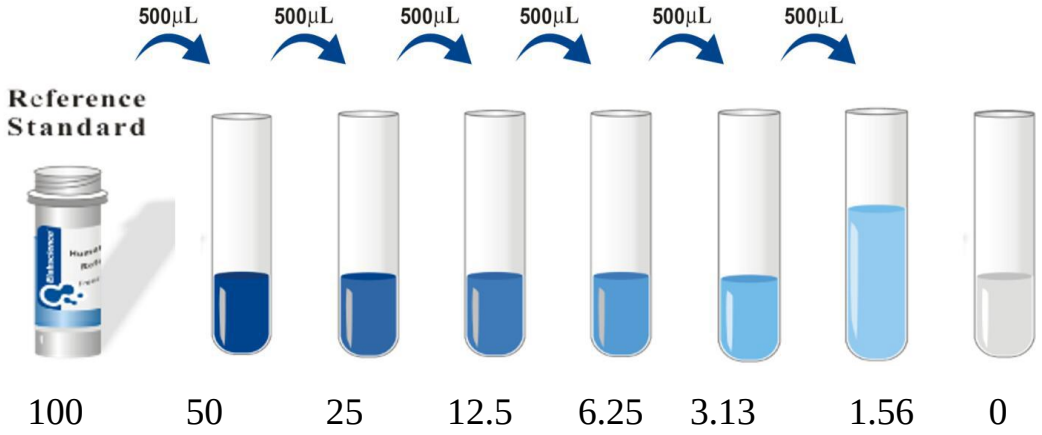

Figure (2.1): Concentration of standards of NAGase

4- The required amount of biotinylated detection Ab working solution was calculated before used (100 µL/well). The stock tube was utilized by centrifuge before used; 100 µL of concentrated biotinylated detection Ab was diluted with 9900 µL biotinylated detection Ab diluent.

5- The required amount of HRP conjugate working solution was calculated before used (100µL/well). 100 µL of concentrated HRP conjugate was diluted with 9900 µL of HRP conjugate diluent.

Assay procedure

1- A volume of 100 µL of standard was added to well standard, antibody was not added to a standard well because the standard solution contains Biotinylated antibody.

2- A volume of 100 µL from sample was added to each sample wells. The wells were mixed. The plate was covered with sealer. Incubate for 90 min, at 37 ° C.

3- Without washing, the liquid was decanted from each well. Each well was filled immediately with 100µL of Biotinylated Detection Ab working solution. The surface of plate was protected by plate sealer. The wells were mixed and incubated for 1 hour at 37° C.

4- The solution was decanted from each well after incubation and added 350 µL of wash buffer to each well. After 1-2 minutes of soaking, the solution was decanted from each well and patted it dry with clean absorbent paper. This wash phase was repeated three times. The ELISA reader was used to complete this phase.

5- Each well was received 100 µL of HRP Conjugate working solution. The surface of plate was protected by plate sealer. The plate was incubated at 37° C, for 30 minutes.

6- The solution was decanted from each well, and then the wash procedure was performed in five times as in step 4.

7- Each well was received 90 µL of Substrate Reagent. The new plate sealer were covered the wells. At 37° C, The plate was incubated for around 15 minutes. The plate was protected from light.

8- The reaction was stopped by adding 50 µL of stop solution to each well, and the color change from the blue to yellow immediately.
9- The optical density (OD value) of each well identified directly after applying the stop solution by utilize a microplate reader set at 450 nm within 10 min.

Calculation of Results

Known concentration of Human NAG standard and its corresponding reading absorbance was plotted on the scale (X-axis) and the scale (Y-axis) respectively. The level of Human NAG in sample is determined by plotting the sample's absorbance on the Y-axis as shown in figure (2-3). The dilution factor must be multiplied by the concentration determined from the standard curve for each sample diluted.

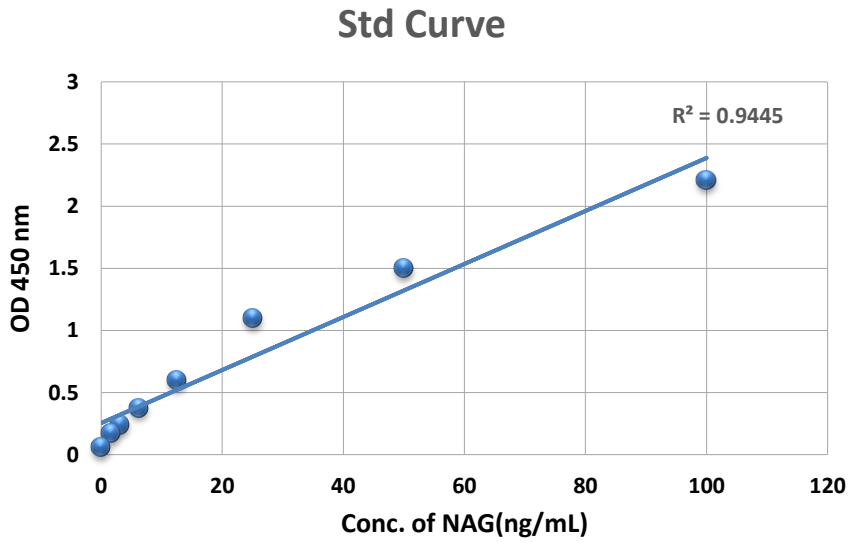

Figure :(2.2) - Standard curve of NAGase

2.4.4. Determination of Urinary Beta2-microglobulin.

Beta2-microglobulin (β2M) level was measured by VIDAS technique.

Principle

The assay is based on a two-stage enzyme immunoassay sandwich method with a final fluorescent detection. The solid phase receptacle (SPR) functions as both the solid phase and the assay's pipetting mechanism. The test reagents are predispensed in sealed reagent strips and are ready to use. The instrument performs all of the assay processes automatically. The β2 microglobulin in the sample binds to the specific monoclonal antibody on the interior of the SPR. During the washing process, unbound components are removed.

An alkaline phosphatase-labeled polyclonal anti-human β2 microglobulin antibody (sheep) reveals the retained β2 microglobulin. During the washing procedure, any unbound conjugate is removed.

The substrate (4-Methylumbelliferyl phosphate) is cycled in and out of the SPR during the final detection stage. The conjugate enzyme catalyzes the hydrolysis of this substrate to produce a fluorescent product (4-Methylumbelliferone) with a 450 nm fluorescence. The fluorescence intensity is proportional to the amount of β2 microglobulin present in the sample[107].

Kit composition

Table: (2.3) - Components of β2M Kit

B2M strips (30 STP)	Ready to use.
B2M SPRs (30 SPR)	SPRs coated with mouse monoclonal anti-B2M antibodies.
B2M Control **C1** (1 Vial)	TRIS buffer (0.05 mol/L) pH 7.4 containing β2 microglobulin of human origin + bovine albumin + 1 g/l sodium azide.
B2M Control **C2** (1Vial)	TRIS buffer (0.05 mol/L) pH 7.4 containing β2 microglobulin of human origin + bovine albumin + 1 g/l sodium azide.
B2M Calibrator **S1**(1Vial)	TRIS buffer (0.05 mol/L, pH 7.4) with human B2M and + bovine albumin + 1 g/l sodium azide.

Procedure

The β2M strips and β2M SPR were inserted into the instrument then 100 µL were added from each sample to wells no.1 of strips. The instrument performs all the assay steps automatically. Calibration was performed each 14 days and its curve is stored in memory while quality

Reference Range. Normal urine β2M: 0.00-0.15 mg/L

2.4.5. Determination of Urine Calcium.

Principle

The metallo-chromogen Arsenazo III interacts with calcium to generate a colored complex at a mildly acidic pH, whose absorbance at 650 nm (640-660) is proportional to the quantity of calcium in the specimen [104,105].

Sample Preparation

To dissolve calcium salts, a volume of 2 to 3 drops of HCl 6 N was added to the sample. The samples were diluted (1+2) with distilled water before testing.

Assay procedure

Table: (2.4) - Procedure of urine calcium.

Pipette into test tubes	Blank	Standard	Sample
Reagent	1mL	1mL	1mL
Distilled water	20 µL		
Standard		20 µL	
Sample			20 µL

The tubes were mixed and then leave stands for about 1 minute at room temperature, then absorbance at 650 nm was measured against reagent blank.

Calculations

The results were calculated as follows:

$$\text{Result} = \frac{\text{Abs(Assay)}}{\text{Abs(standard)}} \text{ x Standard concentration (10 mg/dL)}$$

- The above result was multiplied by dilution factor 3.
- The results of urine calcium were divided on urine creatinine.

Reference Range. Urine calcium: urine creatinine ratio is <0.14.

2.4.6. Determination of Serum Creatinine.

Principle

This procedure is based upon a modification of the original picrate reaction (Jaffe). Creatinine under alkaline conditions reacts with picrate ions forming a reddish complex. The formation rate of the complex measured through the increase of absorbance in a prefixed interval of time is proportional to the concentration of creatinine in the sample [110].

Creatinine + Picric acid $\xrightarrow{\text{pH >12}}$ Red complex

Reagents Composition.

Table: (2.5) - Reagents composition of Creatinine Kit.

Reagent 1	R1	Disodium Phosphate 6.4 mmol/L Sodium hydroxide 150 mmol/L
Reagent 2	R2	Sodium dodecyl sulfate 0.75 mmol/L Picric acid 4.0 mmol/L
Standard	R3	Standard 177 µmol/L (2 mg/dL)

Procedure

All reagents and specimens were placed at room temperature before using.

Table: (2.6) - Procedure of serum creatinine.

Working Reagent (R1+R2)	1000 µL
Serum	1000 µL

The contents were mixed and the kinetic test were performed at 37° C. After 30 seconds, the absorbance A1 was read and 120 seconds later, the absorbance A2 was read against distilled water at 490 nm (490-510).

Calculations

The results were calculated as follows:

$$\text{Result} = \frac{\text{Abs(Assay(A2 - A1) Assay -(A2-A1) Blank)}}{\text{(A2 - A1) Standard-(A2-A1) Blank)}} \text{ x (2 mg/dL)}$$

Reference Intervals[111]**.**
Male 0.9 -1.3 mg/dL
Female 0.6 - 1.1 mg/dL

2.4.7. Determination of Blood Urea.

Principle

Urea is hydrolyzed by urease into ammonia and carbon dioxide. The ammonia generated forms with chloride and salicylate a blue – green complex. The intensity of the color formed is proportional to the concentration of urea in the sample [112].

Reagents Composition.

Table: (2.7) - Reagents composition of Urea Kit.

Reagent 1	R1	Salicylate Nitroprussiate	31 mmol/L 1.67 mmol/L
Reagent 2	R2	Urease	≥ 15 UI/L
Reagent 3	R3	Sodium hypochloride Sodium hypooxide	7 mmol/L 62 mmol/L
Standard	R4	Standard urea	40 mg/dL

Procedure

The reagents and specimens were placed at room temperature before using.

Table: (2.8) - Procedure of blood urea.

Pipette into test tubes	**Blank**	**Standard**	**Sample**
Working Reagent (R1+R2)	1mL	1mL	1mL
Distilled water	10 µL		
Standard		10 µL	
Serum			10 µL
The tubes were mixed and incubated for 2 min at 37°C			
Reagent 3 (R3)	1mL	1mL	1mL

The contents were mixed and let stand for 8 min at room temperature or 5 min at 37° C. the absorbance was read at 600 nm against blank.

Calculations

The results were calculated as follows:

$$\text{Result} = \frac{\text{Abs(Assay)}}{\text{Abs(standard)}} \text{ x Standard concentration (40 mg/dL).}$$

2.4.8. Determination of Serum Ferritin.

Principle

The principle includes a one-step enzyme immunoassay sandwich method with a final fluorescent detection. One well containing anti -ferritin antibody is labeled with alkaline phosphate (conjugate).The antigen (serum ferritin) binds to antibodies coated on interior of SPR and to the conjugate forming a "sandwich". In the final step the conjugate enzyme

catalyzes the substrate into fluorescent product which is measured at 450 nm[113].

Kit composition

Table: (2.9) - Components of Ferritin Kit

FER Strips (60 STP)	
FER SPRs (60 SPR)	SPRs coated with mouse monoclonal anti-ferritin antibodies.
FER Control **C1** (1 Vial)	TRIS buffer (0.1 mol/L, pH 7.4) with human spleen ferritin and protein and chemical stabilizers
FER Calibrator **S1**(1Vial)	TRIS buffer (0.1 mol/L, pH 7.4) with human spleen ferritin and protein and chemical stabilizers
FER Dilution Buffer **R1(1Vial)**	TRIS buffer (0.1 mol/L, pH 7.4) and protein and chemical stabilizers.

Procedure

The ferritin strips and ferritin SPRS were inserted into the instrument then 100 µL were added from each sample to wells no.1 of strips. The instrument performs all the assay steps automatically. Calibration is performed each 14 days and its curve is stored in memory while quality control is performed each new kit opening.

2.5. Statistical Analysis.

Statistical analysis was carried out using SPSS version 21. Continuous variables were given as (Mean ± SD) while categorical variables were provided as frequencies and percentages. Student t-test was used to compare means between two groups and the correlation test (Pearson test) was performed to find the association between variables. P-value less than 0.05 was considered as significant.

Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of NAG, B2M and ACR. The sensitivity and specificity of biochemical parameter and calculate the optimal cutoff according to "Youden Index" by select the point that is closest to the top-left corner of the ROC curve[114]. The area under the curve (AUC) provides a useful tool to compare different biomarkers as Table 2-10.

Table (2-10) List of AUC ranges and their classification levels [115]**.**

AUC Range	Classification Level
0.90 - 1.00	Excellent
0.80 - 0.90	Good
0.70 - 0.80	Fair
0.60 - 0.70	Poor
0.50 - 0.60	Failure

3. Results and Discussion.

3.1. Demographic Characteristics in Patients and Control.

3.1.1. Age Distribution in Patients and Control Groups.

The distribution of patients with β- thalassemia major according to age shown in Table (3.1). Total of patients with thalassemia were 60 whose age ranged (6-35) years. These patients included in this study divided into two subgroup according to age, group I were less than 18 year, with mean ± SD (14.00 ± 2.75) year, group II were equal or more than 18 years with mean ± SD (23.13 ± 4.45) year. Control group (60) apparently healthy subjects with an age range (6-32) years; These control group divided in to two groups according to age, group I were less than 18 year, with mean±SD (12.76 ± 2.86) year, group II were equal or more than 18 years with mean ± SD (22.09 ± 4.22) year. This grouping and distribution of age as two subgroup is similar to many previous studies such as Quinn , *et al*. 2011[1] and different with others.

Table: 3.1- Age distribution in patients and control.

Age group	N	Mean ± SD		P-value
		Patients	controls	
Group I (< 18 years)	38	14.00 ± 2.75	12.76 ± 2.86	0.059
Group II (≥18 years)	22	23.13 ± 4.45	22.09 ± 4.22	0.429

P value ≤ 0.05 was significant

This matching of age between patients and control is important to eliminate any effects on the results that may arise from the difference in these characteristics.

3.1.2. Gender Distribution in Patients and Control Groups.

Amongst sixty patients with β-thalassemia who contributed to this study, there were 33 males and 27 females, and this represents 55% and 45% of patients respectively, as shown in figure (3.1).

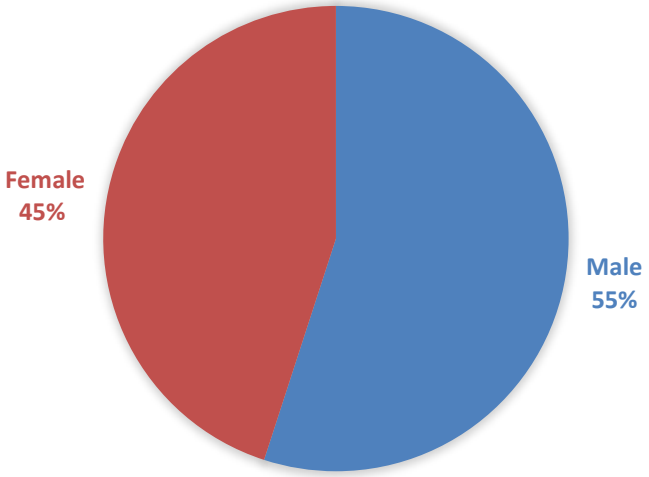

Fig :(3.1) - Rate of male to female among patients.

3.1.3. Iron Chelation Treatment.

From 60 patients with beta thalassemia major, 44 patients used deferasirox as iron chelators and 16 patients used Desferrioxamine.

3.2. Biochemical Parameters.

3.2.1. Albumin Creatinine Ratio.

The findings demonstrated a significant difference in ACR levels between patients and their control group (P< 0.05). The means, standard deviation, and statistical parameters as shown in the Table (3.2), (3.3).

Table: 3.2- Comparison of ACR level in patients and control groups.

Parameter	Study group	N	Mean ± SD	P-Value*
ACR(mg/g)	Patients	60	51.2 ± 23.14	**0.005**
	controls	60	15.5 ± 6.31	

Table: 3.3- Comparison of ACR level in males and females patients, group I and group II, and patients on iron chelation therapy.

Parameter	Groups	N	Mean ± SD	P-Value*
ACR(mg/g)	Males patients	33	35.0 ± 18.85	0.184
	Females patients	27	46.5 ± 27.30	
	Group I patients	38	36.4 ± 18.12	0.515
	Group II patients	22	42.3 ± 26.75	
	Deferasirox	44	41.6 ± 23.8	0.573
	Desferrioxamine	16	36.1 ± 21.96	

In this study, the result revealed that there were significant differences in ACR levels between patients and control group, but there were no significant differences in ACR level between other groups, as shown above. Several studies that have looked for urine albumin/creatinine ratios in beta-thalassemia patients reported that this parameter was significantly higher in beta-thalassemia patients than in healthy subjects, similar to our findings [110-112].

Albuminuria in patients with BTM may be due to chronic anemia, that lowers systemic vascular resistance, which leads to a rise in renal blood flow and glomerular filtration rate. These modifications can contribute to glomerular capillary stretching and subsequent capillary damage, as well as macromolecule transudation into the mesangium, which is linked to glomerular dysfunction. Furthermore, apoptosis, cytokine release, tubulointerstitial damage, and glomerulosclerosis are all caused by persistent hypoxia and heavy iron overload in tubular cells [119]. In the long-term, such modifications may result in a gradual decline in glomerular filtration rate [72,114].

Albuminuria was attributed to the destruction of the glomerular filtration membrane, which was thought to be caused by massive iron deposition in the tissues, which resulted in an increase in free radical production, which led to cell death by binding cell proteins and interfering with their production [120]. Moreover, albuminuria can also be caused by chronic anemia, persistent hyperfiltration, and prostaglandin secretion [121].

3.2.2. Urinary N-Acetyl-beta-D-Glucosaminidase.

The findings demonstrated a significant difference in NAG levels between patients and their control group (P< 0.05), as shown in the Table (3.4). There was no significant difference between males and females patients groups, group I and group II, and between patients on deferasirox treatment and patients on desferrioxamine treatment (p-value > 0.05), as shown in Table (3.5).

Table: 3.4- Comparison of urinary NAG level in patients and control groups.

Parameter	Study group	N	Mean ± SD	P-Value*
NAG(ng/mL)	Patients	60	131.5 ± 55.2	< 0.001
	controls	60	77.0 ± 25.1	

Table: 3.5- Comparison of urinary NAG level in males and females patients, group I and group II, and patients on iron chelation therapy.

Parameter	Groups	N	Mean ± SD	P-Value*
NAG(ng/mL)	Males patients	33	126.9 ± 53.4	0.47
	Females patients	27	137.3 ± 57.8	
	Group I patients	38	134.9 ± 53.8	0.54
	Group II patients	22	125.8 ± 58.4	
	Deferasirox	44	130.1 ± 50.7	0.74
	Desferrioxamine	16	135.4 ± 67.8	

The result in Table (3.4) show comparison of the level of NAG in urine between the patient group and the healthy control group that revealed a high significant increase in the patient group ($p < 0.05$). Increased NAG level in the urine indicate the presence of an injury to the renal tubules, as previous studies have shown, Koliakos *et al*.2003 and Mohkam *et al*.2008, they investigated that increasing urine NAG in individuals with β-thalassemia major, which they attributed to iron overload as the main cause of this dysfunction [66, 111].

The rise of urinary NAG in urine is attributed to tubular dysfunction and is regarded a sensitive and reliable measure of proximal tubular toxicity and a probable predictor of proteinuria since NAG is not of plasmatic origin and is not filtered via the glomeruli [122].

Other previous published studies demonstrating the urine of healthy humans contains a small amount of NAG comparing with patients. Increased excretion of NAG in urine has been associated with tubular dysfunction, which is in agreement with our findings [116-118].

On the other hand, the present study show that there was no significant difference in urine NAG excretion among patient groups based on age. These results do not agree with the results of some studies that showed a significant relationship between urinary NAG and age of thalassemia patients that may be due to duration of desferrioxamine therapy or duration of receiving blood transfusions [117]. The reason for this difference between studies could be due to the sample size included in the study or the different in groups of the patients according to age.

3.2.3. Urinary Beta2-Microglobulin.

The results revealed that there was a significant difference in the level of β2M between patients and their control group (p-value <0.05), the means, standard deviation, and statistical parameters are listed in the Table (3.6).

Table: 3.6- Comparison of urinary β2M level in patients and control groups.

Parameter	Study group	N	Mean ± SD	P-Value*
β2M (mg/L)	Patients	60	0.12 ± 0.048	**0.003**
	controls	60	0.06 ± 0.035	

Table: 3.7- Comparison of urinary β2M level in males and females patients, group I and group II, and patients on iron chelation therapy.

Parameter	Groups	N	Mean ± SD	P-Value*
β2M (mg/L)	Males patients	33	0.096 ± 0.054	0.146
	Females patients	27	0.072 ± 0.042	
	Group I patients	38	0.6 ± 0.03	**0.04**
	Group II patients	22	0.11 ± 0.06	
	Deferasirox	44	0.13 ± 0.06	**0.01**
	Desferrioxamine	16	0.06 ± 0.05	

The results revealed that there was no significant differences in the level of β2M between males patients and females patients groups (p-value >0.05), while significant differences between group I and group II patients, and significant differences between patients according to iron chelation therapy received (p-value < 0.05), the means, standard deviation, and statistical parameters are listed in the Table (3.7).

The results in the Table (3.7) show highly significant difference between patients and control at (p-value 0.003), which similar to Ong-ajyooth *et al*.1998, the first report of renal tubular defects found associated with beta-thal/Hb E disease. The authors explained that the mechanism leads to the damage is not clear, but they think that it may be related to the increase in oxidative stress secondary to the accumulation of iron in the tissues, as indicated by an increase levels of serum and urine malondialdehyde (MDA) [126].

In our study, there statistically significant differences were found between patients subgroup according to age, this result similar to study was done by Jalaly *et al*. 2011, demonstrated that renal impairment in thalassemia major patients can be increased by advanced age, higher blood transfusion frequency, and hypercalciuria [78].

Furthermore, the present study investigate that there was significant difference in urine β2M among patient groups according to iron chelation treatment as shown in table (3.7). The level of β2M in patients on Deferasirox was higher than patients on Desferrioxamine, these results are in agreement with Annayev *et al.* 2018, they found positive correlations between the β2M levels and DFX doses and suggested that DFX might cause dose-dependent tubulopathy [127].

3.2.4. Calcium Creatinine Ratio.

The results revealed that there was a significant difference in the level of UCa/Cr between patients and control group (p-value <0.05), the means, standard deviation, and statistical parameters are listed in the Table (3.8).

Table: 3.8- Comparison of urinary Ca/Cr in patients and control groups.

Parameter	Study group	N	Mean ± SD	P-Value*
UCa/Cr mg/mg	Patients	60	0.099 ± 0.06	**0.001**
	controls	60	0.066 ± 0.02	

The results revealed that there no significant difference in the level of UCa/Cr between males patients and females patients groups, group I and group II, and between patients on deferasirox treatment and patients on desferrioxamine treatment (p-value > 0.05), as shown in Table (3.9).

Table: 3.9.-Comparison of urinary Ca/Cr in **males and females patients, group I and group II, and patients on iron chelation therapy.**

Parameter	Groups	N	Mean ± SD	P-Value*
UCa/Cr mg/mg	Males patients	33	0.086 ±0.04	0.24
	Females patients	27	0.09 ±0.05	
	Group I patients	38	0.091 ±0.04	0.72
	Group II patients	22	0.096 ±0.05	

	Deferasirox	44	0.092 ±0.04	0.94
	Desferrioxamine	16	0.094 ± 0.04	

One of the most serious adverse effects of blood transfusion is organ dysfunction in patients with thalassemia major [116]. Some data on heart and liver problems is available in this area, but research on renal failure is restricted since early diagnosis is regarded a major issue [128].

Although measuring urine calcium excretion using a 24-hour urinary analysis is necessary for diagnosing hypercalciuria, it is sometimes difficult to perform in patients. To overcome these difficulties, the random urine calcium/creatinine ratio (UCa/Cr) has been utilized [98]. However, some reports have found strong correlations between (24hCa) with UCa/Cr [129]. In our study, seven patients have hypercalciuria (11.6 %). The hypercalciuria in present study was similar to study was performed by Sadeghi *et al*. 2008, Ali *et al*. 2014, reported the renal dysfunctions such as hyperfiltration, proteinuria, and hypercalciuria were common findings in patients compared with the control group [110,113].

3.2.5. Serum Creatinine and Blood Urea.

The results revealed that there was a significant difference in the level of S-Cr and urea between patients and control group (p-value <0.05), the means, standard deviation, and statistical parameters are listed in the Table (3.8).

Table: 3.10- Comparison of serum Cr, blood urea level in patients and control groups.

Parameter	Study group	N	Mean ± SD	P-Value*
Creatinine mg/dL	Patients	60	0.57 ± 0.1	**0.04**
	Controls	60	0.51 ± 0.1	
Blood Urea mg/dL	Patients	60	31.7 ± 5.8	**< 0.01**
	Controls	60	20.6 ± 5.13	

The results revealed that there significant difference in the level of creatinine between males patients and females patients groups, group I and group II, but no between patients on deferasirox treatment and patients on desferrioxamine treatment (p-value > 0.05), as shown in table(3-11).

There is no significant difference in the level of urea between males patients and females patients groups, group I and group II, and between patients on deferasirox treatment and patients on desferrioxamine treatment (p-value > 0.05), as shown in Table (3.11).

Table: 3.11-Comparison of serum Cr, blood urea in males and females patients, group I and group II, and patients on iron chelation therapy.

Parameter	Groups	N	Mean ± SD	P-Value*
Creatinine mg/dL	Males patients	33	0.59 ± 0.12	**0.03**
	Females patients	27	0.50 ± 0.09	
	Group I patients	38	0.54 ± 0.08	**0.012**
	Group II patients	22	0.61 ± 0.11	
	Deferasirox	44	0.57 ± 0.09	0.89
	Desferrioxamine	16	0.56 ± 0.12	
Blood Urea mg/dL	Males patients	33	32.8 ± 6.5	0.11
	Females patients	27	30.4 ± 4.6	
	Group I patients	38	31.0 ± 4.6	0.23
	Group II patients	22	32.9 ± 7.4	
	Deferasirox	44	32.0 ± 6.1	0.57
	Desferrioxamine	16	31.0 ± 4.9	

Although the level of urea and creatinine is within the normal limit, there is a significant difference between the patients and the control group. Moreover, there is a significant difference for creatinine between the male and female group and between group I and group II of patients, and this is similar to the study conducted by Helin *et al* .1998, Ali *et al*. 2008 [123,124].

The raise in the level of urea and creatinine in β-thalassemia patients when compared to the control group may be due to glomerular capillary wall expansion and consequent endothelial and epithelial damage can lead to glomerular dysfunction and a steady reduction in GFR [74].

Creatinine is a final result of skeletal muscle catabolism and excreted through the kidney. Creatinine was affected by muscle mass, gender and age.

Excess iron and shortened red cell lifespan cause functional and physiological problems in numerous organ systems, with significant frequency of renal tubular abnormalities in beta thalassemia patients [132]. The damage was related to the degree of anemia, with the damage being least severe in patients receiving hypertransfusion and iron chelation therapy, suggesting that the damage was caused by anemia and increased oxidation induced by excess iron deposits[133].

In contrast to the current study, Sen *et al*. 2015, they reported that no significant differences were found in serum urea and creatinine levels between children with βTM and control subjects [125]. We think that the inconsistency with this study is due to the fact that serum creatinine and blood urea are affected by several factors such as ; muscle mass, protein intake, inflammatory disease and liver disorder [134].

3.3- Correlation between Age, ACR, and NAG ,B2M ,UCa/Cr, ferritin and number of previous blood transfusion in BTM and control, shown in Table (3.12).

Table: 3.12-Correlation between variables.

		ACR mg/g	NAG ng/mL	B2M mg/L	Ca/Cr mg/mg	Cr mg/dL	Urea mg/dL	Ferritin ng/mL	No of blood transfusion
Age Year	**r**	0.31	- 0.36	0.26	0.06	0.32	0.30	0.13	0.09
	P	**0.00**	0.69	**0.004**	0.48	**0.00**	**0.001**	0.14	0.48
	N	120	120	120	120	120	120	120	60
ACR mg/g	**r**	----	0.04	0.39	0.32	0.01	0.1	0.06	0.06
	P	----	0.63	**0.00**	**0.00**	0.84	0.25	0.48	0.61
	N	----	120	120	120	120	120	120	60
NAG ng/mL	**r**	----	----	0.23	-0.05	0.2	0.34	0.51	0.008
	P	----	----	**0.008**	0.52	**0.02**	**0.00**	**0.00**	0.95
	N	----	----	120	120	120	120	120	60
B2M mg/L	**r**	----	----	----	0.09	0.003	0.15	0.5	0.32
	P	----	----	----	0.31	0.97	0.08	**0.00**	**0.01**
	N	----	----	----	120	120	120	120	60
UCa/Cr	**r**	----	----	----	----	0.05	0.14	-0.06	0.17
	P	----	----	----	----	0.56	0.11	0.94	0.18
	N	----	----	----	----	120	120	120	60
Ferritin ng/mL	**r**	----	----	----	----	----	----	----	0.34
	P	----	----	----	----	----	----	----	**0.006**
	N	----	----	----	----	----	----	----	60

r: correlation coefficient.

In this study significant positive correlation between age with ACR and β2M p-value=0.000, p-value = 0.004 respectively, as seen in figures (3.2), (3.3). It is possible that the increase in the renal dysfunction markers with increasing the age of patient is a result of the increase duration of disease and therefore the number of blood transfusions.

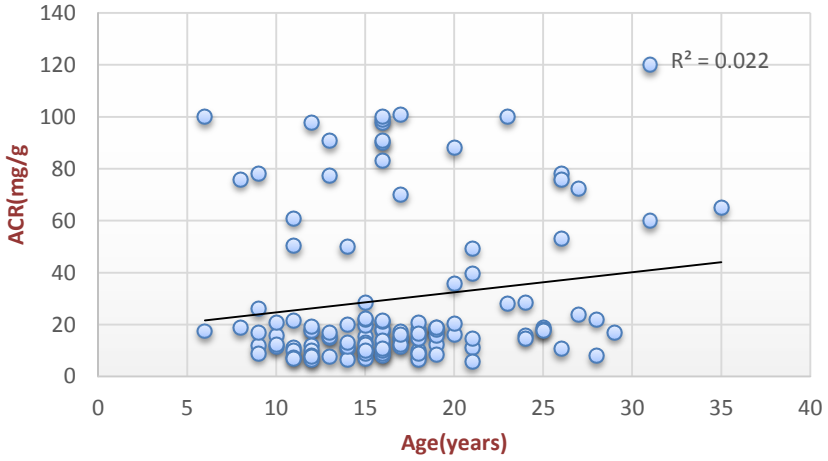

Figure: (3.2) - Correlation between age and ACR level.

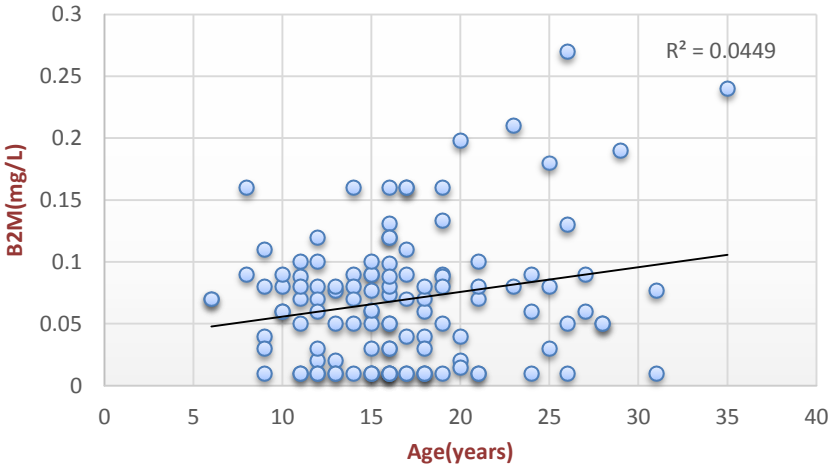

Figure: (3.3) - Correlation between age and B2M level.

Significant positive correlation between ACR and β2M, UCa/Cr level as seen in figure (3.4), (3.5) at p-value = 0.00. An increase in UCa/Cr and β2M is evidence of tubular dysfunction (reabsorption of calcium and β2M) that may be a consequence of chronic anemia, hypoxia or nephrotoxicity [85].

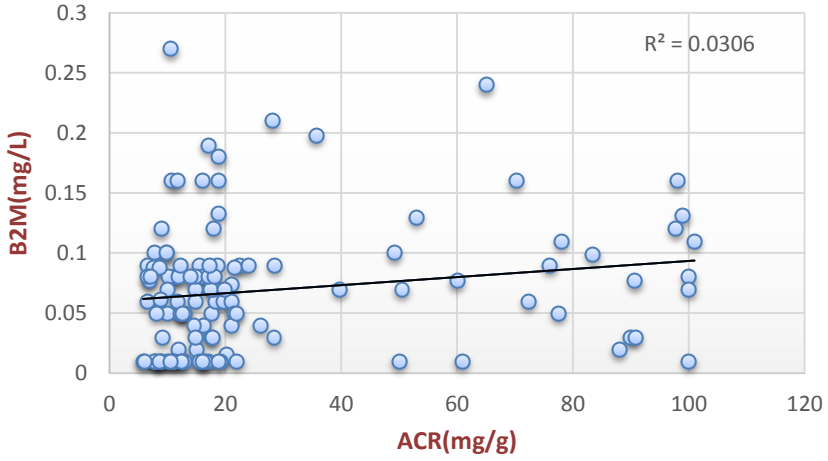

Figure: (3.4) - Correlation between ACR and B2M level.

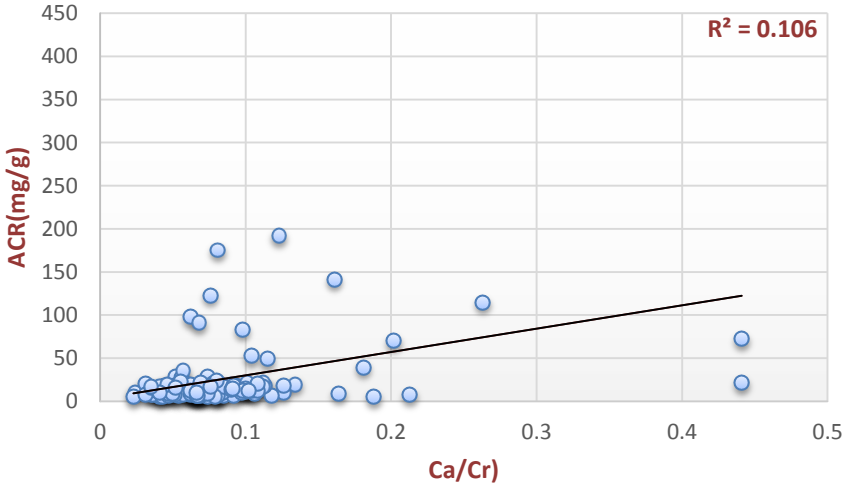

Figure :(3.5) - Correlation between ACR and UCa/Cr.

Although it is weak, there is a statistically significant positive correlation between the ferritin level and the number of previous blood transfusions (p-value= 0.006, r=0.34), as shown in figure (3.6). We can explain the relationship between these variables to the fact of each unit of packed red blood cells contains 200 to 250 mg of iron; and frequent transfusions lead to an overload of iron [5].

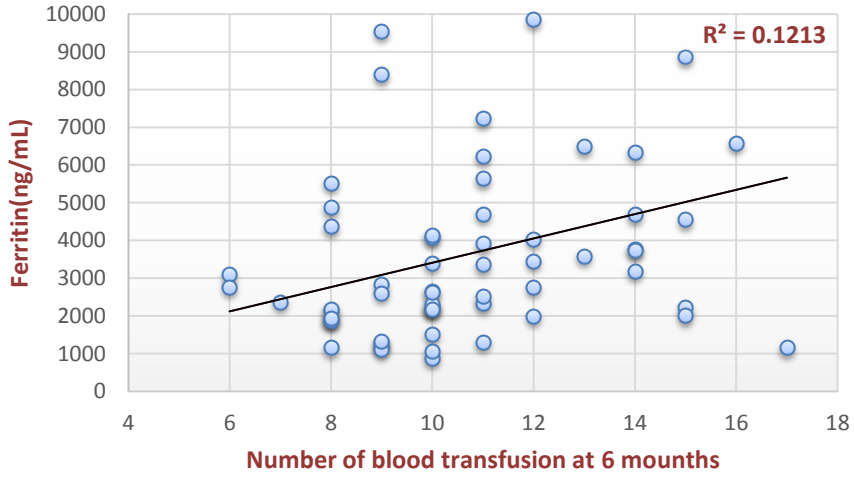

Figure: (3.6) - Correlation between ferritin and number of blood transfusion.

Moderate positive significant correlation between ferritin with NAG and β2M, as shown in figure (3.7), (3.8), and (3.9).

Several methods to assess kidney iron overload, kidney biopsy would provide a wealth of information concerning iron deposits, but it is an invasive method that comes with several risks and severe patient discomfort [85]. Now, kidney magnetic resonance imaging (MRI) is essential for noninvasive assessment of renal iron overload. Hashemieh *et al*, they found statistically moderate significant correlation between serum ferritin and kidney T2* relaxation (correlation coefficient = −0.446, $p < .001$) [135].

In another study, Koliakos *et al*. 2003, they assessed liver MRI T2* values and blood ferritin concentrations to evaluate renal iron overload in thalassemic patients and its relationship with tubular dysfunction. They discovered a statistically significant correlation between β2-microglobulin in urine and NAG activity, as well as serum ferritin concentration and liver iron deposition. These authors hypothesized that iron overload was the cause of renal impairment in homozygous β-thalassemia patients, but the researchers did not examine the kidneys for iron deposits directly [68].

Anemia, as well as the possibility for persistent hypoxia, can cause oxidative stress, lipid peroxidation, and tubular dysfunction. Furthermore, hypoxia causes tubular cells to differentiate into myofibroblasts, while also activating fibroblasts, changing the extracellular matrix metabolism of renal cells, and obliterating peritubular capillaries [136].

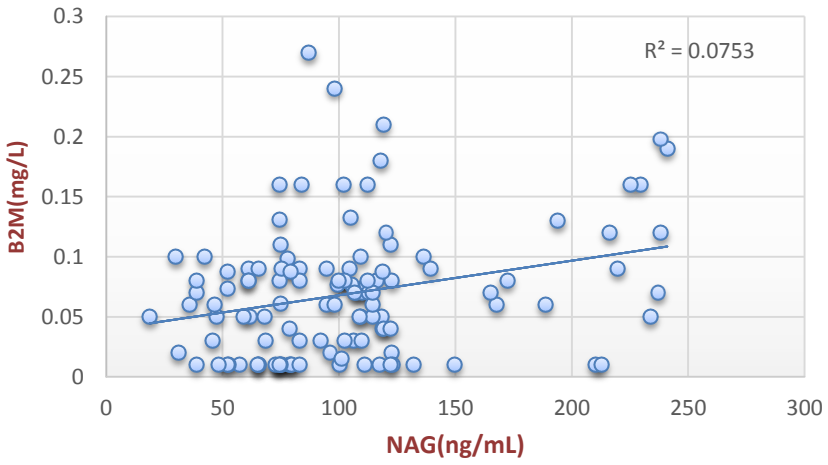

Figure: (3.7) - Correlation between NAG and B2M.

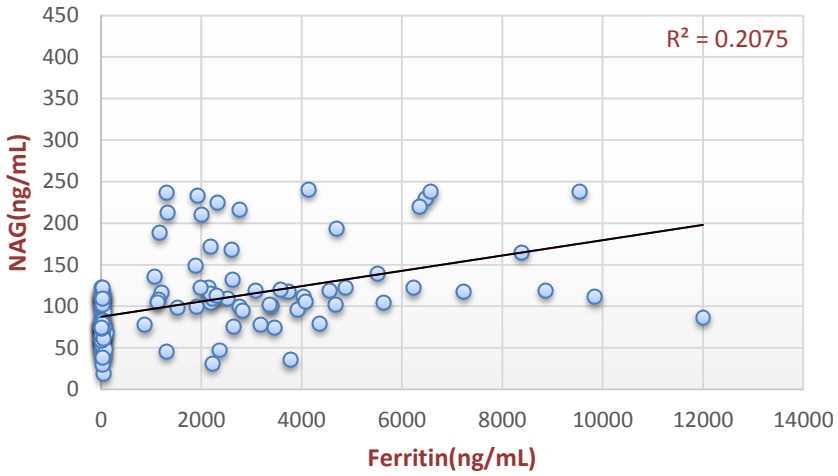

Figure: (3.8) - Correlation between NAG and Ferritin.

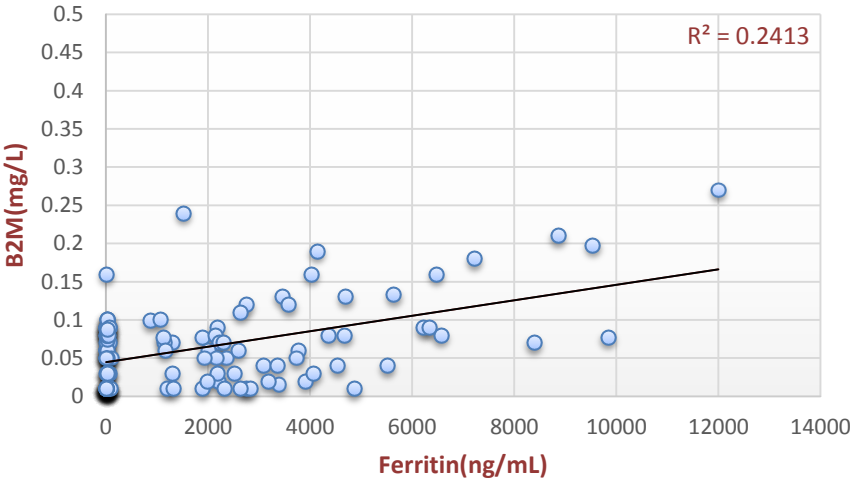

Figure: (3.9) - Correlation between B2M and Ferritin.

3.4. ROC curve of biochemical parameters.

3.4.1. ROC Curve of N-Acetyl-beta-D-Glucosaminidase.

ROC curve for the sensitivity and specificity of NAG (ng/mL) for diagnosis of renal dysfunction in beta thalassemia major, (Cut-off point was ≥ 93.3 (ng/mL)) , AUC=0.83, P= <0.001*, 95% CI (0.755-0.904), the sensitivity and the specificity was 83.3 %, 71.7 % respectively, positive predictive value(PPV) was 74.6%, negative predictive value(NPV) was 81.1%,, as shown in figure (3.10)

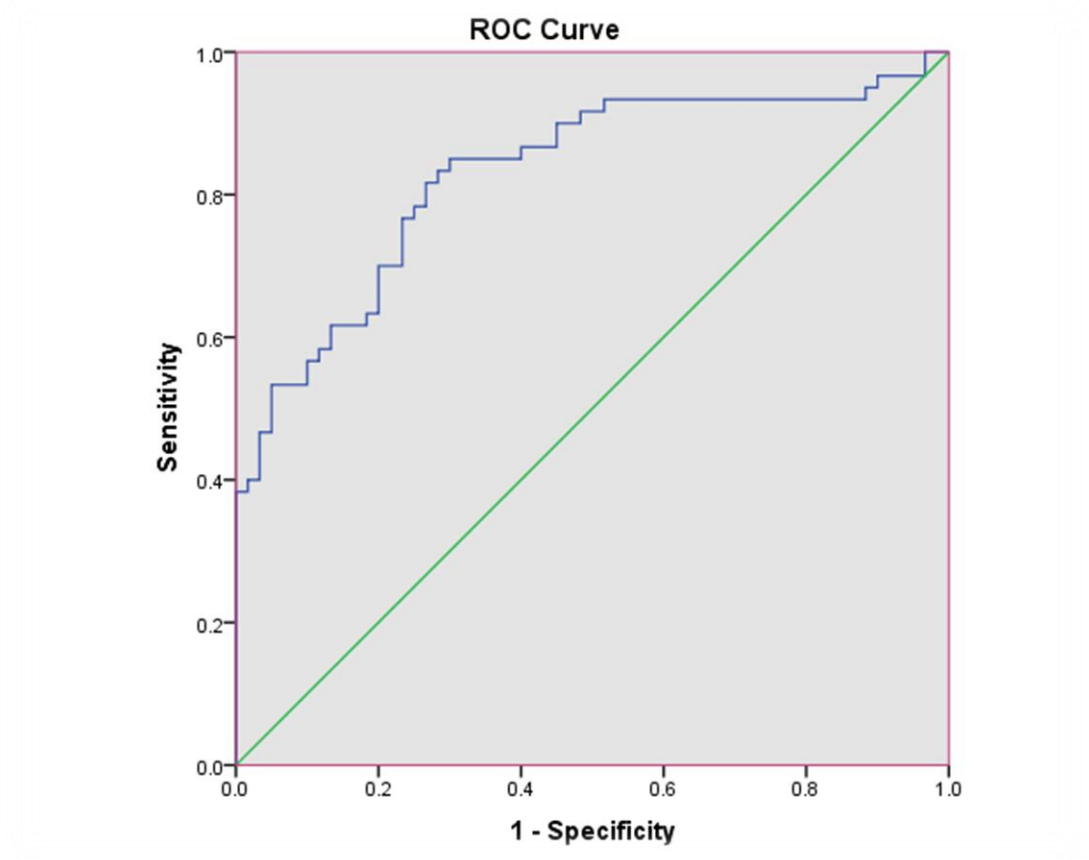

Figure :(3.10) - ROC curve of N-acetyl-beta-D-glucosaminidase (NAG).

For NAG, The results showed there that good diagnostic value in the diagnosis of renal dysfunction in BTM.

3.4.2. ROC Curve of Beta2-microglobulin.

ROC curve for the sensitivity and specificity of B2M (mg/L) for diagnosis of renal dysfunction in beta thalassemia major , (Cut-off point was ≥ 0.07 (mg/L)) , AUC=0.62, P 0.023, 95% CI (0.521-0.720),), the sensitivity and the specificity was 71.7 %, 56.7 % respectively, positive predictive value was 55.8 %, negative predictive value was 60.4 %, as shown in figure (3-11).

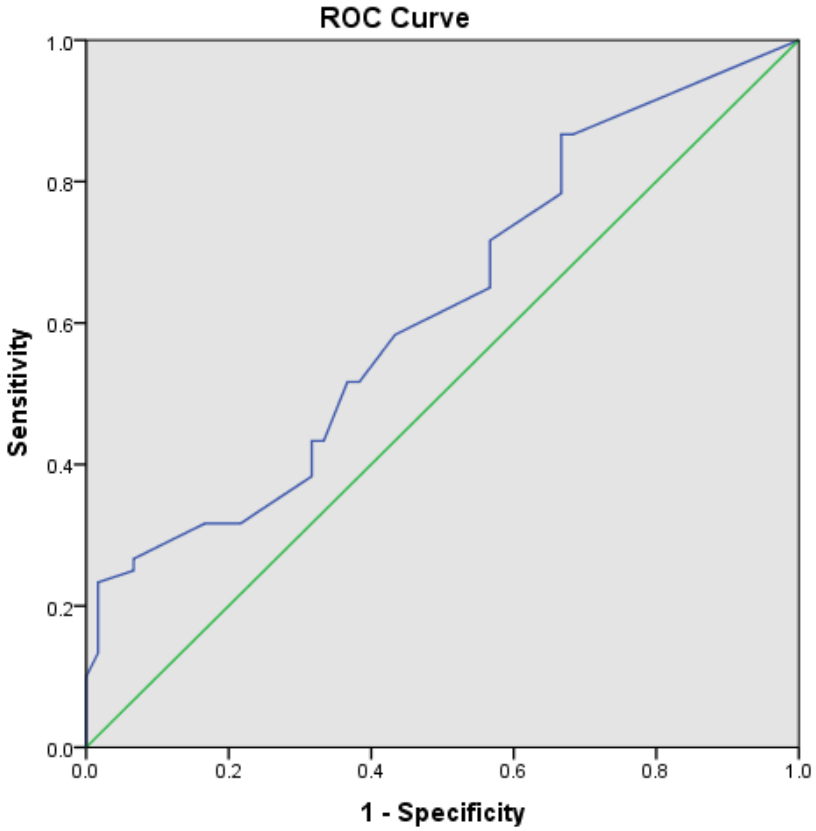

Figure: (3.11) - ROC curve of Beta2-microglobulin (B2M).

For ACR, our results stated that poor diagnostic value in the diagnosis of renal dysfunction in patients with BTM

3.4.2. ROC Curve of Albumin Creatinine Ratio.

ROC curve for the sensitivity and specificity of ACR (mg/g) for diagnosis of renal dysfunction in beta thalassemia major was non-significant, p-value 0.231 as shown in figure (3-13).

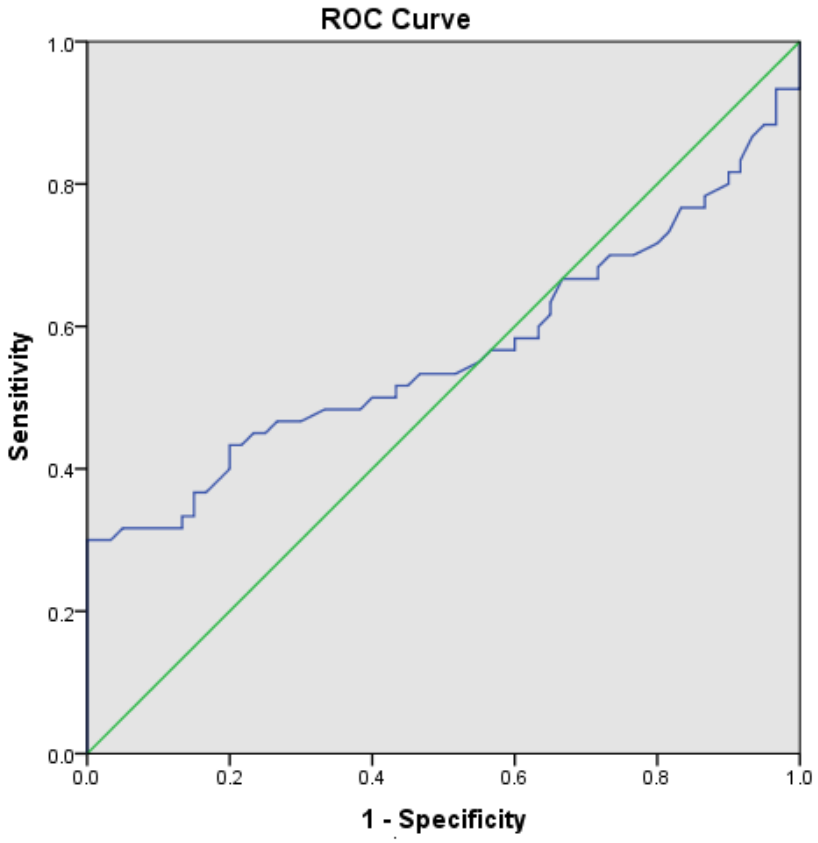

Figure :(3.12) - ROC curve of Albumin Creatinine Ratio (ACR).

Conclusion

Renal hemosiderosis and asymptomatic renal dysfunction are prevalent among β-thalassemia major patients with repeated blood transfusion, which are not found in routine renal investigations.

Urinary NAG are sensitive, specific, and highly predictive early indicators for acute renal injury in individuals with BTM when subclinical kidney damage or dysfunction is expected before serum creatinine increases.

Recommendations

- A future study is to be done on larger sample size to give results that are more accurate.
- Other early markers of renal dysfunction (such as, Cystatin c and AKM1 and NGAL) are needed to be measured.
- Assessment of oxidative stress secondary to iron deposition.
- Measurement of hemoglobin level for patients is required to determine severity of anemia.

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الخلاصة

يعد مرض الثلاسيميا نوع بيتا أكثر أنواع اعتلال الهيموغلوبين الوراثي انتشارًا في العالم، ويحدث بسبب انخفاض أو عدم إنتاج سلسلة بيتا غلوبين التي عادة ما تكون جزءًا من هيموغلوبين البالغين (HbA، وهو $\alpha 2\beta 2$). ينتج عن ذلك المرض الجيني التكوين غير الفعال لكريات الدم الحمراء التعويضية، وفقر الدم الحاد، وتسارع معدل دوران كرات الدم الحمراء وغالبًا ما تصاحب متلازمة الثلاسيميا مجموعة كبيرة من المشكلات الناتجة عن المرض نفسه والعلاجات المستخدمة.

يهدف هذا البحث الى دراسة المضاعفات الكلوية في مرضى الثلاسيميا الكبرى نوع بيتا الذين يحتاجون الى استلام دم بشكل متكرر. وبعد ذلك تقديم المقترحات حول الفحوصات المبكرة لوظائف الكلى للتقليل من مضاعفات المرض.

تضمنت هذه الدراسة ٦٠ مريضاً مصاباً بالثلاسيميا بيتا الكبرى، ٣٣ منهم ذكور و٢٧ إناث، و٦٠ شخصا يبدو أنهم أصحاء ٣٣ منهم ذكور ٢٧ إناث. قسمت مجموعة المرضى ومجموعة الأصحاء الى مجموعتين ضمنية، الأولى اقل من ١٨ سنة وهم ٣٨ مريض والثانية أكثر من او مساوي لعمر ١٨ سنة وهم ٢٢ مريض. استثنى من هذه الدراسة مرضى السكري، مرضى اعتلال الكلى، امراض الكبد المزمنة، النساء الحوامل وأي مرضى مصابين بالأنواع الأخرى من اعتلال الهيموغلوبين.

أجريت هذا الدراسة في مختبرات كلية الطب فرع الكيمياء الحياتية. وكل عينات المرضى جمعت من شهر تشرين الثاني سنة ٢٠٢١ الى شهر كانون الثاني سنة ٢٠٢٢ في مركز امراض الدم الوراية في بابل. وتم فحص انزيم N-استايل كلوكوزامينايديز، بيتا-٢-مايكروغلوبين، نسبة الالبومين الى الكرياتنين في الادرار، نسبة الكالسيوم الى الكرياتنين في الادرار، كرياتنين المصل، ويوريا الدم.

أظهرت نتائج الدراسة فرق معنوي في كل الفحوصات أعلاه في مقارنة المرضى مع مجموعة الاصحاء.

الاستنتاجات، ان ضعف وظائف الكلى بدون الأعراض منتشر بين مرضى الثلاسيميا نوع بيتا الذين يحتاجون الى استلام دم بشكل متكرر والتي لم يتم العثور عليها في فحوصات الكلى الروتينية، ويعزى ذلك إما إلى المرض نفسه او عمليات نقل الدم المتكررة، بالتالي ترسب الحديد اوالى العلاج المستخدم. كما يوصى بأجراء الفحوصات اللازمة والمنتظمة بعلامات مبكرة للخلل الكبيبي والأنبوبي للحد من مضاعفات المرض.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل/ كلية الطب
فرع الكيمياء والكيمياء الحياتية

تقييم مستوى انزيم NAG في الادرار ووظائف الكلى في المرضى المعتمدين علي نقل الدم في مركز امراض الدم الوراثية في بابل

رسالة

مقدمة إلى عمادة كلية الطب في جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير

في العلوم/ كيمياء حياتية سريرية

من قبل

أمجد حسين جواد كاظم

بكالوريوس علوم الكيمياء/ جامعة بابل سنة ٢٠١٠

اشراف

الأستاذ الدكتور
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الأستاذ المساعد الدكتور
ربيع محسن حساني

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١٤٤٣هـ