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Evaluation of Reproductive Hormones and Bones Metabolism in Patients with β-Thalassemia Major in Misan Province

A Thesis

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By

Noor AL-Huda Salah Hashim AL-Zuhairy

B.Sc. Biology 2013

Supervised by

Prof. Dr. Zainab Abdul Jabbar Ridha Al- Ali

2020 A.D. **1441 A.H.**

Supervisor's Certificate

We certify that this thesis entitled **"Evaluation of Reproductive Hormones and Bone Metabolism in Patients with β-Thalassemia Major in Misan Province"**

Has been prepared under our supervision at the College of Science, University of Misan; as a partial fulfillment of the requirements for the degree of Master of Biology

Signature

Prof. Dr. Zainab Abdul Jabbar Ridha Al- Ali

Department of Biology

College of Science/Misan University

Date: / / 2020

Recommendation of Head of Biology Department

I view of the available recommendations; I forward this thesis debate by the Examining Committee.

Signature

Dr. Maytham AbdulKadhim Dragh

Head of Department of Biology

College of Science/Misan University

Date: / /2020

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ قْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (١) خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ (٢) اقْرَأْ وَرَبُّكَ الْأَكْرَمُ (٣) الَّذِي عَلَّمَ بِالْقَلَمِ (٤)عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ(٥) ﴾

صدق الله العلي العظيم

سورة العلق (١–٥)

Dedication

To whom made my life a shine

My Parents and My Sisters

Noor

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In the name of **God**, the most Gracious, the Most Merciful. And peace and blessing be upon the most honorable of the prophets and the seal of messengers, our master Mohammed, upon him and his family, the best of blessing and peace.

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List of Abbreviations

Abbreviation	Meaning
$(NH_4)_6\ Mo_7O_{24}$	Ammonium molybdate tetrahydrate
°C	Degree Celsius
µL	Microliter
1, 25(OH) $_2$ D_3	1, 25 dihydroxycholecalciferol
AHSP	Alpha hemoglobin stabilizing protein
ALP	Alkaline phosphatase
Aγ	Gamma-A
BCL11A	Protein Coding gene
BPG	Biphosphoglycerol
C_3	Complement 3
Ca	Calcium
CBC	Complete blood count
CO_2	Carbon dioxide
df	Degrees of freedom
dL	Deciliter
DNA	Deoxyribonucleic acid
E_2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
ELFA	Enzyme Linked Fluorescent Assay
EPO	Erythropoietin
Fe	Ferritin
FSH	Follicle-stimulating hormone
g	Gram
GH-IGF	Growth hormone - Insulin-like Growth Factors
GnRH	Gonadotrophin releasing hormone
Gγ	Gamma -G
H^+	Hydrogen ion
Hb	Hemoglobin
Hb A_2	Adult hemoglobin two
Hb C	Hemoglobin C
Hb S	Hemoglobin S
HbA	Adult hemoglobin
HbE	Hemoglobin E
HbF	Fetal hemoglobin
HbH	Hemoglobin H

HIF	Hypoxia – inducible factor
HPFH	Hereditary persistence of fetal hemoglobin
HPG	Hypothalamic-pituitary-gonadal
HPLC	High performance liquid chromatography
HSCT	Hematopoietic stem cell transplantation
IgG	Immunoglobulin Gamma
kDa	Kilodalton
kg	Kilogram
L	Litter
LH	Luteinizing hormone
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
mg	Milligram
Mg	Magnesium
mIU	Milli-international units
ml	Milliliter
MLE	Master Lot Entry
mmol	Millimole
mRNA	Messenger ribonucleic acid
N	Nitrogen
ng	Nanogram
nm	Nonometer
NO	Nitric oxide
No.	Number
NTBI	Non-transferrin-bound iron
NTDT	Non–Transfusion-Dependent Thalassemia
p	Shorter arm of human chromosome
PCV	Packed cell volume
pg	Picogram
Pi	Isoelectric point
PO_4	Phosphate
PRBC	Packed red blood cell
PRL	Prolactin
PTH	Parathyroid hormone
QTLs	Quantitative trait loci
RBC	Red blood cell

RFV	Relative Fluorescence Value
RIA	Radioimmunoassay
SE	Standard Error
SPR®	Solid Phase Receptacle
SPSS	Statistical Package for Social Science
STR	Short Tandem Repeat analysis
TDT	Transfusion-Dependent Thalassemia
TIBC	Total iron binding capacity
TRH	Thyrotropin-releasing hormone
Vit. D	Vitamin D
WBC	White blood cell
xg	Standard acceleration due to gravity at the Earth's surface
α	Alpha
β	Beta
β^+	Reduced beta chain
β++	Mild absence beta chain
β^0	Absence beta chain
γ	Gamma
δ	Delta
ε	Epsilon
ζ,	Zeta
μg	Microgram
X^2	Chi-square
ψB_1,θ	Pseudogenes

Summary

This study was conducted, from December 2018 to the end of May 2019, on the sample consisting 50 patients and 50 healthy controls were involved in this study. A 50 (30 male and 20 female) adolescents with β- thalassemia major patients with ages range between 11 to 16 years attended Misan Thalassemia Center, and an equal number of age and sex matched healthy adolescents as control group were included in this case control study. The patients and control were further divided into 2 more subgroups based on age from 11-13 years, and 14-16 years to measure the serum ferritin, reproductive hormones and some biochemical bone markers levels.

The results revealed: a significant increase ($p<0.05$) in serum ferritin level (4094.78±492.55ng/ml) in male and female (3603.20±564.88 ng/ml) patients in comparison to male and female control groups, but the gender of patients had a non-significant influence on serum ferritin level.

Male β-thalassemia major patients have significant decreased ($p< 0.05$) levels of serum FSH (1.52±0.26 mIU/ml), LH (0.92±0.16 mIU/ml), testosterone (1.31±0.20 ng/ml), estradiol (23.67±3.54pg/ml), PTH (35.01±5.19 pg/ml), vitamin D (12.22±0.67 ng/ml) and calcium (7.90±0.19 mg/dl). While serum phosphorous (4.97±0.32 mg/dl) and ALP (212.20±19.40 U/L) were statistically significant ($P<0.05$) increased, whereas serum magnesium (2.18±0.38 mg/dl) was increased non significant in male patients compared with control. While female patients have significant ($P<0.05$) decreased serum vitamin D (12.70±0.84 ng/ml) and calcium (7.55±0.23 mg/dl) levels, but phosphorus (4.61±0.52 mg/dL) and ALP (210.30±33.54 U/L)

levels were significant ($p<0.05$) increased, but serum FSH (3.38±0.60 mIU/ml) and testosterone (1.08±0.20 ng/ml) and magnesium (2.04±0.36 mg/dl) levels were non-significant , whereas LH (2.54±0.78 mIU/ml), estradiol (48.13±6.57 pg/ml) and PTH (31.39±5.69 pg/ml) levels were not significantly decreased in comparison to control.

In β-thalassemia major patients group, female patients as compared to male patients have significant levels of FSH (3.38±0.60 mIU/ml) and LH hormones; moreover estradiol (48.13±6.57 pg/ml) and vitamin D (12.70±0.84 ng/ml) levels were non-significantly increased level, but serum testosterone (1.08±0.20 ng/ml), PTH (31.39±5.69 pg/ml), calcium (7.55±0.23 mg/dL), phosphorus (4.61±0.52 mg/dL), ALP (210.30±33.54 U/L) and magnesium (2.04±0.36 mg/dL) levels were non-significant decreased. Prolactin level was decreased without significance in male (12.91±1.11ng/ml) and female patients (13.97±1.09 ng/ml) compared with control. While it level was increased in female patients (13.97±1.09 ng/ml) as compared to male patients, but gender of patients had non-significant influences on its level.

Serum ferritin as a dependent variable had an inverse correlation with testosterone (- 0.123), E2 (-0.023), FSH (-0.135), LH (-0.124) PRL (-0.154), PTH (-0.136), calcium (-0.275), and magnesium (-0.093), but it's significantly (P value <0.05) negative with vitamin D (-0.303), whereas it had a positive correlation with ALP (0.158) and significant positive with phosphorus (0.554).

The results have been discussed physiologically according to the iron overload increase in thalassemia patients and the effects of the

iron overload on each of the hormonal parameters and on the biochemical bone markers.

Chapter One
Introduction

1. Introduction:

The thalassemia syndromes are a heterogeneous group of inherited forms of anemia caused by mutations that affect the synthesis of hemoglobin, and the most monogenic disease in man (Sankaran *et al.*, 2015; and *Weatherall, 2016*).

The primary defect in thalassemia is usually quantitative, consisting of the reduced or absent synthesis of normal globin chains, but there are mutations resulting is structural variants produced at reduced rate (e.g., HbE, Hb Lepore) and mutations producing hyperunstable hemoglobin variant with thalassemia phenotype (thalassemic hemoglobinopathies), (Borgna and Galanello, 2014).

Thalassaemia was not recognized as a clinical entity until 1925, when Cooley and Lee described a syndrome occurring early in life that was associated with splenomegaly and bone deformities. The term thalassemia was coined by George Whipple in 1936, and the thalassemia derived from a combination of two Greek words: *Thalassa* meaning the sea that is the Mediterranean and *anemia* “weak blood” (Cooley and Lee, 1925; Cooley *et al.*, 1927; and Bradford and Dye, 1936).

The thalassemia have a high incidence in a broad area extending from the Mediterranean basin and parts of Africa, throughout the Middle East, the Indian subcontinent, Southeast Asia, and Melanesia in to the Pacific Islands (Ladis *et al.*, 2013; Nigam *et al.*, 2017; and Tari *et al.*, 2018).

In populations in which malaria is (or was) endemic, 3 to 40% of individuals carry one significant variants, and the prevalence of hemoglobin disorders ranges from 0.3 to 25 per 1000 live births, this high frequency of thalassemia gene in these areas due to natural selection

against malaria is Plasmodium falciparum (Ladis *et al.*, 2013; and Tari *et al.*, 2018).

In almost all Arab countries β-thalassemia is encountered in polymorphic frequencies with carrier rates ranging from 1 to 11% (Hamamy and Al-Allawi, 2013).

While, In Iraq there is little data on epidemiology and burden of thalassemia, but the prevalence of thalassemia in Iraq was 37.1/100,000 population (Kadhim *et al.*, 2017) .

Whereas, the prevalence of thalassemia in neighboring countries Saudi Arabia "7.25%" (Alenazi *et al.*, 2015), Qatar "2-3.5%" (Al-Obaidli *et al.*, 2007), Iran 3.6% (Khodaei *et al.*, 2013), and in Southeast Turkey "2.44%" (Incebiyik *et al.*, 2014).

In thalassemia the primary defect is usually quantitative, it occurs when there is decreased or absent production of one of the types of globin chains (most commonly either α or β), that cause insufficient amount of normal structure of globin chains. This results in an imbalance between α - and β -chains and causes the clinical features of thalassemia; it can be separated into two major types such as α -thalassemia and β –thalassemia (Higgs *et al.*, 2001; Borgna and Galanello, 2014; Sankaran *et al.*, 2015; Weatherall, 2016; and Sharma *et al.*, 2017).

Thalassemia syndrome is classified according to genetic basis into α, β, γ, δβ, δ, and εγδβ, depending on which globin chain is under produced. Beta- thalassemia is caused by a decrease in the production of *β* -globin chains, and the heterogeneous nature of β-Thalassemia is well known, with more than 350 β-thalassemia mutations and, rarely, by deletions has been reported. Many mutations eliminate β-gene expression, and there is no β- chain production (β^0), whereas others cause a variable decrease in

the level of β-gene expression, and cause partial deficiency of β- chain production (β^{+}). There are four clinical syndromes of β-thalassemia according to increasing severity: silent carrier, thalassemia trait, thalassemia intermedia, and thalassemia major. (Olivieri, 1999; Higgs *et al.*, 2001; Kountouris *et al.*, 2014; Borgna and Galanello **,** 2014; Sankaran *et al.*, 2015; and Weatherall, 2016).

Clinical presentation of thalassemia major occurs between 6 months and 2 years of age. These children exhibited severe anemia (hemoglobin concentration of 3 to 7 g/dL), progressive enlargement of the abdomen due to hepatosplenomegaly, and severe growth retardation. In addition, marrow expansion results in characteristic deformity of skull and face, such as frontal bossing and maxillary prominence, gave the patients characteristic faces (Higgs *et al.*, 2001; Rund and Rachmilewitz 2005; Borgna and Galanello, 2014; Sankaran *et al.*, 2015; Nigam *et al.*, 2017; and Sharma *et al.*, 2017).

The mainstay of treatment available for sever β-thalassemia are regular blood transfusions, iron chelation therapy to prevent iron overload, splenectomy in case complicated by hypersplenism, and general supportive pediatric care (Weatherall, 2016; Nigam *et al.*, 2017; Sharma *et al.*, 2017; and Tari *et al.*, 2018) .

The cause of the complications of thalassemia is multifactorial and variable and depends on the genotype, other known and unknown phenotypic modifiers, and availability and adherence to therapy (Joshi, and Phatarpekar, 2013, Hamidi, 2016; Sharma *et al.*, 2017; De Sanctis, 2018; and Tari *et al.*, 2018).

Hypothalamic-pituitary-gonadal axis dysfunction are the most frequently registered endocrine complication in β- thalassemia major

despite regular transfusions and optimal chelation therapy ranges between 30 and 80% of patients in various studies (Parijat et al., 2014; and De Sanctis et al., 2018).

Parathyroid dysfunction and biochemical bone markers disease is mainly due to repeated blood transfusion results in citrate toxicity and iron overload with deposition in parathyroid cells and tissue fibrosis, and chronic anemia (Galanello and Origa, 2010; and Hamidi, 2016).

Aims of the study:

This study is designed to known about the effect of iron overload increment on some reproductive hormones and biochemical bone markers in thalassemia major patients in misan province

1. Assess the serum reproductive hormones (FSH, LH, estradiol, testosterone) in addition to prolactin hormone.
2. Assess the serum PTH.
3. Asses the biochemical bone markers (serum calcium, phosphorus, alkaline phosphatase enzyme, vitamin D and magnesium).

Chapter Tow
Literature Review

2. Literatures Review:

2.1. Thalassemia Syndrome:

2.1.1. Historical Review:

The name thalassemia derived from a combination of two Greek words: *Thalassa* meaning the sea that is the Mediterranean and anemia "weak blood". Another term found in literature, although infrequently, is Cooley's anemia after the name of Professor Cooley Thomas, a pediatrician in the USA and Lee first described the clinical characteristics of severe anemia that occurred early in life and was associated with splenomegaly and bone changes in patients of Italian origin in 1925. In 1932, George H. Whipple and William L. Bradford published a comprehensive account of the pathologic findings in this disease, and the actual term of thalassemia was coined by George H. Whipple (Cooley and Lee, 1925; Cooley *et al.*, 1927; Bradford and Dye, 1936; and Whipp*l*e and Bradford, 1936).

After the Second World War, independent studies in Italy and in Mediterranean immigrants in the United States showed that there was a remarkably high frequency of thalassemia in these populations, and the term thalassemia major and minor were used by Valentine and Neel in 1944(Valentine and Neel, 1944), later the term thalassemia intermedia was used to describe disorders that were milder than the major form but more severe than the traits (Weatherall, 2004).

Work in 1940s, again carried out independently in the United States of America and Italy showed that the disease is inherited in a Mendelian recessive fashion. But, until the 1950s, there was no understanding of the underlying cause (Weatherall, 2004).

During the 1950s, there was rapid progress toward an understanding of the structure and function of human hemoglobin. During the 1960s a

genetic basis of the thalassemia diseases was proposed, linking them to unbalanced globin chain synthesis. The stage was set for further progress. Simpler methodology was developed that made it possible for routine laboratories to analyze levels of hemoglobin A_2 and confirm the diagnosis of thalassemia. Other observations on the alterations of hemoglobin patterns in patients with thalassemia led to the discovery of HbH (β_4) and Hb Barts (γ_4), which later became established markers of alpha-thalassemia (Weatherall, 2004; and Marengo-Rowe, 2007).

During 1960s a method was developed for measuring in vitro synthesis globin chains in quantitative fashion, when David Weatherall and associates labeled reticulocytes of thalassemic patients with radioactive amino acids in vitro and were able to demonstrate that in patients with alpha- and beta-thalassemia, alpha- or beta-chain production was defective because of unbalanced globin chain synthesis (Weatherall *et al.*, 1965; Weatherall, 2004; and Marengo-Rowe, 2007).

Although, until the mid-1970s, it was not possible to analyze globin genes directly, some progress was made toward an understanding of what might be happening at the DNA level, and by the end of 1970s, it became possible to clone and sequence the globin genes; there were few surprises, and the order that had been predicted by simple family studies over the previous 20 years proved to be correct (Weatherall, 2004).

During 1980s, the application of Southern blotting, together with the development of the rapid DNA-sequencing methods, led to extensive studies of the molecular pathology of the disease, the discovery of the strong association between different mutations of the α- and β-globin genes with restriction fragment–length polymorphisms of the α- and β-globin gene clusters (Orkin *et al.*, 1982; and Higgs *et al.*, 1986).

It is now known that there are over 200 different β-globin gene mutations that underlie β thalassemia and a large number of different deletions and nondeletional forms of a thalassemia (Weatherall, 2004).

2.1.2. Prevalence and Geographic Distribution:

The thalassemia syndromes are heterogeneous group of congenital anemias that have in common decrease synthesis of one or more of the globin chain subunits of the normal human hemoglobin. As a group, they represent the commonest recessive monogenic, single genetic disorder in human according to Mendel's laws (Giardina and Rivella, 2013; Weatherall, 2016; De Sanctis *et al.*, 2017; and Tari *et al.*, 2018).

Thalassemia affects both sexes equally, occurring approximately in 4.4% of every 10,000-live birth and accounting for about 60,000–70,000 children each year who born with different types of thalassemias (Muncie and Campbell, 2009; Weatherall, 2010; and De Sanctis *et al.*, 2017).

Thalassemias are prevalent worldwide, have been encountered in virtually every ethnic group and geographic location, and in many parts of the world, they constitute major public health problems with 25,000 deaths in 2013. They are occur in a particularly high frequency in a broad belt extending from the Mediterranean basin through the Middle East, Indian subcontinent, Burma, Southeast Asia, Melanesia, and the islands of the Pacific. The “thalassemia belt” extends along the shores of the Mediterranean and throughout the Arabian Peninsula; Turkey; Iran; India; and southeastern Asia, especially Thailand, Cambodia, and southern China, and thalassemia trait affects 5-30% in these ethnic group, figure (2. 1), (Giardina and Rivella, 2013; Borgna and Galanello, 2014; Sankaran *et al.*, 2015, De Sanctis *et al.*, 2017; and Sharma *et al.*, 2017).

Diseases caused by *α*-thalassemia are encountered commonly in Southeast Asia and China with up to 40% of the regional population being carriers, while 3% of the world’s populations carry *β*-thalassemia

genes. Maldives has the highest incidence of thalassemia in the world with carrier rate of 18% of the population, Cyprus (16%), Sardinia (12%), and South East Asia (Borgna and Galanello, 2014; Nigam *et al.*, 2017; and Sharma *et al.*, 2017).

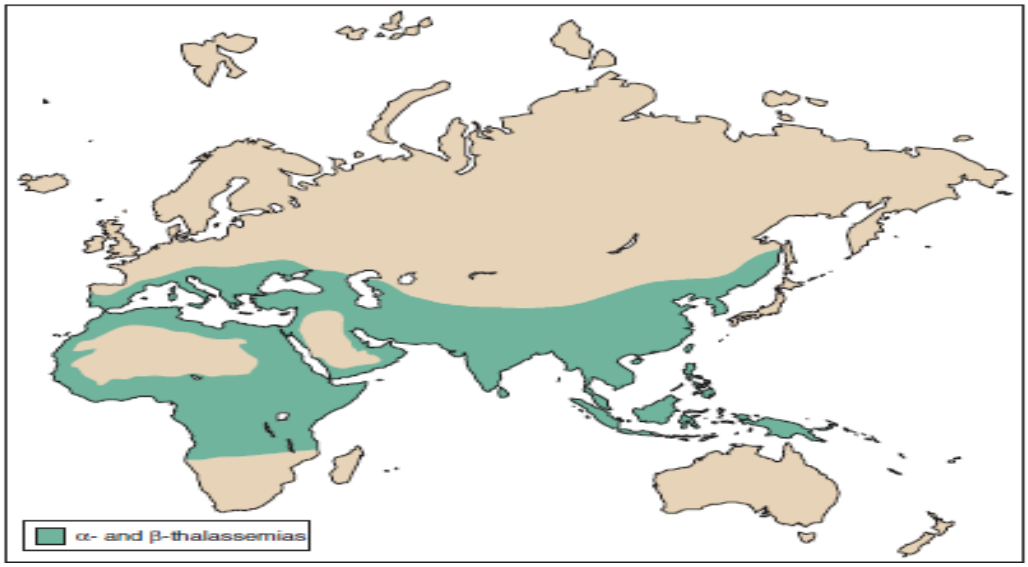


Figure (2.1): Geographic distribution of thalassemia (Borgna and Galanello, 2014).

Thalassemia syndromes are known to be prevalent in most Arab countries with varying prevalence, α-thalassemia encountered in the majority of Arab countries in frequencies ranging from 1 to 58 % with the highest frequencies reported from Gulf countries, while β-thalassemia carrier rates of 1–11 % (Hamamy and Al- Allawi, 2013).

Regarding Iraq, (Kadhim *et al.*, 2017), estimated the prevalence of thalassemia 37.1/100,000 population, and β-thalassemia major represented 73.9% of all types of thalassemia with a prevalence of 27.4/100,000 population, with the highest prevalence of thalassemia was in Basra governorate (54.5/100,000 population) followed by Dohuk governorate (54.1/100,000 population), and the lowest prevalence was in Muthanna (19.7/100,000 population).

2.1.3. Pathophysiology of Thalassemia Syndromes:

The pathophysiology of the thalassemia syndromes can be due to the deleterious effects of the globin-chain subunits that are produced in excess. In β- thalassemia, excess α-chain cause destruction to the red cell precursors and red cells and lead to severe anemia. This causes expansion of the ineffective marrow, with severe effects on development, bone formation, and growth. The major cause of morbidity and mortality is the effect of iron overload on endocrine organs, liver, and heart, which results from increased intestinal absorption and the effects of frequent blood transfusions. The pathophysiology of the α-thalassemia is different because the excess β chains that result from defective α-chain production form β^4 molecules, or hemoglobin H, which is soluble and does not precipitate in the marrow. However, it is unstable and precipitates in older red cells. So that's, the anemia of α-thalassemia is hemolytic rather than dyserythropoietic (Weatherall, 2016).

The clinical syndromes associated with thalassemia are heterogeneous due to the many possible mutations affecting the human globin chain loci. These mutations include those of gene deletions, as well as globin chain initiation, translation, and termination (Marengo-Rowe, 2007), and they are arise from the combined consequences of decrease in erythrocyte hemoglobin production and imbalanced accumulation of globin subunits. The former leads to hypochromia and microcytosis; and the latter results in ineffective erythropoiesis and hemolytic anemia. This heterogeneity arises from the variable severities of the primary biosynthetic defects and coinherited modifying factors, such as increased synthesis of fetal globin subunits or diminished or increased synthesis of α-globin subunits (Giardina and Rivella, 2013; and Tari *et al.*, 2018).

2.1.4. Classification of Thalassemia Syndromes:

According to the chain whose production is impaired, thalassemia is classified according to genetic basis into α, β, γ, δβ, δ, and εγδβ, depending on which globin is under produced (Higgs *et al.*, 2001; Giardina and Rivella , 2013; Weatherall, 2016; and Sharma *et al.*, 2017).

Alpha-thalassemias can be divided into four types on the basis of genotype and the total number of abnormal genes that result: silent carrier state (one inactive a gene), α-thalassemia trait (two inactive α genes), Hb H disease (three inactive α genes), and hydrops fetalis with Hb Bart (four inactive α genes), (Turgeon, 2012).

The β-thalassemias include four clinical syndromes of increasing severity: silent carrier, thalassemia trait, thalassemia intermedia, and thalassemia major, in addition to that the expression of mutated beta globin genes can result in reduced or absent beta-globin production, according to these finding, β-thalassemia can be phenotypically classified into 2 types: β^0-thalassemia where no beta globin chains are synthesized and β^+-thalassemia where some beta-globin chains are synthesized (Ho and Thein, 2000; Muncie and Campbell, 2009; Sankaran *et al.*, 2015; Weatherall, 2016; and Sharma *et al.*, 2017).

Recently in 2012, the new terminology for a phenotypic and clinical classification of thalassemia {Transfusion-Dependent Thalassemia (TDT) and Non–Transfusion-Dependent Thalassemia (NTDT)} was proposed and then adopted by the Thalassemia International Federation in their recent guidelines and publications. Differentiation of a new thalassemia patient as either (TDT) or (NTDT) requires a careful clinical evaluation based on patients' clinical severity whether they require regular blood transfusions to survive and hematological parameters, particularly baseline hemoglobin levels, figure (2.2), (Taher *et al.*, 2013; Cappellini *et al.*, 2014; and Viprakasit and Ekwattanakit 2018).

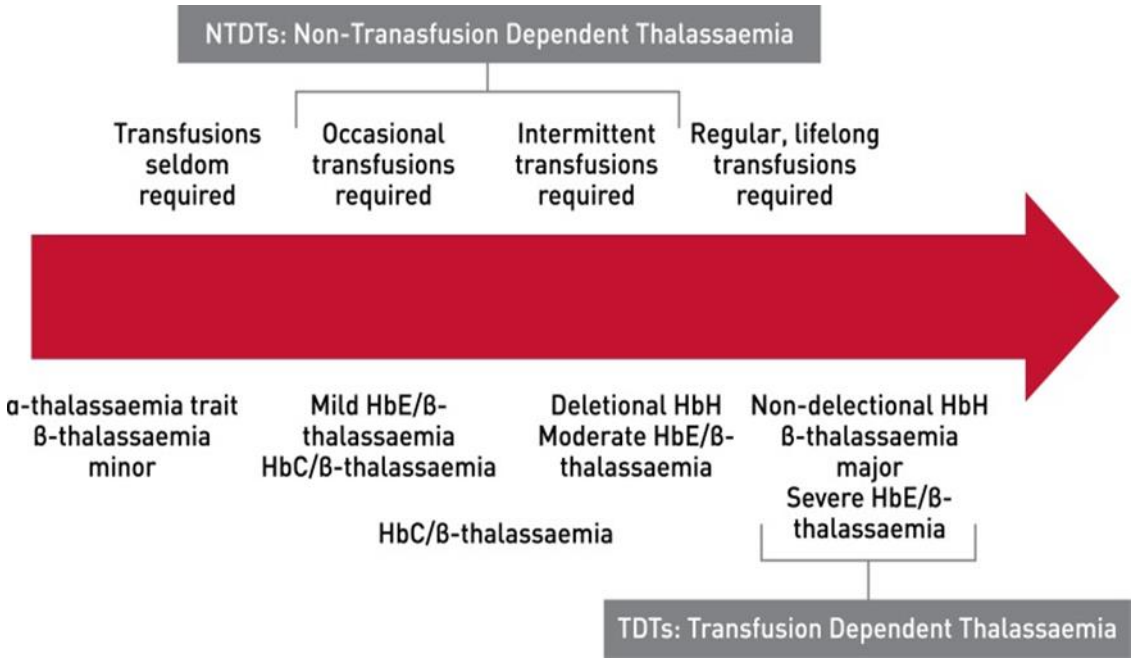


Figure (2.2): Phenotypic classification of thalassemia syndromes based on clinical severity and transfusion requirement (Cappellini *et al.*, 2014).

2.2. Beta-thalassemia:

Beta-thalassemia is a heterogeneous group of hereditary blood disorders characterized by decreased or deficient beta-globin chain synthesis. The β-thalassemia is much more diverse than the α-thalassemia due to the diversity of the mutations that produce the defects in the β-globin gene. The severity of β-thalassemia relates to the degree of imbalance between the α- and non-α-globin chains. The β-globin gene maps in the short arm of chromosome 11(p15.5), in a region also containing the: delta globin gene, embryonic epsilon gene, fetal gamma genes, and a pseudogene (ψB_1). (Flint *et al.*, 1988; Rund and Rachmilewitz, 2005; and Cao and Galanello, 2010).

To date, more than 350 β-thalassemia mutations have been reported and (rarely) deletions of the two genes and the mutations are population-

specific (Muncie and Campbell, 2009; Qari *et al.*, 2013; and De Sanctis *et al.*, 2017).

The expression of mutated beta-globin genes can result in reduced or absent beta-globin production. According to these finding, β-thalassemia can be phenotypically classified into 2 types: β^0-thalassemia where total absence of beta-globin chains production and β^+-thalassemia where partial deficiency of beta-globin chains production. In β^+- thalassemia, there is a 5% to 30% reduction of beta-globin chains from normal levels and hypochromia and microcytosis characterize all types of thalassemia (Ho and Thein, 2000; Gibbons *et al.*, 2001; Marengo-Rowe 2007; and Muncie and Campbell, 2009)

Nearly all β-thalassemia variants are inherited in a Mendelian recessive manner, but there is a small subgroup of β-thalassemia alleles that behave in a dominant fashion (Higgs *et al.*, 2001). The hallmark of β-thalassemia is the decreased production or absence of adult hemoglobin A (α^2 β^2), reactivation of fetal hemoglobin F ($\alpha^2\gamma a^2$), and importantly, accumulation of excess α-globin chains which appears to underlie the main physiopathology of the disease (Leecharoenkiat *et al.*, 2016).

2.2.1. Epidemiology of β- Thalassemia:

Considering the current distribution of β- thalassemia, the wide diversity of mutations and the small number of specific mutations in individual populations, it seems unlikely that β-thalassemia originated in a single place and time (De Sanctis *et al.*, 2017). About 3% of the world's populations (150 million people) carry β-thalassemia genes (Borgna and Galanello, 2014), and approximately 1.5% of the global populations are heterozygotes (carriers) of the β-thalassemias (Higgs *et al.*, 2012).

There is a high incidence of β-thalassemia in populations from the Mediterranean basin, throughout the Middle East, the Indian subcontinent, Southeast Asia, and Melanesia to the Pacific Islands, figure (2.3), (Galanello and Origa, 2010; Weatherall, 2016; De Sanctis *et al.*, 2017; Tari *et al.*, 2018).

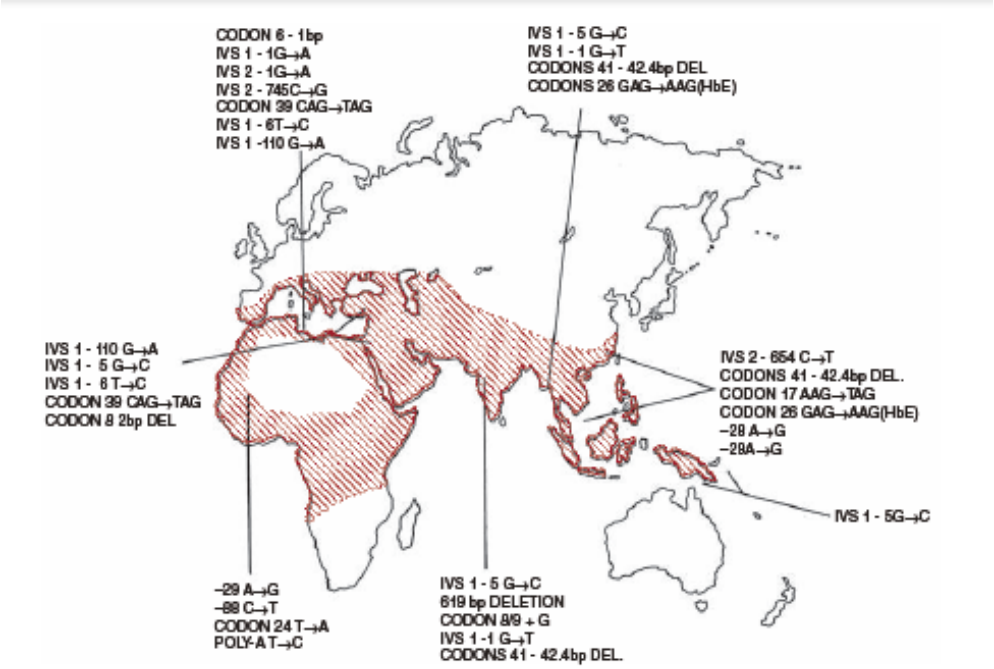


Figure (2.3): World distribution of β-thalassemia (Weatherall, 2016).

Regarding Iraq, there is little data on epidemiology and burden of β-thalassemia major or intermedia, however a number of studies were conducted in Iraq concerning the frequency of β- thalassemia minor in different governments; (Yahya *et al.*, 1996) noticed that (4.4%) were found to have ß- thalassemia minor in Baghdad. While (Kadhim *et al.*, 2017) reported that the prevalence of thalassemia syndromes in Iraq was 37.1/100,000 populations and β-thalassemia major represented 73.9% of all types of thalassemia.

In south of Iraq, (Hassan *et al.*, 2003) observed that the overall carrier frequency of β-thalassemia in Basra Province was 4.6%, while in Missan Province, (Al-Ali and Faraj, 2016) studied the prevalence of β-

thalassemia patients, and the results demonstrated that males were significantly more affected than females, and β-thalassemia major (79%) was more frequent than thalassemia intermedia, with the highest representation of β-thalassemia patients was observed between (1-3) years of age group. Moreover, (Almosawi and Al-Rashedi, 2018) found distribution of β-thalassemia major patients significantly more common in male (62.7%) than female and prevalence of β- thalassemia was significantly high (52%)in age ranging (1-10) years.

In Mousl and Iraqi Kurdistan Region the prevalence of β-thalassemia minor was estimated to be 4.14 -7.7 (Jalal *et al.*, 2008; Alnakshabandi and Muhammad, 2009, and Khaleel *et al.*, 2009).

2.2.2. Nomenclature of β-Thalassemia:

Many different mutations cause β-thalassemia and its related disorders, such as δβ-thalassemia and the silent carrier state. Three clinical and hematological conditions of increasing severity are recognized, i.e., the β-thalassemia carrier state, thalassemia intermedia, and thalassemia major (Galanello and Origa. 2010; and Giardina and Rivella, 2013).

Beta-thalassemias can be subdivided into: Nigam *et al*., (2017) and Galanello and Origa , (2010)

- β-thalassemia
  - Thalassemia major
  - Thalassemia intermedia
  - Thalassemia minor
- β-thalassemia with associated Hb anomalies
  - HbC/β-thalassemia
  - HbE/ β –thalassemia
  - HbS/ β -thalassemia (clinical condition more similar to sickle cell disease than to thalassemia major or intermedia)

- Hereditary persistence of fetal hemoglobin and β-thalassemia
- Autosomal dominant forms
- β -thalassemia associated with other manifestations
- β -thalassemia-tricothiodystrophy
- X-linked thrombocytopenia with thalassemia.

2.2.3. Laboratory Findings in Homozygous β- Thalassemia Major:

In β-thalassemia major the laboratory results are (Qari *et al*., 2013; and Sankaran *et al.*, 2015):

- Complete blood count (CBC):
  - False elevated WBC because of nucleated red blood cells (RBC).
  - Severe anemia with Hb as low as 3-4 g/dL.
  - Microcytic hypochromic picture with fragmented RBC, tear drops, target cells, and RBC with inclusion bodies.
- Reticulocyte count low.
- Serum iron to total iron binding capacity (TIBC) is high, as well as high serum ferritin.
- Hb electrophoresis: absent Hb A with remaining as Hb F and Hb A_2.

2.2.4.Treatment Homozygous β- Thalassemia Major:

Blood transfusion during lifelong is an inevitable treatment in β-thalassemia major patients. Several different regimens like hyper transfusion regimen (the pre-transfusion Hb is maintained as >10 g/dl with mean Hb of about 12 g/dl immediate post transfusion) , super transfusion regimen (the pre-transfusion Hb is maintained at ≥11 g/dl with a mean Hb of about 14 g/dl), while in moderate transfusion regimen the pretransfusion Hb is maintained at the values between 9 and 10 g/dl and to reach a posttransfusion level of 13 to 14 g/dl have been proposed over the years. In general, the amount of transfused RBC should not

exceed 15 to 20 ml/kg/day, infused at a maximum rate of 5 ml/kg/hour to avoid a fast increase in blood volume. Over time, due to complications of frequent blood transfusions and iron overload, iron chelators (like Dessferrioxamine B) are used to help remove the excess iron (Galanello and Origa, 2010; Hamidi, 2016; Sharma *et al*., 2017; and De Sanctis, 2018).

Splenectomy is indicated when patients have hypersplenism, which characterized by splenic enlargement, leukopenia and/or thrombocytopenia, increasing iron overload despite good chelation, and the calculated annual transfusion requirement more than 200 - 220 ml packed RBCs/kg/year with a hematocrit (PCV) value of 70% is to reduce the transfusion requirements and iron overload. Splenectomy also prevents extramedullary hematopoiesis (Galanello and Origa, 2010).

Inducing the synthesis of fetal hemoglobin can be increases lifespan of red blood cells, and ameliorate the severity of β-thalassemia. Drugs such as 5-azacytidine, hydroxyurea, and various butyrate derivatives have been used for this purpose (Rund and Rachmilewitz, 2005; and Tari *et al.*, 2018).

Hematopoietic stem cell transplantation (HSCT), and bone marrow transplant are the most confident treatments for β-thalassemia major patients , which by theory makes absolute cure for this disease and has been increasingly adopted, but is not available for all patients (Qari *et al.*, 2013; Angelucci *et al.*, 2014; Hamidi, 2016; and Eshaqh-hosseini *et al.*, 2018).

Very recently genome-wide association studies have demonstrated a gene (BCL11A) encoding a zinc-finger protein on chromosome (2p15) that modulates hemoglobin F levels. These types of therapies remain highly experimental; accordingly, their clinical potential remains uncertain (Schechter, 2008; and Tari *et al.*, 2018).

2.2.5 Parathyroid hormone, Calcium, Phosphorus, Vitamin D, and Alkaline Phosphatase Interaction:

The major regulation of bone and bone mineral metabolism results from the interactions of four hormones – parathyroid hormone (PTH), vitamin D , fibroblast growth factor 23 (FGF23) and calcitonin at three target organs bone, kidneys, and GI tract to regulate three bone minerals calcium, magnesium, and phosphorus. Other hormones also play a role, and skin is a participating organ system. The deviations from this normal regulatory scheme that occur in disease states can be evaluated, diagnosed, and, in most cases, effectively managed by the clinician when considered in the light of bone and mineral homeostasis (Bruder *et al.*, 2001).

Parathyroid hormone (PTH), also called parathormone or parathyrin, is a hormone secreted by the parathyroid glands. The central function of PTH is to regulate ionized calcium levels by concerted effects on three basic target organs: bone, intestinal mucosa, and kidney. The effect of PTH on intestinal calcium absorption is indirect, resulting from increased renal production of the intestinally active vitamin D metabolite 1, 25 dihydroxycholecalciferol (1, 25$(OH)_2 D_3$). When serum levels of calcium fall, the signal is transduced through the calcium-sensing receptor and secretion of PTH increases. PTH stimulates activity of 1α-hydroxylase in the kidney, enhancing production of 1, 25$(OH)_2 D_3$, (Kliegman *et al.*, 2020).

The increased level of 1, 25$(OH)_2 D_3$ induces synthesis of a calcium-binding protein in the intestinal mucosa with resultant absorption of calcium. PTH also mobilizes calcium by directly enhancing bone resorption, an effect that requires 1, 25$(OH)_2 D_3$. The effects of PTH on bone and kidney are mediated through binding to specific receptors on

the membranes of target cells and through activation of a transduction pathway involving a G-protein coupled to the adenylate cyclase system (Peacock, 2010; and Kliegman *et al.*, 2020).

Parathyroid hormone also play important role to maintained serum phosphorus level within a narrow range through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption, (Takeda *et al*, 2004; and Goretti and Alon, 2012).

Alkaline phosphatase (ALP) helps in the deposition of the hydroxyapatite crystals between the collagen fibrils of bone, and it is responsible for promoting bone mineralization by increasing the local concentration of inorganic phosphorus (Orimo, 2010).

2.2.6 complications of β-Thalassemia Major:

2.2.6.1 Iron Overload:

Complications in β-thalassemia major are linked to overstimulation of the bone marrow, dysfunctional erythropoiesis, iron overload which can be either from enhanced dietary absorption of iron or from frequent blood transfusions , imbalance of oxidant/antioxidant ratio, and chelation of essential elements (Qari *et al.*, 2013).

Although blood transfusions are crucial in the survival of β-thalassemia major, but the most serious disadvantage of life-saving transfusions is the inexorable accumulation of iron within tissues and blood-borne infections (Hamidi, 2016; and De Sanctis, 2018).

Since hemostasis of iron in normal human achieved by controlling absorption, and a human have no physiologic mechanism for active elimination of excess iron, and loses only 1–2 mg iron per day (Ganz and Nemeth, 2012; and Nienhuis and Nathan, 2012). Each milliliter of transfused blood contains about one mg of iron, thus receipt of a unit of

packed red cells typically results in the deposition of 200 mg of iron ultimately in the tissues following red cell senescence. So in β-thalassemia major patient, who receives regular PRBC transfusions of 25 units of blood / year accumulates 5 g of iron each year in the absence of any iron chelation therapy and are at risk for iron toxicity (Ganz and Nemeth, 2012; and Leecharoenkiat *et al.*, 2016).

In addition to multiple blood transfusions and hemolysis of red blood cells, the increased gastrointestinal iron absorption due to paradoxical hepcidin suppression from dyserythropoiesis lead to iron overload in β-thalassemia major patients. (Gardenghi *et al.*, 2010; and Leecharoenkiat *et al.*, 2016).

As a consequence, iron deposits in thalassemia patients can exceed from the storage and detoxification capacity of ferritin and also fully saturates transferrin, the transferrin saturation increases to 75%–100% and non-transferrin-bound iron (NTBI), and leads to the formation of free iron which accumulates in blood and tissues. This free iron will cause the formation of very harmful compounds, such as hydroxyl radical (OH) that is highly reactive, and attacks lipids, proteins and DNA and contributes to oxidative stress (Raghuveer *et al.*, 2009; and Nienhuis and Nathan, 2012).

Excess iron is extremely toxic to all cells of the body and can cause serious and irreversible end-organ damages as these β- thalassemia major children grow into adolescence and adulthood if untreated (Hamidi, 2016; Leecharoenkiat, *et al* 2016; and Khan *et al.*, 2017).

The harmful effects of excessive iron deposition are primarily observed in the heart, liver, skin, and endocrine organs like pituitary- gonadal axis, growth hormone - Insulin-like Growth Factors (GH-IGF) axis, thyroid, parathyroid, pancreas and adrenals. Therefore, the endocrine complications have emerged as an important cause of morbidity and an

important determinant of quality of life in children with β-thalassemia major (Singh and Seth, 2017).

The degree and the severity of iron toxicity depends upon the frequency and /or need for transfusions determined by genotype of the patient, the age at initiation of chelation therapy and compliance to it (Shalitin *et al.*, 2005; Merchant *et al.*, 2011; and Singh and Seth, 2017).

Serum ferritin is the most commonly used indicator for monitoring iron overload despite its inaccuracy and limitation in assessing the body iron overload. It is a valuable method to roughly assess the long-term overall status of iron overload and to monitor response to chelation therapy, but its level may be influenced by other factors such as inflammation, liver damage, and vitamin C deficiency (Olivieri and Brittenham, 1997; and De Sanctis *et al.*, 2018).

Iron overload is generally defined as serum ferritin consistently ≥ 1000 ng/L and high serum ferritin level during puberty (> 2,500 ng/ mL) is a risk factor for endocrine dysfunctions (Morrison *et al.*, 2003; and Shalitin *et al.*, 2005).

2.2.6.2 Hypothalamic-Pituitary-Gonadal (HPG) Axis and Prolactin Hormones Profiles:

The key factor implicated is the iron overload that mediates its oxidative damage (through iron generated free radicals) to the HPG axis leading to failure of adequate production of LH and FSH. Direct gonadal damage by iron overload is much less likely. The direct effect of iron on the ovaries and testes is currently unknown. The ovarian reserve is preserved in the majority of female thalassemia patients, even in women with amenorrhea, while in males, histological examination of testicular tissues from autopsies demonstrated testicular interstitial fibrosis and

small, heavily pigmented, undifferentiated seminiferous tubules and an absence of Leydig cells (Srisukh *et al*., 2016; and Singh and Seth, 2017).

In addition to free radical oxidative stresses, chronic hypoxia, zinc deficiency, liver disorder and diabetes mellitus, may too contribute to hypogonadism in β- thalassemia major patients (AL Hashmi *et al.*, 2008).

There are three main clinical presentations of the HPG axis impairment in β- thalassemia major, including delayed puberty (absence of any pubertal signs by 14 years in boys and 13 years in girls), arrested puberty and hypogonadism. In addition, HPG axis dysfunction can manifest as low estradiol or testosterone with low to normal serum LH and FSH as commonly seen in hypogonadotropic hypogonadism (hypothalamic-pituitary lesion). While low estradiol and testosterone accompanied with increased serum LH and FSH indicates primary gonadal failure due to direct gonadal damage (Srisukh *et al.,* 2016; and Singh and Seth, 2017).

Hypothalamic-pituitary-gonadal axis dysfunction are the most frequently registered endocrine complication in β- thalassemia major despite regular transfusions and optimal chelation therapy ranges between 30 and 80% of patients in various studies (Parijat *et al*., 2014; and De Sanctis *et al*., 2018).

Both genders of β- thalassemia major patients have HPG axis derangement and the reported prevalence was 45- 70% in boys and 39 - 75% in girls without significant differences (Najafipour *et al*., 2008, and Parijat *et al*., 2014).

Hagag *et al*., (2016), in their study, found that female patients with β-thalassemia with iron overload may have gonadal hormones including serum FSH, LH, estrogen deficiency with significant negative correlations between gonadal hormones and serum ferritin.

Vahidi *et al*., (2003), in their cross-sectional, controlled study ,They found that mean levels of FSH and LH were significantly lower in cases

than in controls for boys and girls, and mean testosterone levels were significantly depressed in male thalassemia major patients compared to controls, as well as a lower level of estradiol in female patients compared to female controls, while comparison of laboratory variables between cases with ferritin levels below 2000 µg/L and cases with ferritin levels of 2000 µg/L or higher did not show any significant difference.

While, Moayeri and Oloomi, (2006), collected the clinical data of 158 patients (82 females and 76 males) with thalassemia major, with mean age: 15.1 ± 4.8 years, showed that 69% (73.2% of males and 64.8% of females) of thalassemia major patients had hypogonadism, with low serum FSH and LH, and this complication more common in patients with serum ferritin levels above 2000 µg/L.

Moreover, Najafipour *et al*., (2008), in their study found that HPG dysfunction occurred in approximately 71% of β-thalassemia patients, and it was present in 73% of girls and 70% of boys without significant differences. LH, FSH, and testosterone in boys and estradiol in girls were lower than normal. No cases of primary hypogonadism due to primary gonadal failure were detected.

Al-Rimawi *et al*., (2005) conducted a prospective study of the function of the HPG axis function ,They were found HPG dysfunction in 15 (48.4%) patients. Twelve patients (38.7%) had hypogonadotropic hypogonadism (low LH, FSH, and estradiol levels) and 5 (16.1%) had ovarian failure (low estradiol level). High levels of serum ferritin had significant affect.

Habeb *et al*., (2013), reported that the hypogonadism was the most common complication detected in 52.7% (19/36) of β- thalassemia major patients of pubertal age group (12 females >13 years and 7 males >14 years), and all patients with hypogonadism have low FSH and LH.

Belhoul *et al*., (2013), reported hypogonadism in 52.7% of β-thalassemia major patients with mean serum ferritin 2719 ng/ml, and it was the most common endocrine complication in their study.

Majeed, (2017) reported that the mean of circulating FSH levels in healthy male in control group were higher than patients group, while circulating levels of LH in male patients and control groups were comparable. Both FSH levels of male and female patients were significant lower than the corresponding values in the healthy. While the values of estradiol, and testosterone in patients group were significant lower than the corresponding values in the control group. Results demonstrated no relationship between all parameters of reproductive hormone versus iron and ferritin levels in β- thalassemia major patients.

Moreover, Jouda *et al*., (2019) mentioned that β-thalassemia major patients aged < 18 years have a low level of FSH, LH, and testosterone compared to older patients, but only the difference in LH and testosterone levels reached the significant.

While , Yenzeel and Salih, (2017) reported a significant decrease in the levels of FSH, LH, and estradiol hormones when compared to control in their study of forty female β-thalassemia major patients aged 17 – 24 years and 20 ages matched healthy control females.

Regarding prolactin, it is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs. It was originally named for its ability to promote lactation in response to the suckling stimulus of hungry young mammals. Prolactin secretion is affected by a large variety of stimuli provided by the environment and the internal milieu. The most important physiological stimuli that elevate pituitary prolactin secretion are suckling, stress, and increased levels of ovarian steroids, primarily estrogen. Thyrotropin-releasing factor (thyrotropin-releasing hormone) has a stimulatory effect

on prolactin release. Pituitary prolactin secretion is regulated by endocrine neurons in the hypothalamus that secrete dopamine causing inhibition of prolactin secretion (Freeman *et al*., 2000).

Livadas *et al*., (1984) mentioned that, in β-thalassemia major the anterior pituitary deficiency exists possibly for the lactotrophs, and they were found the basal prolactin values did not differ significantly between the β-thalassemia major patients and control groups, but after TRH administration the increment was significantly lower in patients than in controls.

Mousa *et al*., (2016) found significantly decreased prolactin level in their study of 38 Egyptian patients with β-thalassemia major compared to control subjects.

AL-Mosawy., (2017) in their study of 41 β -thalassemia major patients (19male and 22 female), in Karbala, reported that the prolactin levels were within the normal range and there were no significant differences between prolactin levels according to the age and sex of the patients, and blood transfusion interval rate in < 3 weeks or ≥ 3 weeks.

Jouda *et al*., (2019) studied 35 males with β-thalassemia major patients, reported that the prolactin levels were within the normal range and there were no significant differences between prolactin levels according to the age of patients.

2.2.6.3 Parathyroid Hormone and other related Bone Metabolism Profiles in β-Thalassemia Major:

2.2.6.3.1 Parathyroid hormone (PTH)

The striking increases in survival of patients with β-thalassemia major over the past decade have focused attention on abnormal endocrine function (Olivieri, 1999). Hypoparathyroidism and metabolic bone

disease with various skeletal complications including osteopenia, osteoporosis, scoliosis, rickets, spinal deformities, and spontaneous fractures are an important cause of morbidity and regularly reported in transfusion dependent β-thalassemia major patients (Salama *et al*., 2006; and Saboor *et al.*, 2014). Parathyroid dysfunction is mainly due to repeated blood transfusion results in citrate toxicity and iron overload with deposition in parathyroid cells and tissue fibrosis, and chronic anemia (Galanello and Origa, 2010; and Hamidi, 2016).

Most of the β-thalassemic major patients with hypoparathyroidism are asymptomatic while a few have mild symptoms, that it may cause various neurological manifestations such as tetany, seizures, carpopedal spasms, and parasthesia. The first evidence of hypoparathyroidism is loss of diurnal variation in PTH levels; low PTH, calcium, vitamin D levels and high phosphate levels (Basha *et al.,* 2014; and Lubis and Yunir, 2018). Hypocalcaemia due to hypoparathyroidism is a recognized complication in β- thalassemia major patients principally due to iron overload and it has a higher incidence in males and usually evident after 10 years of age (Parijat De *et al*., 2014; and Shah, 2015).

Many studies have been done to assess the prevalence and frequency of parathyroid dysfunction and biochemical markers of metabolic bone disease in β- thalassemia major patients, but the prevalence and results of abnormalities reported very different in different studies (Shazia *et al*., 2012; Hamidi, 2016; and Dejkhamron *et al.*, 2018).

Shamshirasaz *et al., (*2003) estimated that 7.6% of patients have low serum calcium, high serum phosphorus and low serum PTH, while 37.2% of patients had low 25-hydroxy vitamin D3 level. As well as, Najafipour *et al*.,(2008) , found that the serum calcium level was lower than normal in 23 patients (41%), and 14 (25%) patients had hyperphosphatemia (60%

of patients with hypocalcaemia had hyperphosphatemia at the same time), and 31 patients (56%) had elevated ALP.

Whereas, Mohey El-Deen *et al*., (2014) showed a highly significant difference between patients and controls regarding PTH, and study pointed out that PTH was significantly lower among patients whose duration of illness was ≥6 years, those required frequent blood transfusion, and here were a highly significant difference between PTH level and serum ferritin, but there were no significant difference in relation to serum calcium and phosphorus levels, and the study also pointed out that there was no significant difference in the PTH level with regard to the sex of the patients. Regarding the serum calcium was within the normal range in (77%) of patients, and below the normal level in (23%) of β- thalassemia major patients with no significant difference in their study. While the serum phosphorus level was normal in (31%) patients and higher than normal in (69%) patients but there was no significant difference between patients and control groups.

In addition, Hagag *et al*., (2015) reported that PTH levels were significantly lower in thalassemia patients, with a significant negative correlation with serum ferritin.

Pirinççioğlu *et al*.,(2017) found serum PTH level was significantly higher than normal range in 23/ 90 (25.6%) patient than those in controls, and they concluded that serum ferritin was significantly correlated with PTH.

El-Nashar *et al*., (2017) reported that the level of PTH in both patients and controls groups were not significantly different with a higher level in patients group . In other study done by , Bahy El-Din *et al*., (2018) included 60 patients with transfusion-dependent β- thalassemia major they found low PTH in 13/60 (21.7%) patients, and PTH level not

statistically influenced by sex, age, age at diagnosis, frequency of blood transfusion/month.

2.2.6.3.2 Vitamin D

Fahim *et al*., (2013) they observed that the mean serum level of 25-OH vitamin D was significantly lower in their patients than in controls, and 37 % had vitamin D deficiency and 54% had vitamin D insufficiency, and only 9% of patients had normal serum 25-OH vitamin D concentration.

Isik *et al., (*2014) in their study of 47 β-thalassemia major patients, reported that vitamin D insufficiency and deficiency observed in (78.2%) of patients. In addition, Hagag *et al*., (2015), they reported that patients with β- thalassemia major had significantly lower levels of serum 25-hydroxy vitamin D levels compared to the control group.

While, Majeed, (2017) studied some biochemical and endocrine profiles in sixty patients with β-thalassemia major, he observed deficiency of vitamin D in 100% (60/60) patients, while vitamin D insufficiency was observed in 25% (15/60) patients and he mentioned that the deficiency of vitamin D may started early in thalassemia major even before hypoparpthyroidism is detected.

2.2.6.3.3 Calcium and Phosphorus Profile

Salama et *al., (*2006). They found that serum calcium level was within normal range and showed no statistical significance when compared to the control group, while serum phosphorus level was significantly higher in patients than the control subjects. Al-Samarrai *et al*., (2008) in their randomized prospective cross-sectional hospital based study to assess the level of some essential elements in 105 thalassemia blood transfusion- dependent patients and 54 healthy controls. They found that, the mean serum levels of calcium and serum phosphate were

within normal limits in patients, and had no significant difference as compared with healthy control group.

Fahim *et al*., (2013) they observed that the mean total serum calcium levels were significantly lower in patients than in controls, and serum phosphorous levels were within the normal range in patients compared to controls. In addition ,El-Adham *et al*., (2013) , they registered hypocalcaemia in 16/40 (40%) patients with no significant difference between them either due to sex or types of chelation modalities, but there was negative correlation between serum calcium and age of patients.

Nawar *et al*., (2014) studied data obtained from 50 β-thalassemia patients, concluded that serum calcium was significantly lower when compared to controls, but they reported significantly higher serum phosphorus. In addition, Hagag *et al*., (2015) studied 60 patients they reported that the significantly lower levels of serum ionized calcium, and significantly higher serum phosphorus level in in β-thalassemia major patients compared to the control group. Al-Hakeim *et al*., (2017) in their study, they found no significant difference in serum calcium levels between control and patients groups.

Pirinççioğlu *et al*.,(2017) they reported significantly lower serum total calcium level in 12.2% of patients and serum ferritin was not correlated with serum total calcium. While serum phosphorus levels were found high in 2/90 (2.22%) patients with β-thalassemia major and low in 4 (4.44%) patients, but the results were not significant compared with control group.

Shawkat *et al*., (2018) in their study designed as a single center cross-sectional observational study. A total of 201 patients with β-thalassemia major enroll in the study, and the patients included fall into three groups: 40 (19.9%) children (age 8–12 years), 78 (38.8%) adolescents (age 13-18years) and 83 (41.3%) adults (age >19 years), but children and

adolescents were considered as a one category (pediatric) during the study, and female patients (53.2%). They were found that serum calcium and vitamin D levels were low in both adults and pediatrics patients without statistical difference in both groups.

2.2.6.3.4 Magnesium Profile

About 99% of total body magnesium is located in bone, muscles and non-muscular soft tissue (Elin, 2010). In bone, magnesium ions bind at the surface of the hydroxyapatite crystals, and magnesium increases the solubility of phosphorus and calcium hydroxyapatite and induces osteoblast proliferation; therefore, magnesium deficiency results in decreased bone formation (Salimi *et al*, 1985; and De Baaij *et al.*, 2015).

Cell magnesium is an important modulator cell volume regulation and affects the activity of various membrane cation transport pathways such as the sodium/ potassium pump, sodium – potassium - chloride cotransport, calcium and potassium channels, and potassium- chloride cotransport, and the physical properties of the erythrocyte membrane are severely affected by changes in cell magnesium content. Magnesium supplementation improves the anemia of β- thalassemia in mice, and it also compensates for the abnormal erythrocyte potassium loss by decreasing the activity of the potassium- chloride cotransport system (De Franceschi *et al.*, 1997).

Regarding magnesium abnormalities in β-thalassemia major. Al-Samarrai *et al*., (2008) in their study found serum magnesium levels slightly lower in thalassemia major patients as compared to controls. While, Genc *et al*., (2016) assessed the levels of magnesium in chronically transfused β- thalassemia major in Antalya; they found magnesium levels were higher in patients than those in the control, with no significant difference between the patients and the control.

Moreover, Ratha *et al*., (2015) , They found serum magnesium level was decreased significantly in patients in comparison to healthy subjects. However, Khaleel *et al*.,(2018) in their study of 60 with β- thalassemia major and 30 healthy volunteers matched for age and sex were studied as control, in Baghdad. They found serum magnesium level was significantly higher in patients than normal control.

As well as, Nafady *et al.,* (2018) in their study of 53 Egyptian patients with β- thalassemia major were divided into two groups according to the ferritin level (≥1000 1000 µg/l and <1000 1000 µg/l) and were compared with 40 healthy control group, with the mean ages of patients with β-thalassemia and control were 8.48±4.07 and 7.11±4.46 years respectively, and sexes were comparable between the two study groups. They showed that serum magnesium level was significantly lower in patients than the healthy control group, and the results showed that the mean serum magnesium level was lower in patients with ferritin level of at least 1000 µg/l than patients with ferritin less than 1000 µg/l.

2.2.6.3.5 Alkaline phosphatase (ALP)

Salama et *al., (*2006), They found that serum alkaline phosphatase was slightly higher in patients than the control group but not statistically significant.

Nawar *et al*., (2014) studied data obtained from 50 β-thalassemia patients, they reported significantly higher serum alkaline phosphatase levels among patients when compared to control group.

El-Nashar *et al*., (2017) in their case-control study, they reported that the serum ALP was significantly higher in patients when compared to control group. While Pirinççioğlu *et al*.,(2017) they reported the ALP it was higher among β-thalassemia major patients compared to the control group, and they observed that β-thalassemia major children with higher

parathyroid hormone levels had significantly higher serum ALP compared with those with normal parathyroid hormone levels.

Chapter Three
Materials and Methods

3.Materials and Methods:

3.1. Study Subjects

This study was conducted over a six –month period, from December 2018 to the end of May 2019, a total 100 sample including a 50 patients and a 50 healthy controls were involved in this study. A 50 (30 male and 20 female) patients with β- thalassemia major patients, and their ages range between 11 to 16 years. The diagnosis of β-thalassemia major was based on clinical history and examination in addition to the usual hematological data (complete blood count, and Hb electrophoresis); all patients were on regular blood transfusion as part of their treatment.

While control group involved a 50 healthy adolescents persons randomly selected from the general population matched for age and sex with patients group

The patients and controls were further divided into 2 more subgroups based on age from 11-13 years, and 14-16 years to study the serum ferritin, reproductive hormones and some biochemical bone markers levels.

A structural questioner was designed to collect the data about studied subjects (Appendix1), and a study project was designed as scheme, figure (3.1).

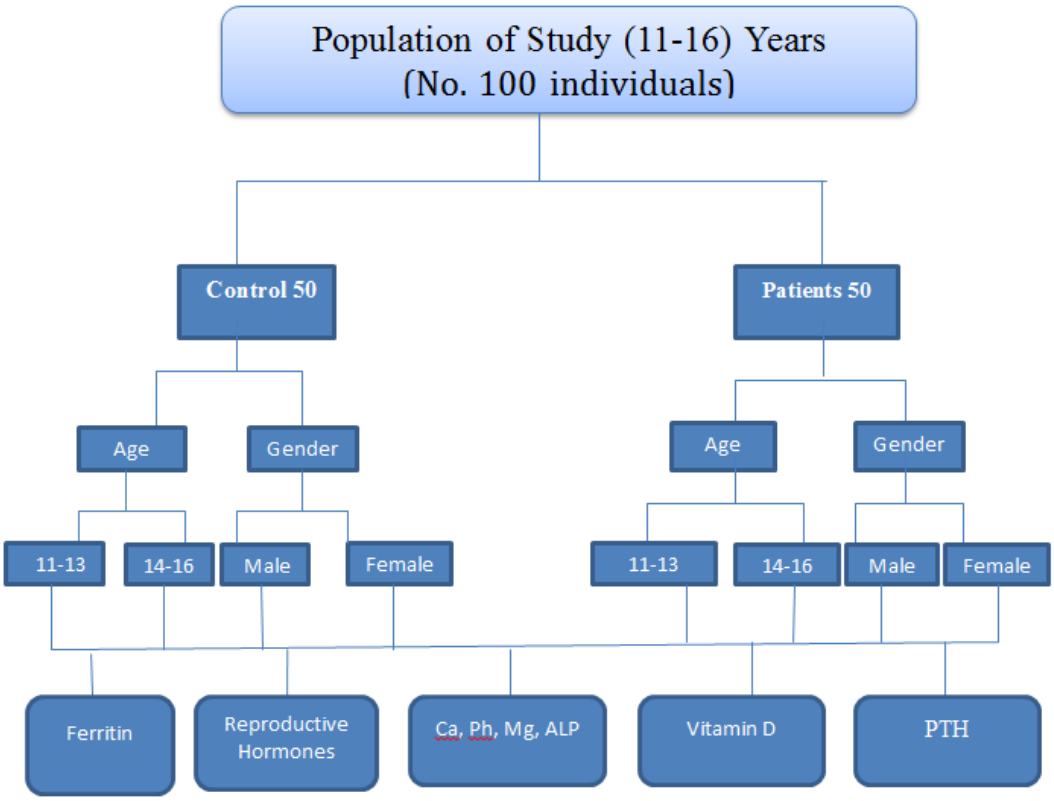


Figure (3.1): Design of the current study scheme

3.2. Materials:

3.2.1. Instruments and Apparatus:

The apparatus and equipment used in this study were shown in table (3.1).

Table (3.1): The apparatus and equipment used in the study

No.	Apparatus	Manufacturer
1	Deep freezer	Hitachi (Japan)
2	EDTA tubes	Medical Technology (China)
3	Elisa	Bio-Rad Laboratories (USA)
4	Hood	C.B.S Scientific (USA)

5	Horizontal Electrophoresis system	Biometra (Germany)
6	Hot Plate	Biobase (China)
7	Jel Tubes	Alibaba (China)
8	Micro-centrifuge	Hettich (Japan) Zentifugen(Germany)
9	Mini Vidas	BioMérieux (France)
10	Oven	Biobase (China)
11	Plain tube	Alibaba (China)
12	Spectrophotometer	Emc Lab (Germany)
13	VIDAS IVA 30/908	BioMérieux (France)

3.2.2. Kits

The kits used in this study are listed in table (3.2).

Table (3.2): The kits used in the study

No.	**Kits and Chemicals**	**Manufacturer**
1	25-Hydroxye Vitamin D Kit	BioMerieux (France)
2	ALP Kit	BioMerieux (France)
3	Calcium Kit	Spinreact (Spain)
4	Estradiol	BioMerieux (France)
5	Ferritin Kit	BioMerieux (France)
6	FSH Kit	BioMerieux (France)
7	LH Kit	BioMerieux (France)
8	Magnesium Kit	Spinreact (Spain)
9	Phosphorus Kit	Spinreact (Spain)
10	Prolactin Kit	BioMerieux (France)
11	PTH Kit	Human PTH ELISA Kit (USA)
12	Testosterone	BioMerieux (France)

3.3. Methods:

3.3.1. Collection of blood and samples preparation:

Ten milliliters of blood have been taken from all subjects collected in two tubes, 7.5 ml in jel tube and 2.5 ml in ethylenediaminetetraacetic acid (EDTA) tube. The serum tubes were centrifuged at 2000 xg for 10 minutes. The serum were separated and divided into three aliquots and stored at (–20 °C) until time of use.

3.3.2. Determination of Serum Ferritin Value:

3.3.2.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection Enzyme Linked Fluorescent Assay (ELFA) according to (Aisen and Listowsky, 1980 and Challand *et al.*, 1990). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. The sample has taken and transferred into the well containing alkaline phosphatase labeled anti-ferritin (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of ferritin present in the sample.

Table (3.3): Content of the ferritin kit - reconstitution of reagents

60 Strips Ferritin	STR	Ready-to-use.
60 Ferritin SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti- ferritin immunoglobulins (mouse).
Ferritin control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distille water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human ferritin + preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
Ferritin calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human ferritin + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and

		the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
Ferritin diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.2.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "ferritin" strip and one "ferritin" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "ferritin" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).

6. For this test, the calibrator, control, and sample test portion is 100 μL.
7. Insert the "vitamin D" SPRs and "ferritin" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.
8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.
10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.
11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 12-140 ng/ml (Kliegman *et al.*, 2020).

3.3.3. Hormonal Parameters Assessment:

3.3.3.1. Determination of FSH Value:

3.3.3.1.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection Enzyme Linked Fluorescent Assay (ELFA) according to (Butt *et al.*, 1983). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready-to-use and pre-dispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

The sample is taken and transferred into the well containing alkaline phosphatase-labeled anti-FSH antibodies (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to the antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-Methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone) the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, the results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

Table (3.4): Contents of the FSH Kit - Reconstitution of Reagents

60 FSH strips	STR	Ready-to-use.
60 FSH SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti-FSH immunoglobulins (mouse).
FSH control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human FSH + preservatives.

		MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
FSH calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human FSH + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
FSH diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (MasterLot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.3.1.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "FSH" strip and one "FSH" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "FSH" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).
6. For this test, the calibrator, control, and sample test portion is 200 µL.
7. Insert the "FSH" SPRs and "FSH" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.
8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.
10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.
11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 0.23–10.37 mIU/ml (Gardner and Shoback, 2007).

3.3.3.2. Determination of LH Value:

3.3.3.2.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection by Enzyme Linked Fluorescent Assay (ELFA) according to (Butt *et al.*, 1983). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready-to-use and pre-dispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times.

The sample is taken and transferred into the well containing alkaline phosphatase-labeled anti-LH antibodies (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to the antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-Methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone) the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, the results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

Table (3.5): Contents of the LH Kit- Reconstitution of Reagents.

60 LH strips	STR	Ready-to-use.
60 LH SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti-LH immunoglobulins (mouse).
LH control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human LH + preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
LH calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human LH + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV

		Range).
LH diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.3.2.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "LH" strip and one "LH" SPR for each sample, control or calibrator to be tested. Make sure that the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "LH" code on the instrument. The calibrator must be identified by "S1", and tested in duplicate. If the control needs to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex-type mixer (for serum or plasma separated from the pellet).
6. For this test, the calibrator, control, and sample test portion is 200 µL.
7. Insert the "LH" SPRs and "LH" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.

8. Initiate the assay as directed in the User Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.
10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.
11. Dispose of used SPRs and reagent strips in an appropriate recipient.

*Expected value = 0.28–29.38 mIU/ml (Gardner and Shoback, 2007)

3.3.3.3. Determination of Testosterone Value:

3.3.3.3.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection by Enzyme Linked Fluorescent Assay (ELFA) according to (Wheeler, 2006; and Vesper *et al.*, 2014). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. The sample is taken and transferred into the well containing alkaline phosphatase labeled anti-testosterone (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a “sandwich”. Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of testosterone present in the sample.

Table (3.6): Content of The Testosterone Kit - Reconstitution of Reagents

60 Strips testosterone	STR	Ready-to-use.
60testosterone SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti- testosterone immunoglobulins (mouse).
Testosterone control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distille water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25 ± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human testosterone + preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
Testosterone calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human testosterone + preservatives. MLE data indicate the concentration in

		mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
Testosterone diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.3.3.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "testosterone" strip and one "testosterone" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "testosterone" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.

5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).

6. For this test, the calibrator, control, and sample test portion is 200 µL.

7. Insert the "testosterone" SPRs and "testosterone" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.

8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.

9. Reclose the vials and return them to the required temperature after pipetting.

10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.

11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 10–800 ng/ml (Gardner and Shoback, 2007).

3.3.3.4. Determination of Estradiol Value:

3.3.3.4.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection by Enzyme Linked Fluorescent Assay (ELFA) according to (Anckaert *et al.*, 2002 and Vesper *et al.*, 2014). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. The sample is taken and transferred into the well containing alkaline phosphatase labeled anti-estradiol (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction

speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a “sandwich”. Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of estradiol present in the sample.

Table (3.7): Content of The Estradiol Kit - Reconstitution of Reagents

60 strips Estradiol	STR	Ready-to-use.
60 **estradiol** SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti- estradiol immunoglobulins (mouse).
Estradiol control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distille water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human estradiol + preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
Estradiol calibrator	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix.

3 x 2 ml (lyophilized)		Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human estradiol + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
Estradiol diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.3.4.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "estradiol" strip and one "estradiol" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.

3. The test is identified by the "estradiol" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).
6. For this test, the calibrator, control, and sample test portion is 200 µL.
7. Insert the "estradiol" SPRs and "estradiol" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.
8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.
10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPR^{s} and strips from the instrument.
11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 5–410 pg /mL (Gardner and Shoback, 2007).

3.3.3.5. Determination of Prolactin Value:

3.3.3.5.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection by Enzyme Linked Fluorescent

Assay (ELFA) according to (L'Hermite, 1976; and Saleem *et al.*, 2018). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. The sample is taken and transferred into the well containing alkaline phosphatase labeled anti-prolactin (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of prolactin present in the sample.

Table (3.8): Content of The Prolactin Kit - Reconstitution of Reagents

60 strips Prolactin	STR	Ready-to-use.
60 prolactin SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti- prolactin immunoglobulins (mouse).
prolactin control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distille water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible. Human serum* + human prolactin +

		preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
prolactin calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human prolactin + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
prolactin diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from www.biomerieux.com/techlib.		

3.3.3.5.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "prolactin" strip and one "prolactin" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "prolactin" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).
6. For this test, the calibrator, control, and sample test portion is 200 µL.
7. Insert the "prolactin" SPRs and "prolactin" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.
8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.

10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.

11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 2.0–18.0 ng/mL (Gardner and Shoback, 2007).

3.3.3.6. Determination of PTH Value:

3.3.3.6.1. Principle:

The assay principle based on competitive radioimmunoassay (RIA). In PTH RIA, radiolabeled hormone (125I-PTH) and unlabeled PTH compete for binding to an immobilized antibody or antisera generated against PTH (Cavalier *et al.*, 2015)

The kit is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of PTH in human serum, plasma and other biological fluids.

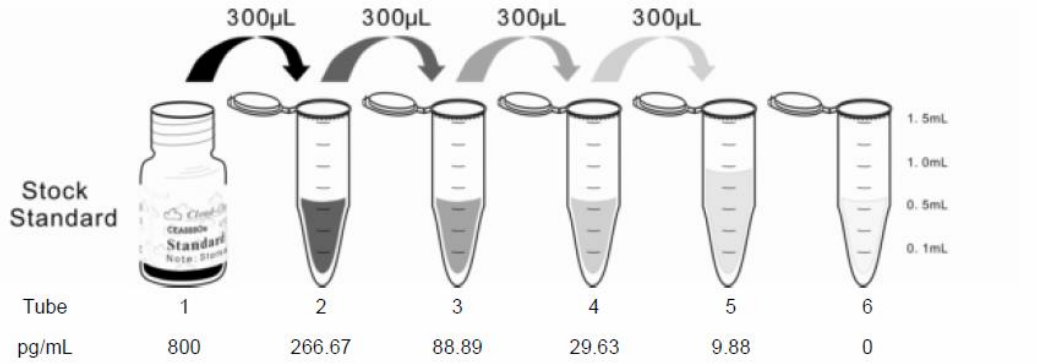
Table (3.9): Content of the PTH Kit – Reconstitution of Reagents

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120µL	Assay Diluent A	1×12mL
Detection Reagent B	1×120µL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

3.3.3.6.2. Reagent Preparation:

1. Bring all kit components and samples to room temperature (18-25oC) before use.
2. Standard - Reconstitute the Standard with 2.0mL of Standard Diluent, kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 800pg/mL. Prepare 5 tubes containing 0.6mL Standard Diluent and

produce a triple dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 5 points of diluted standard such as 800pg/mL, 266.67pg/mL, 88.89pg/mL, 29.63pg/mL, 9.88pg/mL, and the last EP tubes with Standard Diluent is the blank as 0pg/mL.



3. Detection Reagent A and Detection Reagent B - Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with Assay Diluent A and B, respectively (1:100).
4. Wash Solution - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. TMB substrate - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

3.3.3.6.3. Procedure:

1. Determine wells for diluted standard, blank and sample. Prepare 5 wells for standard points, 1 well for blank. Add 50µL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50µL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37oC. Detection Reagent

A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.

2. Aspirate the solution and wash with 350µL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
3. Add 100µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37oC after covering it with the Plate sealer.
4. Repeat the aspiration/wash process for total 5 times as conducted in step 2.
5. Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37oC (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
6. Add 50µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

*Expected value = 18.2 – 76.5 pg/ml (Aloia *et al.*, 2006).

3.3.3.7. Determination of 25- Hydroxy Vitamin D Value:

3.3.3.7.1. Principle:

The assay principle combines an enzyme immunoassay sandwich method with a final fluorescent detection by Enzyme Linked Fluorescent Assay (ELFA) according to (Holick, 2007; and Zipitis and Akobeng, 2008). The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device for the assay. The sample is taken and transferred into the well containing alkaline phosphatase labeled anti-vitamin D (conjugate). The sample/conjugate mixture is cycled in and out of the SPR several times to increase the reaction speed. The antigen binds to antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of vitamin D present in the sample.

Table (3.10): Content of The Kit Vitam D - Reconstitution of Reagents

60 strips Vitamin D	STR	Ready-to-use.
60 vitamin D SPRs 2 x 30	SPR	Ready-to-use. Interior of SPRs coated with monoclonal anti- vitamin D immunoglobulins (mouse).
vitamin D control 1 x 3 ml (lyophilized)	C1	Reconstitue with 3 ml of distille water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at - 25 ±

		6°C. 5 freeze/thaw cycles are possible. Human serum* + human vitamin D + preservatives. MLE data indicate the confidence interval in mIU/mL (milli-international units per milliliter) ("Control C1 Dose Value Range").
vitamin D calibrator 3 x 2 ml (lyophilized)	S1	Reconstitute with 2 ml of distilled water. Leave to stand for 5 to 10 minutes, then mix. Stable after reconstitution for 14 days at 2-8°C or until the expiration date on the kit at -25 ± 6°C. 5 freeze/thaw cycles are possible Bovine serum + human vitamin D + preservatives. MLE data indicate the concentration in mIU/mL ("Calibrator (S1) Dose Value") and the confidence interval in "Relative Fluorescence Value ("Calibrator (S1) RFV Range).
vitamin D diluent 1 x 3 ml (liquid)	R1	Ready-to-use. Bovine serum + 1 g/l sodium azide.
Specifications for the factory master data required to calibrate the test: • MLE data (Master Lot Entry) provided in the kit, or • MLE bar code printed on the box label.		
1 Package insert provided in the kit or downloadable from		

www.biomerieux.com/techlib.

3.3.3.7.2. Procedure:

1. Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
2. Use one "vitamin D" strip and one "vitamin D" SPR from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.
3. The test is identified by the "vitamin D" code on the instrument. The calibrator must be identified by "S1" and tested in duplicate. If the control is to be tested, it should be identified by "C1".
4. If necessary, clarify samples by centrifugation.
5. Mix the calibrator, control and samples using a vortex- type mixer (for serum or plasma separated from the pellet).
6. For this test, the calibrator, control, and sample test portion is 100 µL.
7. Insert the "vitamin D" SPRs and "vitamin D" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent strips match.
8. Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.
9. Reclose the vials and return them to the required temperature after pipetting.

10. The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.

11. Dispose of the used SPRs and strips into an appropriate recipient.

*Expected value = 13–67 ng/ml (Gardner and Shoback, 2007).

3.3.3.8. Determination of Calcium Value:

3.3.3.8.1. Principle:

The method is based on specific binding of cresolfalein complexone (OCC), a metallochromic indicator and calcium at alkaline pH with the resulting shifts in the absorbtion wavelength of the complex. The intensity of the cromophore formed is proportional to the concentration of total calcium in the sample (Henry, 1984; Tietz, 1984, and Tieiz, 1995).

$$\text{OCC} + \text{Calcium} \xrightarrow{\text{pH } 10.7} \text{OCC-calcium complex}$$

3.3.3.8.2. Preparation of Reagent:

Working reagent, Mix 1 volume of R1+ 1volume of R2. Stable 5 days at 2-8 ^{0}C. Recap reagents immediately after use.

3.3.3.8.3. Procedure:

	Blank	Sample	Standard
Working reagent	1.0 ml	1.0 ml	1.0 ml
Sample	-	10 µl	-
Standard	-		10 µl

Mix and let the tubes stand 2 minutes at room temperature. The absorbance (A) of the samples, and the standard, at 570 nm against their agent blank.

3.3.3.8.4. Calculation:

$$\text{Calcium (mmol/ l)} = \frac{\text{A Sample}}{\text{A Standard}} \times \text{Concentration of Standard}$$

*Expected Value = 8.4-10.2 mg/dl (Tieiz, 1995).

3.3.3.9. Determination of Inorganic Phosphate:

3.3.3.9.1. Principle:

Inorganic phosphate reacts with molybdic acid forming a phosphomolybic complex. Its subsequent reduction in alkaline medium originates a blue molybedenum colour which intensity is proportional to the amount present in the sample (Drewes, 1972).

$$Po_4 + H^+ + (NH_4)_6\, Mo_7O_{24} \xrightarrow{\text{Pi}} \text{phosphomolybdic Complex}$$

$$\text{Phosphomolybdic Complex} \xrightarrow[\text{Reductant}]{\text{pH> 10}} \text{Molybdenum blue}$$

3.3.3.9.2. Reagent Preparation:

Working reagent: Mix 1 volume of R1 + 1volume of R2. Stable for 8 hours at 2-8 ^{0}C protected from light.

3.3.3.9.3. Procedure:

	Blank	Sample	Standard
Working reagent	1.0 ml	1.0 ml	1.0 ml
Sample	_	50 µl	_
Standard	_	_	50 µl

Mix, let stand the tubes for 1 minute and then pipette:

R3 Developer	**0.5 ml**	**0.5 ml**	**0.5 ml**

Mix and let the tubes stand 10 minutes at room temperature. Read the absorbance (A) of the sample and the standard at 740 nm against the reagent blank.

3.3.3.9.4. Calculations:

$$\text{Phosphorus (mmol/l)} = \frac{\text{Sample}}{\text{Standard}} \times \text{Concentration of Standard}$$

*Expected value = 2.8 – 4.5 mg/dl (Ghosh and Joshi, 2008).

3.3.3.10. Determination of ALP Value:

3.3.3.10.1. Principle:

Colorimetric determination of Alkaline Phosphatase Activity according to the following reaction:

$$\text{Phenylphosphate} \xrightarrow[\text{pH10}]{\text{ALP}} \text{phenol + phosphate}$$

The liberated phenol was measured in the presence of sodium arsenate in the reagent stop the enzymatic reaction (Belfield and Goldberg, 1971).

3.3.3.10.2. Procedure:

	Serum sample	**Serum blank**	**Standard**	**Reagent blank**
R1	2 mL	2 mL		2 mL
The content of tubes were incubated for 5 minutes at 37 C^o				
R2	-	-	50 µL	-
Serum	50 µL	-	-	-
The content of tubes were incubated for 15 minutes at 37 C^o				
R3	0.5 mL	0.5 mL	0.5 mL	0.5 mL
The content of tubes were mixed well				
R4	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Serum	-	50 µL	-	--
Distilled water	-	-	-	50 µL

The content of tubes was mixed well and stands for 10 minutes in the dark.

The content of tubes were mixed, the absorbance of the sample was measured against the sample blank at 510 nm.

3.3.3.10.3. Calculations:

$$\text{Alkaline phosphatase} = \frac{\text{Optical Density of sample} - \text{Optical Density of bank}}{\text{Optical Density of standard}} \times n$$

*Expected Value = 21- 92 U/l (Belfield and Goldberg, 1971)

3.3.3.11.Determination of Magnesium Value:

3.3.3.11.1. Principle:

The method is based on the specific binding of calmagite, a metallochromic indicator and magnesium at alkaline pH with the resulting shift in the absorbtion wavelength of the complex. The intensity of the cromophore formed is proportional to the concentration of magnesium in the sample (Lindstrom and Diehl, 1960)

$$\text{Calmagite} + \text{Magnesium} \xrightarrow{\text{pH 11.5}} \text{Calmagite - Magnesium complex}$$

3.3.3.11.2. Procedure:

	Blank	Sample	Standard
R1. Reagent	1.0 ml	1.0 ml	1.0 ml
Sample	_	10 µl	_
Standard	_	_	10 µl

Mix and let the tubes stand 2 minutes at room temperature. Read the absorbance (A) of the samples and the standard at 520 nm against the reagent blank.

3.3.3.11.3. Calculations

$$\text{Magnesium (mmol/l)} = \frac{\text{A Sample}}{\text{A Standard}} \times \text{Concentration of Standard}$$

*Expected value =0.66- 1.23 mmole/l =1.6- 3.0 mg/dl, **(**Tieiz, 1995).

3.4. Statistical Analysis

Data were analyzed by T. test was performed by using Statistical Package for Social Science (SPSS) version 23 for windows , the difference were considered to be significant at ($p<0.05$) , the data are presented as mean ± standard error, the correlation coefficient pearson was calculated to examine association parameters, (saville,2015).

Chapter Four
Results

4. Results:

4.1. Evaluation of Serum Ferritin Level According to Gender and Age:

Results revealed a significant (P <0.05) increase in serum ferritin level (4094.78±492.55ng/ml) in male and female (3603.20±564.88 ng/ml) β- thalassemia major patients in comparison to male and female in control groups (94.96±8.17 ng/ml, 74.82±6.44 ng/ml respectively), table (4.1).

According to the age subgroups in control and β- thalassemia major individuals groups, male patients in both age subgroups have a significant (P <0.05) increase in serum ferritin level (11–13 years =3716.03±535.76 ng/ml, and 14-16 years = 4473.50±835.21ng/ ml) in comparison to male control groups (86.86±10.51 ng/ml, and 103.07±12.53 ng/ml respectively), and so female patients in both age subgroups have a statistically significant (P <0.05) increase in serum ferritin (11-13 years = 3894.90±894.56 ng/ml, and 14-16 years=3311.40±726.72 ng/ml), table (4.2).

Table (4.1): Serum ferritin value in healthy and β-thalassemia major patients according to gender

Variable	Gender	Control Group	Patients Group
Ferritin (ng/ml)	**Male**	94.96±8.17[a]	4094.78±492.55[b]
	Female	74.82±6.44[a]	3603.20±564.88[b]

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level (p<0.05).

The similar letters refer to a non-significant difference (between the control and patients groups) .

Table (4. 2): Serum ferritin value in healthy and β- thalassemia patients according to gender and age groups

Variables	Gender	Age	Control Group	Patients Group
Ferritin (ng/ml)	**Male**	**11-13**	86.86±10.51[a]	3716.03±535.76[b]
		14-16	103.07±12.53[a]	4473.50±835.21[b]
	Female	**11-13**	79.77±8.45[a]	3894.90±894.56[b]
		14-16	69.88±9.91[a]	3311.40±726.72[b]

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level ($p<0.05$).

The similar letters refer to a non-significant difference (between the control and patients groups) .

In thalassemia major patients group, table (4.3) showed that the male β-thalassemia major patients had a high serum ferritin (4094.78±492.55ng/ml) than female patients (3603.20±564.88ng/ml), but there was no statistical significant difference (P value =0.84), but female in age subgroup 11 – 13 years, have non-significant increase serum ferritin levels (3894.90±894.56 ng/ml) compared to male patients (3716.03±535.76 ng/ml) of same age subgroup, while serum ferritin in male patients of 14- 16 years subgroup was higher (4473.50±835.21 ng/ml) compared to female patient (3311.40±726.72 ng/ml in the same age subgroup, but non statistically significant difference.

Table (4. 3): Serum ferritin value in β- thalassemia patients according to gender and age groups

Variables	Age	Male	Female
Ferritin (ng/ml)	11-16	4094.78±492.55[a]	3603.20±564.88[a]
	11-13	3716.03±535.76[a]	3894.90±894.56[a]
	14-16	4473.50 ±835.21[a]	3311.40 ±726.72[a]

Value represented mean ± SE.

The different letters refer to significant difference between male and female patients at level ($p<0.05$).

The similar letters refer to non-significant difference between male and female patients.

4.2. Evaluation of Serum Reproductive and Prolactin Hormones According to Gender and Age:

The results of our study revealed male patients have significant ($P<0.05$) decreased levels of FSH (1.52±0.26 mIU/ml), LH (0.92±0.16 mIU/ml), testosterone (1.31±0.20 ng/ml) and estradiol hormones (23.67±3.54 pg/ml) levels in comparison to male in control group. While, in female the FSH (3.38±0.60 mIU/ml), and testosterone (1.08±0.20 ng/ml) in β-thalassemia patients was not significantly compared with control group, while LH (2.54±0.78 mIU/ml), and estradiol (48.13±6.57 pg/ml) levels were not significantly low in thalassemia patients in comparison to control. Regarding prolactin level it was not significantly decreased in male (12.91±1.11 ng/ml), and female (13.97±1.09 ng/ml) patients compared with control, table (4. 4).

Table (4.4): Reproductive and prolactin hormones in healthy and β-thalassemia patients according to gender

Hormone	Gender	Control Group	Patients Group
FSH (mIU/ml)	Male	2.67±0.17^{a}	1.52±0.26^{b}
	Female	2.72±0.21^{a}	3.38±0.60^{a}
LH(mIU/ml)	Male	2.64±0.20^{a}	0.92±0.16^{b}
	Female	2.73±0.18^{a}	2.54±0.78^{a}
Testosterone (ng/ml)	Male	5.64±1.62^{a}	1.31±0.20^{b}
	Female	0.80±0.10^{a}	1.08±0.20^{a}
Estradiol (pg/ml)	Male	54.79±5.18^{a}	23.67±3.54^{b}
	Female	72.92±17.83^{a}	48.13±6.57^{a}
Prolactin (ng/ml)	Male	13.84±0.61^{a}	12.91±1.11^{a}
	Female	17.44±1.86^{a}	13.97±1.09^{a}

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level ($p<0.05$).

The similar letters refer to a non-significant difference (between the control and patients groups) .

Regarding age subgroups; the results revealed there were significant ($P < 0.05$) decreased levels of FSH (1.47±0.39 mIU/ml), LH (0.88±0.25 mIU/ml), testosterone (1.40±0.37 ng/ml), and estradiol (23.89±5.13 pg/ml) in male patients compared with control in 11 – 13 years subgroups, and so in female of same age subgroup FSH (2.71±0.84 mIU/ml), LH (1.24±0.42 mIU/ml), and estradiol (59.45±9.42 pg/ml) levels were decrease in thalassemia major patients compared with control, but FSH and estradiol had no significant difference, while testosterone level in female patients group (1.52±0.31 ng/ml) was higher than in control (0.92±0.12 ng/ml) non significant difference.

In 14 – 16 years subgroups our results showed that FSH, LH and estradiol hormones levels in male patients were significantly ($p<0.05$) decreased (1.57±0.35 mIU/ml, 0.96±0.20 mIU/ml, and 23.44±5.06 pg/ml) respectively, as so testosterone level (1.21±0.17 ng/ml) was lower but non significant difference compared with their levels in male control subjects (2.66±0.26 mIU/ml, 2.63±0.32 mIU/ml, 58.85±8.40 pg/ml and 7.30±3.21 ng/ml), table (4.5)

While hormones levels in female of same age subgroup FSH (4.06±0.88 mIU/ml), and LH (3.83±1.41mIU/ml) were high without significant differences in comparison to female control (2.39±0.20 mIU/ml, and 2.82±0.32mIU/ml respectively), but testosterone (0.65±0.18 ng/ml) and estradiol (36.81±8.06 pg/ml) levels were measured low, but

Table (4.5): Reproductive and prolactin hormones values in healthy and β-thalassemia patients according to gender and age groups

Hormone	Gender	Age	Control Group	Patients Group
FSH (mIU/ml)	**Male**	**11-13**	2.67±0.24[a]	1.47±0.39[b]
		14-16	2.66±0.26[a]	1.57±0.35[b]
	Female	**11-13**	3.06±0.35[a]	2.71±0.84[a]
		14-16	2.39±0.20[a]	4.06±0.88[a]
LH (mIU/ml)	**Male**	**11-13**	2.65±0.27[a]	0.88±0.25[b]
		14-16	2.63±0.32[a]	0.96±0.20[b]
	Female	**11-13**	2.64±0.18[a]	1.24±0.42[b]
		14-16	2.82±0.32[a]	3.83±1.41[a]
Testosterone(ng/ml)	**Male**	**11-13**	3.98±0.45[a]	1.40±0.37[b]
		14-16	7.30±3.21[a]	1.21±0.17[a]
	Female	**11-13**	0.92±0.12[a]	1.52±0.31[a]
		14-16	0.68±0.16[a]	0.65±0.18[a]
Estradiol (pg/ml)	**Male**	**11-13**	50.73±6.19[a]	23.89±5.13[b]
		14-16	58.85±8.40[a]	23.44±5.06[b]
	Female	**11-13**	93.93±34.21[a]	59.45±9.42[a]
		14-16	51.91±8.58[a]	36.81±8.06[a]
Prolactin (ng/ml)	**Male**	**11-13**	13.30±1.02[a]	12.33±1.08[a]
		14-16	14.38±0.67[a]	13.49±1.97[a]
	Female	**11-13**	18.14±3.63[a]	13.76±1.58[a]
		14-16	16.75±1.15[a]	14.17±1.57[a]

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level ($p<0.05$).

The similar letters refer to a non-significant difference (between the control and patients groups) .

not significantly differed in female patients compared to same gender of control group.

Regarding prolactin hormone levels were lower non significant differences (12.33±1.08 ng/ml) in male and female (13.76±1.58 ng/ml) patients compared with male and female controls (13.30±1.02 ng/ml, and 18.14±3.63 ng/ml respectively) of same age subgroup (11 -13), and its levels also reported not significantly decreased in male (13.49±1.97 ng/ml) and female (14.17±1.57 ng/ml) patients compared to control in 14 – 16 years subgroup (14.38±0.67 ng/ml, and16.75±1.15 ng/ml respectively), table (4.5)

When we measured reproductive hormones levels β-thalassemia major patients group, table (4.6), showed that FSH (3.38±0.60 mIU/ml), LH (2.54±0.78 mIU/ml) hormones levels were significantly higher in female compared to male patients (1.52±0.26 mIU/ml, 0.92±0.16 mIU/ml respectively) and so estradiol (48.13±6.57 pg/ml) and prolactin (13.97±1.09 ng/ml) levels were high in female patients but non significant difference ($P > 0.05$) in comparison to male (23.67±3.54 and 12.91±1.11 ng/ml respectively), while testosterone levels was higher in male (1.31±0.20 ng/ml) than female patients (1.08±0.20 ng/ml), but non significant difference .

While in 11- 13 subgroup patients, all hormones levels including FSH (2.71±0.84 mIU/ml), LH (1.24±0.42 mIU/ml), testosterone (1.52±0.31 ng/ml), estradiol (59.45±9.42 pg/ml), and prolactin (13.76±1.58 ng/ml) were high in female patients compared with male (1.47±0.39 mIU/ml, 0.88±0.25 mIU/ml, 1.40±0.37 ng/ml, 23.89±5.13 pg/ml, and 12.33±1.08 ng/ml respectively) in same age subgroup, but only FSH had a significant difference.

Regarding 14 -16 years subgroup patients, our results revealed that female patients have an increased levels of FSH (4.06±0.88 mIU/ml), LH(3.83±1.41 mIU/ml), estradiol (36.81±8.06 pg/ml) and prolactin (13.49±1.97 ng/ml) hormones in comparison to male patients (1.57±0.35 mIU/ml, 0.96±0.20 mIU/ml, 23.44±5.06 pg/ml, and14.17±1.57 ng/ml respectively) of same age subgroup, but only FSH and LH have a significant differences, while testosterone level was had a high level without significant difference in male (1.21±0. 17 ng/ml) compared to female patients (0.65±0.18 ng/ml) in same age subgroup.

Table (4.6): Reproductive and prolactin hormones value in β-thalassemia patients according to gender and age groups

Hormone	Age	Male	Female
FSH (mIU/ml)	11-16	1.52±0.26[a]	3.38±0.60[b]
	11-13	1.47±0.39[a]	2.71±0.84[b]
	14-16	1.57±0.35[a]	4.06±0.88[b]
LH (mIU/ml)	11-16	0.92±0.16[a]	2.54±0.78[b]
	11-13	0.88±0.25[a]	1.24±0.42[a]
	14-16	0.96±0.20[a]	3.83±1.41[b]
Testosterone (ng/ml)	11-16	1.31±0.20[a]	1.08±0.20[a]
	11-13	1.40±0.37[a]	1.52±0.31[a]
	14-16	1.21±0. 17[a]	0.65±0.18[a]
Estradiol (pg/ml)	11-16	23.67±3.54[a]	48.13±6.57[a]
	11-13	23.89±5.13[a]	59.45±9.42[a]
	14-16	23.44±5.06[a]	36.81±8.06[a]
Prolactin (ng/ml)	11-16	12.91±1.11[a]	13.97±1.09[a]
	11-13	12.33±1.08[a]	13.76±1.58[a]
	14-16	13.49±1.97[a]	14.17±1.57[a]

Value represented mean ± SE.

The different letters refer to significant difference between male and female patients at level ($p<0.05$).

The similar letters refer to non-significant difference between male and female patients.

4.3. Evaluation of Parathyroid Hormone and Biochemical Bone Markers According to Gender and Age:

Regarding biochemical bone markers in β-thalassemia major, our study demonstrated that male β-thalassemia major patients have a significant ($p<0.05$) low serum PTH (35.01±5.19 pg/ml), vitamin D (12.22±0.67 ng/ml), and calcium (7.90±0.19 mg/dL) levels in comparison to male control subjects (74.76±7.30 pg/ml, 54.30±4.69 ng/ml, and 9.84±0.23 mg/dL respectively), while serum phosphorous (4.97±0.32 mg/dL) and ALP (212.20±19.40 U/L) were statistically significant ($p<0.05$) increase in male patients compared with male control (1.96±0.14 mg/dL and 83.62±7.11U/L respectively), also serum magnesium (2.18±0.38 mg/dL) was increased non statistical significant in male patients compared with control (1.83±0.09 mg/dL), table (4.7).

Table (4.7): Biochemical bone markers in healthy and β - thalassemia patients according to gender

Variables	Gender	Control Group	Patients Group
PTH (pg/ml)	Male	74.76±7.30[a]	35.01±5.19[b]
	Female	36.64±6.57[a]	31.39±5.69[a]
Vitamin D (ng/ml)	Male	54.30±4.69[a]	12.22±0.67[b]
	Female	40.38±4.66[a]	12.70±0.84[b]
Calcium (mg/dL)	Male	9.84±0.23[a]	7.90±0.19[b]
	Female	9.59±0.24[a]	7.55±0.23[b]
Phosphorus (mg/dL)	Male	1.96±0.14[a]	4.97±0.32[b]
	Female	2.75±0.21[a]	4.61±0.52[b]
ALP (U/L)	Male	83.62±7.11[a]	212.20±19.40[b]
	Female	91.36±4.55[a]	210.30±33.54[b]
Magnesium (mg/dL)	Male	1.83±0.09[a]	2.18±0.38[a]
	Female	1.76±0.12[a]	2.04±0.36[a]

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level ($p<0.05$).

The similar letters refer to a non-significant difference (between the control and patients groups) .

In comparison female β-thalassemia major patients with control subjects table (4.7) revealed that serum PTH level (31.39±5.69 pg/ml) decreased non statistical significant, while vitamin D (12.70±0.84 ng/ml) and calcium (7.55±0.23 mg/dL) levels were highly significant ($p<0.05$) decreased in patients compared with control females (40.38±4.66 ng/ml and 9.59±0.24 mg/dL). Phosphorus (4.61±0.52 mg/dL), ALP (210.30±33.54U/L), and magnesium (2.04±0.36 mg/dL) levels were measured higher, but only phosphorus level and ALP was significant ($P <0.05$) in female patients in comparison to female control group (2.75±0.21mg/dL, 91.36±4.55 U/L, and 1.76±0.12 mg/Dl respectively).

Analysis of our studied patients and control subjects according to age subgroups, our results in table (4.8) showed that in 11 – 13 years subgroup male patients have statistically significant ($p<0.05$) decreased serum levels of PTH (30.43±4.77 pg/ml), vitamin D (12.02±0.84 ng/ml), and calcium (7.93±0.25 mg/dL), but serum magnesium level (1.86±0.43mg/dL) was decreased non statistical significant in comparison to male control subgroup (77.06±11.56 pg/ml, 57.17±7.09 ng/ml, 9.67±0.36 mg dL, 1.94±0.18 mg/dL respectively). While serum phosphorus (4.67±0.47 mg/dL), and ALP (190.87±34.76 U/L) levels were measured statistically significant ($p<0.05$) in male patients compared with control male of same age subgroup (1.78±0.12 mg/dL, 83.25±11.89 U/L) respectively.

Table (4.8): Biochemical bone markers in healthy and β- thalassemia patients according to gender and age groups

Variables	Gender	Age (years)	Control Group	Patients Group
PTH (pg/ml)	**Male**	**11-13**	77.06±11.56[a]	30.43±4.77[b]
		14-16	72.45±9.30[a]	39.60±9.28[a]
	Female	**11-13**	28.08±8.45[a]	29.37±5.39[a]
		14-16	45.20±9.37[a]	33.40±10.34[a]
Vitamin D (ng/ml)	**Male**	**11-13**	57.17±7.09[a]	12.02±0.84[b]
		14-16	51.43±6.31[a]	12.44±1.08[b]
	Female	**11-13**	40.13±5.24[a]	13.48±1.32[b]
		14-16	40.63±8.01[a]	11.92±1.06[b]
Calcium (mg/dL)	**Male**	**11-13**	9.67±0.36[a]	7.93±0.25[b]
		14-16	10.01±0.31[a]	7.87±0.29[b]
	Female	**11-13**	9.73±0.40[a]	7.25±0.39[b]
		14-16	9.46±0.29[a]	7.85±0.22[b]
Phosphorus (mg/dL)	**Male**	**11-13**	1.78±0.12[a]	4.67±0.47[b]
		14-16	2.13±0.24[a]	5.27±0.42[b]
	Female	**11-13**	3.24±0.15[a]	5.75±0.57[b]
		14-16	2.26±0.34[a]	3.46±0.72[a]
ALP (U/L)	**Male**	**11-13**	83.25±11.89[a]	190.87±34.76[b]
		14-16	83.99±8.24[a]	233.54±16.91[b]
	Female	**11-13**	97.13±4.35[a]	231.00±50.99[b]
		14-16	85.58±7.83[a]	189.62±45.32[b]
Magnesium (mg/dL)	**Male**	**11-13**	1.94±0.18[a]	1.86±0.43[a]
		14-16	1.72±0.06[a]	2.50±0.64[a]
	Female	**11-13**	1.89±0.18[a]	2.47±0.70[a]
		14-16	1.64±0.16[a]	1.61±0.14[a]

Value represented mean ± SE.

The different letters refer to significant difference (between the control and patients groups) at the level ($p<0.05$).

The similar letters refer to a non-significant difference (between the control and patients groups) .

Our results documented that female in 11 – 13 years subgroups of β-thalassemia major patients have a higher estimated levels of PTH (29.37±5.39 pg/ml), phosphorous (5.75±0.57 mg/dL), ALP (231.00±50.99 U/L), and magnesium (2.47±0.70 mg/dL), but only phosphorus and ALP have a statistical significant in comparison to control female subjects (28.08±8.45 pg/ml, 3.24±0.15 mg/dL, 97.13±4.35U/L, and 1.89±0.18 mg/dL respectively). While serum vitamin D (13.48±1.32 ng/ml) and calcium (7.25±0.39 mg/dL) levels in female patients reported a statistically significant ($p<0.05$) decreased in comparison to female control subjects (40.13±5.24 ng/ml and 9.73±0.40 mg/dL) of same 11- 13 years subgroup, table (4. 8).

Regarding 14 – 16 years subgroup, in comparison between male patients and male control adolescents, the study results revealed that male patients have lower statistical significant ($p<0.05$) serum vitamin D (12.44±1.08 ng/ml) and calcium (7.87±0.29 mg/dL) levels, moreover serum levels of PTH (39.60±9.28 pg/ml)level was lower but non significant than that in control group (51.43±6.31ng/ml, 10.01±0.31 mg/dL and72.45±9.30 pg/ml), while serum magnesium was higher in patients (2.50±0.64 mg/dL) than in control subject (1.72±0.06 mg/dL) but non significant difference. Serum phosphorus (5.27±0.42 mg/dL) and ALP (233.54±16.91U/L) levels were higher with statistically significance ($p<0.05$) in male patients compared to male control individuals (2.13±0.24 and 83.99±8.24 respectively), table (4. 8).

While female β-thalassemia major patients in the same age subgroup (14 – 16 years) have a decreased levels of PTH (33.40±10.34 pg/ml),

vitamin D (11.92±1.06 ng/ml), calcium (7.85±0.22 mg/dL), and magnesium (1.61±0.14 mg/dL), but only vitamin D and calcium levels were have significant differences (P <0.05) in comparison to female control subjects (45.20±9.37 pg/ml, 40.63±8.01 ng/ml, 9.46±0.29 mg/dL, and 1.64±0.16 mg/dL respectively). While serum phosphorus (3.46±0.72 mg/dL) and ALP (189.62±45.32 U/L) levels were measured higher in female patients, but only ALP had a statistical significant difference in comparison to female control adolescents (2.26±0.34 mg/dL, and 85.58±7.83 U/L respectively), table (4.8).

In our study demonstrated that the serum levels of biochemical bone markers in male β-thalassemia major patients group including PTH (35.01±5.19 pg/ml), calcium (7.90±0.19 mg/dL), phosphorus (4.97±0.32mg/dL), ALP (212.20±19.40 U/L), and magnesium (2.18±0.38 mg/dL) were measured higher than in female patients(31.39±5.69 pg/ml, 7.55±0.23 mg/dL, 4.61±0.52 mg/dL, 210.30±33.54 U/L, and 2.04±0.36 mg/dL respectively), but non significant difference, while vitamin D levels in male (12.22±0.67 ng/ml) was lower non statistical significant difference than that in female patients (12.70±0.84 ng/ml), Table (4.9)

In β- thalassemia major 11 – 13 years subgroup, the male patients have a lower serum levels but non significant difference of vitamin D (12.02±0.84 ng/ml, phosphorus (4.67±0.47 mg/dL), ALP (190.87±34.76 U/L), and magnesium (1.86±0.43 mg/dL) in comparison to female patients (13.48±1.32 ng/ml, 5.75±0.57 mg/dL, 231.00±50.99 U/L, and 2.47±0.70 mg/dL), while serum PTH (30.43±4.77 pg/ml) and calcium (7.93±0.25 mg/dl) levels were non significant difference in male than female patients (29.37±5.39 pg/ml, and 7.25±0.39 mg/dL) of same subgroup, table (4.9).

Table (4.9): Biochemical bone markers in β- thalassemia patients according to gender and age groups

Variables	Age	Male	Female
PTH (pg/ml)	11-16	35.01±5.19[a]	31.39±5.69[a]
	11-13	30.43±4.77[a]	29.37±5.39[a]
	14-16	39.60±9.28[a]	33.40±10.34[a]
Vitamin D (ng/ml)	11-16	12.22±0.67[a]	12.70±0.84[a]
	11-13	12.02±0.84[a]	13.48±1.32[a]
	14-16	12.44±1.08[a]	11.92±1.06[a]
Calcium (mg/dL)	11-16	7.90±0.19[a]	7.55±0.23[a]
	11-13	7.93±0.25[a]	7.25±0.39[a]
	14-16	7.87±0.29[a]	7.85±0.22[a]
Phosphorus (mg/dL)	11-16	4.97±0.32[a]	4.61±0.52[a]
	11-13	4.67±0.47[a]	5.75±0.57[a]
	14-16	5.27±0.42[a]	3.46±0.72[a]
ALP (U/L)	11-16	212.20±19.40[a]	210.30±33.54[a]
	11-13	190.87±34.76[a]	231.00±50.99[a]
	14-16	233.54±16.91[a]	189.62±45.32[b]
Magnesium (mg/dL)	11-16	2.18±0.38[a]	2.04±0.36[a]
	11-13	1.86±0.43[a]	2.47±0.70[a]
	14-16	2.50±0.64a	1.61±0.14[b]

Value represented mean ± SE.

The different letters refer to significant difference between male and female patients at level ($p<0.05$).

The similar letters refer to non-significant difference between male and female patients.

Regarding 14 -16 years, all serum levels of biochemical bone markers (PTH 39.60±9.28 pg/ml, vitamin D 12.44±1.08 ng/ml, calcium 7.87±0.29 mg/dl, phosphorus 5.27±0.42 mg/dl, ALP 233.54±16.91U/L and magnesium (2.50±0.64mg/dl) were increased, in male β-thalassemia major patients compared to females patients (33.40±10.34 pg/ml, 11.92±1.06 ng/ml , 7.85±0.22 mg/dl, 3.46±0.72 mg/dl, 189.62±45.32 U/L, and 1.61±0.14) , with only ALP and magnesium had a significant difference ($P < 0.05$), table (4.9).

4.4. Correlation of Serum Ferritin Level Versus Reproductive, Prolactin, PTH and Biochemical Bone Markers in β-thalassemia Major Patients:

Linear regression analysis was applied to evaluate the relationship between the levels of serum ferritin with hormones and biochemical Bone Markers variables in β-thalassemia major patients included in our study.

The results indicated that serum ferritin as a dependent variable had an inverse correlation with testosterone (- 0.123), E_2 (-0.023), FSH (-0.135), LH (-0.124) PRL (-0.154), PTH (-0.136), calcium (-0.275), and magnesium (-0.093), but it's significantly (P value <0.05) negative with vitamin D (-0.303), whereas it had a positive correlation with ALP (0.158), but non significant (P > 0.05) and highly significant (P value <0.01) positive with phosphorus (0.554), table (4.10).

On the other hand testosterone had a negative correlation with FSH (-0.043), LH (-0.033), phosphorus (-0.055) and ALP (-0.133); while it had positive correlation with E2 (0.000), PRL (0.012), PTH (0.265), calcium (0.109), magnesium (0.122), but it had significantly (P <0.05) positive with vitamin D (0.320).

However, estradiol had a negative correlation with LH, PTH, vitamin D, calcium and magnesium (-0.011, -0.238, -0.043, -0.158, and -0.003) respectively, while it had a positive correlation with FSH, PRL, phosphorus, and ALP (0.044, 0.091, 0.065, and 0.096) respectively, table (4.10).

Regarding FSH, it had an inverse correlation with PTH (-0.147), vitamin D (-0.151), calcium (-0.129), and phosphorus (-0.056), but it had a positive correlation with PRL (0.095), ALP (0.093), magnesium (0.005), and a highly significant (P <0.01) with LH (0.676).

Table (4.10): Correlation between serum ferritin level versus reproductive, prolactin, PTH and biochemical bone markers in β-thalassemia major patients

Variables	Ferritin	Testosterone	E_2	FSH	LH	prolactin	PTH	Vit D	Calcium	Phosphorus	ALP
Testosterone	-.123	1									
E_2	-.023	.000	1								
FSH	-.135	-.043	.044	1							
LH	-.124	-.033	-.011	.676**	1						
Prolactin	-.154	.012	.091	.095	.155	1					
PTH	-.136	.265	-.238	-.147	-.148	.112	1				
Vit. D	-.303*	.320*	-.043	-.151	-.148	.004	.205	1			
Calcium	-.275	.109	-.158	-.129	.017	-.002	.111	.287*	1		
Phosphorus	.554**	-.055	.065	-.056	-.167	.008	-.137	-.147	-.583**	1	
ALP	.158	-.133	.096	.093	-.100	.102	.097	.060	-.091	.019	1
Magnesium	-.093	.122	-.003	.005	-.035	.232	.091	.238	.430**	-.028	.073

* Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed).

In our study there was a negative correlation between LH with PTH, vitamin D, Phosphorus, ALP, and magnesium (-0.148, -0.148, -0.167, -0.100, and -0.035) respectively, but it had a positive correlation with PRL (0.155), and calcium (0.017).

While PRL hormone showed a negative correlation with calcium (-0.002) only, but it had a positive correlation with PTH, vitamin D, phosphorus, ALP, and magnesium (0.112, 0.004, 0.008, 0.102, and 0.232) respectively.

On the other hand, the level of PTH had a negative correlation with phosphorus (-0.137), while it had a positive correlation with vitamin D (0.205), calcium (0.111), ALP (0.097) and magnesium (0.091).

Moreover, the results in our study reported that vitamin D had an inverse correlation with phosphorus (-0.147), but it positively correlated with ALP (0.060), and magnesium (0.238), and significantly positive ($P<0.05$) with calcium (0.287).

While calcium in our study results reported a negative correlation with ALP (-0.091), and a high significant negative (P <0.01) correlation with phosphorus (-0.583), but it had a highly significant positive (P <0.01) with magnesium (0.430).

The level of phosphorus had an inverse correlation with magnesium level (-0.028), but it had a positive correlation with ALP (0.019) in our patients results.

Lastly the level of serum ALP in our study showed a positive correlation with level of serum magnesium (0.073).

Chapter Five
Discussion

5. Discussion:

5.1. Evaluation of Serum Ferritin Level According to Gender and Age:

Our study results showed that all our patients have iron overload demonstrated by a high statistically significant increase in serum ferritin levels among male (4094.78±492.55 ng/ml) and female (3603.20±564.88 ng/ml) patients compared with same genders in control group, with serum ferritin not statistically influenced by gender of β-thalassemia patients, table (4.3).

This high serum ferritin level most likely due to frequent blood transfusions and hemolysis of red blood cells in our β-thalassemia patients.

Serum ferritin has in general been found to correlate with body iron stores and overload (Brittenham *et al*., 1993), and as iron overload is generally defined as serum ferritin consistently ≥ 1000 ng/ml and high serum ferritin level during puberty (> 2,500 ng/ ml) is a risk factor for endocrine dysfunctions (Morrison *et al*., 2003; and Shalitin *et al*., 2005).

In addition, (Gardenghi *et al*., 2010; and Leecharoenkiat *et al*., 2016) mentioned that iron overload in β-thalassemia major patients as predicted by high serum ferritin level was mainly due to multiple blood transfusions, hemolysis of red blood cells, and increased gastrointestinal iron absorption due to paradoxical hepcidin suppression from dyserythropoiesis.

Moreover, our result of a high serum ferritin level among β-thalassemia major patients consistent with (Mourad *et al*., 2003; Habeb *et al*., 2013) results, when they noticed a higher level of serum ferritin in thalassemia major patients than healthy children and the serum ferritin

level was twenty times higher than normal (5506 ±635 ng/ml) in their study.

Our study result of high serum ferritin was comparable to that reported the high mean serum ferritin among β-thalassemia major patients in thalassemia major patients with endocrine complications, (Shamshirasaz *et al*., 2003, Najafipour *et al*., 2008; Eshaqh-hosseini *et al*., 2018, Ratha *et al*., 2015; AL-Mosawy, 2017;Hagag *et al*., 2015;and Hagag *et al*., 2016).

As well as, in study done by (Yaman *et al*., 2013), found a high mean serum ferritin (2515±1221 ng/ml) among β-thalassemia major patients, and serum ferritin values were higher with increasing age but was not statistically significant and this result was comparable with our results regarding male patients.

Uniquely, (Abdulzahra *et al*., 2011, Majeed, 2017; and Yenzeel and Salih, 2017) mentioned a significant increase in serum ferritin in thalassemia major patients in comparison with control children, and mean concentration of serum ferritin was more than eight times higher than normal.

In contrast, (Belhoul et al., 2013) found the mean serum ferritin level among β-thalassemia major patients was high (2597.2 ng/ml) and there was a significantly positive correlation between serum ferritin level with age, and female patients had statistically significant low serum ferritin.

5.2. Evaluation of Serum Reproductive and Prolactin Hormones Levels According to Gender and Age:

Analysis of our study documented abnormalities in reproductive hormones and hypothalamic-pituitary-gonadal (HPG) axis. Compared to healthy male control group, male β-thalassemia major patients group have significantly lower mean serum levels of FSH, LH, testosterone and

estradiol. While female patients group in comparison to female control have statistically non-significant higher mean serum levels of FSH and testosterone, but decreased levels of LH and estradiol without significant effect, table (4.4).

our results show serum FSH and LH hormones levels were significantly higher in female compared to male in β-thalassemia major group and so estradiol level was high in female patients but without significant difference in comparison to male patients, while testosterone levels was higher in male than female patients but non significant difference.

The abnormalities in the reproductive hormones in present study most likely due to iron overload in our patients, which mediates its oxidative damage to the HPG axis. Thus, the cause is usually damage to gonadotrophs in anterior pituitary leading to failure of adequate production of gonadotrophins LH and FSH.

Endocrine glands have extreme sensitivity to iron toxicity, because they have high levels of transferrin receptors that promote iron accumulation and hence increase vulnerability of these glands to iron toxicity, even small amounts of iron concentration in early periods of life may cause permanent injury. Iron stored in endocrine glands binds to intracellular transferrin and as the storage capacity of transferrin gets exceeded, pathological quantities of metabolically active iron catalyses formation of free radicals (Shalitin *et al.*, 2005; Merchant *et al.*, 2011; and Singh and Seth, 2017).

These variations in the levels of reproductive hormones in our male and female β-thalassemia major patients are comparable with observations found by (Najafipour *et al.,* 2008; and Parijat *et al*., 2014), that both genders of β- thalassemia major patients have HPG axis

dysfunction and the reported prevalence was 45- 70% in boys and 39 - 75% in girls without significant differences.

Eshaqh-hosseini *et al*., (2018) observed the endocrine complications have emerged as an important cause of morbidity and an important determinant of life quality in children with β-thalassemia major. Moreover, (Parijat *et al*., 2014; Srisukh *et al*., 2016; and De Sanctis *et al*., 2018) reported that, abnormalities in reproductive hormones and HPG axis are the most frequently registered endocrine complication in β-thalassemia major despite regular transfusions and optimal chelation therapy.

In comparison to our current study, (Sutay *et al*., 2017) in their study, they found the difference was not significant in the FSH levels in the age group of 8-12 years, and the FSH values of cases >12 years were significantly lower than those of controls, but LH values were significantly lower in patients as compared to control in both age groups, and estrogen values were significantly lower in thalassemia patients as compared to control in both age groups. Also, they found FSH, LH and estrogen levels were significantly lower in the girls with thalassemia as compared to control in age group of 12-16 years. However the FSH levels although lower in cases in the age group of 8- 12 years as compared to controls the difference was not statistically significant.

In comparison to our results, (Al-Rimawi *et al*., 2005 Vahidi *et al*., 2003; Hagag *et al*., 2016; and Moayer and Oloomi ,2006), conducted a prospective study about the function of the HPG axis function , They found HPG dysfunction in had low LH, FSH, and estradiol levels, but high levels of serum ferritin had significant affect., and mean testosterone levels were significantly depressed in male thalassemia major patients compared to controls.

Moreover, (Jouda *et al*., 2019) mentioned that β-thalassemia major patients aged < 18 years have a low level of FSH, LH, and testosterone compared to older patients, but only the difference in LH and testosterone levels reached the significant. While(Yenzeel and Salih, 2017), reported a significant decrease in the levels of FSH, LH, and estradiol hormones when compared to control in their study of forty female β-thalassemia major patients aged 17 – 24 years and 20 ages matched healthy control females.

Compare to our results, (Majeed , 2017) reported that the mean of FSH levels in healthy male in control group were higher than patients group, but levels of LH in male patients and control groups were comparable, but FSH levels of male and female patients were significant lower than the corresponding values in the healthy. While the values of estradiol, and testosterone in patients group were significant lower than in the control group.

In our study, the mean serum prolactin level did not differ significantly between patients and controls in both genders, although β-thalassemia major patients have low prolactin levels. However in comparison between male and female in β-thalassemia major group, prolactin levels were higher without significant in female patients as compared to male patient in the same age subgroup .

In accordance to our results (Livadas *et al*., 1984) mentioned in β-thalassemia major the anterior pituitary deficiency exists possibly for the lactotrophs, because of it is sensitivity to oxidative stress due to the iron overload, and they found the basal prolactin values did not differ significantly between the β-thalassemia major patients and control groups.

Equally, (Abdulzahra *et al*., 2011) documented prolactin values did not differ significantly between patients and control groups, although

10% of the patients have low prolactin levels, as well as (Mousa *et al*., 2016) found significantly decreased prolactin level in their study of 38 Egyptian patients with β-thalassemia major compared to control subjects.

In contrast to our study, (Majeed, 2017), found significantly higher level of prolactin hormone among female β-thalassemia major patients in comparison to control, but he did not found significant difference among male patients in both groups. While (AL-Mosawy, 2017) in their study reported that the prolactin levels were within the normal range and there were no significant differences between prolactin levels according to the sex of the patients, but (Jouda *et al*., 2019) reported that the prolactin levels among β-thalassemia major patients were high without significant differences between prolactin levels according to the age of patients.

5.3. Evaluation of PTH and Biochemical Bone Markers According to Gender and Age:

Results of our study demonstrated that male patients in β-thalassemia major group have low statistical significant mean serum PTH, vitamin D and calcium levels, but mean serum phosphorus and alkaline phosphatase levels significantly higher as compared to the male control of comparable groups. However, in comparison female β-thalassemia major patients with female control individuals, we observed low without statistical significant serum PTH level, whereas vitamin D and calcium levels were highly significant reduced, but phosphorus and ALP levels were significant increased, table (4.7).

Regarding β- thalassemia major group, current study show male patients have non-significant higher levels of PTH, calcium, phosphorus, and ALP, whereas vitamin D level was non-significantly low in male patients as compared to female patients. .

Low PTH level among our patients is mainly due to involvement of parathyroid gland by iron overload, as well as its oxidative damage, and the low serum calcium and high serum phosphorus levels among our patients most likely due to low PTH attributed to excessive iron overload deposition in parathyroid gland and deficiency of vitamin D due to liver involvement.

The lower serum levels of vitamin D among β-thalassemia major patients can be due to decreased serum PTH level in our patients as a result of parathyroid gland involvement by iron overload.

Uniquely, (Gutteridge and Halliwell, 1989; and Goyal et *al*., 2010) observed that parathyroid gland involvement by iron overload occurs particularly after 10 years of age in β-thalassemia major patients, there are numbers of possible mechanisms have been described to be responsible for the damage of parathyroid glands through iron overload, which include free radical formation and lipid peroxidation resulting in mitochondrial, lysosomal and sarcolemmal membrane damage. In addition, (Aleem *et al*., 2000) suggested that parathyroid gland dysfunction in β-thalassemia major with iron overload is due to chelation therapy.

Hence, many researchers have reported abnormalities in PTH and biochemical bone markers values among β- thalassemia major patients (De Franceschi et al., 1997; Shazia et al., 2012; Hamidi, 2016; and Dejkhamron et al., 2018).

In agreement with our results,(De Sanctis et al., 1992; and Aleem et al., 2000) observed low PTH among β- thalassemia major patients

In agreement to our findings, (Basha *et al*., 2014; Saboor *et al*., 2014; Mohey El-Deen *et al*., 2014; and Bahy El-Din *et al*., 2018; and Majeed, 2017; and Hagag *et al*., 2015), in their study found significant decrease

in serum PTH, and calcium, but significant increase in serum phosphorus and ALP in β-thalassemia major patients when compared to control.

However, (Goyal *et al*., 2010; Anju and Jain, 2017) noticed serum PTH and serum calcium significantly reduced in β-thalassemia major patients that are similar to our results, but their serum ALP and phosphorus were not significantly altered when compared to the respective mean values for the control group.

While, (Fahim *et al*., 2013; Shawkat *et al*., 2018), in their case control study observed that the mean serum calcium and 25-OH vitamin D levels were significantly lower in patients compared to controls which are in agreement to our results, but serum phosphorous levels were within the normal without significant difference.

In concordance to our findings, (Ridha et al., 2018) noticed in their study that ALP and phosphorus were significantly increased, but serum vitamin D and calcium levels were decreased as compared to healthful control, whereas they observed serum PTH significantly increased which is incomparable to our results.

In contrast to our findings, (Agrawal *et al*., 2016) observed that mean serum PTH was significantly higher and so calcium level but without significant difference, while phosphorus level was non-significantly lower in thalassemia patients compared to control, although vitamin D value in their study is in agreement to our results was significantly lower in patients compared to control group, but there was no significant correlation was found between vitamin D level and sex, while in our study we found a significant reduced level of vitamin D in male and female patients group compared to male and female control groups, but among β-thalassemia patients group and 11 – 13 years subgroup patients vitamin D levels were lower in male without significant difference compared to female patients of corresponding groups, while its level was

not significantly higher among male patients in comparison to female patients in 14 – 16 years subgroup. While in other study by (Aggarwal *et al*., 2018), found significant low vitamin D levels, despite serum PTH levels were not significantly different between cases and controls, while the serum level of calcium was found in normal range in both groups, although the phosphorus and ALP levels were found to be significantly high in cases in comparison to controls.

In disparity to our results, (Pirinççioğlu et al., 2017) noticed mean PTH value was significantly higher in patients than that in control, yet mean calcium value was significantly lower and vitamin D level was low in all β-thalassemia major enrolled in their study.

Our findings do not concur with those of (El-Nashar *et al*., 2017) who observed level of PTH was higher non significant difference in patients compared to control, despite they found serum phosphorus and ALP levels were significantly higher, and significant reduction in serum calcium level in patients compared to the control group that are similar to our observation.

Our findings are incomparable to (Al-Samarrai *et al*., 2008; Al-Hakeim *et al*., 2017) observations, when they found the mean serum levels of calcium and serum phosphate were within normal limits in patients, and had no significant difference as compared with healthy control group.

Alkaline phosphatases levels in our study were statistically significantly high in cases than in controls. Consequently, it basically represents the presence of high bone turnover rate in the cases. High level of ALP may have association with bone changes including osteomalacia and liver impairment, that frequently seen in patients with β-thalassemia major. These complications increase with regular blood transfusion,

secondary hemosiderosis, iron chelation therapy, erythroid hyperplasia and nutritional deficiency (Salama *et al*., 2006; and Tantawy *et al*., 2008).

Regarding magnesium values, in this study we observed that magnesium levels were increased without significant difference in male and female patients compared to male and female control groups, and magnesium levels not statistically influenced by gender of patients compared to control. While in thalassemia major group magnesium levels not statistically effected by gender of patients except in 14 – 16 years age subgroup when male patients have statistically significant higher levels than female patients.

The finding of serum magnesium in our thalassemia patients may result from diet, chelation therapies, bone involvement (as 50% of total body magnesium resides in bone), and increased rate of hemolysis among our patients (as magnesium is one of the major intracellular ions).

However, our results are in consistent with that of (Khaleel et al., 2018; and Ridha et al., 2018) when they observed serum magnesium level was significantly higher in patients than normal control.

At variance with our findings, (Arcasoy and Cavdar, 1975; and Fahmy *et al*., 2019) observed a normal serum magnesium level in β-thalassemia patients, while (Al-Samarrai *et al*., 2008; Ratha *et al*., 2015; and Nafady *et al*., 2018), in their study showed that serum magnesium level was significantly lower in patients than the healthy control group.

Hyman *et al., (*1980), has been speculated that hypomagnesemia could be due to chelation by citrate in chronically transfused patients, or could just be a consequence of the cellular iron overload, but, (Genc *et al*., 2016) observed that level of magnesium had no significant difference between controls and patients with β-thalassemia major regardless type of chelating therapies.

5.4. Correlation of Serum Ferritin Level Versus Reproductive, Prolactin, PTH and Biochemical Bone Markers in β-thalassemia Major Patients:

Our study results indicated that serum ferritin had an inverse correlation with FSH, LH, estradiol, testosterone, prolactin, PTH, calcium and magnesium; moreover it's significantly negative with vitamin D, whereas it had a positive correlation with ALP, as well as highly significant positive with phosphorus. These findings highlight the importance of iron overload in the development of endocrinopathy in the β-thalassemia major patients, due to iron deposition in secretory cell of endocrine glands such as gonadotrophin cell of pituitary gland leading to impairment of prolactin and gonadal hormones including FSH, LH, estradiol and testosterone. In addition, iron overload deposition in parathyroid gland lead to abnormalities in PTH, with subsequent abnormalities in biochemical bone markers, due to defect in bone mineralization.

Comparison to our results (Abdulzahra *et al.*, 2011) observed significant correlation between serum ferritin with LH and FSH, but no significant correlation between serum ferritin with prolactin and testosterone.

In our study PTH had no significant correlation with calcium and phosphorous, that’s similar to (Mohey El-Deena *et al.*, 2014) observations, when they were noticed no significant correlation of serum calcium and phosphorus with PTH.

In agreement to our findings, (Hagag *et al*., 2015) reported a significant negative correlation between PTH level and ferritin. As well as, (Hagag *et al.*, 2016) observed significant negative correlations between FSH, LH, and estrogen with serum ferritin. (Yenzeel and Salih, 2017) show in their

study significant negative correlation of prolactin and estradiol with ferritin, while FSH and LH had non-significant negative correlation with ferritin.

Moreover, in our study, ferritin was significantly negative with vitamin D, but it had no significant negative correlation with PTH, as well as vitamin D had significant correlation with calcium but, it had no significant correlation with ALP.

In agreement to our results (Ridha *et al*., 2018) noticed vitamin D had significant correlation with calcium and ALP, while no significant correlation between PTH with the vitamin D, calcium, phosphorus, ALP, and magnesium, however he found no significant correlation between PTH and vitamin D with ferritin.

Variations in our results in comparison to other researcher's studies can be explained on the basis of differences in age group studied, genotype of β-thalassemia major, frequency and extent of transfusion, age at the beginning of transfusion, and type of iron chelation therapy, in addition to the compliance of patients for follow-up and to the therapy (Sirsukh *et al.*, 2016).

Conclusion and Recommendation

Conclusion:

1. There was a significant increase in serum ferritin level in patients in comparison to control groups. However the gender of patients had a non-significant influence on serum ferritin level.
2. Male and female β-thalassemia major patients have significant dysfunction of both hypothalamic –pituitary- gonadal axis and parathyroid glands, as reflected by a significant decreased levels of serum FSH, LH, testosterone, estradiol, PTH, vitamin D and calcium. While serum phosphorous and ALP were statistically significant increase as compared with control group and Prolactin level was decreased without significance in male and female patients compared with control.
3. In β-thalassemia major patients group, female patients as compared to male patients have higher significant levels of FSH and LH hormones; moreover estradiol and vitamin D levels were non-significantly higher level, but serum testosterone, PTH, calcium, phosphorus, ALP and magnesium levels were non-significant lower.

Recommendation

1. Further studies on large sample of β-thalassemia major patients are required to know the impacts of iron overload on other endocrine glands such as thyroid and adrenal glands as early recognition and hence prevention of these complications might help improve quality of life for these patients.
2. Further studies to evaluate the toxic impact of the iron overload and oxidative stress in beta thalassemia major patients
3. evaluation of serum ferritin for β-thalassemia major patients is crucial (every 3 months) for early detection of iron overloads to initiate early therapeutic interventions, in addition to prevent its harmful toxicity on endocrine glands like pituitary, gonads and parathyroid glands.
4. Recommended to using bone mineral density (BMD) as a good index of bone status in these patients.
5. Developing a preventive program, encompasses carrier screening, genetic counseling along with prenatal, postnatal and premarital diagnosis. This approach is cost-effective and successful in reducing the frequency of β-thalassemia in our country.
6. Knowledge of the spectrum and distribution β-thalassemia mutations in Misan Province is a recommendation.

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Appendices

Appendix -1

Questioners for:

Evaluation of Reproductive Hormones and Bones Metabolism in Patients with Beta –Thalassemia Major in Misan Province

Patient (control) name:

Age: Years:

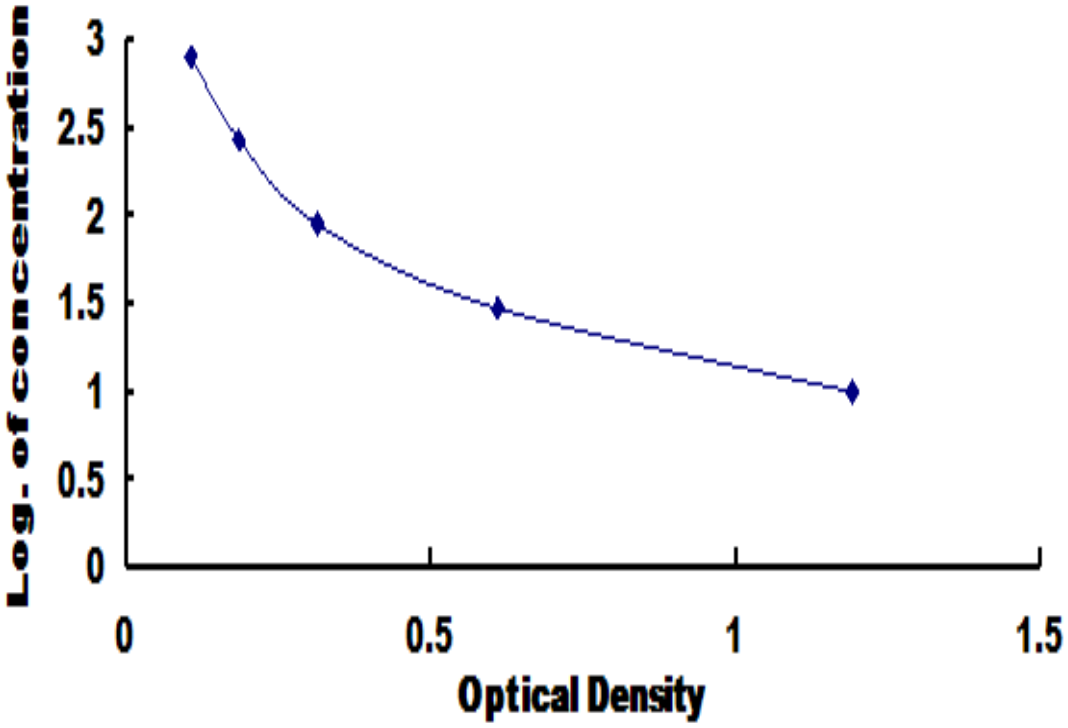
Gender: Male Female

Serum ferritin : ng/ml

Variable	Serum level
FSH hormone	
LH hormone	
Testosterone hormone	
Estradiol hormone	
Prolactin hormone	
Parathyroid hormone	
Vitamin D	
Calcium	
Phosphorus	
Alkaline phosphate	
Magnesium	

By: Noor AL-Huda Salah AL-Zuhairy, and Supervisor: Prof .Dr. Zainab A.J.R.Al-Ali

Appendix- 2



Typical Standard Curve for PTH, Canine ELISA.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ميسان
كلية العلوم
قسم علوم الحياة

تقييم الهرمونات التكاثرية وأيض العظام لدى مرضى بيتا فقر دم البحر الابيض المتوسط الكبرى في محافظة ميسان

رسالة

مقدمة إلى مجلس كلية العلوم – جامعة ميسان

وهي جزء من متطلبات نيل شهادة الماجستير في علوم الحياة

من قبل
نور الهدى صلاح هاشم الزهيري
بكالوريوس علوم الحياة ٢٠١٣
بإشراف
الأستاذ الدكتور زينب عبد الجبار رضا العلي

١٤٤١ هـ ٢٠٢٠ م

الخلاصة

تم أجراء هذه الدراسة ، للفترة مِنْ كانون الأولِ 2018 إلى نهاية ايار ٢٠١٩ ، على عينه مؤلفه من ٥٠ مريضًا و ٥٠ شخصا من الأصحاء ، مجموعة المرضى تتكون من ٣٠ ذكر و٢٠ أنثى من المصابين بمرض بيتا فقر دم البحر الابيض المتوسط الكبرى و بمدى اعمارٍ بين ١١ إلى ١٦ سنةٍ ومن المراجعين لمركزَ ميسان لفقر دم البحر الابيض المتوسط ،اما مجموعة السيطرة فتتكون من عددَ مساويَ ومتوافق بالعمر والجنسِ من الاصحاء . قسمت مجموعتي المرضى والسيطرة إلى مجموعتين فرعيتين اعتمادا على العُمرِ، المجموعة العمرية الاولى مِنْ ١١-١٣ سَنَوات، و المجموعة العمرية الثانية من ١٤ -١٦ سَنَوات وذلك لدراسة المعايير ذات العلاقة (مصل الحديد، الهورمونات التكاثرية والبرولاكتين ، اضافة الى معايير العظام البايوكيمياوية.

اظهرت النتائج وجود ارتفاع معنوي ($P <0.05$) في مستوى مصل الحديد لكل من المرضى الذكور والإناث، مقارنة مع مجموعتي السيطرة من الذكور والإناث ، بينما جنس المرضى ليس له تأثير كبير على مستوى مصل الحديد.

اما الصورة الهرمونية فقد اظهرت ان ذكور مرضى بيتا فقر دم البحر الابيض المتوسط العظمى لديهم مستويات منخفضه معنويا ($P <0.05$) لكل من هرمونات FSH ، LH ، التستوستيرون ، استراديول ،هرمون جار الدرقية، وكذلك فيتامين D والكالسيوم. بينما كانت مستويات الفوسفور وانزيم الفوسفاتيز القلوي مرتفعة معنويا ($P <0.05$) ، بينما قيمة المغنيسيوم لم تصل الى مستوى المعنوية في المصل عند المقارنة مع ذكور مجموعة السيطرة. اما بالنسبة الى الإناث المريضات فلوحظ لديهم مستويات منخفضة معنويا ($P <0.05$) لفيتامين D و الكالسيوم في المصل، ومرتفعة معنويا ($P<0.05$) لكل من الفوسفور و انزيم الفوسفاتيز القلوي في المصل ، بينما مستويات FSH وهرمون التستوستيرون والمغنيسيوم في المصل مرتفعة ولكن لم تصل الى مستوى المعنوية ، في حين أن مستويات LH، والإستراديول و هرمون الجار الدرقية كانت منخفضة بشكل غير معنوي مقارنة مع مجموعة السيطرة في الاناث.

واظهرت النتائج مستويات مرتفعة معنويا ($P<0.05$) لدى المرضى الاناث لكل من FSH ، LH علاوة على ذلك كانت مستويات الاستراديول وفيتامين D مرتفعة ولكن لم تصل الى مستوى المعنوية ، بينما مستويات هرمون تستوستيرون، هرمون جار الدرقية، الكالسيوم،

الفوسفور ، انزيم الفوسفاتيز القلوي والمغنيسيوم منخفضه بدون تأثير معنوي ملحوظ مقارنة بالمرضى الذكور . بينما مستوى البرولاكتين منخفض بشكل غير معنوي عند كل من المرضى الذكور والإناث مقارنة مع مجموعة السيطرة. على الرغم من أن مستوى البرولاكتين كان مرتفعًا عند المرضى الإناث مقارنةً بالمرضى الذكور ، إلا أن جنس المرضى كان له تأثيرات غير مهمة على مستواه.

اظهر الحديد في المصل علاقة سالبه غير معنويه مع التستوستيرون، الاستراديول، FSH، البرولاكتين، LH، هرمون جار الدرقية ، كالسيوم، والمغنيسيوم، وعلاقه سالبه معنويه ($P <0.05$) التأثير مع فيتامين دي ، في حين كان له علاقة موجبه غير معنويه مع انزيم الفوسفاتيز القلوي وتأثير إيجابي عالي المعنوية ($P<0.05$) مع الفسفور.

تمت مناقشة النتائج من الناحية الفسيولوجية وفقا لزيادة الحديد الحاصلة لدى مرضى التلاسيميا وتأثيرات زيادة الحديد هذا على كل من المعايير الهرمونية وعلى علامات العظام البيوكيمائيه .