EFFECT OF ANTERIOR PITUITARY IRON OVERLOAD IN BETA THALASSEMIA MAJOR PATIENTS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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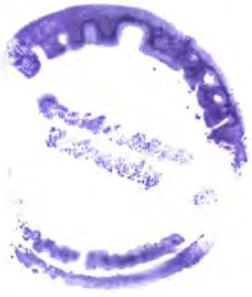

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TO

After thanking ALLAH almighty who always showers his inexplicable blessings over me. It's my immense pleasure to dedicate all my Research work and professional carrier to my beloved parents for their unfailing support and love which always boosted me up whenever I needed them. They made me realize that I had so much strength and courage to coup with the cold facets of life even when I felt lost.

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List of Abbreviations	
RBC	Red blood cells
ROS	Reactive oxygen species
HPG	Hypothalmic pituitary gonadal
BMI	Body mass index
IGF	Insulin Like Growth Factor
TSH	Thyroid stimulating hormone
T_3	Triiodothyronine
T_4	Thyroxine
GH	Growth hormone
PIMS	Pakistan Institute of Medical Sciences
ELISA	Enzyme-Linked Immunosorbent Assay
FTL	Abcam's Ferritin
EDTA	Ethylene diamine tetra acetic acid
SA-HRP	Streptavidin-horseradish peroxidase
mAbs	Monoclonal antibodies
TMB	Tetramethylbenzidine
MEIA	Microparticle Enzyme Immunoassay
RV	Reaction vessel
FPIA	Fluorescence Polarization Immunoassay
RLUs	Relative light units
RES	Reticuloendothelial system
TM	Thalassemia major
MRI	Magnetic resonance imaging
Ca	Calcium
Kisp	Kisspeptin
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
Hb	Hemoglobin
E_2	Estradiol
T	Testosterone
DFO	Deferoxamine
GnRH	Gonadotropin-releasing hormone
GABA	Gamma-amino butyric acid
NMDA	N-methyl-D-aspartate
GPR54	G protein-coupled receptor
HGH	Human growth hormone
CMIA	Chemiluminescent Microparticle Immunoassay
ALT	Serum Alanine Transaminase
EIA	Enzyme Immunoassay

SMI	Science with mission
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C Virus
NKB	Kisspeptin, neurokinin B
DHEAS	Dehydroepiandrosterone sulfate
HP	Hypothalmic pituitary
IGFBP	Insulin like growth factor binding protein
GHR	Growth Hormone receptor
MBH	Medial basal hypothalamus
NTBI	Non-transferrin-bound iron
LTCCs	L type Ca^2 channels
β	Beta
α	Alpha
MCV	Mean Corpuscular Volume
s.c.	subcutaneous
LIC	liver iron content
SQUID	Superconducting quantum interference device

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General Abstract

Background: Thalassemia major is a hereditary haemolytic anaemia which is treated with repeated blood transfusions. About 240 million beta thalassemia carriers are present all over the world. Every year about 100,000 children are born with the disease of thalassemia. Thalassemia major is a homozygous trait in which there is defective Hemoglobin synthesis that leads to the development of severe anemia. Periodic transfusions along with chelation therapy have markedly improved the life span of thalassemic patients. Citrate toxicity and consequent iron overload leads to increase prevalence of endocrine complications in children, adolescents and young adults. Transfusion with chelation therapy has significantly extended life expectancy but, leads to complications due to iron overload. Beta thalassemia patients are more prone to develop different organ damage due to metabolic dysfunction, the actual mechanism is not clear but anemia and iron overload are most important factors leading to increase mortality and morbidity rate along with lipid peroxidation, oxidative stress and free radical release that cause such condition.

Materials and Methods: The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control). Height, Body Mass Index BMI, Hemoglobin and serum Ferritin levels were analyzed. Kisspeptin (Kp), Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and sex steroids were analyzed and correlation of these hormones with body mass index (BMI), serum Ferritin and Hemoglobin (Hb) levels in beta thalassemic patients undergoing regular blood transfusion was studied. Growth hormone (GH), thyroid stimulating hormone (TSH), Triiodothyronine (T_3), Thyroxine (T_4) were analyzed and correlation of these hormones with body mass index (BMI), serum Ferritin, Hemoglobin (Hb) and Kisspeptin levels were done. Hepatitis B and C were detected in all thalassemic groups along with estimation of serum ALT levels. Correlation of serum Alanine Transaminase ALT levels with BMI, Hemoglobin and serum Ferritin levels was done along with estimation of prevalence of hepatitis and liver enlargement.

Results: All groups had reduced height, BMI, Hb and high Ferritin levels as compare to the control groups. Strong positive (P<0.001) correlation of BMI with Hemoglobin was seen in ≥ 13 years females. While <13 years thalassemic males had significant (P<0.01) negative correlation of BMI with Hemoglobin. Thalassemic females ≥ 13 years had significant (P<0.001) positive correlation of BMI with serum Ferritin levels. All groups had significantly reduced (P<0.001) BMI and Hb as compared to control group. Serum Ferritin levels were significantly (P<0.001) high in all thalassemic groups on comparison with control. Kisspeptin levels in thalassemic females of <13 years females and males were significantly (P<0.001) low as compared to the control group. While thalassemic males of ≥13 years had significantly (P<0.01) high Kisspeptin levels as compared to the control group. FSH levels in <13 and ≥13 years thalassemic male and female patients were significantly raised (P<0.001) as compared to the control group. LH levels in thalassemic males in <13 years were significantly high (P<0.001) as compared to the control group. While ≥13 years thalassemic males had significantly (P<0.001) reduced LH levels as compared to the control group. Significantly high Testosterone (P<0.001) were observed in thalassemic males of <13 years as compared to the control group. While Estradiol levels were significantly low (P<0.001) in <13 years thalassemic females. In <13 years thalassemic females FSH had a negative significant correlation (P<0.001) with Ferritin (r= -0.511). In thalassemic males of <13 years FSH had a significant (P<0.05) positive correlation with BMI (r= 0.296) and negative significant correlation (P<0.001) with Hemoglobin (r= -0.479). In <13 years female LH had a significant (P<0.01) negative correlation with Hemoglobin (r=-0.386). Estradiol in ≥ 13 year females had a positive significant (P<0.05) correlation with BMI (r= 0.318) and Hemoglobin (r=0.286). Growth hormone levels were significantly reduced in <13 years male and female thalassemic patients while the levels were significantly raised in ≥13 male and female thalassemic patients. T_3 levels in ≥ 13 years thalassemic females were significantly raised as compared to control. T_4 and TSH in <13 year thalassemic male were significantly reduced than control and ≥13 thalassemic males had significantly raised as compared to the control.GH in <13 years had a positive correlation with Kisspeptin (r=0.310). T_3 in ≥13 years thalassemic females had a significant negative correlation with BMI(r=-0.408) and (P<0.05) with Hb (r=0.329) and a positive correlation with Kisspeptin (r=0.317).In <13 years thalassemic males T_3 had a positive correlation with Ferritin (r=0.523).T_4 in ≥13 thalassemic female had a positive correlation with BMI (r=0.333), Ferritin (R=0.317) and Hb(r=0.328). There was a positive correlation of T_4 with

Hb(r=0.422) in <13 year thalassemic males. TSH in ≥13 thalassemic females had a positive correlation (P<0.001) with BMI (r=0.487), Ferritin(r=0.531) and (P<0.01) with Hb (r=0.394). In ≥13 thalassemic males TSH had a positive correlation (P<0.01) with Ferritin (r=0.446). All groups had significantly reduced (P<0.001) BMI and Hb as compared to control group. Serum Ferritin levels were significantly (P<0.001) high in all thalassemic groups on comparison with control. Serum ALT levels in all four thalassemic groups are significantly (P<0.001) raised as compared to control groups. Significant correlation exist between serum ALT and Ferritin levels. Prevalence of hepatitis is greater than 50 % in all four thalassemic groups.

Conclusion: In beta thalassemic patients growth disturbance or delay is the main clinical feature that effects the life and wellbeing of these individuals. Our study has revealed that patients with beta thalassemia suffer from reduced height, BMI which is enhanced in patients having high levels of serum Ferritin (ng/mL) and low Hemoglobin (gm/dl). The growth retardation seen in these patients with thalassemia major is multifactorial, it can be due to under-nutrition, hypogonadism, hypothyroidism, and other complications of thalassemia such as tissue hypoxia and side effects of chelating therapy with desferrioxamine. So, lifelong care and management of such patients is mandatory which requires significant cost for proper treatment of these patients in all aspects. Our study revealed that the levels of Kisspeptin in thalssemic females and males of < 13 years had reduced levels but, ≥13 years were raised but, at the same time the levels FSH and LH were significantly deranged. These findings are suggestive that hormone production by hypothalmus was correctly secreting Kisspeptin according to the pubertal time frame but, the levels of hormones secreted by anterior pituitary and the gonads were deranged due to damage caused by iron overload at pituitary and gonadal level. Along with disturbed hypothalamic pituitary gonadal axis there was decreased BMI, low Hemoglobin and raised serum Ferritin levels. These findings are indicating that treatment with double chelation from early life should be considered for better outcomes in thalassemic patients. Regular blood transfusion followed by iron chelation therapy is just a supportive treatment for the disease of thalassemia which is associated with serious complications. But, during this supportive treatment, the magnitude of the body iron burden is the principal determinant of clinical outcome for the prime goal i.e of iron-chelating therapy in patients with thalassemia major is to control body iron. The optimal body iron should be reduced both to prevent the adverse effects from the iron-chelating agent to

prevent the risk of complications from iron overload. The apparent facts is that upon reaching age of puberty thalassemic patients develop growth retardation and pubertal failure. Thalassemic patients are short, have low growth rate and BMI and have either delayed or absent pubertal spurt, which is related to low Hemoglobin and high Ferritin levels and sub-optimal iron chelation therapy. These defects start early in life but, become becomes obvious after the age of 8 years. In developing countries, poor socio-economic background adds up to the problem. Therefore, effective alternate chelation regimens should be considered to improve the complication resulting from chelation therapy. Serum Ferritin concentration is an important determinant of liver enzyme levels, and increased serum Ferritin level is an independent predictor of liver damage in thalassemic patients, so it is useful to identify patients at risk of steatohepatitis and advanced fibrosis. Hepatitis also effects the condition of such patients along with anemia and low BMI which reduce the ability of thalassemic patients to fight against such infections. Careful monitoring should be done at early stage of disease and serum Ferritin levels should also be maintained and frequently monitored to prevent further liver damage during the course of treatment.

General Introduction

Thalassemia is the term used for the group of genetic disorders in which there is inadequate and defective synthesis of Hemoglobin. It is also named as Mediterranean anemia (Dogherty *et al.*, 1997). Most common genetic defects in the world are thalassemia and other globin gene defects are which produce a wide clinical spectrum of anemia and other cardiovascular sequelae (Wood *et al.*, 2010).

For red blood cells to perform correctly and supply adequate amount of oxygen to all cells of the body the structure of Hemoglobin carries great importance. If the body does not produce suffient alpha and beta chains of globin the oxygen carrying capacity gets effected and leads to the development of anemia. Onset of anemia is in early childhood and lasts throughout life. There are two main types of thalassemia, alpha and beta that are named according to the two protein chains that are present in the structure of Hemoglobin (McLaren *et al.*, 1983).

About 240 million beta thalassemia carriers are present all over the world. Every year about 100,000 children are born with the disease of thalassemia. Thalassemia has been considered as a major health burden by World Health Organization (Weatherall, 2010).On diagnosis of a child with thalassemia homozygous there is a lifelong sequence of blood transfusion every three weeks along with chelation therapy and dealing with complications due to iron overload and transfusion transmitted infections. Despite the treatment, transfused children either die due to transfusion related overload and they usually do no survive beyond the age of 25 years. These patients start suffering from parenchymal tissue damage due to iron overload which starts within 1 year after the onset of repeated blood transfusions (Taksande *et al.*, 2012).

South-East Asia is the part of the world where majority of the cases of thalassemia are detected, in which the defect is in the imbalance in the rate of alpha and beta globin chain synthesis in hemoglobin of red blood cells. The clinical presentation varies from death in utero, through severe transfusion dependent anemia which is the reduction of Hemoglobin in blood (Weatherall, 2010). In thalassemia major defective gene are inherited from both parents and clinical features appear by 6 to 24 months of age. Transfusion with chelation therapy has significantly extended life expectancy but, leads to complications due to iron overload (Borgna *et al.*, 2004).

Thalassemic infants fail to thrive and become ultimately become pale. Thalassemic patients develop feeding problems, diarrhea, irritability, recurrent bouts of fever, and progressive enlargement of the abdomen caused by spleen and liver enlargement. Skeletal changes also develop in thalassemic patients which include deformities in the long bones of the legs and typical craniofacial changes (bossing of the skull, prominent malar eminence, depression of the bridge of the nose, tendency to a mongoloid slant of the eye, and hypertrophy of the maxillae, which tends to expose the upper teeth). For intiation of normal growth and development a regular transfusion program has to be intiated which maintains a minimum Hb concentration of 9.5 to 10.5 g/dl (Wood *et al.,* 2010).

Thalassemic patients under going repeated transfusions may develop complications related to iron overload. Various complications due to iron overload develop in these children which include growth retardation and failure or delay of sexual maturation. Later in life these children develop iron overload related complications which cause involvement of the heart (dilated myocardiopathy or rarely arrythmias), liver (fibrosis and cirrhosis), and endocrine glands (diabetes mellitus, hypogonadism and insufficiency of the parathyroid, thyroid, pituitary, and, less commonly, adrenal glands) (Borgna *et al.,* 2004).

Full blood count and analysis of the red cell indices are the initial screening tests done for detection of thalassemia. Various national programs are carried out for this purpose which direct such screening to adolescence or mothers attending the antenatal clinic (Dormandy *et al.,* 2010). Increased ineffective erythropoiesis leading to down regulation of hepcidin and increased iron absorption (primary hemochromatosis) are features that develop due to some mutations. While some mutations may present with ineffective erythropoiesis or severe anemia such patients may need chronic blood transfusions, producing secondary hemochromatosis. Clinical severity and presentation of primary and secondary hemochromatosis is often different but the main defect of, iron overload remains a lifelong problem(McLaren *et al.,* 1983).

For adequate erythropoietic function, oxidative metabolism and cellular immune response an essential micronutrient Iron is, required. As there is no active iron excretion of iron absorption of dietary iron (1-2 mg/day) is tightly regulated, and just balanced with losses (Brugnara *et al.,* 2006).

Approximately 50 mg/kg in men and 40 mg/kg body weight in women is the concentration of iron normally maintained in the human body and is distributed among functional (e.g., red blood cells RBCs), transport, and storage compartments (Conte *et al.*, 1986).

Haem (10%) and non-haem (ionic, 90%) are the two forms of dietary iron that are absorbed from the is found in apical surface of duodenal enterocytes via different mechanisms. Iron is exported after being absorbed from the basolateral membrane of the enterocyte into the circulation from where after binding with transferring it is transported to the sites for usage and storage.
Target cells mainly erythroid cells, immune and hepatic cells receive the Transferrin-bound iron through a process of receptor-mediated endocytosis. Senescent RBCs are phagocytosed by reticuloendothelial system (RES) macrophages and the haem is metabolised by heme-oxygenase, and iron is stored as Ferritin. This stored iron in the form of Ferritin, one molecule of which can store up to about 4500 ferric ions (Looker *et al.*, 1997) Later iron is released from macrophages and is bounded to transferrin, which is ultimately, transported to bone marrow. About (20-30 mg/day) of iron is required for the process erythropoiesis which is achieved through this internal turnover (Guggenheim, 1995).

There is no role for transferrin within the lumen of the intestine so the iron obtained from food is not bound to transferring. ingested iron is dissolved by the low pH of gastric effluent which makes a proton-rich milieu. This facilitates enzymatic reduction of ferric iron to its ferrous form by a brush border ferrireductase. Although the amount of iron extracted from the diet is small, the regulation of the intestinal absorption of iron is critical because humans have no physiologic pathway for excretion (Moore and Sears, 1994). Uptake of heme iron is through a separate process that is not well characterized. Inside the absorptive enterocyte, iron can follow two routes: it may be stored as Ferritin, or it may be transferred across the basolateral membrane and end up in the plasma. The determining factor is probably an iron absorption "set point" that is there from the time the enterocyte developed from a crypt cell. Iron present in Ferritin form as the enterocyte completes its limited life cycle will be sloughed with the old cells and will exit the body through the gastrointestinal tract. Which is an important mechanism of iron loss (Heilmeyer *et al.*, 1961).

Lifelong transfusion and chelation therapy is required to avoid premature death due to organ damage by hemosiderosis in thalassemia major (TM) patients. The major cause of death is due to cardiac failure, but along with this many patients suffer from endocrine damage related to iron overload (Walter *et al.*, 2008; Fung *et al.*, 2006). Cumulative iron overload unavoidably and invariably develop in patients receiving regular RBC transfusions, and are therefore, at risk for iron toxicity (Malcovati, 2007).

Iron stores are chiefly present in the body in the form of Ferritin, minimum amount is secreted in the plasma. Amount of iron overload is strongly associated with quantity of serum Ferritin, greater the quantity of iron overload greater the level of serum Ferritin. The levels of serum Ferritin differ with age and sex of an individual. There are raised levels at the time of birth till the first 2 months which than drop in later part of infancy (Domellof *et al.*, 2002).These levels again increase at 1 year of age which continue till adulthood however the males have a higher level of serum Ferritin than females and this pattern persist through adulthood (Gibson, 2005).

Despite the fact that blood transfusions are mandatory for the treatment of patients suffering from anemia, this repeated transfusions lead to iron overload as human beings can not remove the extra accumulated iron (Cappellini, 2007). Amount of iron that is present in one unit of blood that is transfused to thalassemic patient is 250 mg while the body can not remove more than 1 mg of iron per day (Ozment and Turi, 2009). In the absence of chelation therapy if a thalassemic patient is transfused 25 units per year there is accumulation of about 5 grams of iron. Along with this there is increased intestinal absorption of iron which further worsens the condition. This results in the development of iron overload (Piomelli, 1995). The iron gets deposited in various organs like liver, heart and endocrine glands. Out of the three the most severe complications result from deposition of iron in the heart which proves to be fatal (Modell *et al.*, 2000). Adolescents and adults develop various types of endocrinopathies like hypogonadism and diabetes mellitus (Rund and Rachmilewitz, 2005). Splenomegaly always develops in symptomatic thalassemic patients. It aggravates anemia and seldom causes neutropenia and thrombocytopenia. After splenectomy the patients develop a hypercoagulable state which makes venous and arterial thromboembolic events very common (Cappellini *et al.*, 2000).

Puberty is the transformation of an individual from childhood to adulthood along with maturation of gametes and beginning of reproductive activity (Harris and Levine, 2003 ; Sisk *et al.*, 2001). Pubertal changes in females start at 11 years of age but, it can also start as early as 6-7 years. While in the case of males the pubertal changes occur by the age of 12 years, which can start as early as 9 years. The process of puberty takes several years and a female is physically mature by age of 14 years and males are mature by the age of 15-16 years. Thalassemia major has always been treated by the pediatricians but, advancement in the treatment regimes has given better prognosis there has been improvement in life span as well as improved quality of life. Therefore, the patients now are in the adulthood so, supervision by adult physicians is required. (Richard *et al.*, 1999).

There is increased prevalence of bone disease in patients suffering from thalassemia major as compared to normal individual (Soliman *et al.*, 2009; Chatterjee and Bajoria, 2009; Shander, 2009). The bone growth depends on the sex steroids which regulate bone maturity. Therefore, gonadal insufficiency effects both male and female individuals. Bone mineral density advances at a constant rate till 12 years of age after which it suddenly takes a peak with pubertal growth spurt. Thalassemia major patients suffer from hypogonadism and fail to achieve their peak bone mass due to the bone disease they develop during the course of their disease (Chatterjee and Bajoria, 2009; De Sanctis *et al.*, 2006).

About 15-40 % of thalassemia major patients suffer from body disproportion i.e. upper short and normal lower body segment (De Sanctis *et al.*, 2006). They suffer from spinal growth impairment which gradually increases with time. Target height is not achieved by most of the thalassemic males. They have reduced sitting height as their spinal height and growth is effected during pubertal years (Soliman *et al.*, 2009; Roth *et al.*, 1997; Chatterjee *et al.*, 1993). Patients suffering from thalassemia major present with short stature in which leg length velocity changes the standing height while growth stunting associated with thalassaemia major is principally due to retarded truncal height (Rodda, 1994; Borgna-Pignatti *et al.*, 1985).

The body mass index (BMI) is based on the difference in body composition according to the level of adiposity and the relationship between weight and height, thus avoiding the dependence on frame size (Mahan, 2000). BMI can be calculated using the following equation:

BMI = weight (kg) per height (m^2). Greater mortality risk is a feature associated with low BMI (Bray, 1988). Underweight and under-nutrition may lead to loss of energy and susceptibility to injury and infection, under-function of multiple endocrine systems, as well as distorted body image and other psychological problems(Mahan, 2000). BMI is scored and interpreted as following: under weight is BMI of less than 5^{th} percentile for age and sex, normal weight is BMI of 5^{th} to less than 85^{th} percentile for age and sex, overweight is BMI of 85^{th} to less than 95^{th} percentile for age and sex, obese is BMI of 95^{th} percentile or more for age and sex (Kuczmarski *et al.*, 2000).

Patients suffering from thalassemia major require repeated lifelong blood transfusion to maintain a Hemoglobin level higher than 9.5 gm/dl. The need for the transfusions begins by the age of six months as anemia reduces normal growth and development (Rund and Rachmilewitz, 2005; Cazzola *et al.*, 1997).

Chronic blood transfusion in patients leads to the development of iron overload so, such patients should be regularly screened for iron overload. The patients who have developed iron overload should be routinely checked to determine the effectiveness of treatment so adjustment can be made in chelating therapy to reduce iron storage. Laboratory and imaging studies are the indirect methods for assessing iron storage while direct method is through tissue biopsy (Vichinsky, 2001).

Serum Ferritin, liver biopsy, magnetic resonance imaging (MRI) assessment of liver and cardiac iron, in conjunction with echocardiography and measures of endocrine function are the different criterion used for monitoring the iron overload in patients undergoing repeated transfusions. Dual energy computed tomography for measurement of liver iron is also done in some centers. All parameters play their own part but, estimating serum Ferritin is very helpful to patients as it can easily be measured and its testing facilities are readily available at various thalassemia centers (Leighton *et al.*, 1988). Serum Ferritin levels are frequently used for estimating iron overload as it corresponds well with the cardiac damage and the survival rate but it does not correspond well with liver damage. Infections, inflammation, malignancy and hepatic damage are those acute phase reactions that also increase the serum Ferritin levels but, because it is

easily measured and is readily available it is most common test done for checking the prognosis of patients (Olivieri and Brittenham,1997).

Levels of serum Ferritin in males are 12–300 ng/mL while in females, the levels are 12–150 ng/mL (Vichinsky, 2001). Serum Ferritin levels above 1000 ng/mL are considered as a iron overload (Morrison *et al.*, 2003). These levels of serum Ferritin are obtained after regular transfusion of about 100 ml packed RBC/kg of body weight (Crichton and Ward, 2003;Vichinsky, 2001; Porter, 2001). The levels of serum Ferritin vary among patients getting multiple transfusion (Files *et al.*, 2002) however, the cutting level at which iron toxicity and organ damage takes place is still not identified (Olivieri and Brittenham, 1997).

Iron chelation therapy is done with various iron chelators like desferrioxamine, deferiprone and deferasirox. The efficacy of chelation is also monitored through serum Ferritin levels as the side effects of the iron chelators can increase when serum Ferritin levels become less than 500 ng/mL, This is the reason why serum Ferritin levels are not reduced below this level (Farmaki *et al.*, 2010; Leighton *et al.*, 1988).

Growth, sexual development, fertility, bone mineral density, diabetes mellitus, hypothyroidism, hypoparathyroidism, and hypoadrenalism are the main issues to be addressed in the long-term follow-up of patients with thalassemia. Iron overload causes most of the associated mortality and morbidity frequently involving complication of endocrine glands and repeated infections.

The biochemical screening such as serum Ferritin and Hemoglobin levels are of paramount importance in all beta thalassemic patients in pediatric and adolescent age group. Underlying increase Ferritin levels and low Hemoglobin levels should be recognized and treated at the earliest for preventing pubertal delay therefore, present study was carried out to determine serum Ferritin and Hemoglobin levels in both male and female thalassemic patients of pubertal age group. Similarly, thalassemic patients undergoing hyper transfusion present with nutritional stunting which contributes to growth failure and impaired mental development therefore, present study was done to determine the effects of iron overload on height (cm), BMI (Kg/m^2), serum Ferritin (ng/mL) and Hemoglobin (gm/dl) along with exploring the correlation of BMI with

serum Ferritin (ng/mL) and Hemoglobin (gm/dl) of beta thalassemic patients of pubertal age group undergoing repeated blood transfusions with chelation therapy.

Puberty is that stage of development during which a number of complex series of physiological events take place that lead to the evolution of reproductive capabilities in an individual. The intial stages of such changes are manifested by the acceleration of GnRH pulse generator activity that are the primary neural signal for the pubertal maturation of the reproductive axis. Endocrine complications encountered are mainly associated with pituitary and gonadal dysfunction which develop due to iron overload. These findings stress the need for regular chelation therapy to prevent damage to the hypothalamic pituitary gonadal axis due to iron toxcity. Prevalence of endocrine complication is common in thalassemic patients receiving repeated blood transfusion with chelation therapy therefore, serum levels of Kisspeptin, gonadotropins (FSH and LH) and sex steroids (Estradiol and Testosterone) were determined during the present study. The interplay of these hormone levels with BMI, serum Ferritin, Hemoglobin and Kisspeptin levels were evaluated by determining the correlation between them.

The excess of iron is carefully regulated mainly by controlling iron absorption and excess of iron is toxic which gets accumulated because there is no well defined mechanism for its excretion. Toxic iron levels lead to organ dysfunction and damage. Children treated with modern transfusion and chelation therapy are entering early adulthood so, evaluation of various endocrine complication secondary to iron overload can be evaluated in such individuals for future interventions. Therefore, in the present study assessment of thalassemic children was done by getting a detail medical history and physical examination and BMI estimation by using growth chart. Endocrine disorders such as short stature, delayed puberty and hypogonadism are major complications in both adolescent and adult patients. Therefore, exploration of the effects of iron overload on Anterior Pituitary Hormones like Growth Hormone (GH), Thyroid Stimulating Hormone (TSH) was done. Similarly, imbalance of thyroid hormone can be damaging at any stage of life, during puberty, thyroid hormone, is required for the rapid growth and sexual development. Thus, a low-functioning thyroid at this stage of life can delay puberty, delay development and effect individuals reproductive function therefore, thyroid hormones i.e Triiodothyronine (T_3) and Thyroxine (T_4) levels were evaluated during the present study. Also

correlation of these hormones with Body Mass Index (BMI), Ferritin and hemoglobin (Hb) and Kisspeptin levels were further evaluated in beta thalassemic patients undergoing regular blood transfusion with chelation therapy.

Increased levels of ALT are associated with the prospective and prognosis of liver disease. In thalassemic patients with pubertal delay the association of ALT levels is still not well defined therefore, our current study was done to determine the effects of iron overload on ALT (U/L) levels. Correlation of ALT with BMI, serum Ferritin and Hemoglobin (gm/dl) of beta thalassemic patients of pubertal age group undergoing repeated blood transfusions with chelation therapy was also explored during the present study. Prevalence of Hepatitis B and C along with liver enlargement among such patient was also determined.

Materials and Methods

The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The study was approved by Ethics committee of Animal Sciences, Quaid-e-Azam University, Islamabad. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control) as shown in Flow Chart-1. It was a case control study carried out at Quaid-e-Azam University, Islamabad in collaboration with Jamila Sultana Foundation Rawalpindi, Thalassemia house Rawalpindi and Pakistan Institute of Medical Sciences (PIMS), Islamabad from 2010-2014.

Inclusion and Exclusion criteria:

The patients selected for the study were diagnosed as beta thalassemia major according to Hemoglobin electrophoresis. These patients were on regular blood transfusion with chelation therapy (desferroxamine injections). The age group of thalassemic patients along with their corresponding control included in the study was greater than equal to 8 years and less than and equal to 22 years. Patients suffering from any other blood disorders other than beta thalassemia major or any other pathology besides spleen and liver enlargement or hepatitis B and C were not included.

Informed consent and a detail proforma including history and clinical examination were filled on the patients visit to the thalassemia center for blood transfusion with chelation therapy as shown in annexure 1and 2. Following variables were explored during the study which are mentioned in detail in respected chapters.

Measurement of Standing Height (cm)

Height in centimeter and weight in kilogram were measured of all total 300 individuals included in the study. Height in centimeters was converted into meters to calculate Body mass index (BMI). Details of measuring Height and weight is given in detail in chapter-1

Body Mass Index (Kg/m^2)

Individuals of all groups had their Height in centimeter (converted to meters) and weight in kilogram recorded and BMI was calculated by using the growth chart as shown in annexure 3and 4. Details of which are mentioned in chapter-1.

The blood samples from controlled individuals were collected in hospital environment and blood from thalassemic patients were collected when they came for their routine blood transfusions with chelation therapy. For collection of blood sample, the sampling area was cleaned with a spirit swab. Blood sample of (3ml) was drawn from the right median cubital vein of both female and male patients and control individuals. Blood was then collected in labeled serum separator tubes containing Ethylene diamine tetra acetic acid (EDTA). The blood samples were centrifuged at 3000 rpm for 10 minutes, and serum separated was stored at 2 - 8 ^{0}C until analyzed.

Quantitative determination of Hemoglobin (gm/dl)

Thalassemic patients (n=200) and control individuals (n=100) had their Hemoglobin estimated by competitive inhibition enzyme immunoassay technique, which was carried out by using Enzyme-linked Immune Assay Kit. *(Cloud-Clone Corp.USA).* This kit in vitro quantitatively measures Hemoglobin in human serum. Hemoglobin estimation is given in detail in chapter-1.

Quantitative determination of Serum Ferritin (ng/mL)

Quantitative measurement of Ferritin concentrations in serum of thalassemic patients (n=200) and control (n=100) individuals was achieved by Abcam's Ferritin (FTL) Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit. *(Abcam® discover more, Germany).* Details of quantitative determination of serum Ferritin is mentioned in chapter-1.

Quantitative determination of Kisspeptin (ng/ml)

Detection of a specific peptide and its related peptides based on the principle of "competitive" enzyme immunoassay was performed by the Enzyme Immunoassay Kisspeptin-10/Metastin (45-54)-Amide (Human) Enzyme Immunoassay (EIA) kit *(Phoenix Pharmaceuticals, Inc.USA)* on

blood sample of thalassemic patients (n=200) and control individuals (n=100). Quantitative determination of Kisspeptin in detail is given in chapter-2.

Quantitative determination of Follicle Stimulating Hormone (mIU/mL)

The AxSYM hormone assay was done for detection of serum FSH levels of thalassemic aptients (n=200) and conrol individuals (n=100) by using Microparticle Enzyme Immunoassay (MEIA) technology. (Abott AxSYM system,USA). Details of quantitative determinaton of serum FSH is mentioned in chapter-2.

Quantitative determination of Luteinizing Hormone (mIU/mL)

Serum LH levels of thalassemic patients (n=200) and control individuals (n=100) was detected by using AxSYM hormone assay which based on Microparticle Enzyme Immunoassay (MEIA) technology. *(Abott AxSYM system,USA).* Quantitative determination of serum LH levels is mentioned indetail in chapter-2.

Quantitative determination of Estradiol (pg/mL)

Microparticle Enzyme Immunoassay (MEIA) was used for quantitative determination of Estradiol in serum of thalassemic patients (n=200) and control individuals (n=100) on AxSYM System. *(Abott AxSYM system,USA).* Details of quantitative determination of serum Estradiol levels is given in detail in chapter-2.

Quantitative determination of Testosterone (ng/mL)

AxSYM hormone assay was done for the quantitative detection of serum Testosterone levels in thalassemic patients (n=200) and control individuals (n=100), which was based on Microparticle Enzyme Immunoassay (MEIA) and was done on AxSYM System. *(Abott AxSYM system,USA).* Quantitative determination of serum Estradiol is mentioned in detail in chapter-2.

Quantitative determination of Growth Hormone (ng/mL)

The Invitrogen HGH-ELSIA is a solid phase Enzyme Amplified Sensitivity Immuno-Assay Kit *(Invitrogen HGH-ELISA, USA)* that was used for the quantitative determination of Human

Growth Hormone (HGH) in serum of thalassemic patients (n=200) and control individuals (n=100). Details of the quantitative determination of Growth Hormone is mentioned in chapter-3.

Quantitative determination of Triiodothyronine (ng/mL)

AxSYM total T_3 is a Micro particle Enzyme Immunoassay (MEIA) kit *(Abbott Total T_3 Kit AxSYM system USA)* that was used for the quantitative determination of total circulating Triiodothyronine (T_3) in serum of thalassemic patients (n=200) and control individuals (n=100). Quantitative determination of T_3 is mentioned in detail in chapter-3.

Quantitative determination of Thyroxine (µg/dL)

Quantitative determination of Thyroxine (T_4) in serum of thalassemic patients (n=200) and control individuals (n=100) was done by using AxSYM total T_4 kit *(Abbott total T4 Kit AxSYM system USA)*, which is based on Fluorescence Polarization Immunoassay (FPIA). Details for the quantitative determination of Thyroxine (T_4) is mentioned in chapter-3.

Quantitative determination of Thyroid Stimulating Hormone (µIU/mL)

The ARCHITECT TSH assay kit *(ARCHITECT system TSH Kit USA)* is based on Chemiluminescent Microparticle Immunoassay (CMIA) which was used for the quantitative determination of (TSH) in serum of thalassemic patients (n=200) and control individuals (n=100). Quantitative determination of TSH is given in detail in chapter-3.

Quantitative determination of Serum Alanine Transaminase (IU/L)

The MaxDiscovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit *(MaxDiscovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit, Biooscientific Corporation; USA)* is based on plate-based colorimetric enzymatic assay. It was used for the quantitative determination of ALT levels in serum of thalassemic patients (n=200) and control individuals (n=100). Details of quantitative determination of Alanine Transaminase enzyme in serum are given in detail in chapter-4.

Qualitative detection of Hepatitis B

Science with mission (SMI) Hepatitis B surface antigen (HBsAg) test strip is a rapid chromatographic immunoassay *(Sharon ,MA)* which was used for the qualitative detection of HBsAg in serum of thalassemic patients (n=200) and control individuals (n=100), details of which are given in chapter-4.

Qualitative detection of Hepatitis C

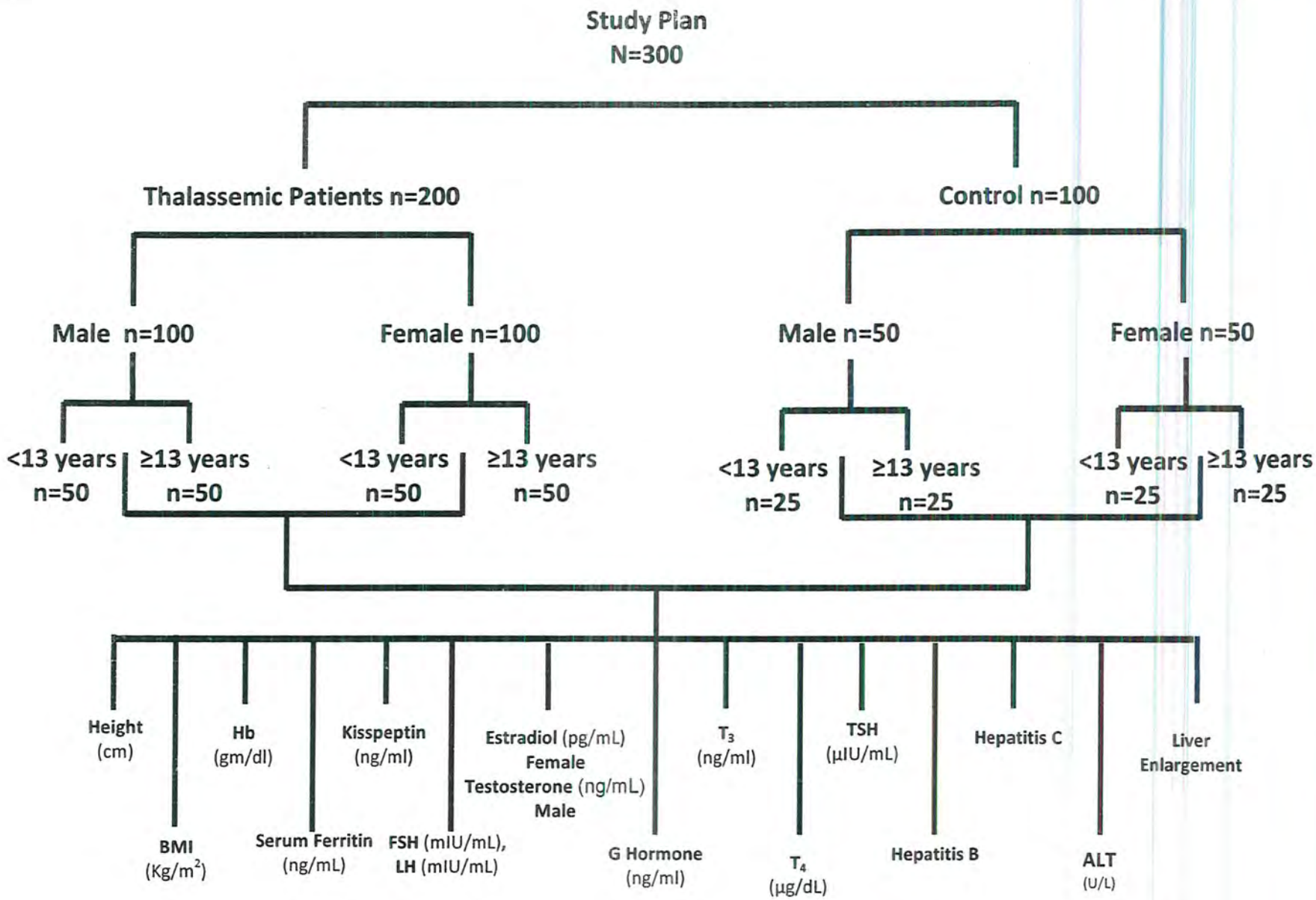
Science with mission (SMI) One-Step Hepatitis C Virus (HCV) Test is a rapid chromatographic immunoassay *(Sharon ,MA)* which was used for the qualitative detection of antibody to Hepatitis C Virus in serum of thalassemic patients (n=200) and control individuals (n=100), details of which are mentioned in chapter-4.

Palpation of liver

The purpose of liver palpation was to determine liver enlargement in thalassemic patients to rule out liver pathology. Liver palpation was also done in control individuals for a comparison with the thalassemic patients. Details of the procedure of liver palpation is given in chapter-4.

Statistical Analysis

Data was analyzed through Graph Pad Prism 5.01. Data was reported as Mean ± SEM. Comparison amongst hormones, BMI, Hemoglobin and serum Ferritin level with the control group was done by using unpaired t-test. Further non parametric co-relation (Spearman) was done for each hormone with rest of the variables through pad prism. $P<0.05$ was considered statistically significant in both cases.



Flow Chart 1: Description of study plan of female and male thalassemic patients with their corresponding control of different age groups in determining various hormone levels along with Height, BMI, serum Ferritin, Hemoglobin, ALT levels, Hepatitis B and C determination and assessment of liver enlargement.

Chapter # 1

Iron overload as a predictor of impaired growth in thalassemic patients undergoing repeated transfusion

Abstract

Background: Thalassemia major is a hereditary haemolytic anaemia which is treated with repeated blood transfusions. About 240 million beta thalassemia carriers are present all over the world. Every year about 100,000 children are born with the disease of thalassemia. Effects of iron overload on Height (cm), Body Mass Index (BMI), Hemoglobin and serum Ferritin levels in beta thalassemic patients undergoing regular blood transfusion was studied.

Materials and Methods: The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control).Height, BMI, Hemoglobin and serum Ferritin levels were analyzed.

Results: All groups had reduced Height, BMI, Hb and high Ferritin levels as compare to the control groups. Strong positive ($P<0.001$) correlation of BMI with Hemoglobin was seen in ≥ 13 years females. While <13 years thalassemic males had significant ($P<0.01$) negative correlation of BMI with Hemoglobin. Thalassemic females ≥ 13 years had significant ($P<0.001$) positive correlation of BMI with serum Ferritin levels.

Conclusion: In beta thalassemic patients growth disturbance or delay is main clinical feature that effects the life and wellbeing of such individuals. Our study has revealed that patients with beta thalassemia suffer from reduced Height, BMI which is enhanced in patients having high levels of serum Ferritin (ng/mL) and low Hemoglobin (gm/dl). The growth retardation seen in these patients with thalassemia major is multifactorial, it can be due to under-nutrition, hypogonadism, hypothyroidism, and other complications of thalassemia such as tissue hypoxia and side effects of chelating therapy with desferrioxamine (DFO). So, lifelong care and management of such patients is mandatory which requires significant cost for proper treatment of these patients in all aspects.

Introduction

Thalassaemia major is a hereditary haemolytic anaemia which is treated with repeated blood transfusions (Argyropoulou and Astrakas, 2007). About 240 million beta thalassemia carriers are present all over the world. Every year about 100,000 children are born with the disease of thalassemia. On diagnosis of a child with thalassemia homozygous there is a lifelong sequence of blood transfusion every three weeks along with chelation therapy and dealing with complications due to iron overload and transfusion transmitted infections. Despite the treatment, transfused children either die due to transfusion related overload and they usually do no survive beyond the age of 25 years. These patients start suffering from parenchymal tissue damage due to iron overload which starts within 1 year after the onset of repeated blood transfusions (Taksande *et al.*, 2012).

Iron stores are chiefly present in the body in the form of Ferritin, minimum amount is secreted in the plasma. Amount of iron overload is strongly associated with quantity of serum Ferritin, greater the quantity of iron overload greater the level of serum Ferritin. The levels of serum Ferritin differ with age and sex of an individual. There are raised levels at the time of birth till the first 2 months which than drop in later part of infancy (Domellof *et al.*, 2002).These levels again increase at 1 year of age which continue till adulthood however the males have a higher level of serum Ferritin than females and this pattern persist through adulthood (Gibson, 2005).

Despite the fact that blood transfusions are mandatory for the treatment of patients suffering from anemia, this repeated transfusions lead to iron overload as human beings can not remove the extra accumulated iron (Cappellini, 2007). Amount of iron that is present in one unit of blood that is transfused to thalassemic patient is 250 mg while the body can not remove more than 1 mg of iron per day (Ozment and Turi, 2009). In the absence of chelation therapy if a thalassemic patient is transfused 25 units per year there is accumulation of about 5 grams of iron. Along with this there is increased intestinal absorption of iron which further worsens the condition. This results in the development of iron overload (Piomelli, 1995). The iron gets deposited in various organs like liver, heart and endocrine glands. Out of the three the most

severe complications result from deposition of iron in the heart which proves to be fatal (Modell *et al.*, 2000). Adolescents and adults develop various types of endocrinopathies like hypogonadism and diabetes mellitus (Rund and Rachmilewitz, 2005). Splenomegaly always develops in symptomatic thalassemic patients. It aggravates anemia and seldom causes neutropenia and thrombocytopenia. After splenectomy the patients develop a hypercoagulable state which makes venous and arterial thromboembolic events very common (Cappellini *et al.*, 2000).

Puberty is the transformation of an individual from childhood to adulthood along with maturation of gametes and beginning of reproductive activity (Harris and Levine, 2003 ; Sisk *et al.*, 2001). Pubertal changes in females start at 11 years of age but, it can also start as early as 6-7 years. While in the case of males the pubertal changes occur by the age of 12 years, which can start as early as 9 years. The process of puberty takes several years and a female is physically mature by age of 14 years and males are mature by the age of 15-16 years. Thalassemia major has always been treated by the pediatricians but, advancement in the treatment regimes has given better prognosis there has been improvement in life span as well as improved quality of life. Therefore, the patients now are in the adulthood so, supervision by adult physicians is required. (Richard *et al.*, 1999).

There is increased prevalence of bone disease in patients suffering from thalassemia major as compared to normal individual (Soliman *et al.*, 2009; Chatterjee and Bajoria, 2009; Shander, 2009). The bone growth depends on the sex steroids which regulate bone maturity. Therefore, gonadal insufficiency effects both male and female individuals. Bone mineral density advances at a constant rate till 12 years of age after which it suddenly takes a peak with pubertal growth spurt. Thalassemia major patients suffer from hypogonadism and fail to achieve their peak bone mass due to the bone disease they develop during the course of their disease (Chatterjee and Bajoria, 2009; De Sanctis *et al.*, 2006).

About 15-40 % of thalassemia major patients suffer from body disproportion i.e. upper short and normal lower body segment (De Sanctis *et al.*, 2006). They suffer from spinal growth impairment which gradually increases with time. Target Height is not achieved by most of the thalassemic males. They have reduced sitting Height as their spinal Height and growth is effected during pubertal years (Soliman *et al.*, 2009;

Roth *et al.*, 1997; Chatterjee *et al.*, 1993). Patients suffering from thalassemia major present with short stature in which leg length velocity changes the standing Height while growth stunting associated with thalassaemia major is principally due to retarded truncal Height (Rodda, 1994; Borgna-Pignatti *et al.*, 1985).

The body mass index (BMI) is based on the difference in body composition according to the level of adiposity and the relationship between Weight and Height, thus avoiding the dependence on frame size (Mahan, 2000). BMI can be calculated using the following equation: BMI = Weight (kg) per Height (m^2). Greater mortality risk is a feature associated with low BMI (Bray, 1988). Underweight and under-nutrition may lead to loss of energy and susceptibility to injury and infection, under-function of multiple endocrine systems, as well as distorted body image and other psychological problems (Mahan, 2000).

BMI is scored and interpreted as following: under Weight is BMI of less than 5^{th} percentile for age and sex, normal weight is BMI of 5^{th} to less than 85^{th} percentile for age and sex, overweight is BMI of 85^{th} to less than 95^{th} percentile for age and sex, obese is BMI of 95^{th} percentile or more for age and sex (Kuczmarski *et al.*, 2000).

Malnutrition secondary to lack of intake of certain foods or malabsorption can result in serious conditions like osteopenia, anemia and syndromes resulting from deficiencies of vitamins, minerals, essential fatty acids and amino acids, and trace elements. Initial and most intense effect of under nutrition is on the vital growth, as muscles are effected more than bones and teeth. Brain cell hyperplasia develops as compared to any effect on myelination and at time of puberty the vital tissues and organs get effected as compared to gonads. Timing of adolescent sexual development depends on the nutritional status of the individual as under nutrition can late onset of menarche (Sinclair,1978).

Patients suffering from thalassemia major require repeated lifelong blood transfusion to maintain a Hemoglobin level higher than 9.5 gm/dl. The need for the transfusions begins by the age of six months as anemia reduces normal growth and development (Rund and Rachmilewitz, 2005; Cazzola *et al.*, 1997). Chronic blood transfusion in patients leads to the development of iron overload so, such patients should be regularly screened for iron overload. The patients who have developed iron overload

should be routinely checked to determine the effectiveness of treatment so adjustment can be made in chelating therapy to reduce iron storage. Laboratory and imaging studies are the indirect methods for assessing iron storage while direct method is through tissue biopsy (Vichinsky, 2001).

Serum Ferritin, liver biopsy, magnetic resonance imaging (MRI) assessment of liver and cardiac iron, in conjunction with echocardiography and measures of endocrine function are the different criterion used for monitoring the iron overload in patients undergoing repeated transfusions. Dual energy computed tomography for measurement of liver iron is also done in some centers. All parameters play their own part but, estimating serum Ferritin is very helpful to patients as it can easily be measured and its testing facilities are readily available at various thalassemia centers (Leighton *et al.*, 1988).

Serum Ferritin levels are frequently used for estimating iron overload as it corresponds well with the cardiac damage and the survival rate but it does not correspond well with liver damage. Infections, inflammation, malignancy and hepatic damage are those acute phase reactions that also increase the serum Ferritin levels but, because it is easily measured and is readily available it is most common test done for checking the prognosis of patients (Olivieri and Brittenham,1997).

Levels of serum Ferritin in males are 12–300 ng/mL while in females, the levels are 12–150 ng/mL (Vichinsky, 2001). Serum Ferritin levels above 1000 ng/mL are considered as a iron overload (Morrison *et al.*, 2003). These levels of serum Ferritin are obtained after regular transfusion of about 100 ml packed RBC/kg of body weight (Crichton and Ward, 2003;Vichinsky, 2001; Porter, 2001). The levels of serum Ferritin vary among patients getting multiple transfusion (Files *et al.*, 2002) however, the cutting level at which iron toxicity and organ damage takes place is still not identified (Olivieri and Brittenham, 1997).

Iron chelation therapy is done with various iron chelators like desferrioxamine, deferiprone and deferasirox. The efficacy of chelation is also monitored through serum Ferritin levels as the side effects of the iron chelators can increase when serum Ferritin levels become less than 500 ng/mL, This is the reason why serum Ferritin levels are not reduced below this level (Farmaki *et al.*, 2010; Leighton *et al.*, 1988).

The biochemical screening such as serum Ferritin and Hemoglobin levels are of paramount importance in all beta thalassemic patients in pediatric and adolescent age group. Underlying increase Ferritin levels and low Hemoglobin levels should be recognized and treated at the earliest for preventing pubertal delay therefore, present study was carried out to determine serum Ferritin and Hemoglobin levels in both male and female thalassemic patients of pubertal age group. Similarly, thalassemic patients undergoing hyper transfusion present with nutritional stunting which contributes to growth failure and impaired mental development therefore, present study was done to determine the effects of iron overload on Height (cm), BMI (Kg/m^2), serum Ferritin (ng/mL) and Hemoglobin (gm/dl) along with exploring the correlation of BMI with serum Ferritin (ng/mL) and Hemoglobin (gm/dl) of beta thalassemic patients of pubertal age group undergoing repeated blood transfusions with chelation therapy.

Materials and Methods

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-2.

The inclusion and exclusion criteria for thalassemic patients and control individuals included in the study are mentioned in detail in general materials and methods. Height in centimeter and Weight in kilogram were measured and BMI was calculated. The blood samples for the tests were collected from thalassemic patients (n=200) and control individuals (n=100) as mentioned in detail as general materials and methods. Enzyme-linked Immunosorbent assay kit was used for quantitative measurement of Hemoglobin (gm/dl) and serum Ferritin was measured by using Ferritin (FTL) ELISA (Enzyme-Linked Immunosorbent Assay) kit.

Measurement of Standing Height (cm)

Standing Height (cm) was measured by using the following equipment i.e Stadiometer, mounting tape, eraser, magnifying glass, lead pencil, vertical board (Kamal, 2010). Children were asked to stand erect on the floor board of the stadiometer with their back to the vertical backboard of the stadiometer. In this position the Weight of the participant was evenly distributed on both feet. The heels of the feet were placed together with both heels touching the base of the vertical board. The buttocks and head were positioned in contact with the vertical backboard. The arms were hanging freely by the sides of the trunk with palms facing the thighs. The children were asked to inhale deeply and stand fully erect without altering the position of the heels. Hair ornaments, buns, braids, etc. were removed to obtain an accurate measurement. The Height was measured with the measuring tape mounted on the wall. The Height measured in centimeter was further converted into meters by the following formula:

$$\text{meters} = \text{centimeters} / 100$$

Measurement of Weight (Kg)

Weight (Kg) was calculated by the help of the following equipment i.e eraser, pencil, level wooden board, magnifying glass, weighing machine (Kamal, 2010). After looking for a well balanced place the Weight machine was placed on clean cemented floor for accurate results. Before weighing was started the reading on the weighing machine was set at zero. To avoid error children were advised to remove everything except briefs or panties and empty their pockets so, Weight could be measured. While children stood upright on the weighing machine with both feet parallel and looking straight, they were asked to breath in deep and hold breath until Weight readings were recorded.

Body Mass Index (Kg/m^2)

Individuals of all groups had their Height in centimeter (converted to meters) and Weight in kilogram recorded during their visit to thalassemia center and the Height and Weight were than used to calculate body mass index (BMI) Kg/m^2 by the following formula:

$$BMI = \frac{\text{Weight in kilogram}}{\text{Height in meters}^2}$$

Quantitative determination of Hemoglobin (gm/dl)

Enzyme-linked Immunosorbent Assay kit estimates Hemoglobin (Hb) by competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of Hemoglobin in human serum. *(Cloud-Clone Corp. USA)*

Assay procedure:

Wells were determined for diluted standard, blank and sample. Five wells for standard points and one for blank were prepared. Then, 50µL of dilutions of standard, blank and samples were added into the appropriate wells, respectively. Then, 50µL of detection reagent A was added to each well immediately. Plates were shaken gently (using a microplate shaker). It was covered with a plate sealer and was then incubated for 1 hour at 37°C. Detection reagent A appeared cloudy, it was warmed to room temperature and was mixed gently until solution appeared uniform.

The solution was aspirated and washed with 350µL of 1X wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser, and was allowed to sit for 1-2 minutes. Remaining liquid was removed from the all wells completely by snapping the plate onto absorbent paper. It was repeated 3 times. After the last wash, any remaining wash buffer was removed by aspiration. The plates were inverted and were blotted against absorbent paper. Detection Reagent B (100µL) working solution was added to each well and was incubated for 30 minutes at 37°C after covering it with the plate sealer. Aspiration / wash process was repeated total 5 times. Substrate solution (90µL) was added to each well. It was covered with new plate sealer. It was incubated for 15 - 25 minutes at 37°C (not exceeding 30 minutes). It was protected from light and the liquid turned blue by the addition of substrate solution. Stop solution (50µL) was added to each well which turned the liquid to yellow color. The liquid was mixed by tapping the side of the plate any drop of water and fingerprint was removed from the bottom of the plate and was confirmed that there was no bubble on the surface of the liquid. Then, it was run at the microplate reader and measurement was conducted at 450 nm immediately.

Quantitative determination of Serum Ferritin (ng/mL)

Abcam's Ferritin Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit was used for the quantitative measurement of Ferritin concentrations in serum.
(Abcam® discover more, Germany)

Principle of the Assay:

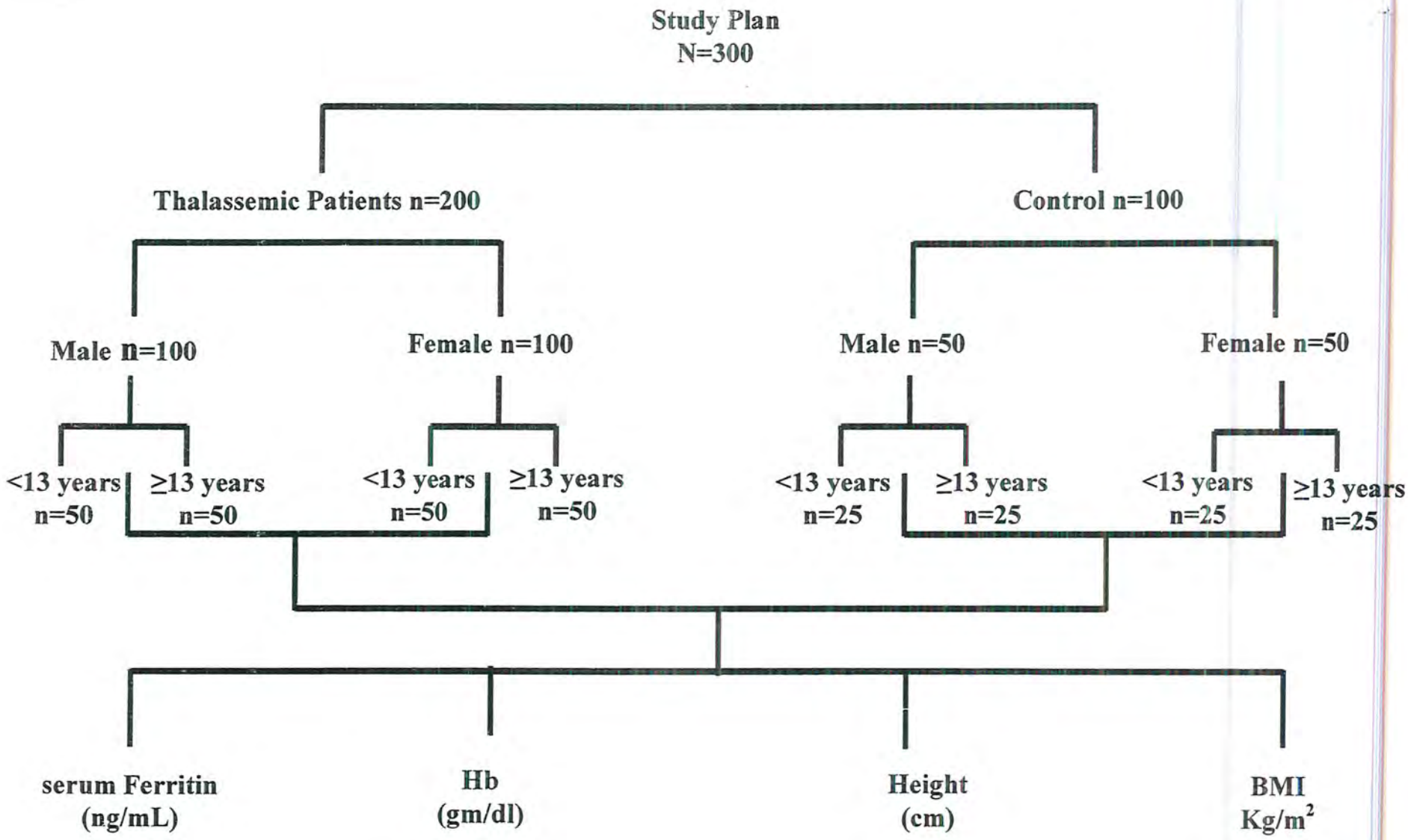
A Ferritin specific antibody were precoated onto 96-well plates and blocked. Standards or test samples were added to the wells and subsequently a Ferritin specific biotinylated detection antibody was added and then it was followed by washing with wash buffer. Streptavidin- Peroxidase Complex was added and unbound conjugates were washed away with wash buffer 3,3',5,5'- tetramethylbenzidine (TMB) solution. TMB was then used to visualize Streptavidin- Peroxidase enzymatic reaction. TMB was catalyzed by Streptavidin- Peroxidase to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow coloration was directly proportional to the amount of Ferritin captured in the plate.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- All samples were free of bubbles prior to analysis.
- Samples generating values higher than the highest standard were further diluted in the appropriate sample dilution buffers.
- Foaming or bubbles were avoided while mixing or reconstituting components.
- Cross contamination of samples or reagents was avoided by changing tips between sample, standard and reagent additions.
- Ensure plates were properly sealed and covered during incubation steps.
- Complete removal of all solutions and buffers was done during wash steps.

Statistical Analysis

Data was analyzed through Graph Pad Prism 5.01. Data was reported as Mean ± SEM. Comparison amongst BMI, Hemoglobin and serum Ferritin levels with the control group was done by using unpaired t-test. Further non parametric co-relation (Spearman) for BMI with serum Ferritin and Hemoglobin was done through pad prism. $P<0.05$ was considered statistically significant in both cases.



Flow Chart 2: Study plan describing female and male thalassemic patients with their corresponding control of different age groups in determining Height, BMI, serum Ferritin, Hemoglobin levels.

Results

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-2

Age

Mean ± SEM of age in male and female patients of < 13 years was 10.3± 0.20 years. Male patients ≥13 years had Mean ± SEM of age 16.7 ± 0.42 years, whereas the female patients of ≥13 years had Mean ± SEM of age 17.8 ± 0.70 years.

Height (cm)

Height in females thalassemic patients of <13 years (123 ± 2.64 cm) was significantly reduced (P<0.001) on comparison to height of female control group 140 ± 2.19 cm. While ≥13 thalassemic females also had similar results of height 149 ± 2.36 cm which was significantly less (P<0.001) compared to the control group having a height of 164 ± 1.23 cm. Similarly thalassemic males of <13 years had height of 128 ± 2.93 cm which was significantly (P<0.001) less than the control group (148 ± 1.67 cm). There was significant (P<0.001) reduction in the height of ≥13 years thalassemic males (141 ± 2.17 cm) on comparison with height of control group 167 ± 1.42 cm. Comparison of height (cm) of male and female thalassemic patients with their corresponding control of different age groups is represented in Figure – 1.1.

Table 1.1: Mean ± SEM of BMI, Hemoglobin and Ferritin of female and male thalassemic patients with their corresponding control of different age groups.

Age (Years)	Gender	Groups	BMI (Kg/m^2)	Hb (gm/dl)	Ferritin (ng/mL)
< 13	Females	Control n=25	19.6 ± 0.51	13.1±0.27	74.8 ± 2 .92
		Thalassemic n=50	16.0 ± 1.42***	7.60±0.17***	3900 ± 301***
≥13		Control n=25	23.1 ± 0.48	13.5 ± 0.26	150 ± 7.02
		Thalassemic n=50	13.7 ± 0.92***	6.71 ± 0.30***	3630 ± 368***
< 13	Males	Control n=25	21.8 ± 0.44	13.5 ± 0.28	75.8 ± 3.00
		Thalassemic n=50	17.2 ± 0.61***	7.06 ± 0.23***	4240 ± 255***
≥ 13		Control n=25	22 ± 0.45	15.00 ± 0.27	130 ± 13
		Thalassemic n=50	18 ± 0.63***	8.00 ± 0.47***	4300 ± 320***

***= P<0.001 is considered significant

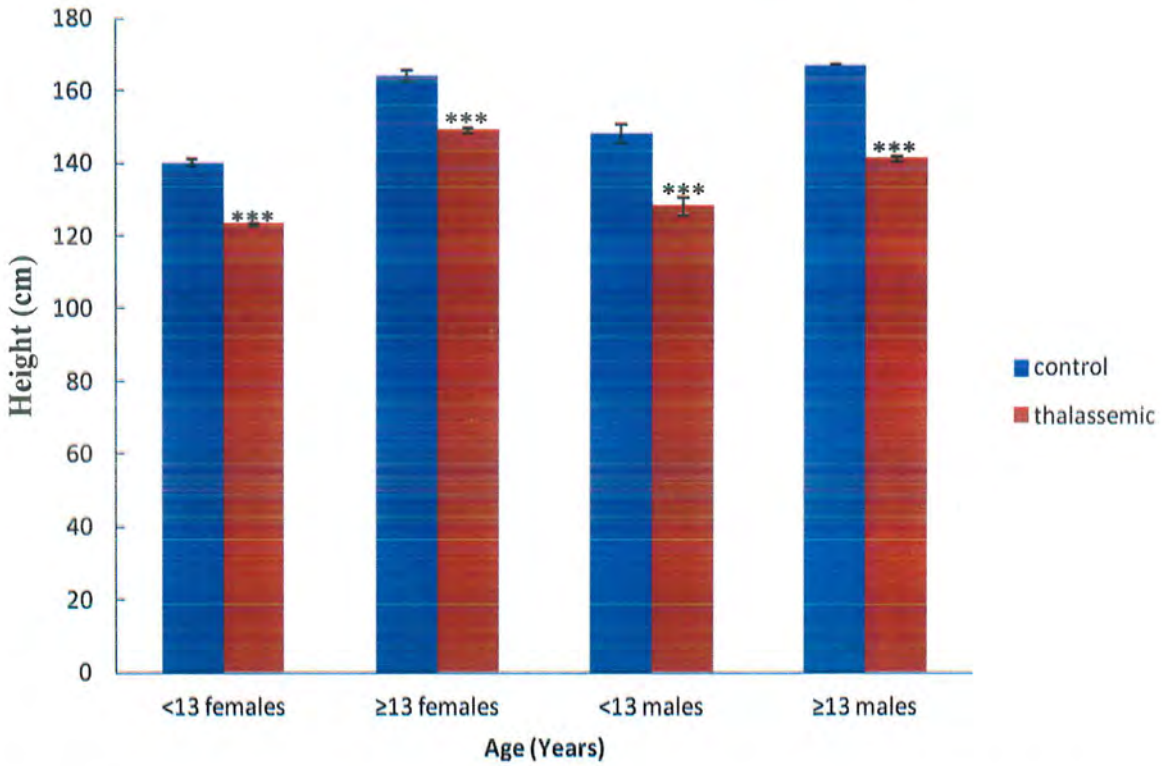


Figure 1.1: Mean ± SEM of height (cm) of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001 (value vs corresponding control)

BMI (Kg/m^2)

Comparison of Body Mass Index (Kg/m^2), in control and thalassemic female and male patients of different age groups is shown in Table-1.1. BMI of thalassemic females of <13 years (16.0 ± 1.42 Kg /m^2) showed significant difference (P<0.001) from the control group which was 19.6 ± 0.51 Kg/m^2. While BMI of thalassemic females of ≥13 years was 13.7 ± 0.92 Kg/m^2 which was significantly, (P<0.001) less than the control group (23.1 ± 0.48 Kg/m^2). Similarly, BMI of <13 years thalassemic males (17.2 ± 0.61 Kg/m^2) was significantly reduced (P<0.001) as compared to the control group (21.8 ± 0.44 Kg/m^2). Thalassemic males of ≥13 years (18 ± 0.63 Kg/m^2) also showed similar results of significantly, reduced BMI (P<0.001) as compared to the control group which was 22 ± 0.45 Kg/m^2.

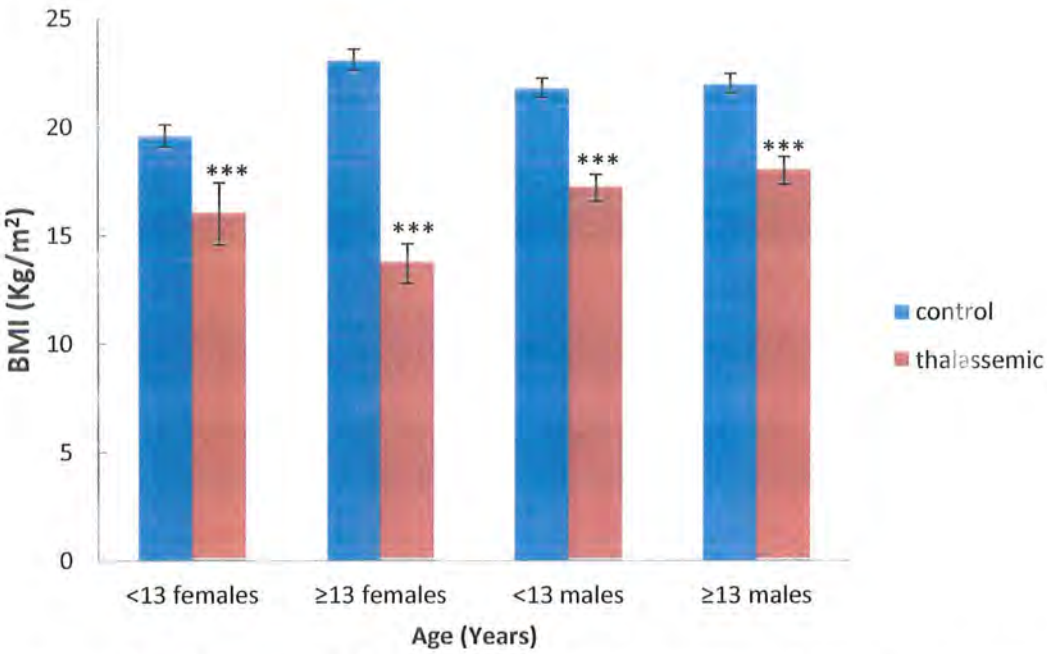


Figure 1.2: Mean ± SEM of BMI (Kg/m^2) of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001 (value vs corresponding control)

Hemoglobin levels (gm/dl)

Hemoglobin levels in < 13 years thalassemic females (7.60 ± 0.17 gm/dl), were significantly less (P<0.001) than the control group which were 13.1 ± 0.27 gm/dl. Similarly, significantly low Hemoglobin levels (P<0.001) were observed in ≥13 year thalassemic females (6.71 ± 0.30 gm/dl) on comparison with the control group (13.5 ± 0.26 gm/dl).

Amongst the male groups Hemoglobin levels showed significant reduction (P<0.001) in < 13 years thalassemic males (7.06 ± 0.23 gm/dl) as compared to the control group (13.5 ± 0.28 gm/dl). Similar results were observed in ≥13 years thalassemic males which also had significantly reduced (P<0.001) Hemoglobin levels (8.00 ± 0.47 gm/dl) on comparison with the control (15.00 ± 0.27 gm/dl). Comparison of Hemoglobin (gm/dl) of female and male thalassemic patients with their corresponding control of different age groups are presented in Figure-1.2.

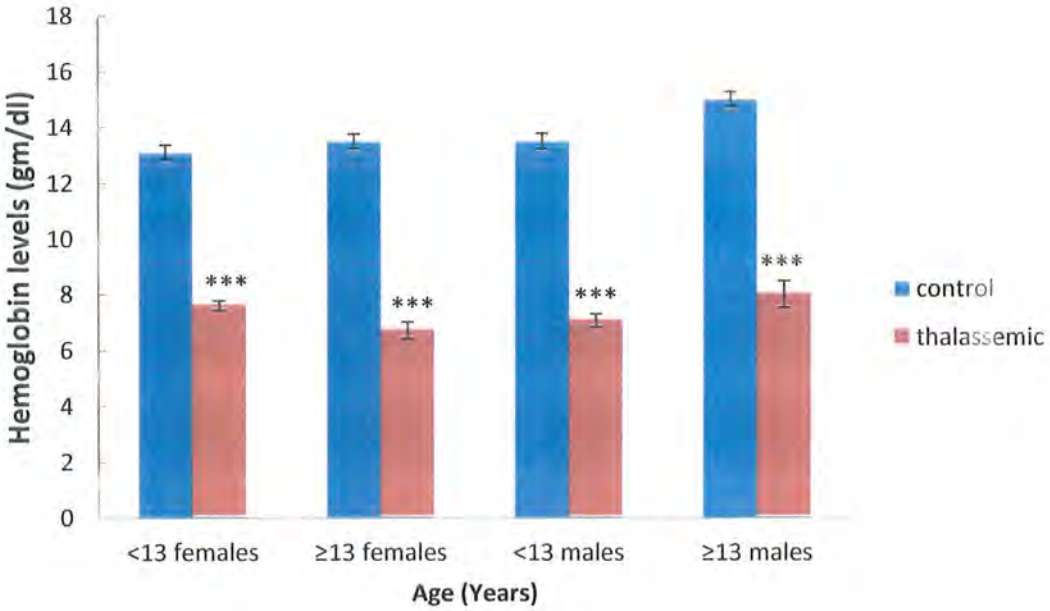


Figure 1.3: Mean ± SEM of Hemoglobin (gm/dl) of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001, value vs corresponding control

Serum Ferritin levels (ng/mL)

Comparison of serum Ferritin (ng/mL) of female and male thalassemic patients with their corresponding control of different age groups are shown in Figure-1.3. Serum Ferritin levels of thalassemic females of < 13 years was observed to be 3900 ± 301 ng/mL which were significantly, raised (P<0.001) as compared to the control group (74.8 ± 2.92 ng/mL). Similarly, ≥13 years thalassemic females had serum Ferritin levels (3630 ± 368 ng/mL) which were significantly raised (P<0.001) as compared to the control group (150 ± 7.02 ng/mL).

While in thalassemic males significantly higher (P<0.001) levels of serum Ferritin (4240 ± 255 ng/mL) were seen in thalassemic males of <13 years of age on comparison with the control group (75.8 ± 3.00 ng/mL). Greater than and equal to 13 year thalassemic males also displayed similar results of having serum Ferritin levels of 4300 ± 320 ng/mL, which were significantly high (P<0.001) as compared to the control group (130 ± 13 ng/mL).

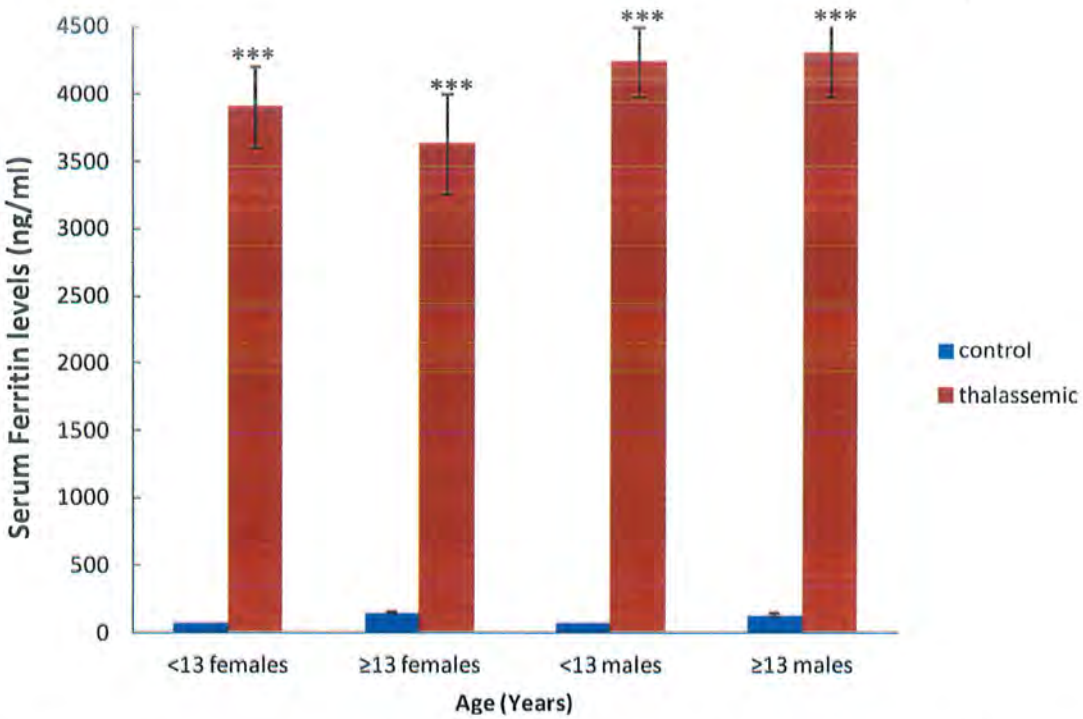


Figure 1.4: Mean ± SEM of serum Ferritin (ng/mL) of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001. value vs corresponding control

Correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) levels

There is a non significant negative correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) levels in < 13 years thalassemic females (r=-0.110). While in ≥13 years thalassemic females BMI (Kg/m^2) had a significant (P<0.001) positive correlation with Hemoglobin (gm/dl), (r=0.558). While thalassemic males of <13 years had a significant (P<0.001) negative correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) levels (r=-0.374). On the contrary BMI (Kg/m^2) ≥ 13 years thalassemic males had a non significant negative correlation with Hemoglobin (gm/dl) levels (r= -0.238).

On calculating correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) in <13 years thalassemic males it was concluded that there was a significant (P<0.01) negative correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) levels (r= -0.374). While ≥ 13 years thalassemic males, BMI (Kg/m^2) had a non significant negative correlation with Hemoglobin (gm/dl) levels (r= -0.238). Correlation of BMI (Kg/m^2) with Hemoglobin (gm/dl) in control and thalassemic female and male patients in different age groups is represented in Table-1.2, Figure-1.4, 1.5, 1.6 & 1.7.

Correlation of BMI (Kg/m^2) with Serum Ferritin (ng/mL) levels

Correlation of BMI (Kg/m^2) with, serum Ferritin (ng/mL) in control and thalassemic female and male patients in different age groups as shown in Table-1.2, Figure-4, 5, 6 & 7. Thalassemic females of < 13 years had a non significant positive correlation of BMI (Kg/m^2) with serum Ferritin (ng/mL) levels (r=0.192) while ≥ 13 years thalassemic females had a significant (P<0.001) positive correlation with serum Ferritin (ng/mL) levels (r= 0.498). Non significant negative correlation (r= -0.118) existed between BMI (Kg/m^2) and serum Ferritin levels in < 13 years thalassemic males. Similarly ≥ 13 years thalassemic males also had a non significant positive correlation (r=0.127) between BMI (Kg/m^2) and serum Ferritin (ng/mL) levels.

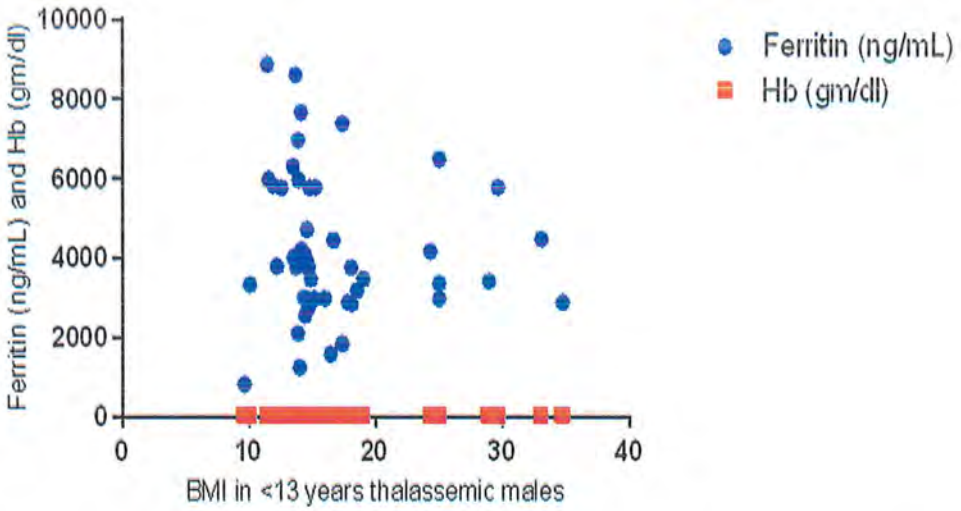


Figure 1.7: Correlation of BMI (Kg/m^2) with serum Ferritin (ng/mL) and Hemoglobin (gm/dl) of thalassemic male patients of <13years.

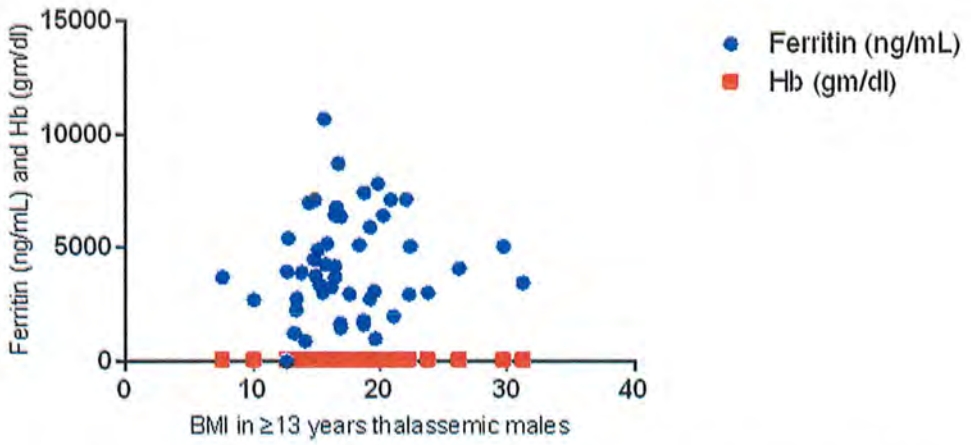


Figure 1.8: Correlation of BMI (Kg/m^2) with serum Ferritin (ng/mL) and Hemoglobin (gm/dl) of thalassemic male patients of ≥13years.

Discussion

Thalassemic patients are dependent on blood transfusions to maintain the levels of Hemoglobin and packed cell volume in their blood. Transfusion and iron-chelation therapy has prolonged and improved the quality of life in these patients (Borgna-Pignatti *et al.*, 2004). Such a treatment, however, leads to chronic iron overload affecting the endocrine glands (Abdulazahra *et al.*, 2011). In our study we observed that patients suffering from thalassemia major present with endocrine disorders and pubertal delay.

In another study carried out by Al-Rimawi *et al.*, 2005 showed that there was a significant difference in the frequency and regularity of using chelation therapy between pubertal and delayed pubertal groups. Whereas in our study the age of starting chelation therapy was 6-8 months and the patients were on regular blood transfusion and chelation therapy.

Najaf *et al.*, (2008) research revealed that 70% of the males and in 73% of female thalassemic patients of 10–27 years suffered from short stature. While Li *et al.*, (2002) observed short stature in 29.7% of patients. The iron overload leading to endocrinopathies, chronic anemia, zinc and folate deficiencies can lead to short stature. These findings are in accordance to our study results in which we observed reduced height in all four groups of thalassemic males and females. Therefore, close observation of growth in such individuals can lead to early detection of complication which can be managed to their full extent so, that the individual can achieve their normal adult height (De Sanctis *et al.*, 1995; Arcasoy *et al.*, 1987).

Underweight and obesity are assessed in a variety of ways, calculating BMI is one of the most ideal methods to access underweight, over weight and obesity in an individual. Underweight and under-nutrition individuals have decreased energy levels and are vulnerable to develop injury and infection, malfunctioning of multiple endocrine systems and psychological problems (Mahan and Escott, 2000). Patients with thalassemia major are exposed to many growth abnormalities as a outcome of the disease or due to the adverse effects of chelating therapy which they receive on regular basis as described by Kattamis *et al.*, (1990).

Work done by Ali and Hamdollah, (2004) on thalassemic patients revealed the reduced BMI was more apparent in greater than 10 years of age, which are similar to our study results. Thalassemic males of <13 and ≥13 years and thalassemic females of ≥13 years in our study had reduced BMI (P<0.001) as compared to the control group. The explanation to these results can be that endocrinopathies which appear as a result of iron overload and development of side effects due to prolong use of chelation therapy can be chief contributing factors in development of underweight thalassemic patients (Ali and Hamdollah, 2004). While considering the prevalence of underweight (low BMI), in males and females, our study revealed that both <13 and ≥ 13 years thalassemic males suffered from low BMI while on the other hand only ≥13 years thalassemic females developed low BMI. Reason for such a finding requires further studies keeping the pathogenesis of underweight and low BMI in thalassemic patients into consideration as also explained by Ali and Hamdollah, (2004). Deena *et al.*, (2014) also showed similar results of 18 (30%) patients who had low BMI. The BMI was significantly lower in the patients group as compared to the control individuals of more than 12 years of age. This finding is indicating that low BMI is highly dependent on disease progression and are in accordance with our present findings.

Growth retardation is a common presentation in thalassemic children which may be attributed to their distraction from proper intake of food, loss of appetite from ineffective erythropoiesis, along with the development of anemia. Frequent blood transfusions normally re establishes the normal growth spurt as explained by Viprakasit *et al.*, (2001). However, despite frequent blood transfusions the adolescent growth spurt is often delayed, except if rigorous iron chelation treatment is commenced at an early age in life (Theodoridis *et al.*, 1998). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overweight and 6.5% were categorized as obese (Adil *et al.*, 2012). Although these results are contrary to our study findings where low BMI and reduced height was detected.

Shalitin *et al.*, (2005) studies revealed that at serum Ferritin levels of 2500 ng/mL there was development of hypogonadism in thalassemic patients and in his studies the serum Ferritin levels were significantly raised as compared to the control groups.

While Bronspiegel *et al.*, (1990) discovered that chelation therapy with DFO initiated before puberty could help children to achieve normal sexual maturation as 90% of thalassemic patients in their study received DFO with mean serum Ferritin (1562 ± 445 ng/mL) before the age of 10 years and had normal sexual development as compared with 38% of patients who received chelation therapy after onset of puberty with mean serum Ferritin levels of 4271 ± 1989 ng/mL.

Shalitin *et al.*, (2005) also observed that thalassemic patients receiving effective chelation therapy in prepubertal years still developed short stature with significantly raised serum Ferritin levels. But these finding were contrary to results obtained by De Sanctis *et al.*, (1994) who detected no significant difference in final height between patients who started chelation therapy during adolescence with high serum Ferritin level and those who started chelation therapy during childhood with low serum Ferritin levels.

Hegazi *et al* ., (2013) observed a significantly low Hb levels and red blood cell count along with significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups. These findings are in accordance with finding of Charles and Linker, (2005); Irshaid and Mansi, (2009) who also reported that Hb levels in thalassemic patients are significantly lower than control. These results are similar to our study results as all thalassemic groups of <13 and ≥13 years had low Hb levels as compared to the control groups.

Hegazi *et al* ., (2013) carried out a study on thalassemic male and female patients of 4-18 years of age, where there was a significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared to control groups. Similarly, Abdulzahra *et al.*, (2011); Patil and Mujawar, (2010); Vahidi, *et al.*, (2003) work also revealed that iron indices were markedly increased in thalassemic patients, and the mean serum level of Ferritin were also raised as compared to control group. Similarly, in our study high serum Ferritin levels were observed in all four groups of <13 and ≥3 years male and female thalassemic patients as compared to the control groups which was similar to the results reported by Adil *et al.*, 2012, suggesting that increased serum Ferritin levels are related to the endocrinopathies. The raised serum Ferritin levels in thalassemic patients were associated with increased prevalence of development of endocrinopathies along with consequent increase in the serum

calcium (Ca), levels, alkaline phosphate and parathyroid hormone levels which was also a similar finding observed by Hussein and Manal in 2013.

Conclusion

In beta thalassemic patients growth disturbance or delay is main clinical feature that effects the life and wellbeing of such individuals. Our study has revealed that patients with beta thalassemia suffer from reduced height, BMI which is enhanced in patients having high levels of serum Ferritin (ng/mL) and low Hemoglobin (gm/dl). The growth retardation seen in these patients with thalassemia major is multifactorial, it can be due to under-nutrition, hypogonadism, hypothyroidism, and other complications of thalassemia such as tissue hypoxia and side effects of chelating therapy with desferrioxamine. So, lifelong care and management of such patients is mandatory which requires significant cost for proper treatment of these patients in all aspects.

Half of the patients with thalassemia major die before reaching an age of 30 years mainly because the conventional iron chelation therapy is too oppressive for full adherence. Patients require an individually made-to-measure treatment plan incorporating new, more tolerable treatment options. By reviewing the transfusion and chelation regimens used for patients we can minimize the iron overload in regularly transfused β-thalassemia patients leading to the normal growth and development along with normal height and BMI.

Chapter # 2

Endocrinological investigation of hypothalamic pituitary gonadal axis in beta thalassemic patients of pubertal age group undergoing chelation therapy

Abstract

Background: Thalassemia major is a homozygous trait in which there is defective Hemoglobin synthesis that leads to the development of severe anemia. Periodic transfusions along with chelation therapy have markedly improved the life span of thalassemic patients. Citrate toxicity and consequent iron overload leads to increase prevalence of endocrine complications in children, adolescents and young adults. Effects of iron overload on Hypothalmic Pituitary Gonadal (HPG) axis was observed.

Materials and Methods: The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control). Kisspeptin (Kp), Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and sex steroids were analyzed and correlation of these hormones with body mass index (BMI), serum Ferritin and Hemoglobin (Hb) levels in beta thalassemic patients undergoing regular blood transfusion was studied.

Results: All groups had significantly reduced (P<0.001) BMI and Hb as compared to control group. Serum Ferritin levels were significantly (P<0.001) high in all thalassemic groups on comparison with control. Kisspeptin levels in thalassemic females of <13 years females and males were significantly (P<0.001) low as compared to the control group. While thalassemic males of ≥13 years had significantly (P<0.01) high Kisspeptin levels as compared to the control group. FSH levels in <13 and ≥13 years thalassemic male and female patients were significantly raised (P<0.001) as compared to the control group. LH levels in thalassemic males in <13 years were significantly high (P<0.001) as compared to the control group. While ≥13 years thalassemic males had significantly (P<0.001) reduced LH levels as compared to the control group. Significantly high Testosterone (P<0.001) were observed in thalassemic males of <13 years as compared to the control group. While

Estradiol levels were significantly low (P<0.001) in <13 years thalassemic females. In <13 years thalassemic females FSH had a negative significant correlation (P<0.001) with Ferritin (r= -0.511). In thalassemic males of <13 years FSH had a significant (P<0.05) positive correlation with BMI (r= 0.296) and negative significant correlation (P<0.001) with Hemoglobin (r= -0.479). In <13 years female LH had a significant (P<0.01) negative correlation with Hemoglobin (r=-0.386). Estradiol in ≥ 13 year females had a positive significant (P<0.05) correlation with BMI (r= 0.318) and Hemoglobin (r=0.286).

Conclusion: Our study revealed that the levels of Kisspeptin in thalssemic females and males of < 13 years had reduced levels but, ≥13 years were raised but, at the same time the levels FSH and LH were significantly deranged. These findings are suggestive that hormone production by hypothalmus was correctly secreting Kisspeptin according to the pubertal time frame but, the levels of hormones secreted by anterior pituitary and the gonads were deranged due to damage caused by iron overload at pituitary and gonadal level. Along with disturbed hypothalamic pituitary gonadal axis there was decreased BMI, low Hemoglobin and raised serum Ferritin levels. These findings are indicating that treatment with double chelation from early life should be considered for better outcomes in thalassemic patients.

Introduction

Thalassaemia major is a homozygous trait in which there is defective Hemoglobin synthesis that leads to the development of severe anemia. Initially, the prevalence of the disease was limited to the Mediterranean countries and the Middle East but later due to migration of individuals from these area to various parts of the world the disease become worldwide. Periodic transfusions along with chelation therapy have markedly improved the life span of thalassemic patients. Citrate toxicity and consequent iron overload leads to increase prevalence of endocrine complications in children, adolescents and young adults (Shamshirsaz *et al.*, 2003).

Chronic anemia causes marked tissue hypoxia as well as it activates the various compensatory mechanisms like increased RBC production from the bone marrow and increased intestinal absorption of iron. All these factors lead to the immense iron deposition (Tiosano and Hochberg, 2001).

Serum Ferritin levels make 1% of the total iron reserve but its levels are not specific for depicting iron overload because it is an acute phase protein and its levels can be raised in various conditions like inflammation, infections, chronic disorders, and liver damage. Despite all, it is widely used as an indirect method of estimating the iron overload because of its availability and simplicity of performing the test (Argyropoulou and Astrakas, 2007).

Increase serum Ferritin levels are indicative that tissues are exposed to iron overload and are bounded to develop damage which is irreversible. There is a 23% increase risk of developing endocrine complications with raised serum Ferritin levels (Chirico *et al.*, 2015).

Thalassemic patients suffer from various complications out of which endocrinopathies are most common but, the incidence rate of developing these complications is not clear as the age of first treatment with chelation therapy as well as their survival and wellbeing is different amongst the thalassemic patients (Cappellini *et al.*, 2000). The statistics regarding the development of endocrinopathies in beta thalassemic patients

are confined to the compliance of the patients with the treatment (De Sanctis *et al.*, 1989).

The purpose of iron chelation therapy is to delay or reverse the development of iron induced damage to various structures as well as enhance the survival of such patients (Miskin *et al.*, 2003). Treatment with deferoxamine (DFO) hampers the development of iron induced endocrine complication to a lesser extent but as the survival rate of beta thalassemic patients is improving therefore, the endocrinopathies have attained great importance (De Sanctis, 2002).

The patients suffer from iron overload that leads to endocrine complications which are most common and most early to appear (Smiley *et al.*, 2008). Out of all the endocrine complication the most prominent is the failure of puberty and growth retardation. Various endocrine structures like pancreas, anterior pituitary, testes/ovaries, thyroid, parathyroids, and adrenals get effected by iron overload leading to various disease states (Ong *et al.*, 2008; Toumba *et al.*, 2007).

Lack of sexual development is a serious complication of thalassemia. Various studies on gonadal and pituitary functions have been carried out, which confirmed that primary gonadal failure takes place due to iron deposition on the gonadal cells (Kuo *et al.*, 1969). The prevalence of failure of pubertal onset is 50% in some studies while 100% is also an observation (Tiosano and Hochberg, 2001).

During the process of puberty the integrity of hypothalamic pituitary gonadal (HPG) axis is very important as any interruption of the axis leads to disturbance in the endocrine functions. During the process of fetal life and infancy the HPG axis is active till it reaches a stage of low functioning that is referred to as the juvenile pause. The factors that regulate the juvenile pause and cause increase secretion of gonadotropin-releasing hormone (GnRH) at the onset of puberty are the main regulating factors for the timings of puberty onset (Wu *el al*, 1996 and Apter *et al*, 1993).

At the onset of puberty their is reappearance of GnRH secretion which cause secretion of FSH and LH from anterior pituitary which bind to the ligand receptors and lead to maturation of gonads and production of Estradiol and Testosterone. This whole

process is termed as gonadarche. The sex steroid along with inhibin, activin, and follistatin control the further activities from hypothalamus and pituitary gland. The process of this transition from childhood to adolesecnt takes place under the effect of GnRH secretion which is a slow process. During this process the FSH and LH levels are high and this change in their pulsatility can be detected as early as 4 years of age. During the pubertal years, the GnRH secretion increases initially during night time and later it is present through out the whole day. The factors which cause reduced secretion of GnRH leading to dampening of the HPG axis during infancy and then reactivation of the HPG axis at puberty are still not very clear but, Gamma-amino butyric acid (GABA) are considered to play an important role in inhibiting GnRH release before the time of puberty (Wood *et al.*, 2010).

The GnRH secretion is enhanced by a major excitatory neurotransmitter in the hypothalamus that works by acting through N-methyl-D-aspartate (NMDA) and kainate receptors. Leptin, norepinephrine, dopamine, tumor growth factor–α, Kisspeptin (that bind to GPR54), neuregulin signaling by means of erbβ4 receptors, and galanin-like peptide are various stimulators of GnRH secretion (Noetzli *et al.*, 2009). The mode of action of the these neurotransmitters is through a complex network of communication between the glial cells and neurons within the hypothalamus (Fujisawa *et al.*, 1988).

Kisspeptin (Kp) is peptide that is encoded by the KISS1 gene. It acts through G protein-coupled receptor GPR54 which are also known as Kiss1 receptor (Roa *et al.*, 2008; de Roux *et al.*, 2003). The underlying mechanism in GnRH pulse generation is not clear there is evidence the KNDy neurons in the arcuate nucleus (also called infundibular nucleus) do play an important role (Lehman *et al.*, 2010). Kisspeptin, neurokinin B (NKB) and dynorphin, send their axons to the median eminence where they join the GnRH fibers which ultimately reach the portal vessel system. Kisspeptin, neurokinin B (NKB) and dynorphin, send their axons to the median eminence where they join the GnRH fibers which ultimately reach the portal vessel system. Loss of function mutations in the genes encoding for Kisspeptin (KISS1) and for NKB or the NKB receptor in humans causes absence of gonadal development at the expected age of puberty that is a state of profound hypogonadotropism (Topaloglu *et al.*, 2012). The basic neuroendocrine unit which comprise of the GnRH pulse

generator and the pituitary gonadotropes is responsible for the LH and FSH secretion (Plant and Witchel, 2006).

Kisspeptin is now being considered as a factor that regulates the initiation of puberty and also development of capability of reproduction (Roseweir and Millar, 2009). These findings were discovered by inactivation of mutations of the GPR54 gene which resulted in failure of puberty onset and that lead to the development of hypogonadotropic hypogonadism(Roa *et al.*, 2008; de Roux *et al.*, 2003). Various studies have proved that kisspeptin acts as a extraordinarily potent factor for the secretion of FSH and LH in a variety of species like rodents, sheep, and primates including humans. It acts mainly on the hypothalmus where it activates the GnRH neurons (d'Anglemont de Tassigny *et al.*, 2009; Seminara *et al.*, 2003).

Previous studies have not clearly localized the anatomical level of HPG axis dysfunction. However in the past it has been proven that permanent damage to the HPG axis takes place and gonadal dysfunction appear as a late finding. However the pituitary T2 and pituitary volume with clinical features suggest the secondary hypogonadism (pituitary level) is the dominant etiology while tertiary hypogonadism (hypothalmus level) has not been explored (Chatterjee and Bajoria, 2010; Chatterjee and Katz, 2000).

Under the effect of gonadal steroid hormones (predominantly Testosterone in males and Estradiol in females) and the adrenal androgens, primarily dehydroepiandrosterone sulfate (DHEAS) sexual maturation occurs during puberty. In both genders the stages of puberty take place in a specific sequence. production of adrenal androgens termed as, Adrenarche, generally occurs 1 to 2 years before the other hormonal changes of puberty. The obvious changes of onset of puberty becomes apparent after thelarche in girls or testicular enlargement in boys, hair growth, adult-type body odor, and occasionally acne in both genders is a separate process from that of the centrally mediated gonadarche (Marshall, 1975). The rate of pubertal development correlates with the levels of sex steroid hormones (DeRidder *et al.*, 1992).

Obvious changes in body composition, including alterations in the relative proportions of water, muscle, fat, and bone, are main features of pubertal maturation

and the result female and male differences specific physical appearance. Along with gonadal steroid hormones the growth hormone, increases in bone mineral content and muscle mass takes place, along with deposition of fat. The changes in the fat distribution of the body (central vs. peripheral, subcutaneous vs. visceral, upper vs. lower body) leads to the development of typical android and gynoid patterns of fat distribution of the older adolescent and adult (Roemmich *et al.*, 2000).

Adequate levels of thyroid hormone and cortisol secretion are mandatory for the normal growth, but out of all the hormones gonadal steroid hormones have the major role. In addition, there is a dramatic activation of the GH/ IGF-1 axis. During adolescence, the gonadal steroid hormones and the GH/IGF-1 axis continue to act and produce their effects but later on the interaction between them leads to the change, in linear growth and body composition. Adequate calcium intake, physical activity, and ethnic background are also important factors taking part during puberty (Attie *et al.*, 1990).

Estrogens rather than androgens take part in many of the growth-promoting effects of the gonadal steroid hormones. There is either direct secretion of estrogen or peripherally located aromatase converts androgens to estrogen (zachmann *et al.*, 1986). Prepubertal human ovary has the ability to secretes Estradiol which is confirmed by ultrasensitive Estradiol bioassay which show significantly increased levels of Estradiol in prepubertal girls than in boys (Paris *et al.*, 2012). During 8-10 years of age the Estradiol levels increase and are similar to the adult females levels at the early follicular phase of the menstrual cycle. These rising levels of serum estrdiol levels cause gonadarche which is stage of puberty when initiation of breast development takes place along with increase in uterine volume (Holm *et al.*, 1995). The levels of Testosterone during peripubertal time period is scanty. The transition from a prepubertal to an adult pattern there is brisk secretion. Although sexual development takes place which include testicular growth during the stage of puberty (Simorangkir *et al.*, 2012).

Puberty is that stage of development during which a number of complex series of physiological events take place that lead to the evolution of reproductive capabilities in an individual. The intial stages of such changes are manifested by the acceleration

of GnRH pulse generator activity that are the primary neural signal for the pubertal maturation of the reproductive axis. Endocrine complications encountered are mainly associated with pituitary and gonadal dysfunction which develop due to iron overload. These findings stress the need for regular chelation therapy to prevent damage to the hypothalamic pituitary gonadal axis due to iron toxcity. Prevalence of endocrine complication is common in thalassemic patients receiving repeated blood transfusion with chelation therapy therefore, serum levels of Kisspeptin, gonadotropins (FSH and LH) and sex steroids (Estradiol and Testosterone) were determined during the present study. The interplay of these hormone levels with BMI, serum Ferritin, Hemoglobin and Kisspeptin levels were evaluated by determining the correlation between them.

Materials and Methods

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-3.

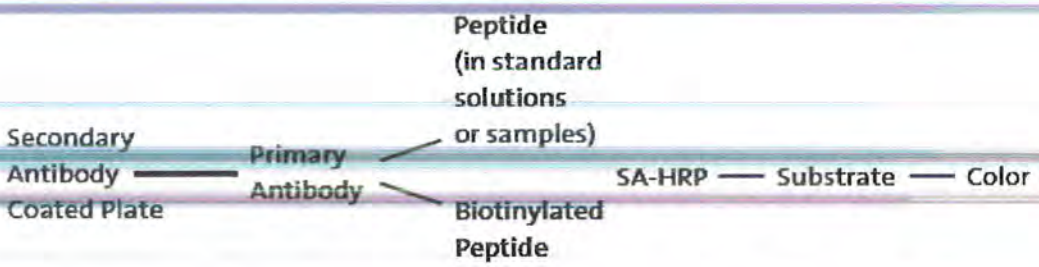
The inclusion and exclusion criteria for thalassemic patients and control individuals included in the study are mentioned in detail in general materials and methods. Height in centimeter and weight in kilogram were measured and BMI was calculated. The blood samples for the tests were collected from thalassemic patients (n=200) and control individuals (n=100) as mentioned in detail as general materials and methods. Enzyme-linked Immunosorbent assay kit was used for quantitative measurement of Hemoglobin (gm/dl) and serum ferritin was measured by using ferritin (FTL) ELISA (Enzyme-Linked Immunosorbent Assay) kit, details of which are mentioned in chapte-1.The blood samples collected and stored were also used for hormonal assay of Kisspeptin (ng/ml), follicle stimulating hormone (FSH) mIU/mL, luteinizing hormone (LH) mIU/mL, Estradiol E_2 (pg/mL) and Testosterone (T) ng/mL.

Quantitative Determination of Kisspeptin (ng/ml)

This Enzyme Immunoassay Kisspeptin-10/Metastin (45-54)-Amide (Human) EIA Kit is designed to detect a specific peptide and its related peptides based on the principle of "competitive" enzyme immunoassay. (*Phoenix Pharmaceuticals, Inc. USA)*

Principle of the Assay:

The immunoplate in the kit were pre-coated with secondary antibody and the nonspecific binding sites were blocked. The secondary antibody bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment competitively was bound by both biotinylated peptide and peptide standard or targeted peptide in samples. The biotinylated peptide interacted with streptavidin-horseradish peroxidase (SA-HRP) that catalyzed the substrate solution. The intensity of the yellow color is directly proportional to the amount of biotinylated peptide- SA-HRP complex but inversely proportional to the amount of the peptide in standard



solutions or samples. This is due to the competitive binding of the biotinylated peptide with the standard peptide or samples to the peptide antibody (primary antibody).

Assay Protocol

Add 50µl/well of standard, sample or positive control, 25 µl primary antibody and 25µl biotinylated peptide.

▼

which was incubated at room temperature (20-230C) for 2 hours

▼

The immunoplate were washed 4 times with 350 µl/well of 1x assay buffer

▼

100µl/well of SA-HRP solution was added

▼

Incubation at room temperature (20-23 ^{0}C) was done for 1 hour

▼

Immunoplate were washed 4 times with 350 µl/well of 1x assay buffer

▼

100µl/well of TMB substrate solution was added

▼

Incubation at room temperature (20-23 ^{0}C) was done for 1 hour

▼

Reaction was terminated with 100µl/well of 2N HCl

▼

Absorbance O.D.at 450nm was read and results were calculated

Quantitative determination of Follicle Stimulating Hormone (mIU/mL)

The AxSYM hormone assay is based on Microparticle Enzyme Immunoassay (MEIA) technology. *FSH kit of Abbott AxSYM system, USA was used*

Principle of the Assay:

Sample and all AxSYM FSH reagents required for one test were pipetted by the sampling probe into various wells of a reaction vessel (RV). The RV was immediately transferred into the processing center. Further pipetting was done in the processing center with the processing probe. Sample, Anti-β FSH coated microparticles and TRIS buffer were pipetted into one well of the RV. The FSH binded to the Anti-β FSH coated microparticles forming an antibody-antigen complex. An aliquot of the reaction mixture containing the antibody antigen complex bound to the microparticles was transferred to the matrix cell. The microparticles binded irreversibly to the glass fiber matrix and the matrix cell was washed with the wash buffer to remove unbound materials. The Anti- FSH subunit specific alkaline phosphatase conjugate was dispensed onto the matrix cell and was allowed to bind with the antibody antigen complex. The matrix cell was washed to remove unbound materials. The substrate, 4-Methylumbelliferyl Phosphate, was added to the matrix cell and the fluorescent product was measured by the MEIA optical assembly.

Reagents:

AxSYM FSH reagent pack (7A60-22)* was used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Luteinizing Hormone (mIU/mL)

The AxSYM hormone kit for luteinizing hormone was sued which is based on Microparticle Enzyme Immunoassay (MEIA) technology *(Abbott AxSYM system, USA).*

Principle of the Assay:

Sample and all AxSYM LH reagents required for one test were pipetted by the sampling probe into various wells of a reaction vessel (RV). The RV was immediately transferred into the processing center. Further pipetting was done in the processing center with the processing probe. Sample, Anti-β LH coated microparticles and TRIS buffer were pipetted into one well of the RV. The LH binded to the Anti-β LH coated microparticles forming an antibody-antigen complex. An aliquot of the reaction mixture containing the antibody antigen complex bound to the microparticles was transferred to the matrix cell. The microparticles binded irreversibly to the glass fiber matrix and the matrix cell was washed with the wash buffer to remove unbound materials. The Anti- LH subunit specific alkaline phosphatase conjugate was dispensed onto the matrix cell and was allowed to bind with the antibody antigen complex. The matrix cell was washed to remove unbounded materials. The substrate, 4-Methylumbelliferyl Phosphate, was added to the matrix cell and the fluorescent product was measured by the MEIA optical assembly.

Reagents:

AxSYM LH reagent pack (7A61-22)* was used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Estradiol (pg/mL)

AxSYM Estradiol is a Microparticle Enzyme Immunoassay (MEIA) for the quantitative determination of Estradiol in human serum on the AxSYM System *(Abbott AxSYM system, USA)*

Principle of the Assay:

Sample and all AxSYM Estradiol reagents required for one test were pipetted by the sampling probe into various wells of a reaction vessel (RV). Sample, Anti-Estradiol coated microparticles, Estradiol assay buffer, and line diluent were combined in a well of the RV. This was the reaction mixture. Estrogen:Alkaline Phosphatase Conjugate was added to a second well of the RV. The RV was immediately transferred into the processing center. Further pipetting was done in the processing center by the processing probe. The reaction mixture was incubated. Estradiol in the sample binded to the anti-Estradiol on the microparticles forming an antibody-antigen complex. After incubation, an aliquot of the reaction mixture was transferred to the matrix cell. The microparticles binded irreversibly to the glass fiber matrix. The matrix cell was washed to remove unbound materials. Estrogen:Alkaline Phosphatase Conjugate was then dispensed onto the matrix cell and incubated. The steroid portion of the conjugate binded to the available sites on the anti-Estradiol coated microparticles. The matrix cell was then washed to remove unbounded materials. The substrate, 4-Methylumbelliferyl Phosphate, was added to the matrix cell and the fluorescent product formed was measured by the MEIA optical assembly.

Reagents:

AxSYM Estradiol reagent pack (7A63-20)[a] was used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Testosterone (ng/mL)

AxSYM Testosterone is a Microparticle Enzyme Immunoassay (MEIA) for the quantitative determination of Testosterone in human serum on the AxSYM System *(Abbott AxSYM system, USA).*

Principle of the Assay:

Sample, Anti-Testosterone coated microparticles and Testosterone displacement agent were pipetted by the sampling probe into one well of a reaction vessel (RV). This is the reaction mixture.Testosterone: Alkaline Phosphatase Conjugate was than added to a second well of the RV. AxSYM Testosterone wash buffer was added to a third well of the RV. The RV was immediately transferred into the processing center. Further pipetting was done in the processing center by the processing probe. The reaction mixture was incubated. Testosterone in the sample binded to the anti-Testosterone on the microparticles forming an antibody antigen complex. Testosterone: Alkaline Phosphatase Conjugate was dispensed into the reaction mixture and incubated. The steroid portion of the conjugate binded to the available sites on the anti-Testosterone coated microparticles. After incubation, an aliquot of the reaction mixture was transferred to the matrix cell. The microparticles bind irreversibly to the glass fiber matrix. The matrix cell was than washed to remove conjugate not bounded to the microparticles. The substrate, 4-Methylumbelliferyl Phosphate, was added to the matrix cell. The alkaline phosphatase-labeled conjugate catalyzeed and removed the phosphate group from the substrate, yielding the fluorescent product, 4-Methylumbelliferone. This fluorescent product was measured by the MEIA optical assembly.

Reagents:

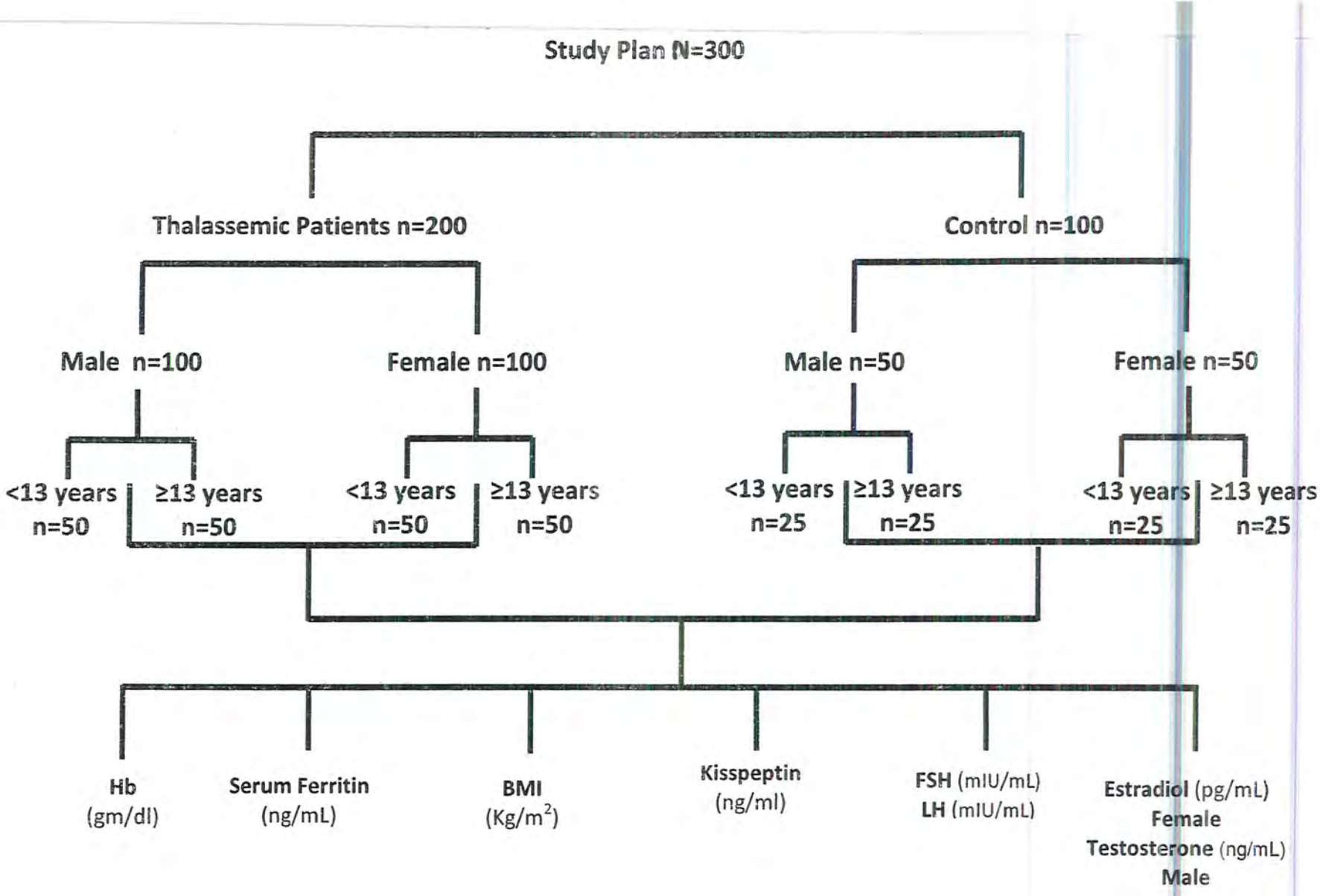
AxSYM Testosterone reagent pack (3C85-20) was used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Statistical Analysis

Data was analyzed through Graph Pad Prism 5.01. Data was reported as Mean ± SEM. Comparison amongst hormones, BMI, Hemoglobin and serum ferritin level with the control group was done by using unpaired t-test. Further non parametric co-relation (Spearman) was done for each hormone with rest of the variables through pad prism. $P<0.05$ was considered statistically significant in both cases.



Flow Chart 3: Study plan describing female and male thalassemic patients with their corresponding control of different age groups in determining serum Kisspeptin, gonadotropin (FSH and LH) and sex steroid (Estradiol and Testosterone) levels along with BMI, serum Ferritin and Hemoglobin levels.

Results

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-3. Results of (BMI) Kg/m^2, Hemoglobin (gm/dl) and serum Ferritin (ng/mL) are mentioned in chapter -1.

Serum Kisspeptin levels (ng/ml)

Comparison of serum Kisspeptin levels in control and thalassemic male and female patients of different age groups are represented in Figure-2.1. Kisspeptin levels in thalassemic females of <13 years (5.60 ± 0.26 ng/ml) were significantly low ($P<0.001$) as compared to the control group (10.1 ± 0.95 ng/ml). Whereas, there was no significant difference in Kisspeptin levels of thalassemic females ≥ 13 years (27.56 ± 0.69 ng/ml) and control group which was 28.35 ± 1.26 ng/ml. There was significant ($P<0.001$) difference in Kisspeptin levels of thalassemic males of < 13 years (5.64 ± 0.42 ng/ml) as compared to control group (10.43 ± 0.89 ng/ml). On comparison of Kisspeptin levels in thalassemic males, ≥ 13 years thalassemic males had significantly high ($P<0.01$) Kisspeptin levels of 28.18 ± 0.74 ng/ml as compared to control group (25.06 ± 0.18 ng/ml).

Table 2.1: Mean ± SEM of Kisspeptin (ng/ml), FSH (mIU/mL), LH (mIU/mL) levels in control and thalassemic female and male patients of different age groups.

Age (Years)	Gender	Groups	Kisspeptin (ng/ml)	FSH (mIU/mL)	LH (mIU/mL)
< 13	Females	Control n=25	10.10 ± 0.959	1.30 ± 0.13	0.58 ± 0.16
		Thalassemic n=50	5.60 ± 0.26***	5.20 ± 0.51***	0.88 ± 0.08
≥ 13		Control n=25	28.35 ± 1.26	1.80 ± 0.41	2.69 ± 0.37
		Thalassemic n=50	27.56 ± 0.69	5.54 ±0.49***	2.58 ± 0.28
< 13	Males	Control n=25	10.43 ± 0.89	1.57 ± 0.17	0.39 ± 0.04
		Thalassemic n=50	5.64 ± 0.42***	4.19 ± 0.54**	0.95 ± 0.05***
≥ 13		Control n=25	25.06 ± 0.18	2.10 ± 0.18	3.25 ± 0.21
		Thalassemic n=50	28.18 ± 0.74**	4.03 ± 0.42**	1.84 ± 0.17***

**=P<0.01, value vs corresponding control, ***=P<0.001, value vs corresponding control

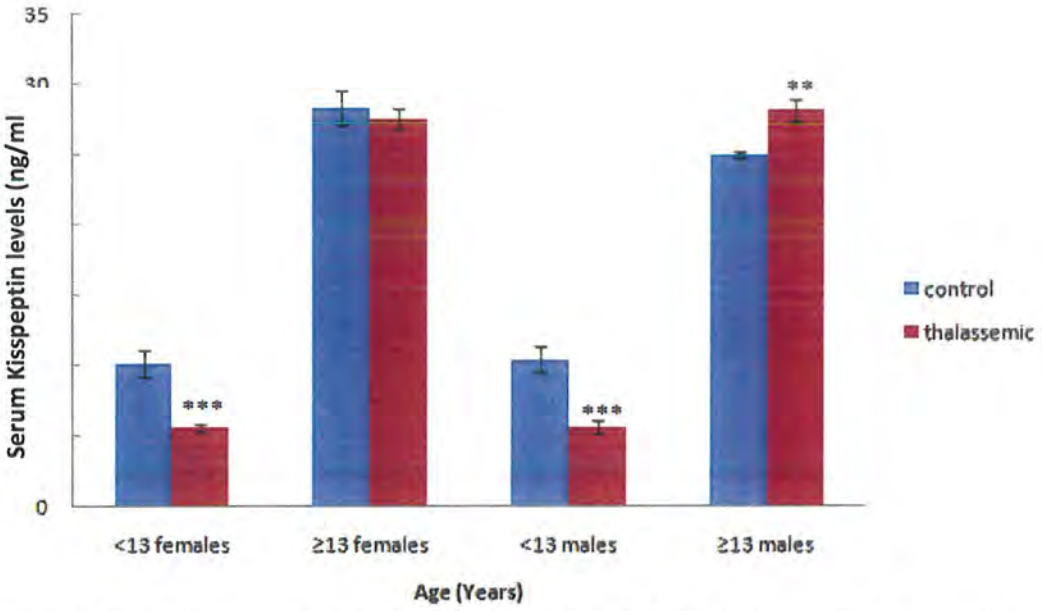


Figure 2.1: Mean ± SEM of serum Kisspeptin of female and male thalassemic patients along with their corresponding control of different age groups. **=P<0.01, value vs corresponding control, ***=P<0.001, value vs corresponding control.

Serum FSH levels (mIU/mL)

Comparison of serum FSH levels of male and female thalassemic patients with their corresponding control of different age groups is shown in Figure 2.2. FSH in < 13 years thalassemic females (5.20 ± 0.51 mIU/mL) was significantly raised (P<0.001) than control (1.30 ± 0.13 mIU/mL). Similarly thalassemic females of ≥ 13 years also had significantly higher (P<0.001) FSH levels (5.54 ± 0.49 mIU/mL) than the control group (1.80 ± 0.41 mIU/mL). On comparison, FSH was 4.19 ± 0.54 mIU/mL in thalassemic males of < 13 years of age, which was higher (P<0.01) than control (1.57 ± 0.17 mIU/mL). Significantly higher (P<0.001) FSH levels were significantly raised (P<0.01) in thalassemic males of ≥ 13 years of age (4.03 ± 0.42 mIU/mL) as compared to control (2.10 ± 0.18 mIU/mL).

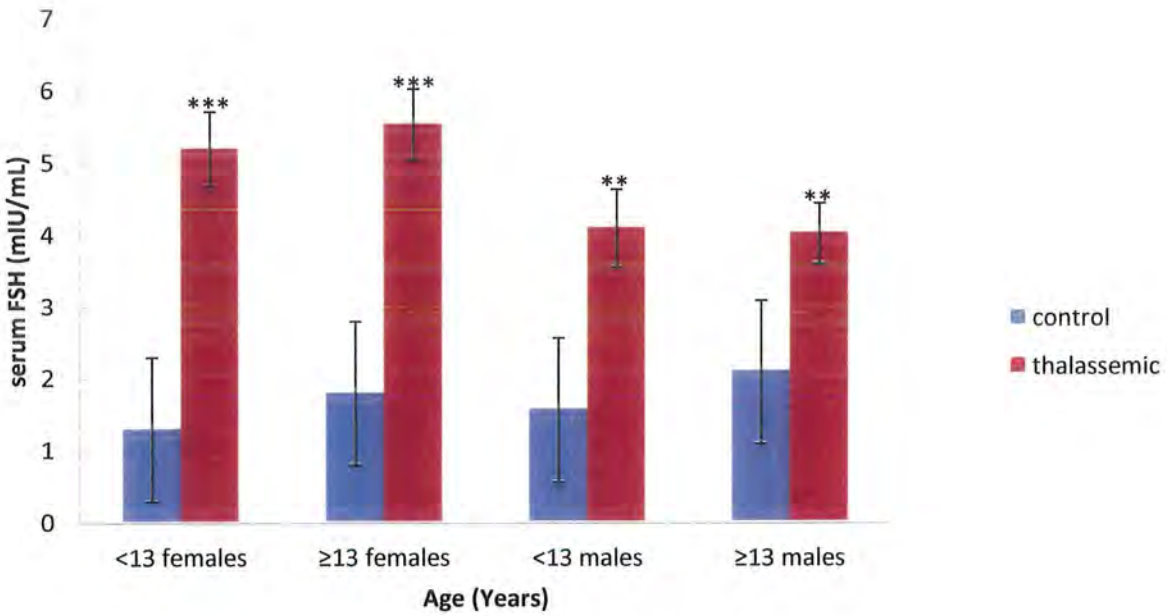


Figure 2.2: Mean ± SEM of serum FSH levels of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001, value vs corresponding control.

Serum LH levels (mIU/mL)

Comparison of serum LH levels of male and female thalassemic patients with their corresponding control of different age groups is represented in Figure-2.3. There was no significant difference in the serum LH levels lf < 13 years thalassemic females (0.88 ± 0.08 mIU/mL) on comparison with the control group (0.58 ± 0.16 mIU/mL). Similarly, serum LH levels of ≥ 13 years thalassemic females (2.58 ± 0.28 mIU/mL) were not significantly different from the control group (2.69 ± 0.37 mIU/mL). LH in < 13 years thalassemic males (0.95 ± 0.05 mIU/mL) was higher (P<0.001) than control (0.39 ± 0.04 mIU/mL). While serum LH levels of thalassemic males (1.84 ± 0.17 mIU/mL) of ≥ 13 years were significantly reduced (P<0.001) than control (3.25 ± 0.21 mIU/mL).

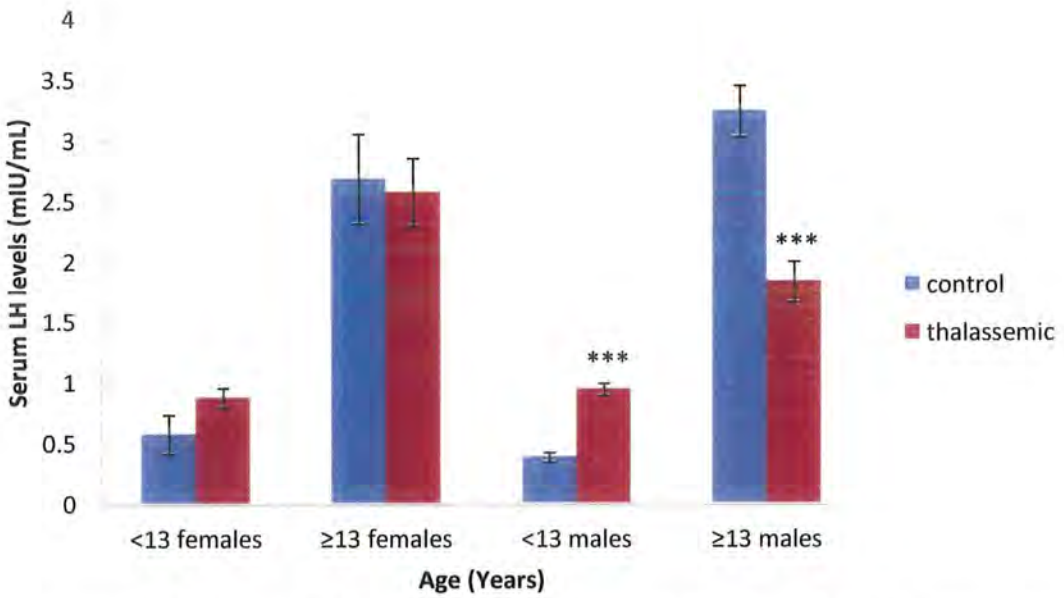


Figure 2.3: Mean ± SEM of serum LH levels of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001, value vs corresponding control

Estradiol levels (pg/mL)

On comparison of serum Estradiol in thalassemic females of < 13 years (3.61 ± 0.33 pg/mL) which was significantly (P<0.001) reduced levels from the control group (22.56 ± 4.64 pg/mL). Similarly, ≥ 13 years thalassemic females serum Estradiol levels (37.82 ± 2.70 pg/mL) were significantly not different from the control group (33.6 ± 1.20 pg/mL). Comparison of serum Estradiol (pg/mL) levels of female thalassemic patients with their corresponding control of different age group is shown in Table-2.2.

Table 2.2: Mean ± SEM of Estradiol (pg/mL) levels of female thalassemic patients with their corresponding control of different age groups.

Age (Years)	Gender	Groups	Estradiol (pg/mL)
< 13	Females	Control n=25	22.56 ± 4.64
		Thalassemic n=50	3.61 ± 0.33***
≥ 13		Control n=25	33.36 ± 1.20
		Thalassemic n=50	37.82 ± 2.70

Values are expressed in Mean ± SEM. ***=P<0.001, value vs corresponding control

Testosterone levels (ng/mL)

High Testosterone levels (P<0.001) were observed in thalassemic males of < 13 years of age (0.95 ± 0.05 ng/mL) as compared to control group (0.39 ± 0.04 ng/mL). While thalassemic males of ≥ 13 years had non significant Testosterone levels (3.97 ± 0.16 ng/mL) as compared to control (3.86 ± 0.13 ng/mL). Comparison of Testosterone levels of male thalassemic patients with their corresponding control of different age groups is represented in Table-2.3.

Table 2.3: Mean ± SEM of Testosterone (ng/mL) levels of male thalassemic patients with their corresponding control of different age groups.

Age (Years)	Gender	Groups	Testosterone (ng/mL)
< 13	Males	Control n=25	0.39 ± 0.04
		Thalassemic n=50	0.95 ± 0.05***
≥ 13		Control n=25	3.86 ± 0.13
		Thalassemic n=50	3.97 ± 0.16

Values are expressed in Mean ± SEM, ***=P<0.001, value vs corresponding control

Correlation of serum FSH (mIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in thalassemic patients of different age groups.

In thalassemic females of < 13 years of age serum FSH (mIU/mL) has a non significant negative correlation (r= -0.197) with BMI and (r= -0.203) with Hemoglobin (gm/dl) levels. It also has non significant positive correlation with Kisspeptin (ng/ml) levels (r= 0.004). While serum FSH (mIU/mL) levels have significant (P<0.001) negative correlation with serum Ferritin (r= -0.511) (ng/mL) levels. In thalassemic patients of ≥ 13 years serum FSH (mIU/mL) shows non significant (P<0.001) negative correlation (r= -0.095) with BMI similarly, there is non significant negative correlation with Hemoglobin (r= -0.188) (gm/dl) and serum Ferritin (ng/mL) levels (r= -0.187) and Kisspeptin (r= -0.198). Serum FSH (mIU/mL) levels in < 13 years thalassemic males has a significant positive correlation with BMI (r=0.296) and non significant negative correlation with Kisspeptin (ng/ml) levels (r= -0.253) while there is non significant negative correlation with serum Ferritin (ng/mL) levels (r= -0.134). There is a significant (P<0.001) negative correlation of serum FSH (mIU/mL) with Hemoglobin (gm/dl) levels (r= -0.479). Serum FSH (mIU/mL) levels in ≥ 13 years thalassemic males show a non significant positive correlation with BMI (r= 0.217) and serum Ferritin (ng/mL) levels (r= 0.033). Similarly serum FSH levels have a non significant negative correlation with Kisspeptin (ng/ml) levels (r= -0.079). There is significant (P<0.05) negative correlation with Hemoglobin levels (r= -0.346). Correlation of serum FSH (mIU/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in control and a thalassemic patient in different age groups is displayed in Figure-2.4,2.5,2.6 and 2.7.

Table 2.4: Correlation of FSH and LH with BMI, serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic female and male patients of different age groups.

Age (Years)	**Gender**	**Hormone (mIU/mL)**	**Groups**	**BMI (Kg/m^2)**	**Hb (gm/dl)**	**Ferritin (ng/mL)**	**Kisspeptin** (ng/ml)
< 13	Females	FSH	Thalassemia n=50	-0.197	-0.203	-0.511***	0.004
≥ 13			Thalassemia n=50	-0.095	-0.188	-0.187	-0.198
< 13	Males		Thalassemic n=50	0.296*	-0.479***	-0.134	-0.025
≥ 13			Thalassemic n=50	0.217	-0.346*	0.033	-0.079
< 13	Females	LH	Thalassemia n=50	-0218	-0.386**	-0.220	-0.132
≥ 13			Thalassemic n=50	0.109	0.110	0.230	0.021
< 13	Males		Thalassemic n=50	0.196	-0.223	0.014	-0.274
≥ 13			Thalassemic n=50	0.096	0.235	-0.034	-0.114

*=P<0.05, **=P<0.01, ***=P<0.001, value are considered significant.

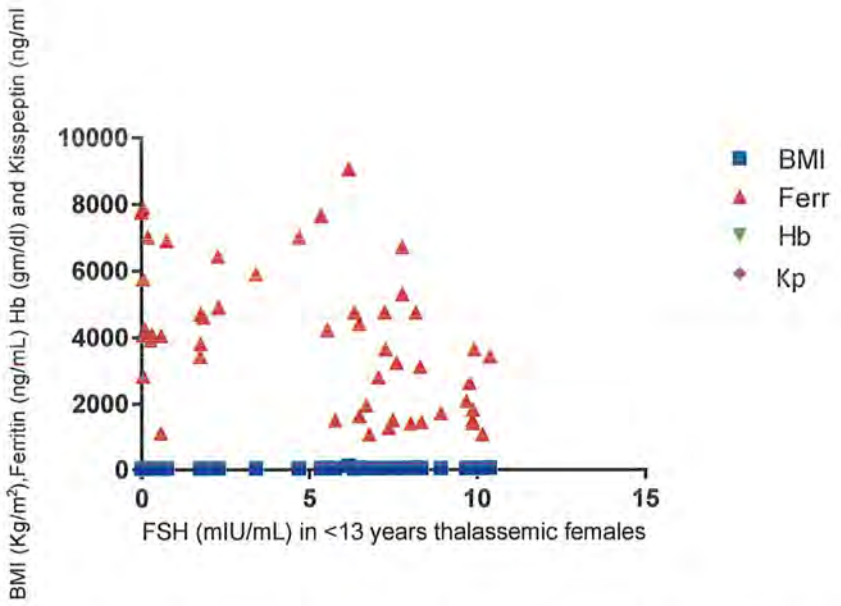


Figure 2.4: Correlation of serum FSH (mIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of <13 years.

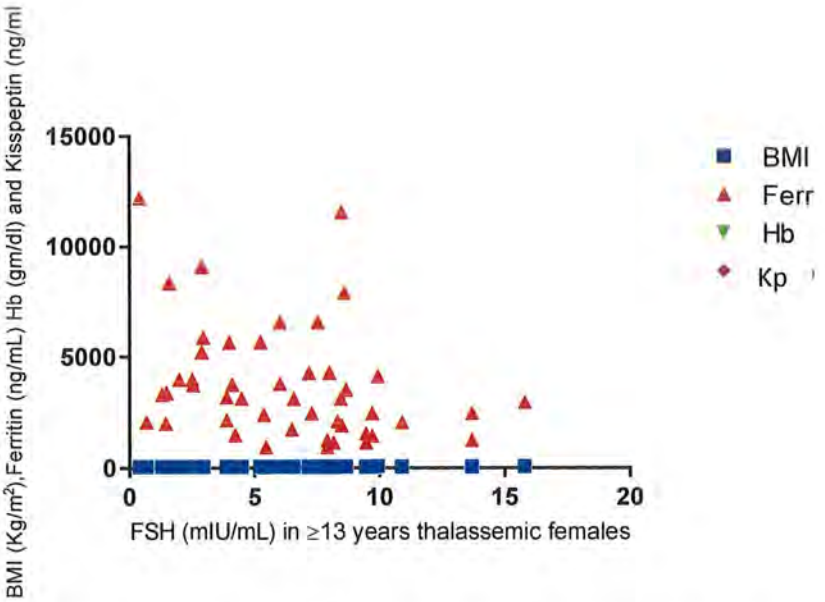


Figure 2.5: Correlation of serum FSH (mIU/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of ≥13 years.

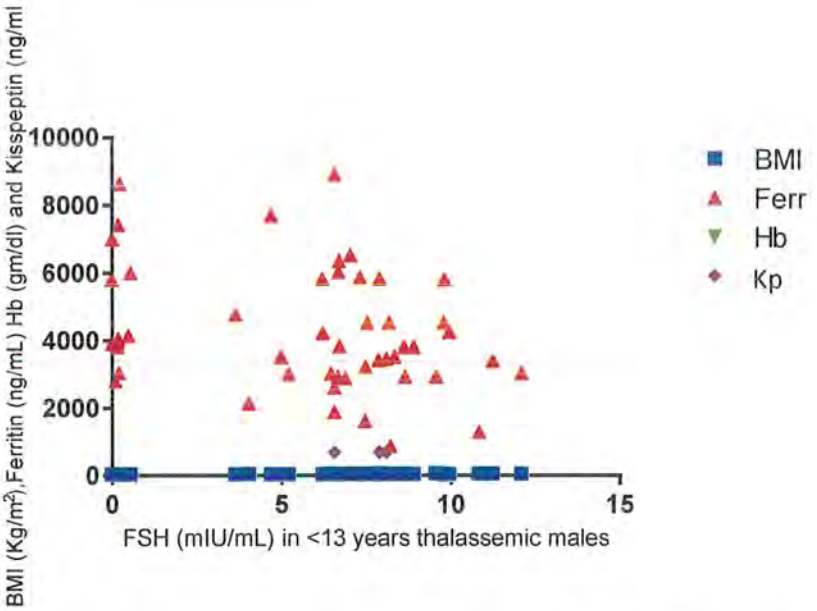


Figure 2.6: Correlation of serum FSH (mIU/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic males of <13 years.

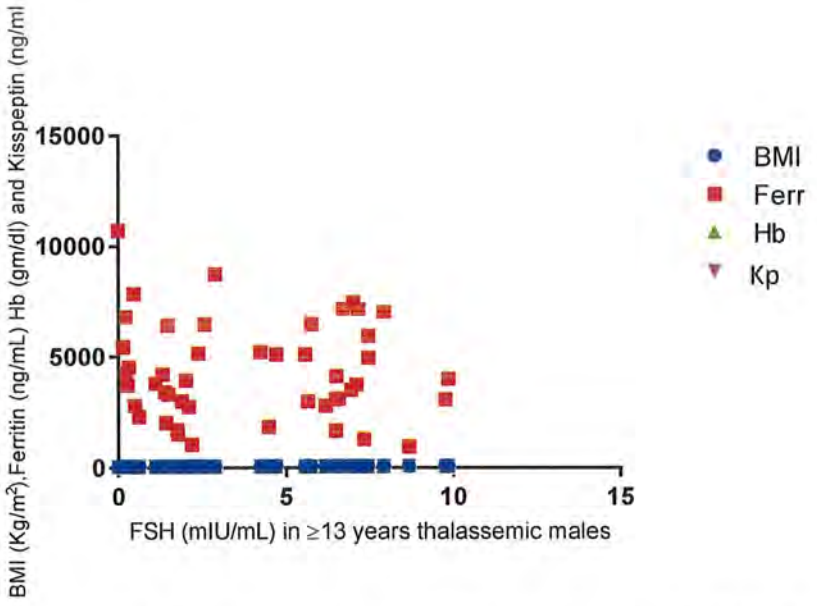


Figure 2.7: Correlation of serum FSH (mIU/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic males of ≥13 years.

Correlation of Serum LH (mIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in thalassemic patients of different age groups

Correlation of serum LH (mIU/mL) with BMI (Kg/m^2), serum Ferritin, Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in control and thalassemic patients in different age groups is presented in Table-2.4 and Figure-2.8, 2.9, 2.10 and 2.11. In thalassemic females of < 13 years age LH (mIU/mL) has a significant ($P<0.01$) negative correlation with BMI (Kg/m^2) (r= -0.386). LH has a non significant negative correlation with BMI (Kg/m^2) (r= -0.218), Ferritin (ng/mL) (r= -0.220) and Kisspeptin (ng/ml) (r= -0.132). While LH has a significant ($P<0.01$) negative correlation with Hemoglobin levels in thalassemic females of ≥ 13 years serum LH (mIU/mL) levels show a non significant positive correlation with BMI (Kg/m^2) (r= 0.109), serum Ferritin (ng/mL) levels (r= 0.230) and Hemoglobin (gm/dl) levels (r= 0.110) and Kisspeptin (ng/ml) levels (r= -0.021).

Serum LH (mIU/mL) levels in < 13 males show a non significant positive correlation with BMI (Kg/m^2) (r= -0.0.196) and Ferritin (ng/mL) (r=0.14). Similarly non significant negative correlation of serum LH (mIU/mL) levels is present with serum Hemoglobin (gm/dl) (r= -0.223) and Kisspeptin (ng/ml) levels (r= -0.274). Serum LH levels in ≥ 13 years thalassemic males had a non significant positive correlation with Hemoglobin (gm/dl) levels (r= 0.235) and BMI (Kg/m^2) (r= 0.096) non significant negative correlation with serum Ferritin (ng/mL) levels (r= -0.034) and Kisspeptin (ng/ml) levels (r= -0.114).

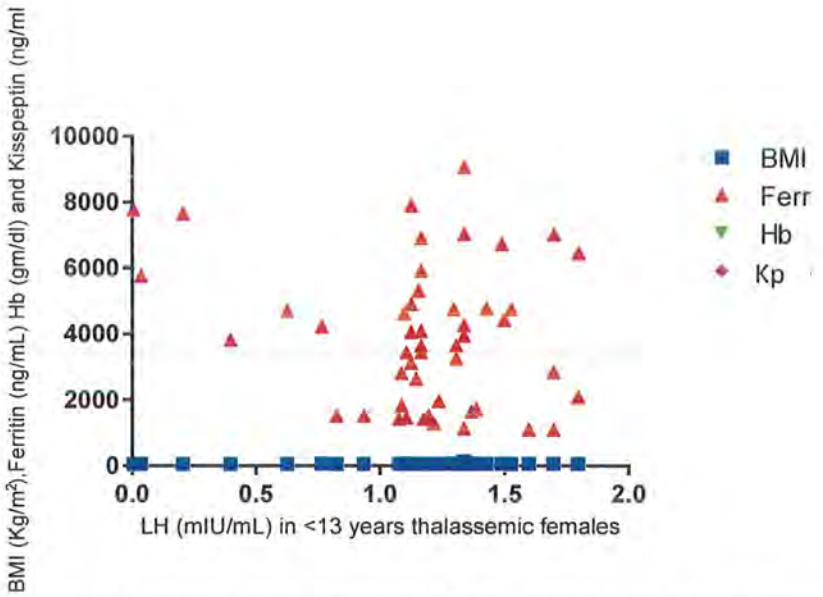


Figure 2.8: Correlation of serum LH (mIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of <13 years.

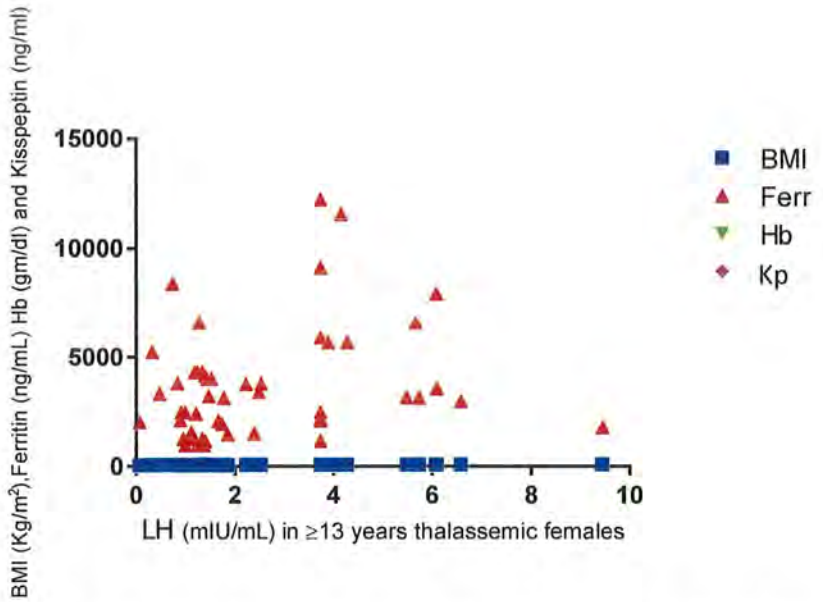


Figure 2.9: Correlation of serum LH (mIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of ≥13 years.

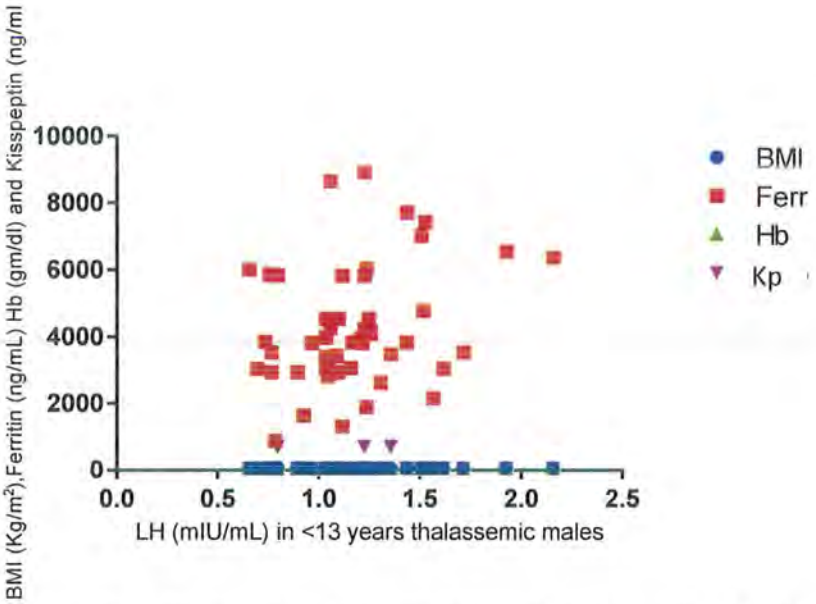


Figure 2.10: Correlation of serum LH (mIU/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic males of <13 years.

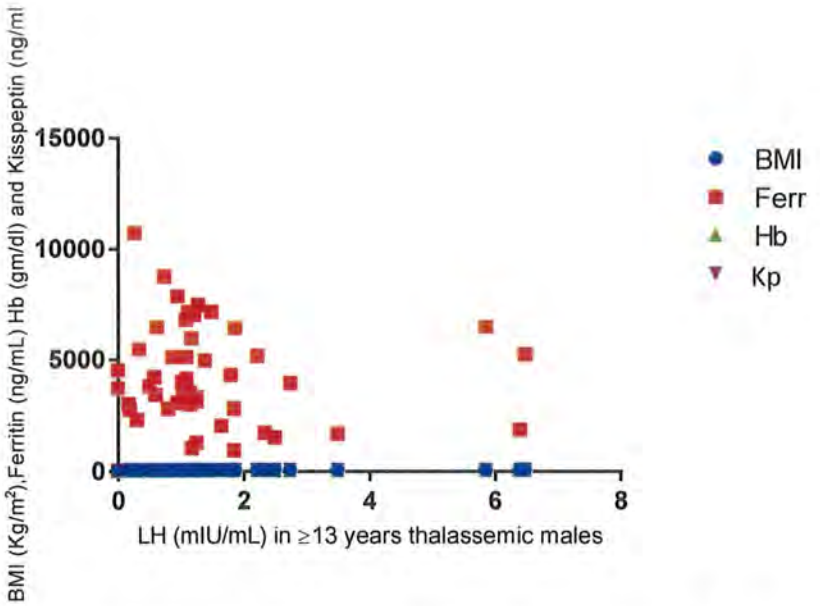


Figure 2.11: Correlation of serum LH (mIU/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic males of ≥13 years.

Correlation of Estradiol (pg/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in control and thalassemic male and female patients in different age groups.

In thalassemic females of <13 years Estradiol levels have a non significant positive correlation with BMI (Kg/m^2) (r= 0.002), serum Ferritin (ng/mL) (r= 0.240), Hemoglobin (gm/dl) levels (r= 0.065) and Kisspeptin (ng/ml) (r= 0.128). In thalassemic females of ≥ 13 years, Estradiol had a positive significant (P<0.05) correlation with BMI (r= 0.318) and Hemoglobin (r=0.286). While a non significant correlation was present between Estradiol and serum Ferritin (r= 0.215) and Kisspeptin (r=0.076) Correlation of Estradiol with BMI, serum Ferritin, Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in control and thalassemic male and female patients in different age groups is shown in Table-2.5 and Figure-2.12 and 2.13.

Correlation of Testosterone (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/mL) levels in control and thalassemic male and female patients in different age groups.

Correlation of testosterone (ng/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) levels in control and thalassemic male and female patients in different age groups is displayed in Table-2.5 and Figure2.14 and 2.15. In <13 years thalassemic males testosterone had a non significant negative correlation with Hemoglobin (gm/dl) (r= -0.223) and Kisspeptin (r= -0.274). While a positive non significant existed between testosterone and BMI (Kg/m^2) (r= 0.196) and serum Ferritin (ng/mL) (r= 0.014). Similarly in ≥ 13 thalassemic males testosterone had a non significant correlation with BMI (r= -0.202) and Ferritin (r= -0.062). Similarly non significant positive correlation existed between testosterone and Hemoglobin (r=0.109) and Kisspeptin (r= 0.197).

Table 2.5: Correlation of Estradiol and Testosterone with BMI, Serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic male and female patients of different age groups.

Age (Years)	**Gender**	**Hormone**	**Groups**	**BMI (Kg/m^2)**	**Hb (gm/dl)**	**Ferritin (ng/mL)**	**Kisspeptin** (ng/ml)
< 13	Females	Estradiol (pg/mL)	Thalassemic n=50	0.002	0.065	0.240	0.128
≥ 13			Thalassemic n=50	0.318*	0.286*	0.215	0.076
< 13	Males	Testosterone (ng/mL)	Thalassemic n=50	0.196	-0.223	0.014	-0.274
≥ 13			Thalassemic n=50	-0.202	0.109	-0.062	0.197

*=P<0.05, value are considered significant.

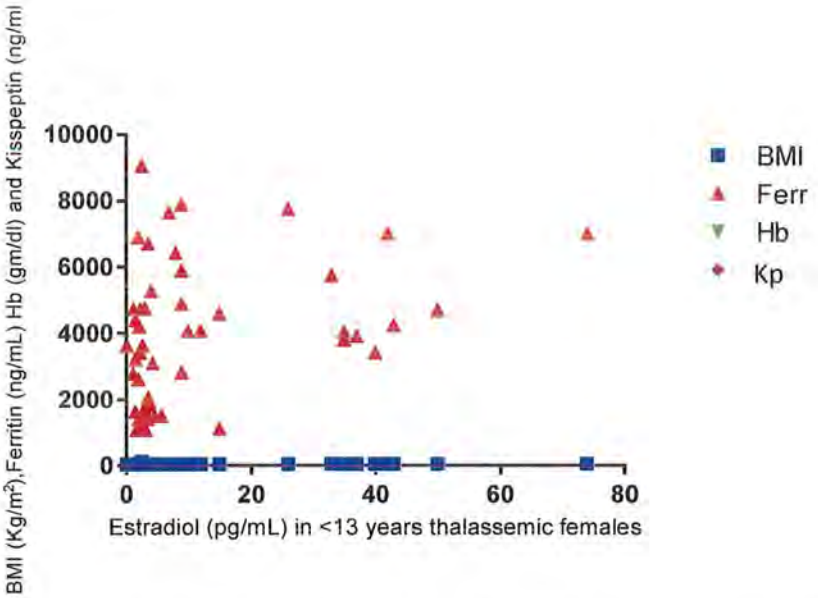


Figure 2.12: Correlation of Estradiol (pg/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of <13 years.

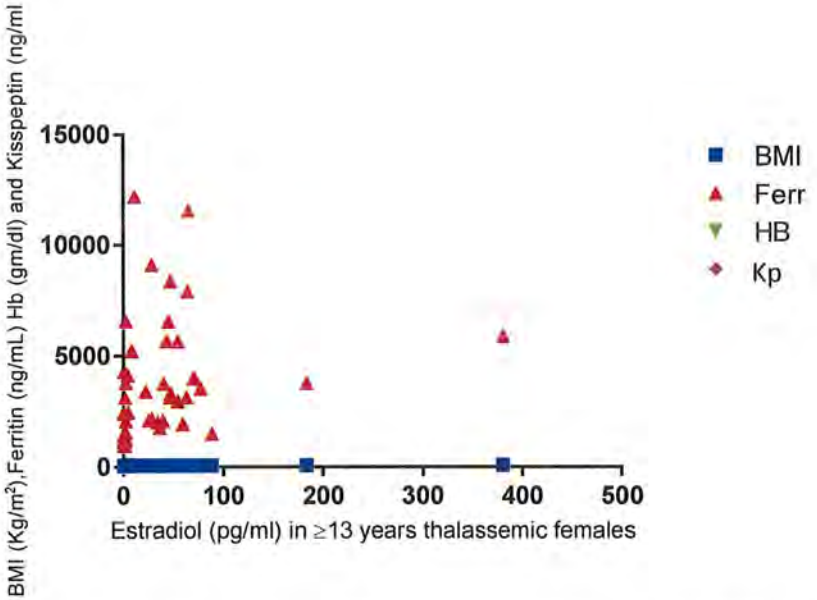


Figure 2.13: Correlation of Estradiol (pg/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of ≥13 years.

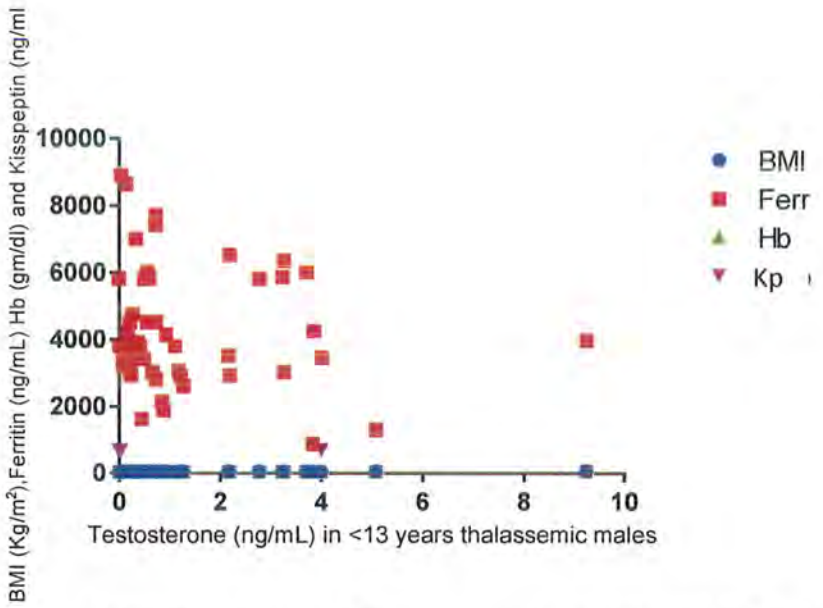


Figure 2.14: Correlation of Testosterone (ng/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic males of <13 years.

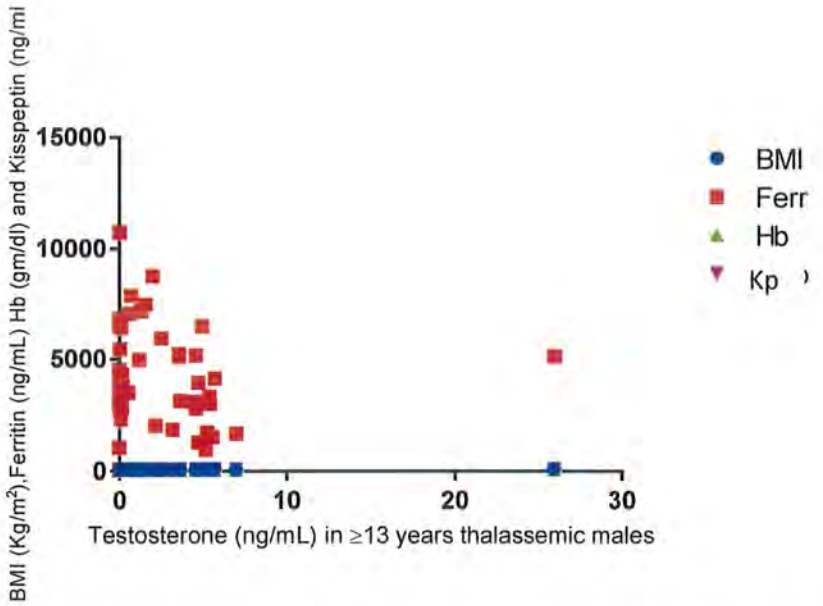


Figure 2.15: Correlation of Testosterone (ng/mL), with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Kisspeptin (ng/ml) thalassemic females of ≥13 years.

Discussion

Thalassemic patients are dependent on blood transfusions to maintain the levels of Hemoglobin and packed cell volume in their blood. Transfusion and iron-chelation therapy has prolonged and improved the quality of life in these patients (Borgna-Pignatti *et al.*, 2004). Such a treatment, however, leads to chronic iron overload affecting the endocrine glands (Abdulazahra *et al.*, 2011). In our study we observed that patients suffering from thalassemia major present with endocrine disorders and pubertal delay.

In another study carried out by Al-Rimawi *et al.*, (2005) showed that there was a significant difference in the frequency and regularity of using chelation therapy between pubertal and delayed pubertal groups. Whereas in our study the age of starting chelation therapy was 6-8 months and the patients were on regular blood transfusion and chelation therapy.

In previous study carried out by Hussein and Mohsen (2013), comparison of measured parameters between male and female thalassemic patients of 3-11 years age group were done which showed high Hemoglobin levels in male patients in comparison with female thalassemic patients. The change in Hemoglobin levels can be easily explained by the genetic changes between male and female, males have higher Hemoglobin levels than females. While comparing with our study Hemoglobin levels of thalassemic males and females were significantly low as compared to the control groups. Similarly, in our study serum Ferritin level were high as compared to the control group which was similar to the results reported by Adil *et al.*, (2012), suggesting that increased serum Ferritin levels are related to the endocrinopathies.

Increased serum Ferritin levels were associated with increased incidence of endocrinopathies along with subsequent increase in the serum levels of calcium (Ca), alkaline phosphate and parathyroid hormone levels (Hussein and Mohsen 2013).

Thalassemic children frequently present with growth retardation which may be attributed to their diversion from caloric resources resulting from ineffective

erythropoiesis, along with the effects of anemia. Since hyper-transfusion has been shown to frequently restore normal growth rates (Viprakasit *et al.*, 2001). However, the adolescent growth spurt is often delayed, even in children who are hyper-transfused, unless intensive iron chelation therapy is instituted early in life (Theodoridis *et al.*, 1998).

Previous study on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overweight and 6.5% were categorized as obese (Adil *et al.*, 2012).Whereas, thalassemic patients males and females of all groups included in our study had reduced BMI ($P<0.001$) as compared to the control group.

A high prevalence of endocrine abnormalities in beta thalassemia major patients is reported by Zervas *et al.*, (2002). Relationship between the level of Ferritin and the development of endocrinopathies suggest that serum Ferritin is used as a prognostic marker for survival of these thalassemic patients, prognosis for survival is excellent when serum Ferritin concentration is below 2500ng/ml in thalassemic patients (Costin *et al.*, 1979).Whereas the study results by Zervas *et al.*, (2002) reported absence of such a relation.

During the course of beta thalassemia major, multiple endocrine disorders may develop mainly due to iron overload. Growth retardation and HPG axis dysfunction represent the commonest disorders of the endocrine system (De Sanctis, 2002).

The major breakthrough in the field of reproduction came in 2003, Seminara *et al.*, (2003) showed that GPR54 was crucial for normal puberty. In our study there were increased levels of serum Kisspeptin in thalassemic females of <13 and ≥13 years as compared to the control group. Whereas, high levels of serum Kisspeptin in ≥13 and ≥13 years thalassemic males were observed as compared to the control group, which can be due the reason that high serum levels of Kisspeptin are required during pubertal years.

Work done by Hegazi *et al.*, (2013) revealed that thalssemic males of 4-12 years of age had high levels of serum iron, Ferritin and low levels of FSH ($P<0.05$).

Meanwhile, no significant difference was detected between LH levels of thalassemic patients when compared with the control group.

Similarly, Dundar *et al.*, (2007) found that the serum level of FSH in male thalassemic patients were significantly lower as compared to control group. Similarly, in our study serum FSH levels in thalassemic males and females <13 years and ≥13 year females were higher than the control group. Whereas, serum FSH levels in thalassemic males <13 years and ≥ 13 years were low as compared with the control group. Serum LH levels in less than 13 years thalassemic male patients were higher ($P<0.001$) than the control group, similarly serum LH levels of thalassemic males ≥13 years were also significantly reduced ($P<0.001$) than the control group.

Findings of previous work done by Hegazi *et al.*, (2013) showed low serum levels of LH, FSH and Testosterone in thalassemic male of 12-18 years of age as compared to control group. These results were in accordance with studies done by (Anoussakis *et al.*, 2008; Dundar *et al.*, 2007; Vahidi *et al.*, 2003). Which is contrary to the results of our study as thalassemic male of <13 years had high serum Testosterone levels when compared with the control group.

Low levels of serum LH, FSH, progesterone and Estradiol were detected in studies done by Hegazi *et al.*, (2013) on thalassemic females of 12-18 years when compared to control group. Our study had a similar finding as significantly low Estradiol levels were observed in <13years thalassemic females as compared to the control group.

In our study negative correlation of FSH levels with serum Ferritin level ($r = -0.511$) in <13 years thalassmic females was observed, this result is supported by the studies done by Hegazi *et al.*,(2013) in 4-12 years thalassemic females. The results demonstrated that there was a significant alteration in the activity of gonadotropins (LH and FSH), in the anterior pituitary gland that takes place early in life and affects the function of the gonads at puberty, due to iron overload. Hegazi *et al.*, (2013) found in thalassemic females of 14-18 years age that there existed a negative correlation between serum Ferritin levels and FSH in accordance with Papadimas *et al.*, (1996). These results are contrary to our study results as in ≥13 years thalassemic females there is a non significant negative correlation of FSH with BMI ($r=-0.455$), serum Ferritin ($r=-0.350$) and Hemoglobin levels ($r=-0.358$).

These results confirmed that there is effect of iron overload on the activity of the pituitary secretion of FSH. Hegazi *et al.*, (2013) revealed a significant elevation of the serum levels of iron and Ferritin and a significant decrease in the mean serum level of FSH ($P<0.05$) in 4-12 years thalassemic males. Our study also showed that FSH had a negative correlation with Hemoglobin in thalassemic males <13 years ($r=-0.479$) and ≥13 years thalassemic males ($r= - 0.346$) and a significant positive correlation with BMI ($r=0.296$) in <13 years thalassemic males.

In our study serum LH also had a negative correlation with Hemoglobin levels ($r = -0.386$) in <13years thalassemic females whereas, Al-Rimawi *et al.*, (2006) observed that when the gonadotropin levels in the thalassemic delayed puberty patients were compared with the constitutional delayed puberty patients, there were no significant difference in the basal hormonal levels, but the response to GnRHa administration was extremely low in the thalassemic delayed puberty group compared with the response ($P<0.001$) in constitutional delayed puberty group. This indicated a defective gonadotropin reserve in the gonadotropic cells in the thalassemic delayed puberty group compared with normal pituitary function in the constitutional delayed puberty group (Al-Rimawi *et al.*,(2006).

In our study Estradiol in ≥13 years thalassemic females had a positive correlation with BMI ($r= 0.318$) and Hemoglobin levels ($r= 0.286$) These finding are similar to results obtained by Hegazi *et al.*, (2013). Other investigators demonstrated through (magnetic resonance imaging) that pituitary gland atrophy in beta thalassemic patients with hemochromatosis (Soliman *et al.*, 2000) and the signal intensity reduction in the anterior lobe of the pituitary gland correlated with serum Ferritin level and the severity of pituitary dysfunction (Sparacia *et al.*, 2000). Furthermore, even a modest amount of iron deposition within the gland could interfere with its function (De Sanctis, 2002).

Patients with transfusional iron overload begin to develop pituitary iron overload in the first decade of life; however, significant iron deposition were observed beginning in the second decade. Heavy pituitary iron deposition were predictive of hypogonadotropic hypogonadism in these patients (Noetzli *et al.*, 2011).This explains our study results as there is development of pituitary dysfunction more in older age patients than younger age patients. In the study carried out by Noetzli *et al.*, (2011),

the serum levels of Testosterone were significantly lower among male thalassemic patients ≥13 years than control (P<0.05) indicating pituitary-gonadal dysfunction. These results are not in accordance with our work as our thalassemic patients of <13 years have significantly high Testosterone levels while ≥ 13 thalassemic males have no significant decrease levels.

Conclusion:

Our study revealed that the levels of Kisspeptin in thalssemic females and males of < 13 years had reduced levels but, ≥13 years were raised but, at the same time the levels FSH and LH were significantly deranged. These findings are suggestive that hormone production by hypothalmus was correctly secreting Kisspeptin according to the pubertal time frame but, the levels of hormones secreted by anterior pituitary and the gonads were deranged due to damage caused by iron overload at pituitary and gonadal level. Along with disturbed hypothalamic pituitary gonadal axis there was decreased BMI, low Hemoglobin and raised serum Ferritin levels. These findings are indicating that treatment with double chelation from early life should be considered for better outcomes in thalassemic patients.

Chapter # 3

Hypothalmic neurosecretory dysfunction of Growth and Thyroid hormone in thalassemic patients of pubertal age group undergoing repeated blood transfusions

Abstract

Background: Beta thalassemia syndrome are a group of hereditary blood disorders characterized by reduced or absent beta globin chain synthesis, resulting in reduced hemoglobin in red blood cells (RBC), decreased RBC production and anemia. Transfusion with chelation therapy has significantly extended life expectancy but, leads to complications due to iron overload.

Materials and Methods: The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control). Growth Hormone (GH), thyroid stimulating hormone (TSH), triiodothyronine (T_3), thyroxine (T_4) were analyzed and correlation of these hormones with body mass index (BMI), serum Ferritin, hemoglobin (Hb) and Kisspeptin levels were done.

Results: Growth Hormone levels were significantly reduced in <13 years male and female thalassemic patients while the levels were significantly raised in ≥13 male and female thalassemic patients. T_3 levels in ≥ 13 years thalassemic females were significantly raised as compared to control. T_4 and TSH in <13 year thalassemic male were significantly reduced than control and ≥13 thalassemic males had significantly raised as compared to the control.GH in <13 years had a positive correlation with Kisspeptin (r=0.310). T_3 in ≥13 years thalassemic females had a significant negative correlation with BMI (r=-0.408) and (P<0.05) with Hb (r=0.329) and a positive correlation with Kisspeptin (r=0.317).In <13 years thalassemic males T_3 had a positive correlation with Ferritin (r=0.523).T_4 in ≥13 thalassemic female had a positive correlation with BMI (R-0.333), Ferritin (R=0.317) and Hb(r=0.328). There was a positive correlation of T_4 with Hb(r=0.422) in <13 year thalassemic males.TSH in ≥13 thalassemic females had a positive correlation (P<0.001) with BMI(r=0.487), Ferritin(r=0.531) and (P<0.01) with Hb (r=0.394).In ≥13 thalassemic males TSH had a positive correlation (P<0.01) with Ferritin (r=0.446).

Conclusion: Regular blood transfusion followed by iron chelation therapy is just a supportive treatment for the disease of thalassemia which is associated with serious complications. But, during this supportive treatment, the magnitude of the body iron burden is the principal determinant of clinical outcome for the prime goal i.e of iron-chelating therapy in patients with thalassemia major is to control body iron. The optimal body iron should be reduced both to prevent the adverse effects from the iron-chelating agent to prevent the risk of complications from iron overload. The apparent facts is that upon reaching age of puberty thalassemic patients develop growth retardation and pubertal failure. Thalassemic patients are short, have low growth rate and BMI and have either delayed or absent pubertal spurt, which is related to low hemoglobin and high Ferritin levels and sub-optimal iron chelation therapy. These defects start early in life but, become becomes obvious after the age of 8 years. In developing countries, poor socio-economic background adds up to the problem. Therefore, effective alternate chelation regimens should be considered to improve the complication resulting from chelation therapy.

Hypogonadotropic hypogonadism is caused by the selective loss of pituitary gonadotropin function. In such thalassemic patients with both GH deficiency and hypogonadism, low dose sexual steroid treatment should be considered either as an alternative or an additional treatment before starting GH therapy. Important functions of GH, not only on growth but also on lipid and protein metabolism and on normal long-term cardiac function, should be considered thalassemic children who may benefit from GH and treat them effectively long-term so that they may have a better quality of life and possibly a longer survival rate.

Introduction

Beta thalassemia syndrome are a group of hereditary blood disorders characterized by reduced or absent beta globin chain synthesis, resulting in reduced Hemoglobin in red blood cells (RBC), decreased RBC production and anemia. In thalassemia major defective gene are inherited from both parents and clinical features appear by 6 to 24 months of age. Transfusion with chelation therapy has significantly extended life expectancy but, leads to complications due to iron overload (Borgna *et al.*, 2004). Iron toxicity causes development of Reactive oxygen species (ROS) that lead to DNA damage (Mehrvar *et al.*, 2008). Iron deposition in gonads (primary), pituitary gland (secondary) and hypothalamus (tertiary) leads to Hypogonadism. Whereas pituitary iron overload is prevalent in thalassemia major (Wood *et al.*, 2010; Farmaki *et al.*, 2010). Early therapeutic intervention can avert irreversible damage (Chatterjee and Bajoria, 2010; Kohgo, 2008).

Disturbance of the Hypothalmic pituitary gonadal (HPG) axis leads to reduced target Height and pubertal delay. Anemia also causes various endocrinopathies (Chatterjee *et al.*, 1993). Gonadotropin releasing hormone (GnRH) is main regulator for onset of puberty (Kyriakou, 2009). Primary excitatory neurotransmitter of GnRH secretion is glutamate along with leptin, Kisspeptins (Chatterjee and Katz, 2000; Apter *et al.*, 1993). Currently, the role of Kisspeptin for the onset of puberty and fertility has been recognized, it functions through the G-protein coupled receptor (GPR54), which stimulates GnRH neurons (Shander *et al.*, 2009; Roseweir and Millar, 2009). Pituitary iron deposition occurs in second decade of life (Marshall and Tanner,1966). Iron toxicity on the adipose tissue causes altered physiological function of leptin that effects body mass index (BMI) and developmental process (d'Anglemont de Tassigny *et al.*, 2007; Plant and Durant, 1997). Serum Ferritin >1000 ng/ml leads to iron overload (Vichinsky, 2001;Olivieri and Brittenham, 1997).

Primary gonadal dysfunction is a late finding during which, irreversible damage to HPG axis has already occurred, strong association has been observed between pituitary volume and appearance of clinical disease which suggest that secondary hypogonadism is the dominant etiology, whereas tertiary hypogonadism has not been explored. Similarly, previous studies have not explored the anatomical level of

hypothalmic pituitary (HP) dysfunction. Biochemical parameters are more reliable than clinical parameters as patients with profound pituitary iron overload may have no clinical proof of hypogonadism (Chatterjee and Bajoria, 2010; Fung *et al.*, 2006). Age of iron deposition and stage at which it causes hypothalmic pituitary dysfunction is unknown, presence of iron in an organ does not necessarily suggest that damage has occurred, a silent period of pituitary siderosis occurs before hypogonadism develops (Chatterjee and Katz, 2000). Definite levels of serum Ferritin causing significant iron toxicity and organ damage are still unknown.

Advancement in medical treatment has improved the survival rate of thalassemic patients (Olivieri *et al.*, 1994). Along with endocrine complications growth retardation is a frequent problem encountered by the thalassemic patients. Etiology of growth retardation is based on a number of factors like disease itself, toxic effects of the drugs used for chelation therapy (Caruso *et al.*, 1998; De Sanctis *et al.*, 1996), toxicity of iron overload and malnutrition and endocrine complications (Fuchs *et al.*, 1997). The mechanism of growth retardation in these patients is not fully exemplified however, regarding endocrine complications along with thyroid and gonadal dysfunction (Roth *et al.*, 1997; Jain *et al.*, 1995), Growth Hormone (GH), insulin like growth factor (IGF),insulin like growth factor binding protein (IGFBP) also play an important role (Soliman *et al.*, 1998; Low *et al.*, 1998).

It also effects the adult life activities and effects the metabolism of the whole body (Michaud *et al.*, 1991; Dunger *et al.*, 1990). Thyroid replacement therapy is advised in all individuals if the level of TSH is >10 (μIU/mL) but special consideration is for the thalassemic patients to prevent the risk of developing cardiovascular problems (De Sanctis *et al.*, 2008).

Cause of retarded growth in thalassemic patient is complex and multi-factorial various factors like chronic hypoxia secondary to anemia (pre-transfusion Hb is below 8.5g/l), deficiency of Growth Hormone due to defective hepatic, biosynthesis of somatomedin, insulin like growth factor-1 (IGF-1) and sex steroid deficiency play main their role. Hypogonadism, Hypothyroidism, hypoparathyroidism, low bone mass, and diabetes mellitus also effect but to a lesser extent (Aleem *et al.*, 2000). GH is also one of the hormones secreted by the anterior pituitary which exerts its action

by directly binding to the Growth Hormone receptor (GHR) to effect growth and cause onset of sexual maturity. Growth Hormone receptors are present in various tissues including ovaries. These receptors are present in the granulosa cells of antral follicles and the corpus luteum (Zaczek *et al.*, 2002).

In thalassemic patients the hormonal cause of growth retardation is complex. Growth Hormone plays an important role in growth retardation. Researchers have reported that reduced serum concentration of IGF-I in the presence of normal GH lead to growth retardation due to GH insensitivity. While other studies have found that GH deficiency and GH neurosecretory dysfunction are cause of abnormal growth (Oerter *et al.*, 1993). Patients suffering from beta thalassemia subnormal GH secretion and response of GH to GHRH is also impaired. Autopsy of the pitutary gland of thalassemic patients have shown decrease number of pituitary cells along with mild to moderate siderosis of pituitary, gonads and thyroid gland. These finding are consistent with the presentation of primary or secondary endocrine gland dysfunction observed in thalassemic patients. Thalassemic patients suffer from under nutrition along with zinc deficiency which can be one of the cause of low IGF-I and associated growth disturbances (Costin *et al.*, 1979).

Before the onset of puberty levels of GH and thyroid hormone change as they are the primary hormones essential for growth. GH leads to synthesis of protein, inhibits the formation of fat and carbohydrate, also plays a major role in the proliferation of cartilage cells at the epiphyseal plate leading to linear growth.

Development of the central nervous system is under the effect of thyroid hormone which works along with GH to promote cartilage and bone formation and carry our normal growth. Regulation of growth through the supply of metabolic substrate to cells and interaction with other growth factors to influence fetal growth takes place under the effect of insulin which is esstential for these functions. Growth failure may occur due to excess of certain hormones like cortisol (e.g., in Cushing disease or syndrome, or secondary to high doses of exogenous glucocorticoids). The interaction of gonadal and adrenal steroid hormones with GH along with their independent

effects becomes essential for the normal adolescent growth spurt and sexual maturation (Tanner, 1989).

At the onset of puberty the hormonal regulation of growth becomes very complex. Along with adequate quantity of thyroid hormones and cortisol, Estradiol and testosterone are prerequisites for normal growth (Martha *et al.*, 1992).Activation of GH/IGF-1 axis during puberty results from increase in mean-24 hour GH levels due to raise secretory rate of GH. The increase in the level of GH varies between male and female individual but once puberty is completed then the level of GH comes to prepubertal levels (Albertsson *et al.*, 1994). Spontaneous Growth Hormone secretion and production are augmented by the rising levels of testosterone during puberty. Although the property of testosterone to augment GH secretion is short lived as GH and IGF-1 levels are reduced during later stages of puberty and adulthood despite the raised levels of gonadal steroids (Martha *et al.*, 1992). Low doses of estrogen also stimulate GH secretory activity through increase production of IGF-1 and higher doses inhibit IGF-1 production at hepatic level (Ho *et al.*, 1987).GH and sex steroids are required for the normal pubertal development and deficient levels of either of the hormone (e.g., hypogonadotropic hypogonadism or isolated GH deficiency) lead to an attenuated growth spurt (Liu *et al.*, 1987).

Critical body weight has to attained for supporting the metabolic processes after which sexual competence is achieved. Leptin and thyroxine (T_4) are two hormones that create a link between metabolic status and reproductive processes in an individuals body (Considine *et al.*, 1996).Thyroid hormone plays an important role in brain development and maturation it also takes part in postnatal growth and skeletal development. Exact relation of thyroid hormone and pubertal development is unclear but in majority of cases hypothyroidism is associated with retarded sexual maturation in childern (Longcope, 2000). During adolescence the circulating levels of thyroid hormone fall (Parra, 1980).However, transient increase and fluctuations in T_3 and T_4 levels are observed that are followed by pubertal changes (Dunger, 1990).Thyroid hormone contributes in the onset of puberty and serves as an effective mechanism for rapid growth and energy requirement during this period (Michaud, 1991).

The site at which thyroid hormone acts to cause its facilitative action to allow pubertal reactivation of pulsatile GnRH release either must reside at the level of the brain to directly interface with the hypothalamic GnRH pulse generator or at a peripheral (somatic) site or sites, which in turn communicate with the hypothalamus via a circulating endocrine signal that convey information to the sites where thyroid hormone dependent somatic development takes place (Plant *et al.*, 1989). Medial basal hypothalamus (MBH)-pituitary unit is the site where thyroid hormone signaling takes place as well as the site for putative thyroid hormone-dependent circulating somatic cues signal to the GnRH pulse generator (Krey *et al.*, 1981).

Thyroid gland is also effected by the excess iron circulating in the body which get deposited in thyroid gland leading to fibrosis and ultimate glandular damage. These tissues are susceptible to excess iron incorporation when non-transferrin-bound iron (NTBI) is present. Increase level of iron in the body, lead to saturation of transferrin, therefore, NTBI species circulate in the plasma. Unbound iron, within cells or in plasma, is labile which are able to redox cycle between Fe^{2+} and Fe^{3+}, leading to the generation of reactive oxygen species, ending in lipid peroxidation (Hashemi *et al.*, 2012). The result of lipid peroxidation, under conditions of iron overload, cause generation of both unsaturated (malondialdehyde and hydroxynonenal) and saturated (hexanal) aldehydes. Both have been implicated in cytotoxicity, cellular dysfunction, and cell death (Gabutti,1996).

TSH increases the beta promoter activity by two mechanisms which either involve calcium ion influx through L type Ca^2 channels (LTCCs) and protein kinase C (Oudit *et al.*, 2003).Studies have shown that (LTCCs) are playing key role for mediating NTBI transport in iron overload conditions. These channels are found in great number in pancreas whereas, they are moderately present in thyrotrophs and gonadotrophs, therefore these tissues are at greater risk of developing iron overload (Kaur *et al.*, 2003). In addition, protein kinase C activity is regulated by iron and is effected by adverse effects of iron overload (Hekmatnia *et al.*, 2010).L type Ca^2 channels (LTCCs) and protein kinase C both activity is compromised by iron overload which explains the defective TSH secretion in response of low T_4 levels in thalassemic patients. Similarly, excess iron gets deposited in the pituitary gland where

it causes deleterious effects on pituitary size and functions (Christoforidis *et al.*, 2007).Therefore, inability to increase TSH levels in response to low T_4 levels are indicative of defective pituitary thyrotrophic function in thalassemic patients (Ashraf *et al.*, 2013). Liver plays a vital role in deiodination to activate and deactivate thyroid hormones along with its transport and metabolism. Studies have proven that there no correlation between ALT and T_4 which proves role of liver to be subtle (Winichagoon *et al.*, 2000).

The degree of severity of these changes lead to thyroid dysfunction ending in overt hypothyroidism. The prevalence and severity of thyroid dysfunction varies (Mehrvar *et al.*, 2008).Elevated Thyroid-Stimulating Hormone (TSH) level and decreased (low) T_4 are observed in primary hypothyroidism while in secondary or central hypothyroidism there is decreased T_4 and low TSH (Malik *et al.*, 2010).

The excess of iron is carefully regulated mainly by controlling iron absorption and excess of iron is toxic which gets accumulated because there is no well defined mechanism for its excretion. Toxic iron levels lead to organ dysfunction and damage. Childern treated with modern transfusion and chelation therapy are entering early adulthood so, evaluation of various endocrine complication secondary to iron overload can be evaluated in such individuals for future interventions. Therefore, in the present study assessment of thalassemic children was done by getting a detail medical history and physical examination and BMI estimation by using growth chart. Endocrine disorders such as short stature, delayed puberty and hypogonadism are major complications in both adolescent and adult patients. Therefore, exploration of the effects of iron overload on Kisspeptin and anterior pituitary hormones like GH, TSH was done. Similarly, imbalance of thyroid hormone can be damaging at any stage of life, during puberty, thyroid hormone, is required for the rapid growth and sexual development. Thus, a low-functioning thyroid at this stage of life can delay puberty, delay development and effect individuals reproductive function therefore, thyroid hormones i.e Triiodothyronine (T_3) and Thyroxine (T_4) levels were evaluated during the present study. Also correlation of these hormones with body mass index (BMI), Ferritin and Hemoglobin (Hb) and Kisspeptin levels were further evaluated in beta thalassemic patients undergoing regular blood transfusion with chelation therapy.

Materials and Methods

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-4.The inclusion and exclusion criteria for thalassemic patients and control individuals included in the study are mentioned in detail in general materials and methods. Height in centimeter and weight in kilogram were measured and BMI was calculated. The blood samples for these tests were collected from thalassemic patients (n=200) and control individuals (n=100) as mentioned in detail as general materials and methods.Enzyme-linked Immunosorbent assay kit was used for quantitative measurement of hemoglobin (gm/dl) and serum Ferritin was measured by using Ferritin (FTL) ELISA (Enzyme-Linked Immunosorbent Assay) kit, details of which are mentioned in chapte-1. The collected blood samples were also used for hormonal assay of Kisspeptin (ng/ml), GH (ng/mL), T_3 (ng/mL), T_4 (µg/dL), TSH (µIU/mL).

Quantitative determination of Kisspeptin (ng/ml)

Detection of a specific peptide and its related peptides based on the principle of "competitive" enzyme immunoassay was performed by the Enzyme Immunoassay Kisspeptin-10/Metastin (45-54)-Amide (Human) EIA kit *(Phoenix Pharmaceuticals, Inc.USA)* on blood sample of thalassemic patients (n=200) and control individuals (n=100). Quantitative determination of Kisspeptin in detail are given in chapter-2.

Quantitative determination of Growth Hormone (ng/mL)

The Invitrogen HGH-ELISA is a solid phase Enzyme Amplified Sensitivity Immuno-Assay for the quantitative determination of Human Growth Hormone (HGH) in human serum *(Invitrogen HGH-ELISA California USA).*

Principle of the Assay:

Invitrogen HGH- ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on a microtiter plate. Samples containing HGH reacted with captured antibodies (mAbs 1) coated on a plastic well with monoclonal antibodies (mAbs 2) labeled with horseradish peroxidase (HRP). After an incubation period, formation of a sandwich took place: the microtiter plate was washed to remove unbound enzyme

labeled antibodies. The substrate solution (tetramethylbenzidine (TMB) – H_{202}) was added and were incubated. The reaction was stopped with H_2SO_4 and the microtiter plate were read at the appropriate wavelength. The amount of substrate turnover was determined colorimetrically by measuring the absorbance which was proportional to the HGH concentration.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Triiodothyronine (ng/mL)

AxSYM total T_3 is a Microparticle Enzyme Immunoassay (MEIA) used for the quantitative determination of total circulating Triiodothyronine (T_3) in human serum. AxSYM System. *(Abbott Total T_3 Kit AxSYM system USA)*

Principle of the Assay:

Sample and all AxSYM total T_3 reagents required for one test were pipetted by the sampling probe into various wells of a reaction vessel (RV). The RV were immediately transferred into the processing center. Further pipetting was done in the processing center with the processing probe. Sample and Anti-T_3 coated microparticles were combined in one RV well. T_3 in the sample binded to the Anti-T_3 coated microparticles forming an antibody-antigen complex.

An aliquot of the reaction mixture, containing the antibody antigen complex binded to the microparticles, and were transferred to the matrix cell. The microparticles binded irreversibly to the glass fiber matrix. The matrix cell was then washed to remove unbound materials. The T_3 Alkaline Phosphatase Conjugate was dispensed onto the matrix cell where it binded to the available sites on the Anti-T_3 coated microparticles. The matrix cell was then washed to remove unbound materials. The substrate, 4-Methylumbelliferyl Phosphate, was added to the matrix cell, and the fluorescent product were measured by the MEIA optical assembly.

Reagents:

AxSYM total T_3 reagent pack (7A52-21)* were used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Thyroxine (µg/dL)

AxSYM total T_4 is a Fluorescence Polarization Immunoassay (FPIA) used for the quantitative determination of Thyroxine (T_4) in human serum. *(Abbott Total T_4 Kit AxSYM system USA)*

Principle of the Assay:

Sample and all AxSYM total T_4 reagents required for one test were pipetted by the sampling probe into various wells of a reaction vessel (RV). Sample, pretreatment solution, T_4 antiserum (antibody), and buffer were further pipetted into one well of the RV. An aliquot of the predilution mixture was transferred to the curette of the RV. The pretreatment solution removed the T_4 from the binding sites on the TBG, prealbumin, and albumin. The RV were immediately transferred into the processing center. Further pipetting was done in the processing center with the processing probe. A second aliquot of the predilution mixture was transferred to the curette along with the T_4 fluorescein tracer. The analytic (T_4) and labeled tracer compete for the sites on the antibody molecule. The intensity of polarized fluorescent light was measured by the FPIA optical assembly.

Reagents:

AxSYM total T_4 reagent pack (7A55-20)* were used.

Precautions:

- Complete clot formation was assured prior to centrifugation.

- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Quantitative determination of Thyroid Stimulating Hormone (µIU/mL)

The ARCHITECT TSH assay is a Chemiluminescent Microparticle Immunoassay (CMIA) for the quantitative determination of human Thyroid Stimulating Hormone (TSH) in human serum *(Architect System TSH kit USA).*

Principle of the Assay:

In the first step, sample, anti- β TSH antibody coated paramagnetic microparticles and TSH Assay Diluent were combined. TSH present in the sample binded to the anti-TSH antibody coated microparticles. After washing, anti- α TSH acridinium labeled conjugate were added in the second step. Pre-trigger and trigger solutions were then added to the reaction mixture and the resulting chemiluminescent reaction was measured as relative light units (RLUs). A direct relationship existed between the amount of TSH in the sample and the RLUs detected by the ARCHITECT optical system.

Reagents:

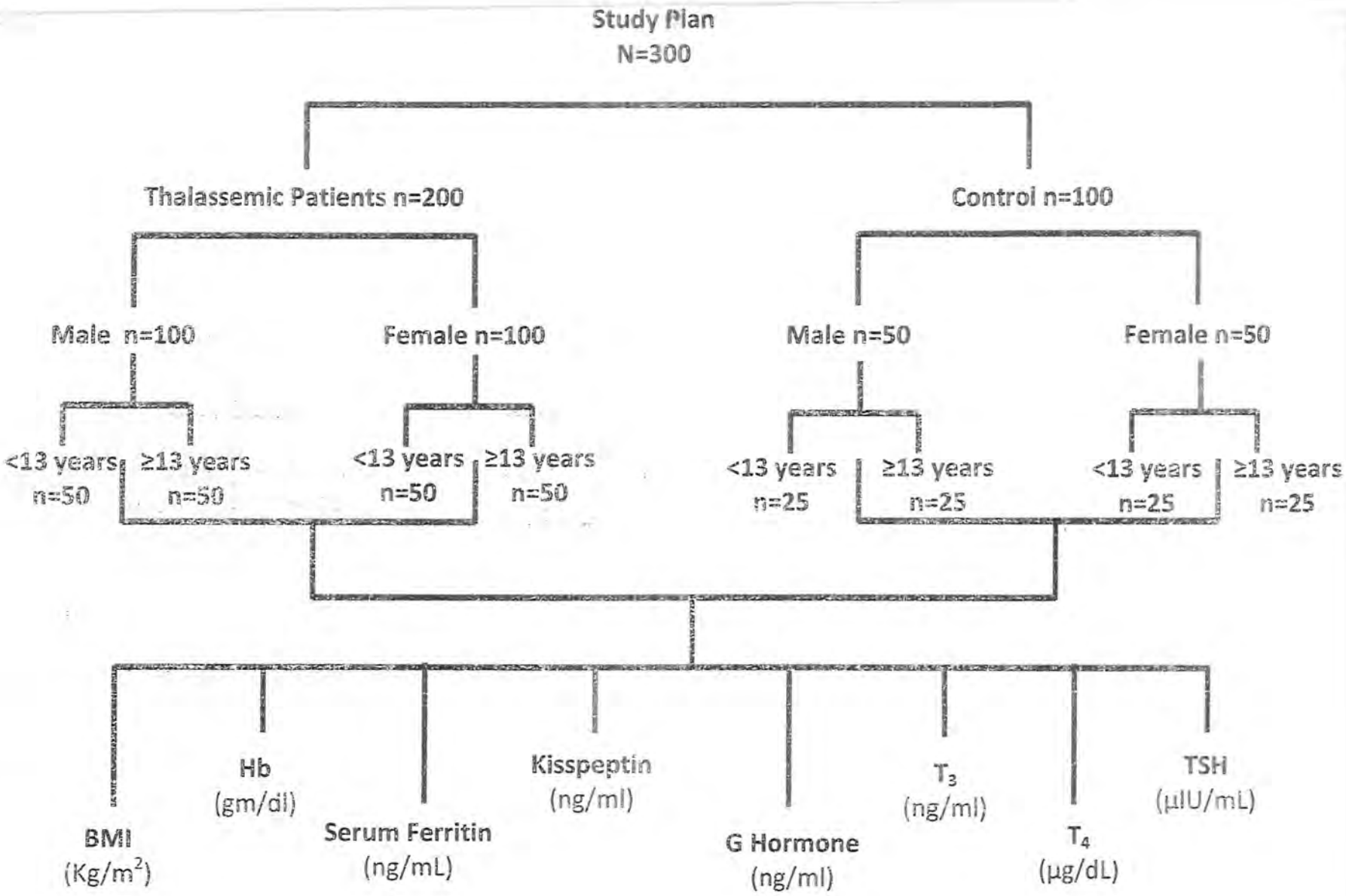
ARCHITECT TSH reagent kit (7K62) was used.

Precautions:

- Complete clot formation was assured prior to centrifugation.
- Specimens were stored for up to 24 hours at 2-8°C prior to being tested.
- To minimize the effects of evaporation, all samples (patient specimens, controls and calibrators) were tested within 3 hours of being placed on-board the AxSYM System.
- All samples were free of bubbles prior to analysis

Statistical Analysis

Data was analyzed through Graph Pad Prism 5.01. Data was reported as Mean ± SEM. Comparison of hormones, BMI, hemoglobin and serum Ferritin level with the control group was done by using unpaired t-test. Further non parametric co-relation (Spearman) was done for each hormone with rest of the variables through pad prism. $P<0.05$ was considered statistically significant.



Flow Chart 4: Study plan describing female and male thalassemic patients with their corresponding control of different age groups in determining BMI, serum Ferritin, Hemoglobin, Kisspeptin, GH, TSH and Thyroid hormones levels.

Results

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-4. Results of (BMI) Kg/m^2, Hemoglobin (gm/dl) and serum Ferritin (ng/mL) are mentioned in chapter -1 while results of serum Kisspeptin (ng/ml) are mentioned in detail in chapter -2.

Serum G H levels (ng/ml)

Serum GH levels in thalassemic male and female patients of different age groups are shown in Figure 3.5. Serum GH levels in thalassemic female of <13 years were (1.33 ± 0.01 ng/ml) which were significantly low (P<0.01) than the control group (1.80 ± 0.27 ng/ml). Whereas, thalassemic females of ≥13 years had Growth Hormone levels (2.29 ± 0.30 ng/ml) which were significantly raised (P<0.01) when compared with the control group (1.02 ± 0.19 ng/ml). Similarly, serum GH levels in thalassemic males of <13 years 0.55 ± 0.01 ng/ml were significantly, low (P<0.001) than the control group 1.44 ± 0.31 ng/ml. While GH levels of thalassemic males of ≥13 years 2.11 ± 0.31 ng/ml were significantly raised (P<0.05) as compared to the control group 1.02 ± 0.19 ng/ml respectively.

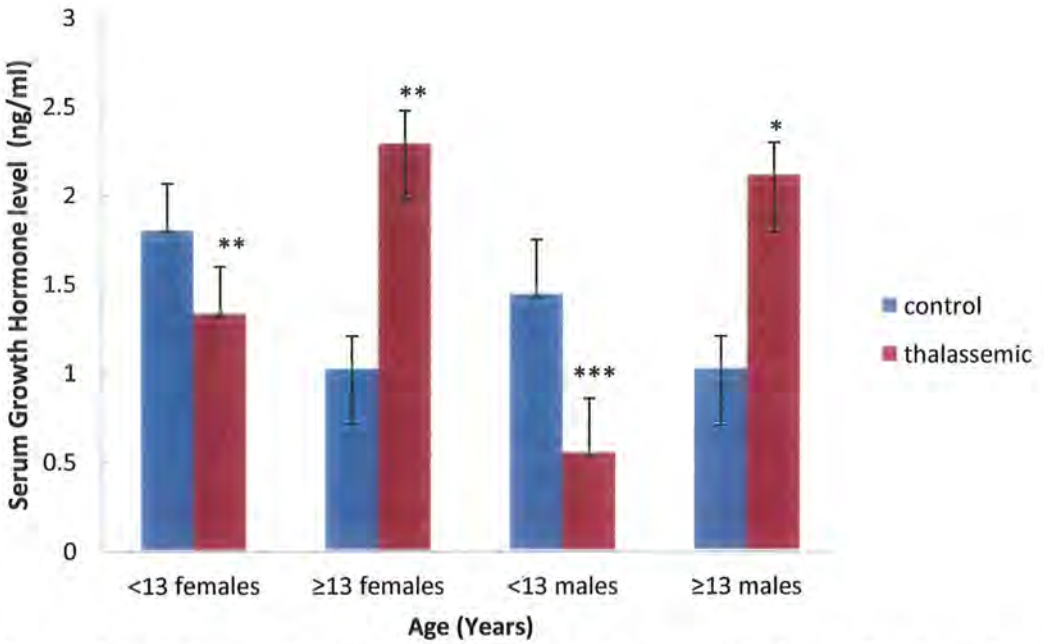


Figure 3.1: Mean ± SEM of Serum Growth Hormone levels of female and male thalassemic patients with their corresponding control of different age groups. **=P<0.001, value vs corresponding control, ***=P<0.001, value vs corresponding control

Serum (T_3) levels (ng/mL)

Serum T_3 levels in < 13 years thalassemic females (1.42 ± 0.07 ng/mL) were significantly reduced than the control group (1.50 ± 0.03 ng/mL), similarly thalassemic females of ≥13 years (1.88 ± 0.11 ng/mL) had significantly higher (P<0.001) T_3 levels than the control group (1.38 ± 0.05 ng/mL). On comparison of serum T_3 levels in thalassemic males of <13 years (1.40 ± 0.30 ng/mL) no significant difference was observed with their control group (1.60 ± 0.03 ng/mL). Similarly serum T_3 levels in thalassemic males of ≥13 years (1.20 ± 0.16 ng/mL) also showed no significant difference on comparison with the control group (1.60 ± 0.05 ng/mL). Comparison of serum T_3 levels of male and female thalassemic patients with their corresponding control of different age groups is shown in Figure 3.2

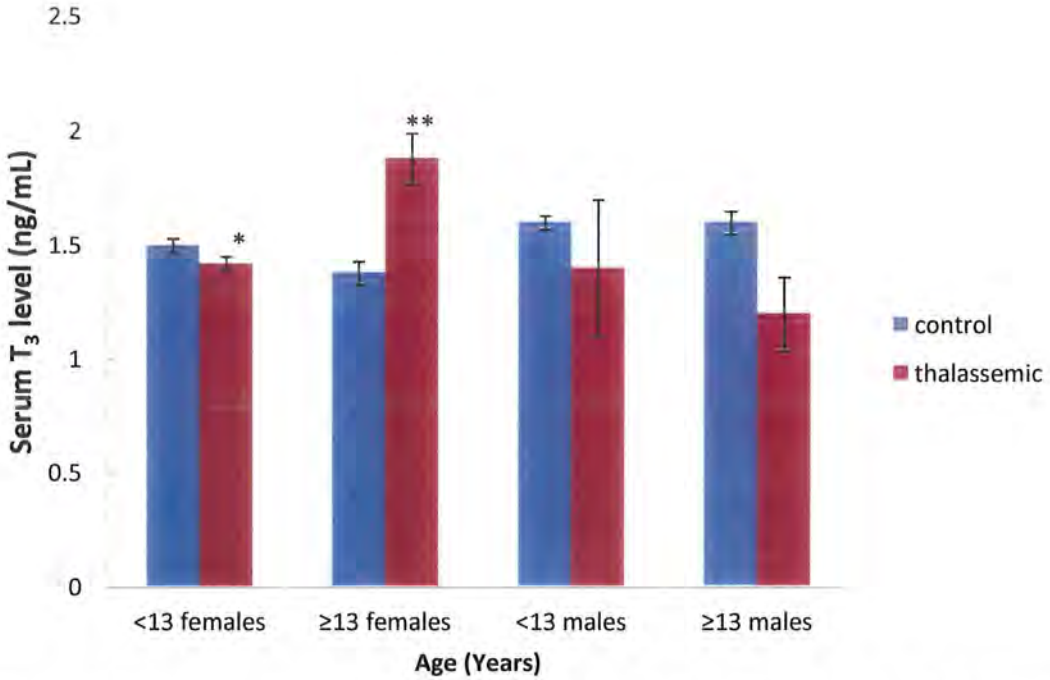


Figure 3.2: Mean ± SEM of Serum T_3 levels of female and male thalassemic patients with their corresponding control of different age groups. *=P<0.01, value vs corresponding control, **=P<0.001, value vs corresponding control

Serum (T_4) levels (µg/dL)

Comparison of serum T_4 levels of male and female thalassemic patients with their corresponding control of different age groups is represented in Figure 3.3 On comparison of serum T_4 levels in < 13 years thalassemic females (6.61 ± 0.47 µg/dL) showed no significant difference with the control group (6.10 ± 0.11 µg/dL). Similarly thalassemic females of ≥13 years (5.72 ± 0.34 µg/dL) also had no significant difference in serum T_4 levels on comparison with their control group (6.56 ± 0.4 µg/dL).

Serum T_4 levels in <13 years thalassemic male patients (6.28 ± 0.24 µg/dL) were significantly low (P<0.001) on comparison with the control group (7.79 ± 0.28 µg/dL), while serum T_4 levels in ≥13 years thalassemic males (5.8 ± 0.31 µg/dL) were significantly not different from the control group (6.70 ± 0.21 µg/dL).

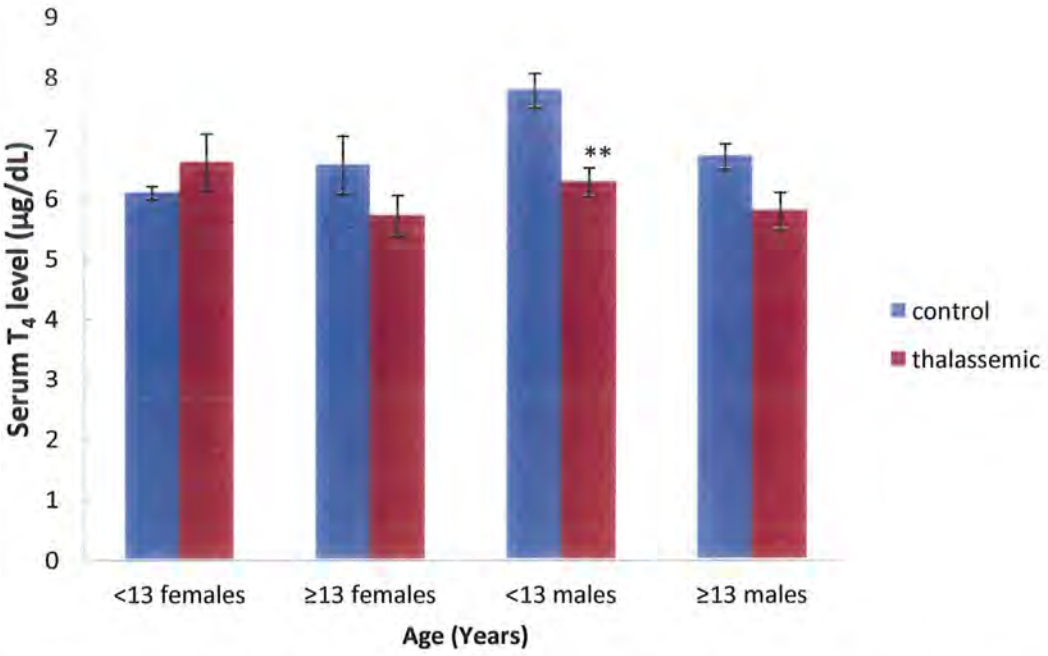


Figure 3.3: Mean ± SEM of Serum T_4 levels of female and male thalassemic patients with their corresponding control of different age groups. **=P<0.001, value vs corresponding control

Serum (TSH) levels (μIU/mL)

Serum TSH levels in thalassemic females of < 13 years (2.84 ± 0.21 μIU/mL) had no significant difference with their control group (2.22 ± 0.43 μIU/mL) on comparison. Similarly non significant results were obtained while comparing serum TSH levels of ≥13 years thalassemic females (2.27 ± 0.25 μIU/mL) with their control group (1.55 ± 0.10 μIU/mL). While serum TSH levels were significantly low (P<0.05) in thalassemic males of <13 years of age (1.50 ± 0.08 μIU/mL) on comparison with the control group (2.20 ± 0.34 μIU/mL). Whereas ≥13 years thalassemic males (3.67 ± 0.33 μIU/mL) had serum TSH levels which were significantly higher (P<0.001) than the control group (1.40 ± 0.11 μIU/mL). Serum TSH levels in thalassemic male and female of different age groups are shown in Figure 3.4

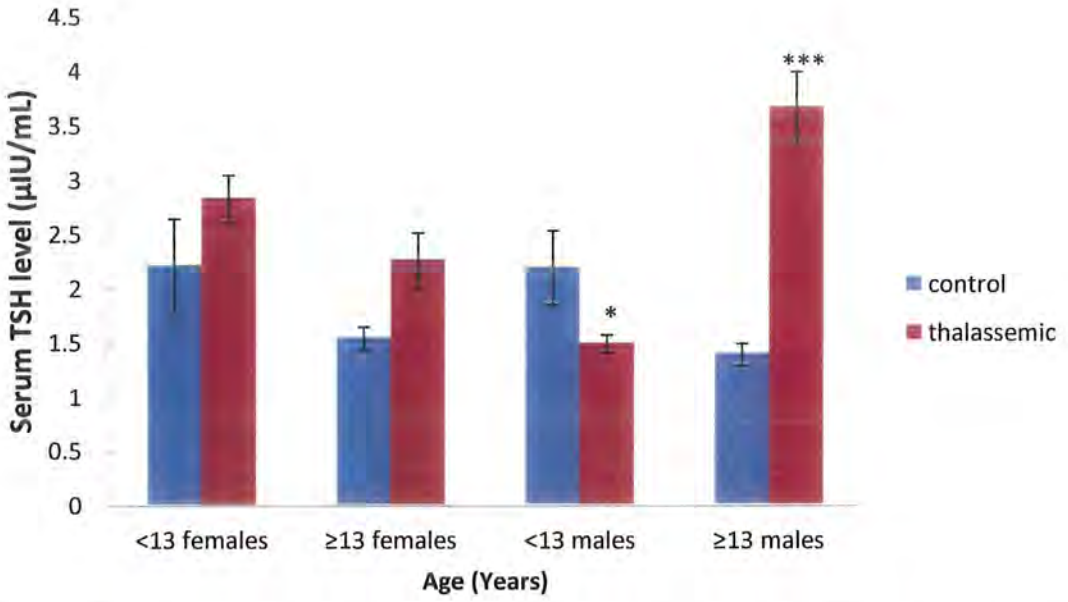


Figure 3.4: Mean ± SEM of Serum TSH levels of female and male thalassemic patients with their corresponding control of different age groups. *=P<0.01, value vs corresponding control, ***=P<0.001, value vs corresponding control

Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) levels in thalassemic male and female patients in different age groups.

Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and serum Kisspeptin (ng/ml) levels in thalassemic males and females patients in different age groups is shown in Table-3.1 Figure-3.5, 3.6, 3.7 and 3.8. While correlating serum Growth Hormone levels (ng/ml) with BMI (Kg/m^2) (r=0.207), serum Ferritin (ng/mL) (r=0.229), Hemoglobin (gm/dl) (r=0.159) and serum Kisspeptin (ng/ml) (r= -0.009) levels, there was no significant correlation observed with any of the variables in female thalassemic patients of <13 years of age. Similarly ≥ 13 years thalassemic females also showed non significant correlation of serum Growth Hormone levels with BMI (Kg/m^2) (r=0.016), serum Ferritin (ng/mL) (r= -0.021), Hemoglobin (gm/dl) (r=0.113) and serum Kisspeptin (ng/ml) r= 0.021) levels. Incase of thalassemic males of <13 years significant (P<0.05) correlation existed between Growth Hormone (ng/ml) and serum Kisspeptin (ng/ml) levels (r=0.310*) while non significant correlation was seen between Growth Hormone (ng/ml) and BMI (Kg/m^2) (r=0.091), serum Ferritin (ng/mL) (r=0.043), Hemoglobin (gm/dl) (r=0.005) levels. Thalassemic males of ≥13 years had non significant correlation between Growth Hormone (ng/ml) and BMI (Kg/m^2) (r=0.020), serum Ferritin (ng/mL) (r=-0.012), Hemoglobin (gm/dl) (r= -0.124) and serum Kisspeptin (ng/ml) r= -0.101) levels.

Table 3.1: Correlation of Growth Hormone with BMI, Serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic female and male patients in different age groups.

Age (Years)	Gender	Hormone	Groups	BMI (Kg/m^2)	Ferritin (ng/mL)	Hb (gm/dl)	Kisspeptin (ng/ml)
< 13	Females	Growth Hormone (ng/ml)	Thalassemia n=50	0.207	0.229	0.159	-0.009
≥ 13			Thalassemic n=50	0.016	-0.021	0.113	0.021
< 13	Males		Thalassemic n=50	0.091	0.043	0.005	0.310*
≥ 13			Thalassemic n=50	0.020	-0.012	-0.124	-0.101

*=P<0.05, value are considered significant.

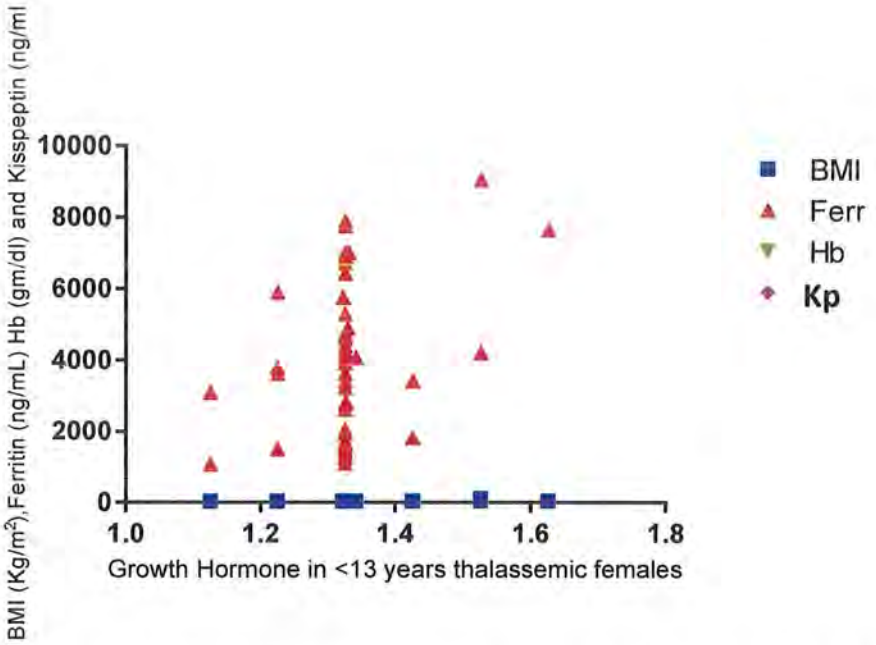


Figure 3.5: Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of <13 years.

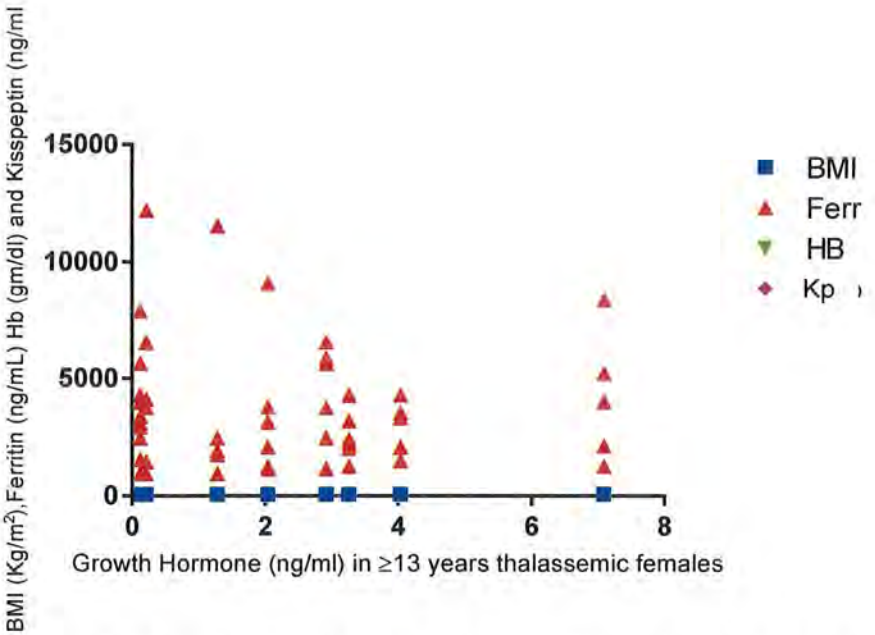


Figure 3.6: Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic females of ≥13 years.

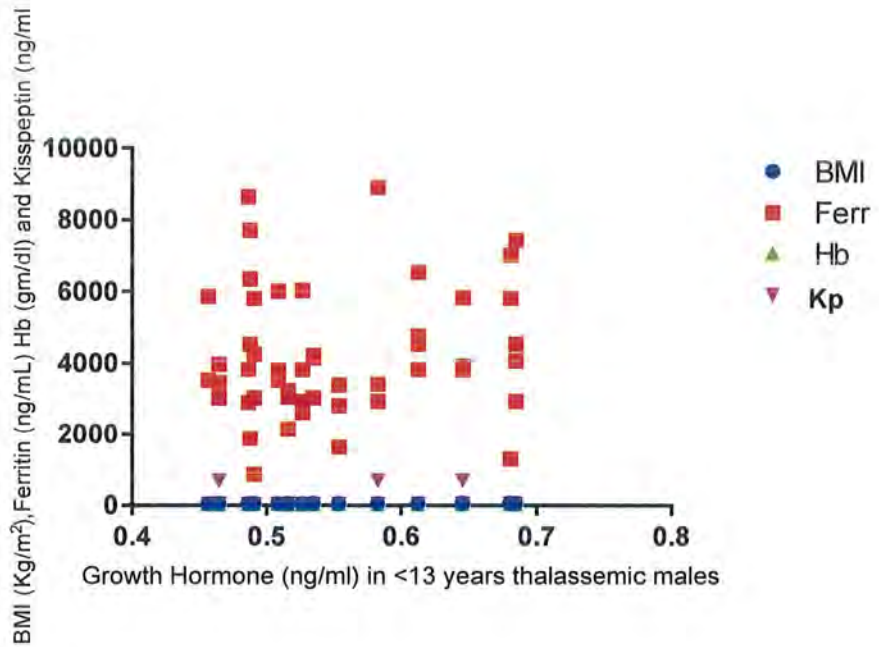


Figure 3.7: Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of <13 years.

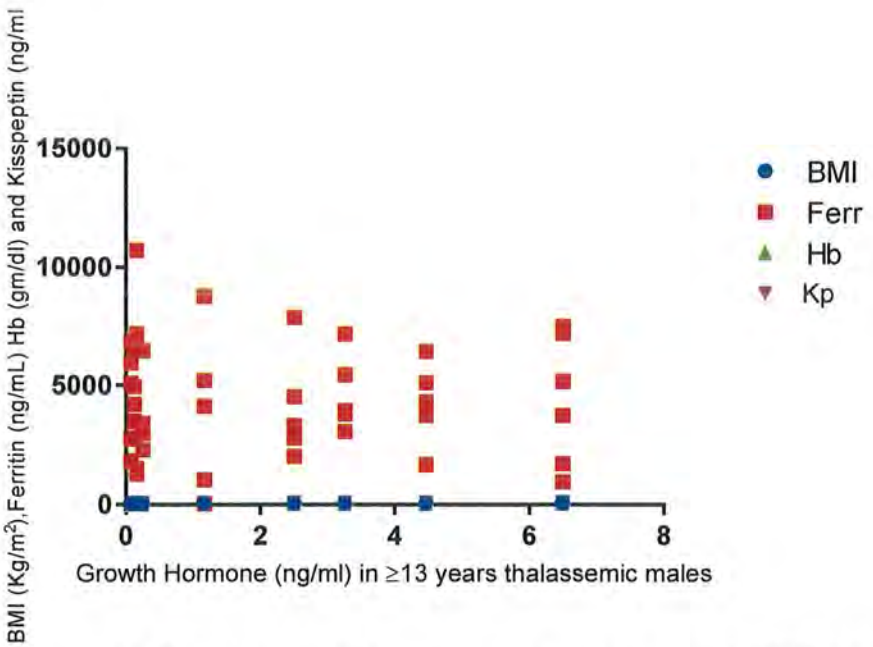


Figure 3.8: Correlation of Growth Hormone (ng/ml) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of ≥13 years.

Correlation of T_3 (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) levels in thalassemic male and female patients in different age groups.

Correlation of T_3 (ng/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and serum Kisspeptin (ng/ml) levels in thalassemic males and females patients in different age groups is shown in Table-3.2 and Figure-3.9, 3.10, 3.11 and 3.12. Thalassemic female patients of <13 years showed a non significant correlation of serum T_3 (ng/mL) with BMI (Kg/m^2) (r=0.148), serum Ferritin (ng/mL) (r= -0.223), Hemoglobin (gm/dl) (r=0.085) and serum Kisspeptin (ng/ml) (r=0.031) levels. Thalassemic female of ≥13 years had a significant (P<0.01) negative correlation of serum T_3 (ng/mL) with BMI (Kg/m^2) (r= -0.408), (P<0.05) with Hb (gm/dl) levels (r= -0.329). While serum T_3 (ng/mL) levels had a non significant correlation with serum Ferritin (ng/mL) (r= -0.038) and serum Kisspeptin (ng/ml) (r= 0.243) levels.

While serum T_3 (ng/mL) has a positive correlation (P<0.001) with serum Ferritin (ng/mL) levels of thalassemic males of < 13 years (r = 0.523). On the other hand serum T_3 has a non significant correlation with BMI (Kg/m^2) (r= -0.263), Hemoglobin (gm/dl) (r= 0.187) and Kisspeptin (ng/ml) (r= 0.202) levels. Significant positive correlation (P<0.05) existed between serum T_3 (ng/mL) and Kisspeptin (ng/ml) (r= 0.317) levels in thalassemic males of ≥13 years. While in this age group T_3 (ng/mL) had a non significant correlation with BMI (Kg/m^2) (r= 0.164) serum Ferritin (ng/mL) (= -0.207) and Hemoglobin (gm/dl) (r= 0.208) levels.

Table 3.2: Correlation of T_3 with BMI, serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic males and females patients in different age groups.

Age (Years)	Gender	Hormone	Groups	BMI (Kg/m^2)	Ferritin (ng/mL)	Hb (gm/dl)	Kisspeptin (ng/ml)
< 13	Females	T_3 (ng/mL)	Thalassemia n=50	0.148	-0.223	0.085	0.031
≥ 13			Thalassemia n=50	-0.408**	-0.038	-0.329*	0.243
< 13	Males		Thalassemic n=50	-0.263	0.523***	0.187	0.202
≥ 13			Thalassemic n=50	0.164	-0.207	0.208	0.317*

*=P<0.05, **=P<0.01, ***=P<0.001, value are considered significant.

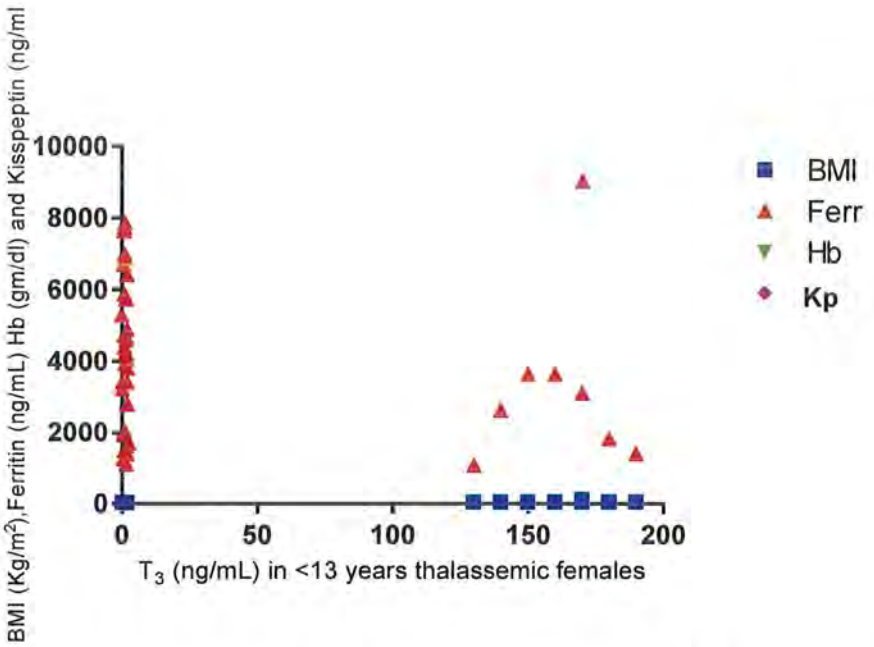


Figure 3.9: Correlation of T_3 (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of <13 years along.

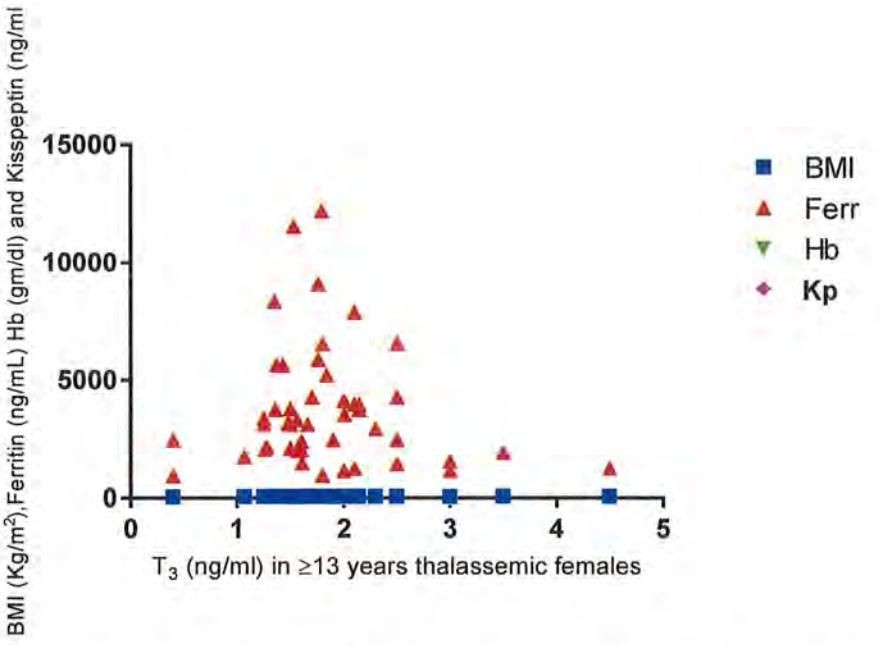


Figure 3.10: Correlation of T_3 (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of ≥13 years.

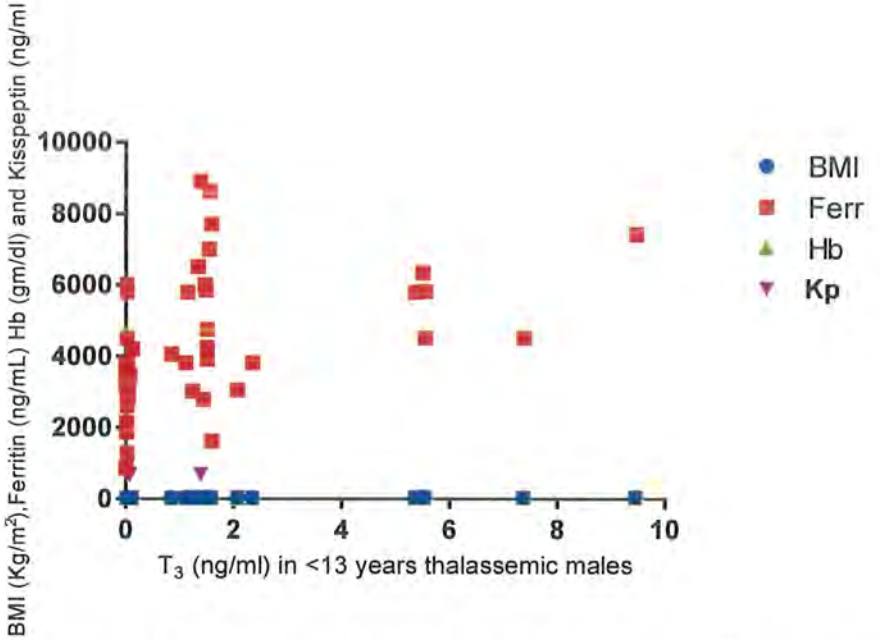


Figure 3.11: Correlation of T_3 (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of <13 years.

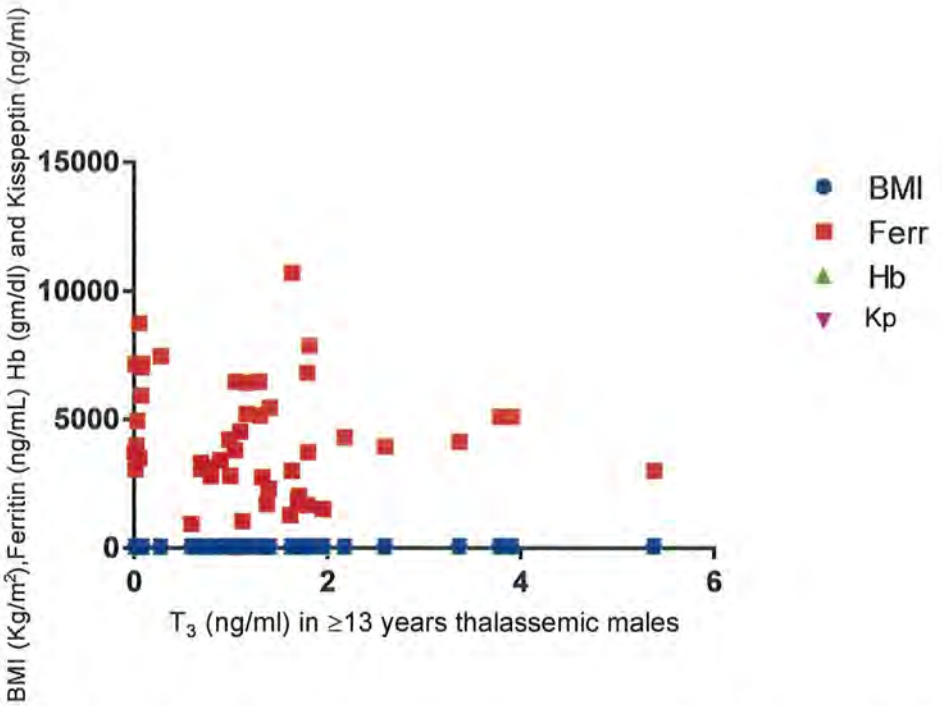


Figure 3.12: Correlation of T_3 (ng/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of ≥13 years.

Correlation of T_4 (µg/dL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) levels in thalassemic male and female patients in different age groups.

T_4 (µg/dL) has a positive correlation ($P<0.05$) with Hb (gm/dl) levels ($r=0.332$) in < 13 years thalassemic females, while T4 (µg/dL) has non significant correlation with BMI (Kg/m^2) ($r= -0.196$), serum Ferritin (ng/mL) ($r= 0.137$) and Kisspeptin (ng/ml) ($r= 0.099$) levels. T_4 (µg/dL) has a significant positive correlation ($P<0.05$) with BMI (Kg/m^2) ($r= 0.333$), serum Ferritin (ng/mL) ($r=0.317$) and Hb (gm/dl) levels ($r=0.328$) in thalassemic females of ≥13 years of age.

Significant ($P<0.01$) positive correlation exist between T_4 (µg/dL) and Hb (gm/dl) ($r= 0.422$) levels in <13 years thalassemic males, while there was non significant correlation observed between T_4 (µg/dL) and BMI (Kg/m^2) ($r= -0.017$), serum Ferritin (ng/mL) ($r= -0.103$) and serum Kisspeptin (ng/ml) ($r= 0.212$) levels in <13 years thalassemic males. On the other hand T4 has a significant positive correlation ($P<0.001$) with Hb levels ($r= 0.516$), while T_4 has a non significant correlation with BMI (Kg/m2) ($r= -0.229$), serum Ferritin (ng/mL) ($r= 0.143$) and Kisspeptin (ng/ml) ($r= 0.064$) levels. Correlation of T_4 (µg/dL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and serum Kisspeptin (ng/ml) levels in thalassemic males and females patients in different age groups is represented in Table-3.3 and Figure-3.13, 3.14, 3.15 and 3.16.

Table 3.3: Correlation of T_4 with BMI, serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic male and female patients in different age groups.

Age (Years)	Gender	Hormone	Groups	BMI (Kg/m^2)	Ferritin (ng/mL)	Hb (gm/dl)	Kisspeptin (ng/ml)
< 13	Females	T_4 (µg/dL)	Thalassemic n=50	-0.196	0.137	0.332*	0.099
≥ 13			Thalassemic n=50	0.333*	0.317*	0.328*	0.061
< 13	Males		Thalassemic n=50	-0.017	-0.103	0.422**	-0.212
≥13			Thalassemic n=50	-0.229	-0.143	0.516***	0.064

*=P<0.05, **=P<0.01, ***=P<0.001, value are considered significant.

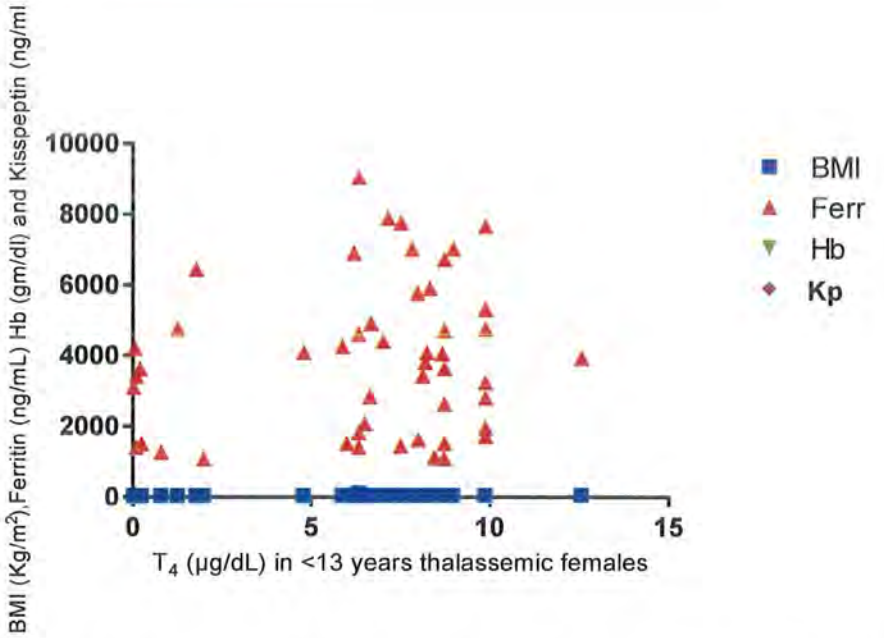


Figure 3.13: Correlation of T_4 (µg/dL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of <13 years.

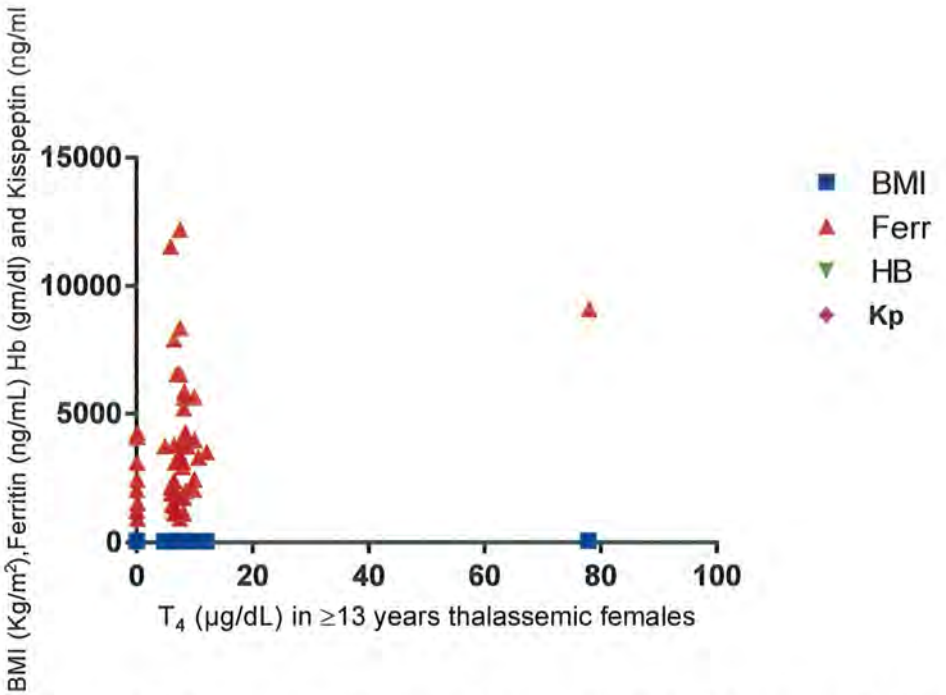


Figure 3.14: Correlation of T_4 (µg/dL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of ≥13 years.

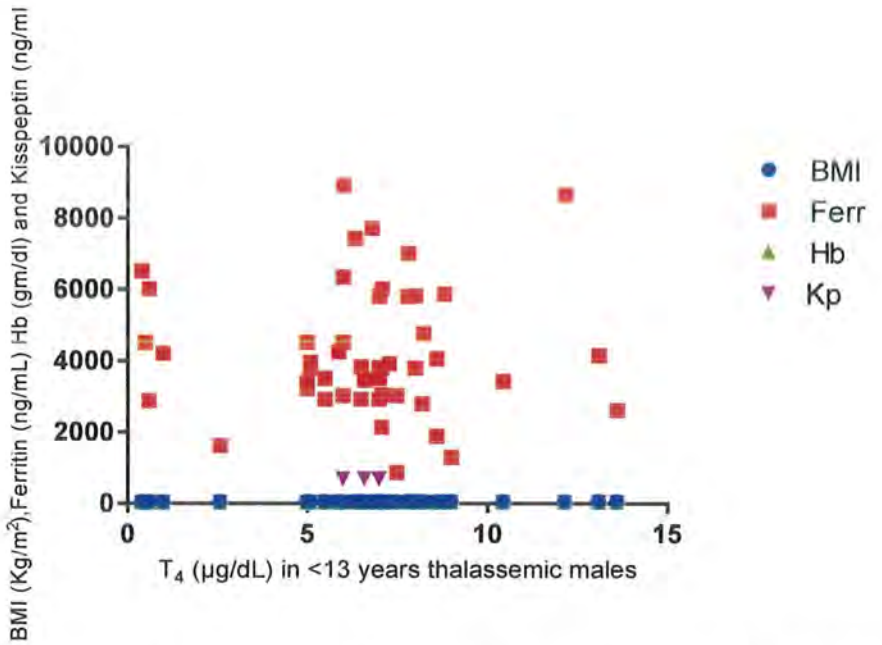


Figure 3.15: Correlation of T_4 (µg/dL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of <13 years.

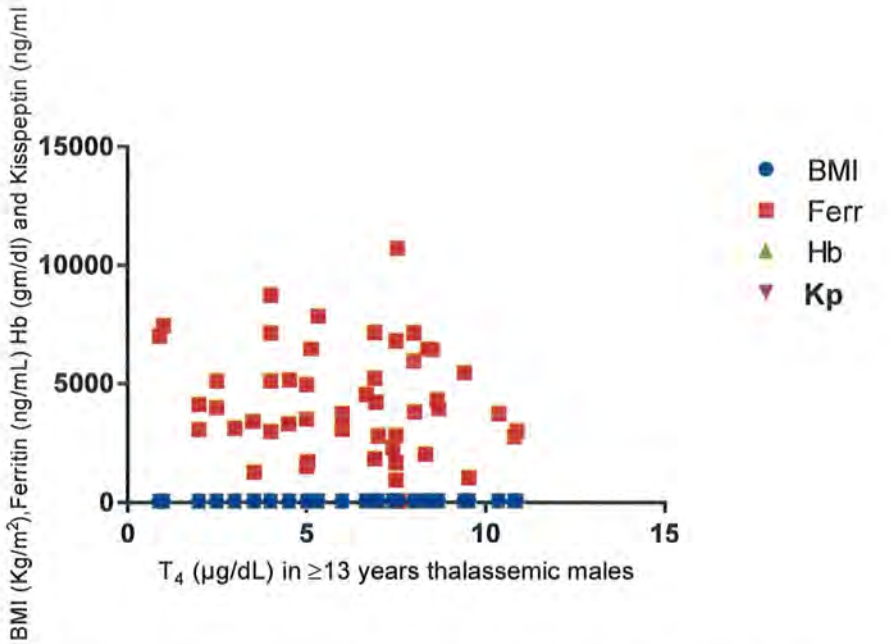


Figure 3.16: Correlation of T_4 (µg/dL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of ≥13 years.

Correlation of TSH (μIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) levels in thalassemic male and female patients in different age groups.

Correlation of TSH (μIU/mL) with BMI (Kg/m^2), serum Ferritin (ng/mL), Hemoglobin (gm/dl) and serum Kisspeptin (ng/ml) levels in thalassemic males and females patients in different age groups is shown in Table-3.4 and Figure-3.17, 3.18, 3.19 and 3.20. Non significant correlation exist between TSH (μIU/mL) levels and BMI (Kg/m^2) (r= -0.062), serum Ferritin (ng/mL) (r= -0.115), Hb (gm/dl) (r= 0.042) and Kisspeptin (ng/ml) (r= -0.052) levels. In thalassemic females of ≥ 13 years of age TSH (μIU/mL) concentration shows highly significant (P<0.001) positive correlation between BMI (Kg/m^2) (r= 0.487) and serum Ferritin (ng/mL) (r= 0.531) level. Similarly, significant positive correlation (P<0.01) was observed with Hb (gm/dl) levels in (r=0.394) in ≥13 years thalassemic females. While non significant correlation exist between TSH (μIU/mL) and Kisspeptin (ng/ml) (r= 0.021) levels. In <13 years thalassemic males TSH (μIU/mL) showed a non significant correlation with BMI (r=0.192), serum Ferritin (ng/mL) (r=0.187), Hb (gm/dl) (r=0.124) and Kisspeptin (ng/ml) (r= -0.056) levels.

TSH (μIU/mL) had a positive correlation (P<0.01) with serum Ferritin (ng/mL) (r=0.446) levels, while a non significant correlation existed between TSH (μIU/mL) and BMI (r=0.064), Hb (gm/dl) (r= -0.197) and Kisspeptin (ng/ml) (r= -0.220) levels.

Table 3.4: Correlation of TSH with BMI, Serum Ferritin, Hemoglobin and Kisspeptin levels in thalassemic male and female patients in different age groups.

Age (Years)	Gender	Hormone	Groups	BMI (Kg/m^2)	Ferritin (ng/mL)	Hb (gm/dl)	Kisspeptin (ng/ml)
< 13	Females	TSH (μIU/mL)	Thalassemia n=50	-0.062	-0.115	0.042	-0.052
≥ 13			Thalassemia n=50	0.487***	0.531***	0.394**	0.021
< 13	Males		Thalassemic n=50	0.192	0.187	0.124	-0.056
≥ 13			Thalassemic n=50	0.064	0.446**	-0.197	-0.220

**=P<0.01, ***=P<0.001, value are considered significant.

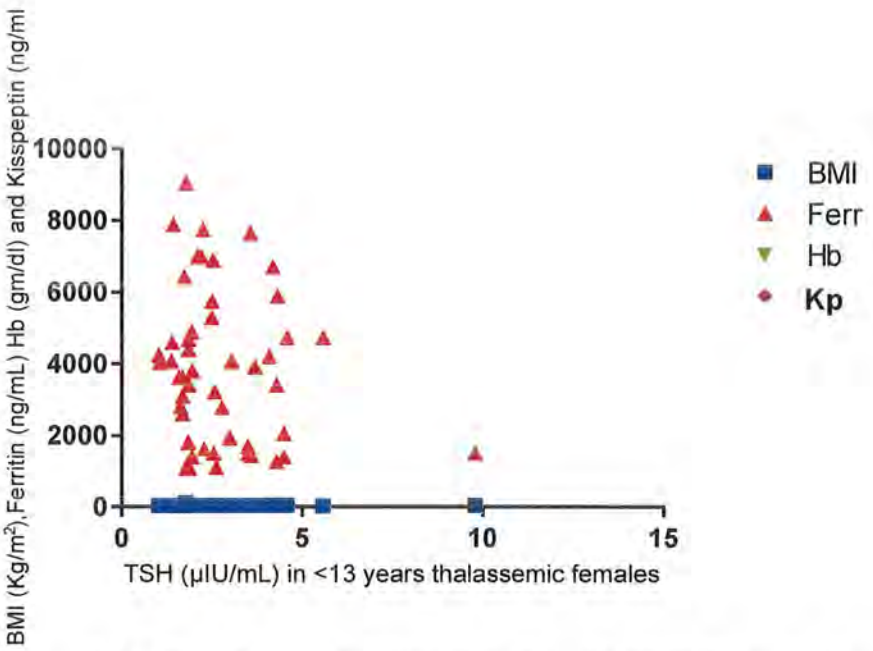


Figure 3.17: Correlation of TSH (µIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of <13 years.

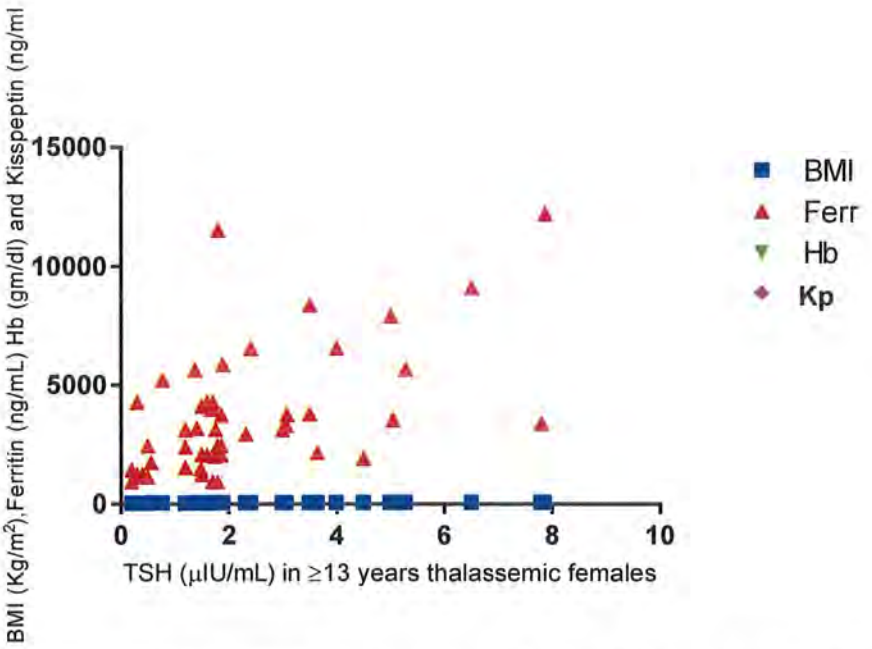


Figure 3.18: Correlation of TSH (µIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic female of ≥13 years.

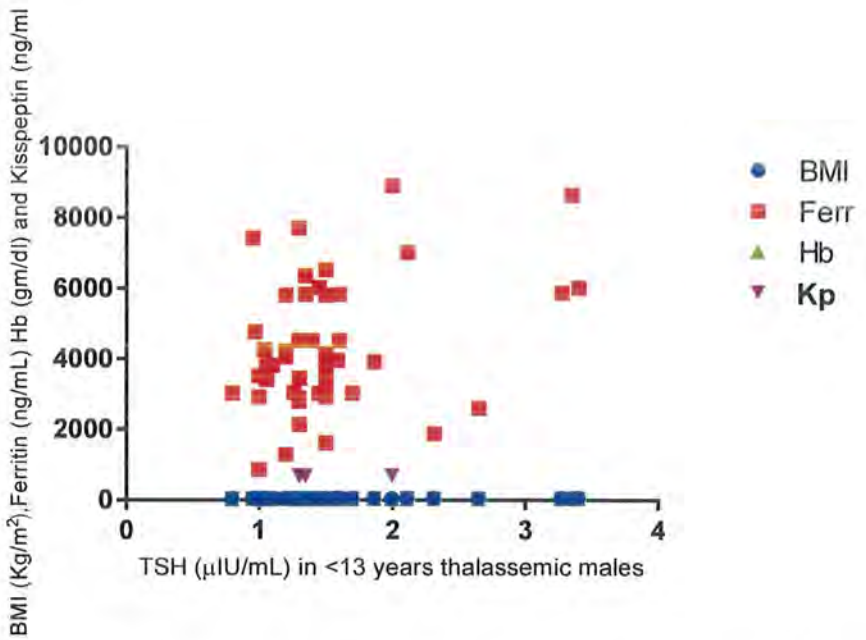


Figure 3.19: Correlation of TSH (µIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of <13 years.

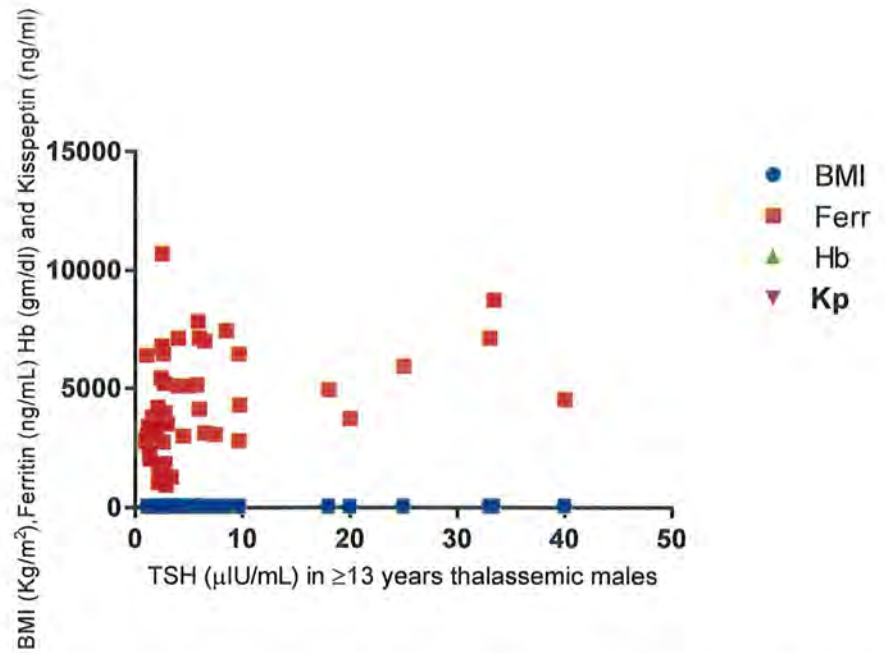


Figure 3.20: Correlation of TSH (µIU/mL) with BMI (Kg/m^2), Serum Ferritin (ng/mL), Hemoglobin (gm/dl) and Serum Kisspeptin (ng/ml) thalassemic male of ≥13 years.

Discussion

Thalassemic patients are dependent on blood transfusions to maintain the levels of Hemoglobin and packed cell volume in their blood. Transfusion and iron-chelation therapy has prolonged and improved the quality of life in these patients (Borgna *et al.*, 2004). Such a treatment, however, leads to chronic iron overload affecting the endocrine glands (Abdulzahra *et al.*, 2011). In our study we observed that patients suffering from thalassemia major present with endocrine disorders and pubertal delay. In another study carried out by Al-Rimawi *et al.*, 2005 showed that there was a significant difference in the frequency and regularity of using chelation therapy between pubertal and delayed pubertal groups (Al-Rimawi *et al.*, 2005).Whereas in our study the age of starting chelation therapy was 6-8 months and the patients were on regular blood transfusion and chelation therapy.

When chelation therapy with deferoxamine is used before the age of 3 years it causes marked stunted growth with a clinical and radiologic rickets-like syndrome.The reason can be due to the fact that along with iron overload deferoxamine also chelates other essential minerals. Therefore, after the age of 10 years adequate levels of Hemoglobin are maintained but, many of the thalassemic childern start developing decelerated growth. Pubertal group of children present with reduced growth spurt with marked deceleration, truncal shortening, most likely due to hypogonadism secondary due to iron overload (De Virgilis *et al.*, 1988; Borgna-Pignattiet *et al.*, 1985).

During the first 10 years of life a Hemoglobin levels above 8.5 g/dl should be maintained because during this period hypoxia due to anemia can be the main factor causing growth retardation. Maintenance of Hemoglobin levels above 10–11 g/dl along with adequate chelation therapy gives the thalassemic childern a chance of normal growth and development (Kattamis and Liakopoulou,1990).In previous study carried out by Hussein and Mohsen 2013, comparison of measured parameters between male and female thalassemic patients of 3-11 years age group were done which showed high Hemoglobin levels in male patients in comparison with female thalassemic patients. The change in Hemoglobin levels can be easily explained by the genetic changes between male and female, males have higher Hemoglobin levels than females (Hussein and Mohsen 2013). While comparing with our study Hemoglobin

levels of thalassemic male and female patients were significantly low as compared to the control groups. Similarly, in our study serum Ferritin level were high as compared to the control group which was similar to the results reported by Adil *et al.*, 2012, suggesting that increased serum Ferritin levels are related to the endocrinopathies (Adil *et al.*, 2012). Increased serum Ferritin levels were associated with increased incidence of endocrinopathies along with subsequent increase in the serum levels of calcium (Ca), alkaline phosphate and parathyroid hormone levels (Adil *et al.*, 2012). Thalassemic children frequently present with growth retardation which may be attributed to their diversion from caloric resources resulting from ineffective erythropoiesis, along with the effects of anemia. Since hyper-transfusion has been shown to frequently restore normal growth rates (Viprakasit *et al.*, 2001). However, the adolescent growth spurt is often delayed, even in children who are hyper-transfused, unless intensive iron chelation therapy is instituted early in life (Theodoridis *et al.*, 1994). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overWeight and 6.5% were categorized as obese (Adil *et al.*, 2012). Whereas, thalassemic patients male and female of all groups included in our study had reduced BMI ($P<0.001$) as compared to the control group. A high prevalence of endocrine abnormalities in beta thalassemia major patients is reported by (Zervas *et al.*,2002). Relationship between the level of Ferritin and the development of endocrinopathies suggest that serum Ferritin is used as a prognostic marker for survival of these thalassemic patients, prognosis for survival is excellent when serum Ferritin concentration is below 2500 ng/ml in thalassemic patients (Costin *et al.*, 1979). Whereas the study results by (Zervas *et al.*, 2002) reported absence of such a relation (Zervas *et al.*,2002). During the course of beta thalassemia major, multiple endocrine disorders may develop mainly due to iron overload. Growth retardation and HPG axis dysfunction represent the commonest disorders of the endocrine system (De-Sanctis *et al.*, 2002). According to Abdulzahra *et al.*, (2011); Pirinççioğlu *et al.*, (2011) thyroid dysfunction is common in thalassemia major, but its prevalence and severity varies. Decreased thyroid levels can lead to growth problems in thalassemic patients as documented by Pirinççioğlu *et al.*, (2011). In study carried out by (Hegazi *et al.*, 2013), thyroid function tests (free

T_4, free T_3 and TSH) showed no significant difference between thalassemic patients and controls (Hegazi *et al.*, 2013).

On the contrary, hypothyroidism was detected in different ratios in other studies done by Kurtoglu *et al.*, (2012); De Sanctis *et al.*, (2006) as they found hypothyroidism in 12.8% of their patients respectively. These results were explained by (Aruratanasirikul *et al.*, 2007) that thyroid dysfunction in thalassemic patients was dependent on many factors like age of studied population, the duration of receiving blood transfusions, the amount of iron overload, the dosage of iron-chelating agent, and the procedure used for evaluation. While in our study serum T_3 levels in <13 years thalassemic females were reduced which are similar to results reported by Karamifar *et al.*, (2003) and Zervas *et al.*, (2002).While ≥13 years thalassemic females had significantly ($P<0.001$) higher levels than the control group.Serum T_4 levels in < 13 years thalassemic male patients were significantly lower ($P<0.001$) than the control group. While TSH levels were significantly lower ($P<0.05$) in thalassemic males of < 13 years of age as compared to the control group. Whereas ≥13 years thalassemic males TSH levels were significantly higher ($P<0.01$) than the control group.

Study carried out by Hegazi *et al.*,(2013), detected a significant positive correlation between age and Ferritin and a significant negative correlation between Ferritin and free T_4.So, thyroid function tests might be affected with progress of age of those patients and increased their serum Ferritin level. Whereas in our study thalassemic female of ≥13 years T_3 had a significant ($P<0.001$) negative correlation with BMI ($r= -0.408$), ($P<0.05$) with Hb levels ($r= -0.329$).While T_3 had a positive correlation ($P<0.05$) with serum Ferritin levels of thalassemic males of < 13 years ($r = 0.523$). Gamberini *et al.*, (2008) observed that serum Ferritin levels of approximately 2,000 ng/mL correlated with hypogonadism while at serum Ferritin of 3,000 ng/mL hypothyroidism, hypoparathyroidism and diabetes mellitus was detected.

Similarly, in our study T_3 has a positive correlation with Kisspeptin levels in thalassemic males of ≥13 years.T_4 had a significant positive correlation with BMI, serum Ferritin and Hb levels in thalassemic females of ≥13 years of age. While T_4 had a positive correlation with Hb levels in < 13 years thalassemic females. Similarly, T_4

has a positive correlation with Hb levels in thalassemic males of ≥ 13 years of age and with Hb levels of <13 years thalassemic male.These findings are in line with studies carried out by Hashemizadeh and Norri, (2012) who found that impaired thyroid function was associated with iron overload. Also, Irshaid and Mansi, (2009) reported that the rate of thyroid dysfunctions increases steadily with advancing age. Controversially, Abdulzahra *et al.*, (2011); Zervas *et al.*, (2002) stated that no statistically significant correlation was found between serum Ferritin levels and thyroid functions. Results of the work done by Ashraf *et al.*, (2013) have proven that there is increase higher prevalence i.e 35% of hypothyroidism was observed in thalassemic patients by age of 18 years They significantly correlated age of thalassemic patients with T_4 and Ferritin levels. While they also proved a negative correlation of Ferritin level with T_4. Through these findings they proved that patients with thalassemia develop progressive deterioration of thyroid function with time,which is due to iron overload. While in our study thalassemic males of <13 years suffered from hypothyroidism and serum levels of T_4 also showed similar negative correlation with serum Ferritin levels.

Elevated thyroid-stimulating hormone (TSH) level and decreased (low) T_4 are observed in primary hypothyroidism while in secondary or central hypothyroidism there is decreased T_4 and low TSH (Malik *et al.*, 2010). Kurtoglu *et al.*, (2012) work proved that there was higher prevalence of primary rather than secondary hypothyroidism. Filosa *et al.*, (2006) reported that there was increase in hypothyroidism over a period of 12 years. Ashraf *et al.*, (2013) patients also had reduced T_4 levels along with corresponding decrease levels of TSH over a period of 12 years This was indicative of the fact that progressive slow dysfunction of the thyroid gland developed with a degree of pituitary insensitivity to the low T_4 level (central component of hypothyroidism).

Higher incidence of secondary (12%) as compared to primary hypothyroidism in thalassemic patients was an observation made by Hashemi *et al.*, (2012). While there was higher prevalence of primary hypothyroidism in thalassemic patients studied by Malik *et al.*, (2010). Thalassemic male of <13 years in our study developed secondary hypothyroidism while ≥13 years thalassemic males suffered from primary

hypothyroidism.This discrepancy in the frequency of pituitary-thyroid axis can be due to difference in the study region, quality of management and treatment protocols (Najafipour *et al.*, 2008).

Whereas, in our study serum GH levels were low in thalassemic male and female of <13 years and the GH levels were high in thalassemic male and females of ≥13 years of age. In our study GH had a positive correlation ($P<0.05$) with Kisspeptin levels in thalassemic male of <13 years. The absence of a pubertal growth spurt during spontaneous or induced puberty has shown required for the attainment of normal final adult Height. Low serum IGF-I and normal GH reserve in short thalassaemic children shows that there is a time when there is relative GH resistance. The rise in IGF-I and changes in growth after giving GH therapy proves that this GH resistance was only partial. In older patients having high serum Ferritin levels present with multiple endocrinopathies, including hypogonadism, hypothyroidism and diabetes mellitus. Better survivals of such individuals is seen after keeping the serum Ferritin below 2000 micrograms/l by regular chelation(Low, 1997).

Conclusion

Regular blood transfusion followed by iron chelation therapy is just a supportive treatment for the disease of thalassemia which is associated with serious complications. The beneficial effects of regular transfusions on wellbeing of patients with thalassemia have been reported and have been subsequently confirmed by further studies. But, during this supportive treatment, the magnitude of the body iron burden is the principal determinant of clinical outcome for the prime goal i.e of iron-chelating therapy in patients with thalassemia major is to control body iron. The optimal body iron should be reduced both to prevent the adverse effects from the iron-chelating agent to prevent the risk of complications from iron overload. With stable transfusion requirements and in the absence of other confounding factors, the lower the level of body iron desired, increased dose of iron chelator is needed. With effective chelation using DFO, normal growth and sexual maturation can be expected. The apparent facts is that upon reaching age of puberty thalassemic patients develop growth retardation and pubertal failure. Thalassemic patients are short, have low growth rate and BMI and have either delayed or absent pubertal spurt, which is related to low Hemoglobin and high Ferritin levels and sub-optimal iron chelation

therapy. These defects start early in life but, become becomes obvious after the age of 8 years. In developing countries, poor socio-economic background adds up to the problem. Therefore, effective alternate chelation regimens should be considered to improve the complication resulting from chelation therapy . Low levels of GH secretion and reduced level of IGF-I in thalassaemic patients are related to a neurosecretory dysfunction which occurs due to iron overload rather than to liver damage. Hypogonadotropic hypogonadism is caused by the selective loss of pituitary gonadotropin function. In such thalassemic patients with both GH deficiency and hypogonadism, low dose sexual steroid treatment should be considered either as an alternative or an additional treatment before starting GH therapy. Important functions of GH, not only on growth but also on lipid and protein metabolism and on normal long-term cardiac function, should be considered thalassemic children who may benefit from GH and treat them effectively long-term so that they may have a better quality of life and possibly a longer survival rate.

Chapter # 4

Prevalence of Hepatitis along with altered ALT levels among multi-transfused Pakistani thalassemic patients.

Abstract

Background: Beta thalassemia patients are more prone to develop different organ damage due to metabolic dysfunction, the actual mechanism is not clear but anemia and iron overload are most important factors leading to increase mortality and morbidity rate along with lipid peroxidation, oxidative stress and free radical release that cause such condition.

Materials and Methods: The present study was conducted on a total of 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group. The total 300 individuals were further divided into 4 groups of <13 years female (50 thalassemic and 25 control), ≥13 years female (50 thalassemic and 25 control), <13 years male (50 thalassemic and 25 control) and ≥13 years male (50 thalassemic and 25 control). Height and BMI were calculated along with analysis of serum Ferritin and Hemoglobin levels. Hepatitis Band C were detected in all thalassemic groups along with estimation of serum ALT levels. Correlation of serum ALT levels with BMI , Hemoglobin and serum Ferritin levels was done along with estimation of prevalence of Hepatitis and liver enlargement.

Results: All groups had significantly reduced ($P<0.001$) BMI and Hb as compared to control group. Serum Ferritin levels were significantly ($P<0.001$) high in all thalassemic groups on comparison with control. Serum ALT levels in all four thalassemic groups are significantly ($P<0.001$) raised as compared to control groups. Significant correlation exist between serum ALT and Ferritin levels. Prevalence of Hepatitis is greater than 50 % in all four thalassemic groups as well as liver enlargement is also observed in all the four groups.

Conclusion: Serum Ferritin concentration is an important determinant of liver enzyme levels, and increased serum Ferritin level is an independent predictor of liver damage in thalassemic patients, so it is useful to identify patients at risk of steatoHepatitis and advanced fibrosis. Hepatitis also effects the condition of such patients along with anemia and low BMI which reduce the ability of thalassemic patients to fight against such infections. Careful monitoring

should be done at early stage of disease and serum Ferritin levels should also be maintained and frequently monitored to prevent further liver damageduring the course of treatment.

Introduction

Worldwide beta (β) and alpha (α) thalassaemias are highly frequent inherited single-gene disorders. To avoid such inheritance premarital counseling and prenatal testing are being done to avoid such occurrence. A large number of childern are born with this disorder and require bone marrow transplantation as a cure for such disorder. This mode of treatment is not affordable by majority of patients. Such patient are left with the option of getting regular blood transfusion and appropriate chelation therapy along with close monitoring and managing the complication developed during the course and management of the disease (Pemde *et al.*, 2011).

Lack of genetic counseling and proper screening (Weatherall,1994) can lead to an alarming situation because it may become a very serious threat in future fifty years as according to thalassemia international federation, currently, about 200,000 patients of β- thalassemia are alive globally(Galanello and Origa , 2010).

The gene frequency of β-thalassemia in Pakistan, has been expected to be 5-8% with 8-10 million carriers (Satwani *et al.*, 2005). Independent origin of β-thalassemia in various populations has also been observed (Flint *et al.*, 1993). Disease thalassemia is associated with a number of clinical features in which moderate to severe anemia is critical one, which is developed in response to hemolysis and ineffective erythropoiesis, which results from the augmented apoptosis of the maturing nucleated erythroid cells (Leung *et al.*, 2005). Thalassemic patients suffer from microcytic and hypochromic anemia with low levels of Mean Corpuscular Volume (MCV) and mean corpuscular Hemoglobin (Cao and Galanello,2010).

There are other various other features which include generalized symptoms like irritability, fever and systemic features which include skeletal and endocrine changes along with gastrointestinal symptoms like diarrhea, feeding problems along with spleen and liver enlargement that develop during the course of the disease (Neufeld, 2010).

The main treatment for the beta thalassemic patients is repeated transfusions along with chelation therapy. Regular blood transfusion with chelation therapy has

markedly improved the quality of life and extended the life expectancy of such patients upto 20 years (Malik *et al.*, 2010). The function of heart, liver and endocrine glands get effected by excess iron deposition. Despite adequate subcutaneous (s.c.) iron chelation therapy with desferrioxamine (DFO),50% of the patients do not survive the age of 35 years because of poor compliance with s.c. chelation regimens. Recurrent transfusions result in iron overload leading to various range of new complications like cholelithiasis and hepatotoxicity which appear in adolescents and young adults (Borgna-Pignatti *et al.*, 2005).

Iron is mainly stored in the liver that retain about 70% of the total body content of iron(Angulo et al., 2008). Ferritin is the major iron storage protein, that is present in various sites like liver, spleen, bone marrow and with minute quantity in the blood. These are the sites where iron is stored in a non-toxic and safe form, and transport it to the target areas when required (Tori, 2002). Repeated blood transfusion lead to iron overload that excel the normal storage and detoxifying capacity of Ferritin. Owing to the multiple blood transfusions in thalassemic patients, iron load may exceed the storage and, sequentially leads to deposition of free iron in blood and tissues (Prabhu *et al.*, 2009). Estimation of serum Ferritin levels is an indicator of total iron in the body. Long term control of serum Ferritin levels are associated with long term survival of patients (Oliveri and Brittenham, 1997).

Cardiomegaly is the main cause of death in thalassemic patients but, recently liver disease is also becoming a critical complication that may lead to death of patients (Zurlo *et al.*, 1989). Chronic hepatitis and/or severe iron overload may lead to Cirrhosis and hepatocellular carcinoma (Aldouri *et al.*, 1987).

Transferrin and Ferritin are synthesized in the liver which is the main site for the storage of iron. Free ferrous iron are extremely toxic and are present in liver bounded with protein. Unbounded iron is catalyzed and free radicals are produced which are involved in development of lipid peroxidation and hepatotoxicity. Lipid peroxidation causes liver damage secondary to iron overload (Kaddah *et al.*, 2005;Larson *et al.*, 2003).

During inflammatory disorders such as hepatitis and ascorbate serum Ferritin levels are raised therefore, they are considered as acute phase reactant. An alternative

approach to measurement of total body stores is the total liver iron content (LIC) This indicator is considered to be more accurate that is why it has been used in diseases like thalassemia major and hereditary haemochromatosis (Angelucci *et al.*, 2000; Brittenham *et al.*, 1994).

Serum Ferritin levels of 1000-3000 µg/L are considered as the cut off points for estimating iron toxicity. Under normal circumstances the value of serum Ferritin in males is (10-220 µg/L) and in females (10-85 µg/L). Although the standard values vary to a wider range in both male and females (Oliveri and Brittenham, 1997). Despite the use of chelation therapy 30–40% of thalassemia major and hemochromatosis patients still develop liver disease. Although serum Ferritin levels are a good parameter for measuring iron overload but for diseases with high serum Ferritin levels like hepatitis C, LIC is considered to be more accurate. Histological grading of haemosiderosis is also done as an alternate measure in centers where LIC is not available (Angelucc *et al.*, 2000).

Hepatitis B and C infections are more prevalent in thalassemia patients(Mohamed et al., 2013; Okada *et al.*, 2000).Significantly, raised liver enzymes are seen in patients suffering from HCV as compared to the non infected individuals (Mohamed et al., 2013). Severe liver iron overload has been observed in chronic hepatitis patients, although the reason of iron overload in such patients is still not clear. damaged hepatocytes as result of hepatic necro-inflammation release iron and Ferritin which gets deposited and leads to increase serum Alanine Aminotransferase (ALT) levels (Nelson and Kowdley, 2004). Another observation is that increase iron levels lead to increase replication of HCV in vitro (Kakizaki *et al.*, 2000).

Increased levels of ALT are associated with the prospective and prognosis of liver disease. In thalassemic patients with pubertal delay the association of ALT levels is still not well defined therefore, our current study was done to determine the effects of iron overload on ALT (U/L) levels. Correlation of ALT with BMI, serum Ferritin and Hemoglobin (gm/dl) of beta thalassemic patients of pubertal age group undergoing repeated blood transfusions with chelation therapy was also explored during the present study. Prevalence of Hepatitis B and C along with liver enlargement among such patient was also determined.

Material and Method

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-5.

The inclusion and exclusion criteria for thalassemic patients and control individuals included in the study are mentioned in detail in general materials and methods. Height in centimeter and weight in kilogram were measured and BMI was calculated. The blood samples for tests were collected from thalassemic patients (n=200) and control individuals (n=100) as mentioned in detail as general materials and methods. The blood samples collected and stored and were used for Enzyme-linked Immunosorbent assay kit was used for quantitative measurement of Hemoglobin (gm/dl) and serum Ferritin was measured by using Ferritin (FTL) ELISA (Enzyme-Linked Immunosorbent Assay) kit, details of which are mentioned in chapte-1 and serum ALT levels.

Quantitative determination of Serum Alanine Transaminase (IU/L)

The MaxDiscovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit is a plate-based colorimetric enzymatic assay for the determination of the Alanine Transaminase enzyme in serum samples. *(MaxDiscovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit, Biooscientific Corporation; USA).*

Reagents:

ALT Reagent Mix, DNPH Color Solution, Pyruvate Control, Pyruvate Dilution Buffer, 0.5 M NaOH

Assay procedure:

Each sample of 10 µL was added to the bottom of the microplate wells. Then 50µL of ALT Reagent Mix was added to the wells. Mixing was ensured and reagent was directly added to the spot in the well where the sample was added. The plates were incubated at 370C for 30 minutes, than 50µL of DNPH Color Solution was added to each well. The plates were again incubated at 37°C for 10 minutes. 0.5 M NaOH was

added to each well and plates were incubated at 37°C for 5 minutes. Absorbance of each sample was measured at 510 nm.

Precautions:

- Do not use the kit past the expiration date.
- Try to maintain a laboratory temperature of (20–25°C/68–77°F). Avoid running assays under or near air vents, as this may cause excessive cooling, heating and/or evaporation. Also, do not run assays in direct sunlight, as this may cause excessive heat and evaporation. Cold bench tops should be avoided by placing several layers of paper towel or some other insulation material under the assay plates during incubation.
- Use only distilled or deionized water since water quality is very important.
- When pipetting samples or reagents into an empty microtiter plate, place the pipette tips in the lower corner of the well, making contact with the plastic.

Qualitative detection of Hepatitis B

Science with mission (SMI) Hepatitis B surface antigen (HBsAg) test strip is a rapid chromatographic immunoassay for the qualitative detection of HBsAg in serum or plasma.

Principle of procedure:

The membrane was pre-coated with anti-HBsAg antibodies on the test line region of the test. During testing, the serum specimen reacted with the particle coated with anti-HBsAg antibody. The mixture migrated upward on the membrane chromatographically by capillary action to react with anti-HBsAg antibodies on the membrane and generated a colored line. The presence of this colored line in the test region indicated that there was positive result, while its absence indicated a negative result. To serve as a procedural control, a colored line always appeared in the control line region indicating that proper volume of specimen had been added and membrane wicking had occurred.

Reagents:

The test device contained anti-HBsAg particles and anti-HBsAg coated on the membrane.

Precautions:

- Do not eat, drink or smoke in the area where the specimens or kits are handled.
- Handle all specimens as if they contain infectious agents. Observe established precautions against microbiological hazards throughout testing and follow the standard procedures for proper disposal of specimens.
- Wear protective clothing such as laboratory coats, disposable gloves and eye protection when specimens are being tested.
- Humidity and temperature can adversely affect results.

Qualitative detection of Hepatitis C

Science with mission (SMI) One-Step Hepatitis C Virus (HCV) Test is a rapid chromatographic immunoassay for the qualitative detection of antibody to Hepatitis C Virus in serum, plasma or whole blood.

Principle of procedure:

The membrane was coated with recombinant HCV antigens on the test line region of the device. During testing, the serum specimen reacted with the HCV antigens coated particles. The mixture migrated upward on the membrane chromatographically by capillary action to react with recombinant HCV antigen on the membrane and generated a colored line. Presence of this colored line indicated a positive result, while its absence indicated a negative result. To serve as a procedural control, a colored line always appeared at the control line region if the test had been performed properly.

Reagents:

The test device contained recombinant HCV antigens coated particles and HCV antigen coated on the membrane.

- Do not eat, drink or smoke in the area where the specimens or kits are handled.
- Handle all specimens as if they contain infectious agents. Observe established precautions against microbiological hazards throughout testing and follow the standard procedures for proper disposal of specimens.

- Wear protective clothing such as laboratory coats, disposable gloves and eye protection when specimens are being tested.
- Humidity and temperature can adversely affect results.

Precautions:

- Do not eat, drink or smoke in the area where the specimens or kits are handled.
- Handle all specimens as if they contain infectious agents. Observe established precautions against microbiological hazards throughout testing and follow the standard procedures for proper disposal of specimens.
- Wear protective clothing such as laboratory coats, disposable gloves and eye protection when specimens are being tested.
- Humidity and temperature can adversely affect results.

Palpation of liver

The purpose of liver palpation is to approximate liver size, feel for tenderness and masses

Technique:

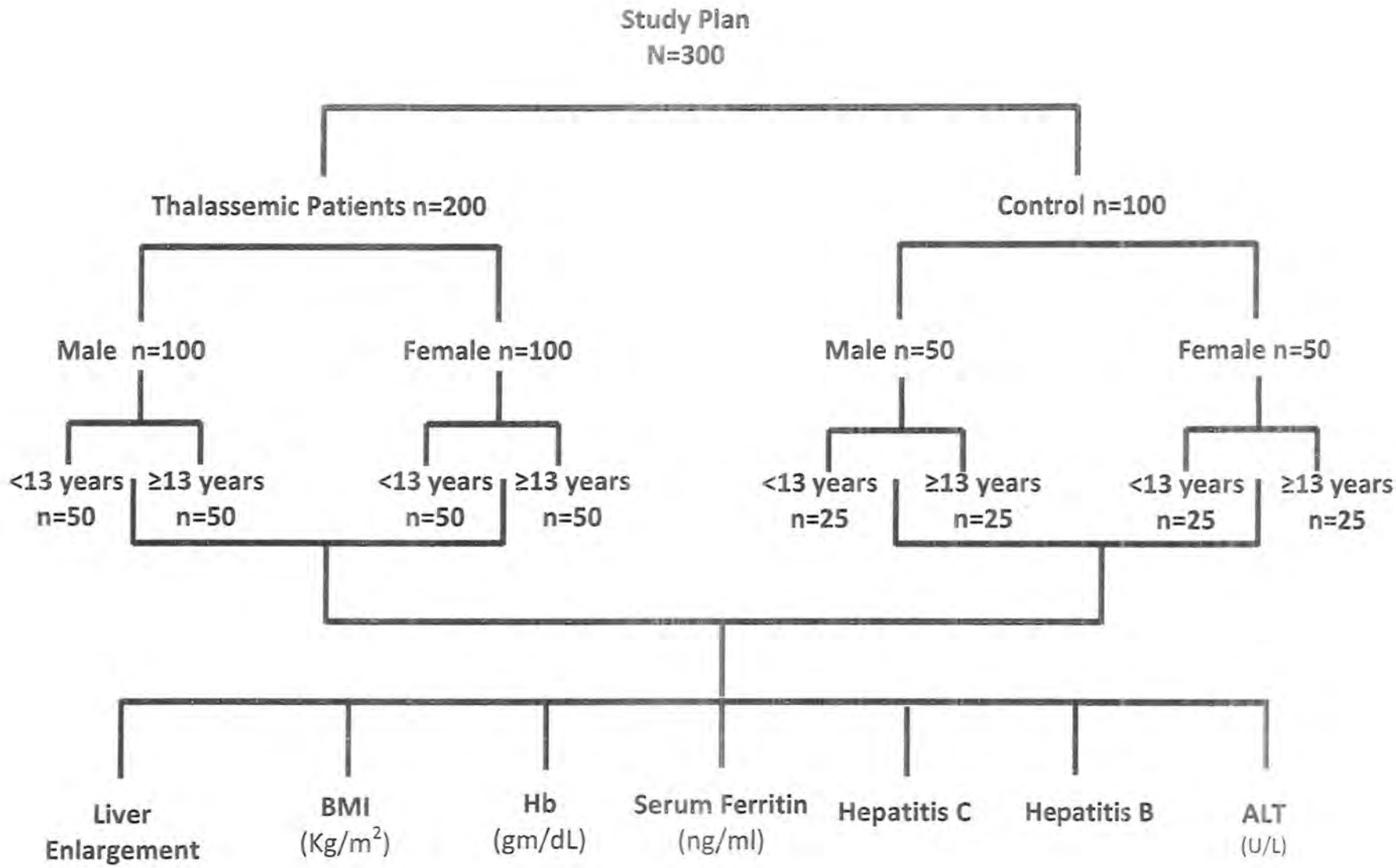
Patients was asked to lie in supine position then right hand was placed on patient's abdomen, just lateral to the rectus abdominis, well below lower border of liver dullness. The patients were asked to take a deep breath and liver edge was felt when it descended during the process of breathing.

Findings:

If liver is enlargement it will come downwards and will meet the finger tips of the palpating hands and will become recognizable.

Statistical Analysis

Data was analyzed through Graph Pad Prism 5.01. Data was reported as Mean ± SEM. Comparison of BMI, Hemoglobin, serum Ferritin and serum ALT levels with the control group was done by using unpaired t-test. Further non parametric co-relation (Spearman) for ALT with BMI, Hemoglobin and serum Ferritin was done through pad prism. $P<0.05$ was considered statistically significant.



Flow Chart 5: Study plan describing female and male thalassemic patients with their corresponding control of different age groups in determining serum ALT, BMI, serum Ferritin and Hemoglobin levels along with prevalence of Hepatitis B & C and assessment of live enlargement.

Results

The present study was conducted on the same total 300 individuals out of which 200 were patients suffering from beta thalassemia major and 100 were control matched for age and gender with the thalassemic group as shown in Flow Chart-5. Results of (BMI) Kg/m^2, Hemoglobin (gm/dl) and serum Ferritin (ng/mL) are mentioned in chapter -1.

Serum ALT levels (U/L)

Serum ALT levels (U/L) in thalassemic females of <13 years of age (79.7 ± 6.88 U/L) was significantly raised (P<0.001) as compared to the control group (24.0 ± 0.32 U/L). On comparison of ALT (U/L) levels (65.9 ± 5.15 U/L) of thalassemic females of ≥13 years were significantly higher than the control group (31.3 ± 0.37 U/L).

Similarly <13 years thalassemic males had serum ALT levels of 101 ± 12.5 U/L which were significantly raised as compared to the control group which had ALT levels of 28 ± 0.32 U/L. Comparison of serum ALT levels (89.2 ± 5.61 U/L) in ≥13 years thalassemic males showed significantly raised levels with respect to the control group (41.9 ± 0.45 U/L). Comparison of serum ALT (U/L) of female and male thalassemic patients with their corresponding control of different age groups is shown in Figure-4.1.

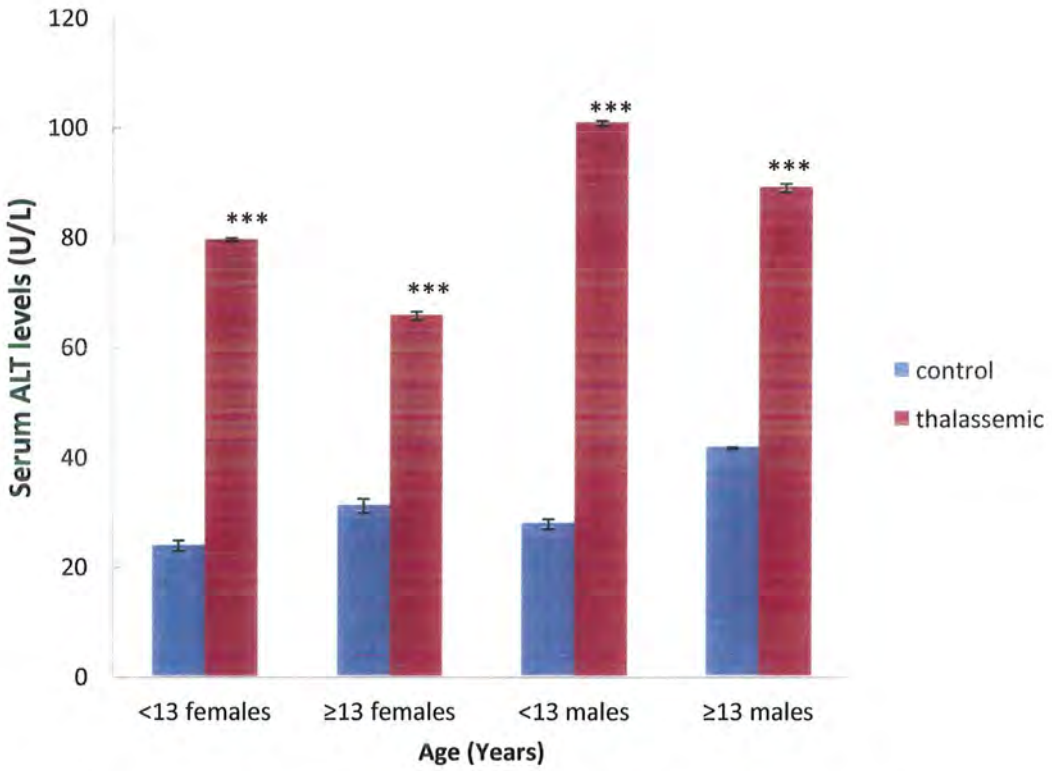


Figure 4.1: Comparison of Serum ALT (U/L) of female and male thalassemic patients with their corresponding control of different age groups. ***=P<0.001 (value vs. corresponding control)

Correlation of ALT (U/L) with BMI (Kg/m^2), Serum Ferritin (ng/mL) and Hemoglobin (gm/dl) levels in thalassemic males and females patients in different age groups.

Correlation of ALT (U/L) with BMI (Kg/m^2), serum Ferritin (ng/mL) and Hemoglobin (gm/dl) levels in thalassemic males and females patients in different age groups is shown in Table-4.1and Figure-4.1,4.2,4.3 and 4.4. In thalassemic females of <13 years serum ALT (U/L) showed a significant (P<0.05) correlation with serum Ferritin levels (ng/mL) (r=0.322). While serum ALT levels (U/L) in this age group had a non significant positive correlation with Hemoglobin (gm/dl) (r=0.024) and non significant negative correlation with BMI (Kg/m^2) (r= -0.009). Correlation of ALT levels (U/L) in ≥13 years thalassemic females with serum Ferritin (ng/mL) (r=0.199), Hemoglobin (gm/dl) (r=0.008) and BMI (Kg/m^2) (r=0.044) was found to be non significant. Similarly in <13 thalassemic males ALT (U/L) showed a non significant correlation with serum Ferritin (ng/mL) (r=0.159), Hemoglobin (gm/dl) (r=0.139) and BMI (Kg/m^2) (r=-0.214). Serum ALT (U/L) levels of thalassemic males of ≥13 years had a significant positive correlation with serum Ferritin (ng/mL) (r=0.399) while a non significant correlation existed with Hemoglobin (gm/dl) (r= -0.023) and BMI (Kg/m^2) (r=0.098).

Table 4.1: Correlation of ALT with BMI, Serum Ferritin, and Hemoglobin levels in thalassemic female and male patients in different age groups.

Age (Years)	Gender	Enzyme	Groups	BMI (Kg/m^2)	Ferritin (ng/mL)	Hb (gm/dl)
< 13	Females	ALT (U/L)	Thalassemia n=50	-0.009	0.322*	0.024
≥ 13			Thalassemia n=50	0.044	0.199	0.008
< 13	Males		Thalassemic n=50	-0.214	0.159	0.139
≥ 13			Thalassemic n=50	0.098	0.399**	-0.023

*=P<0.05, **=P<0.01, value are considered significant.

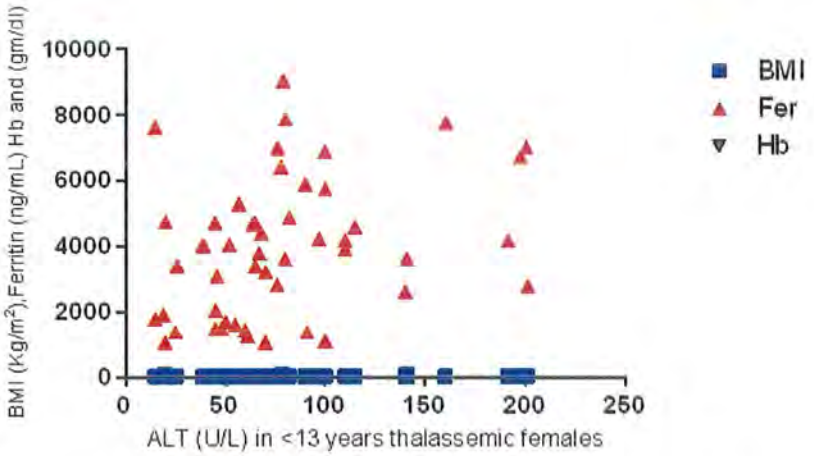


Figure 4.2: Correlation of ALT (U/L) with BMI (Kg/m^2), serum ferritin (ng/mL) and hemoglobin (gm/dl) thalassemic females of <13 years.

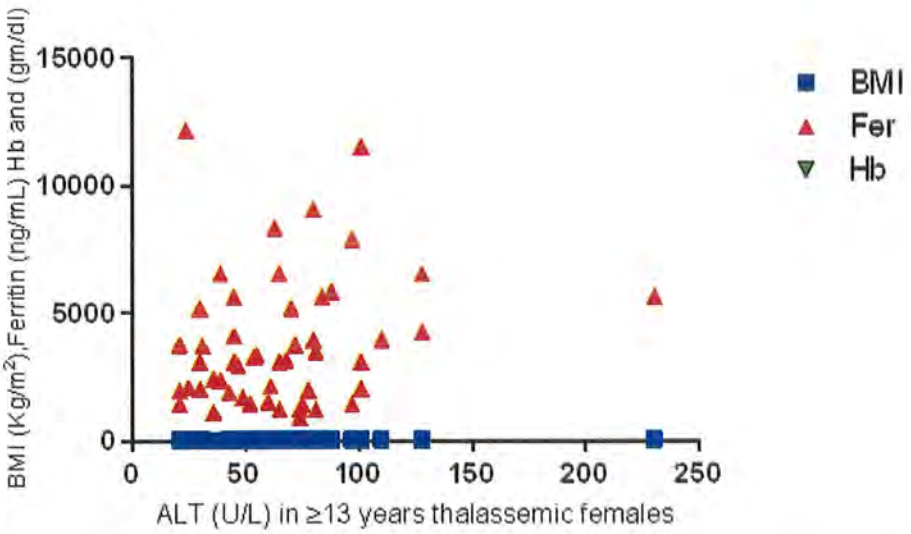


Figure 4.3: Correlation of ALT (U/L) with BMI (Kg/m^2), serum ferritin (ng/mL) and hemoglobin (gm/dl) thalassemic females of ≥13 years.

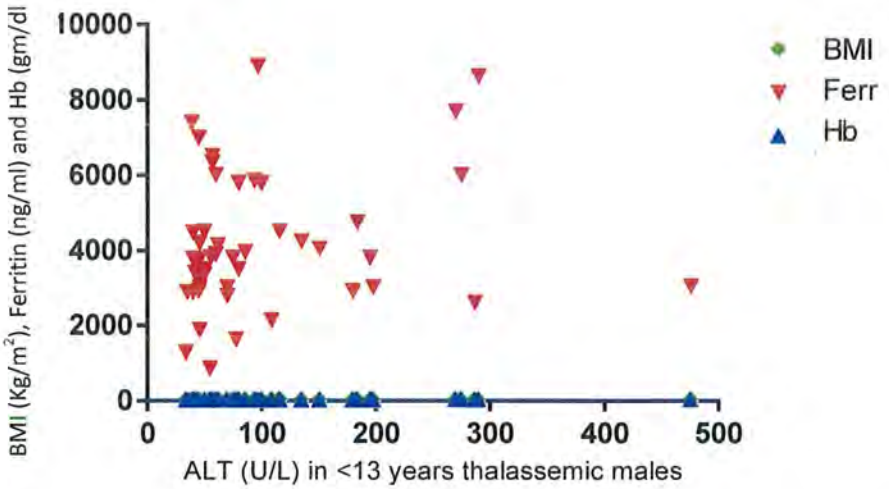


Figure 4.4: Correlation of ALT (U/L) with BMI (Kg/m^2), serum ferritin (ng/mL) and hemoglobin (gm/dl) thalassemic males of <13 years.

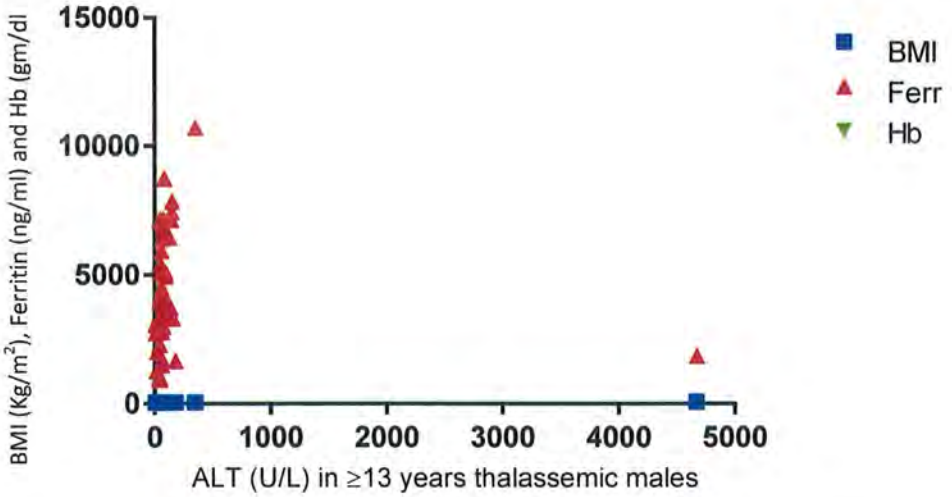


Figure 4.5: Correlation of ALT (U/L) with BMI (Kg/m^2), serum ferritin (ng/mL) and hemoglobin (gm/dl) thalassemic males of ≥13 years.

Prevalence of hepatitis and liver enlargement along with ALT levels (U/L) in female and male thalassemic patients with their corresponding control of different age groups.

Prevalence of hepatitis C in thalassemic patients of <13 years was 51% while liver enlargement which was present in 62% of the patients. Levels of serum ALT (79.7 ± 6.88 U/L) were also significantly raised (P<0.001) of the control group (24.0 ± 0.329 U/L). Similarly 30% of thalassemic females of ≥13 years suffered from hepatitis B & C. Also liver enlargement was observed in 57% of the patients. On comparison of ALT (U/L) levels (65.9 ± 5.15 U/L) of thalassemic females of ≥13 years were significantly higher than the control group (31.3 ± 0.37 U/L).

Hepatitis C was positive in 46% of thalassemic males of <13 years of age, while liver enlargement was observed in 60% of the patients. Similarly <13 years thalassemic males had serum ALT levels of 101 ± 12.5 U/L which were significantly raised as compared to the control group which had ALT levels of 28 ± 0.32 U/L. On the other hand 46% of thalassemic males of ≥13 years suffered from hepatitis B & C along with liver enlargement which was observed in 56% of these patients. Comparison of serum ALT levels in ≥13 years thalassemic males showed significantly raised levels (89.2 ± 5.61 U/L) with respect to the control group (41.9 ± 0.45 U/L). Prevalence of hepatitis and liver enlargement along with ALT levels (U/L) in female and male thalassemic patients with their corresponding control of different age groups is shown in Table-4.2.

Table 4.2: Prevalence of hepatitis and liver enlargement along with ALT levels (U/L) in female and male thalassemic patients with their corresponding control of different age groups.

Age (Years)	Gender	Groups	ALT Levels (U/L)	Hepatitis	Liver Enlargement
< 13	Females	Control n=25	24.0 ± 0.329	Negative	Absent
		Thalassemic n=50	79.7 ± 6.88***	51% Positive	62 % Present
≥ 13		Control n=25	31.3 ± 0.379	Negative	Absent
		Thalassemic n=50	65.9 ± 5.15***	30 % Positive	57 % Present
< 13	Males	Control n=25	28.0 ± 0.329	Negative	Absent
		Thalassemic n=50	101 ± 12.5***	46 % Positive	60 % Present
≥ 13		Control n=25	41.9 ± 0.45	Negative	Absent
		Thalassemic n=50	89.2 ± 5.61***	46 % Positive	56 % Present

Values are expressed in Mean ± SEM, ***=P<0.001 (value vs. corresponding control)

Discussion

Beta thalassemia patients are more prone to develop different organ damage due to metabolic dysfunction, the actual mechanism is not clear but, anemia and iron overload are most important factors leading to increase mortality and morbidity rate along with lipid peroxidation, oxidative stress and free radical release that cause such condition(Walter *et al.*, 2008).

Frequent blood transfusions normally re establish the normal growth spurt as explained by Viprakasit *et al.*, (2001). However, despite frequent blood transfusions the adolescent growth spurt is often delayed, except if rigorous iron chelation treatment is commensed at an early age in life (Theodoridis *et al.*, 1998). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overWeight and 6.5% were categorized as obese (Adil *et al.*, 2012). Although these results are contrary to our study findings where low BMI was detected.

Thalassemic patients develop the tendency of reduced or inadequate nutrient intake that leads to undernutrition resulting in stunting of children. In our study Height was measured in thalassemic groups and all four groups suffered from significantly reduced Height as compared to the control. Similarly, ≥13 years thalassemic female and <13 and ≥13 years thalassemic male patients also had reduced BMI as compared to control groups. These results are consistant with work done by Fuchs *et al.*, (1997) in which thalassemic children had reduced Height and BMI but, changes in Weight, length and body compartments were observed after period of recovery from under nutrition by nutritional support period. Work done by Zamar *et al.*, (2015) also showed that male and female thalassemic patients had reduced Height and BMI, which is consistant with our study results. The reason for such findings is attributed to the fact that childern with thalaseemia suffer from iron deposition in many major organs of the body leading to multiple endocrinopathies, which contribute to reduced Height and low BMI in these children as compared to the healthy children who have strong immune systems which combat such development.

Hegazi *et al* ., (2013) observed a significantly low Hb levels and Red blood cell count along with significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups. These findings are in accordance with finding of Charles and Linker, (2005); Irshaid and Mansi, (2009) who also reported that Hb concentration values in thalassemic patients are significantly lower than controls. These results are similar to our study results as all thalassemic groups of <13 and ≥13 years had low Hb. levels as compared to the control groups.

Hegazi *et al* ., (2013) carried out a study on thalassemic male and female patients of 4-18 years of age, where there was a significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups was observed . Similarly, Abdulzahra *et al.,* (2011); Patil and Mujawar, (2010); Vahidi, *et al.,* (2003) work also revealed that iron indices were markedly increased in thalassemic patients, and the mean serum level of Ferritin were also raised as compared to control group. Similarly, in our study high serum Ferritin level were observed in all four groups of <13 and ≥3 years male and female thalassemic patients as compared to the control groups which was similar to the results reported by (Adil *et al.,* 2012), suggesting that increased serum Ferritin levels are related to the endocrinopathies. The raised serum Ferritin levels in thalassemic patients were associated with increased prevalence of development of endocrinopathies along with consequent increase in the serum calcium (Ca), levels, alkaline phosphate and parathyroid hormone levels was also a finding observed by Hussein and Manal, (2013).

Alanine aminotransferase (ALT) is an enzyme belonging to the family of transaminases, produced mainly in the liver for catalyzing the transfer of amino groups between L-alanine and glutamate. Hepatic injury and before the appearance of hepatic diseases like jaundice the ALT levels rise (Dufour *et al.,* 2000). Cheema and Dilshad, 2011 found high level of ALT in thalassemic patients receiving multiple blood transfusions. Similarly, we have also found the elevated levels of ALT in the current study, Hence, the results of the present study, are in line with these studies.

High Ferritin levels and the age when transfusions was started are related to abnormal liver function (Kaddah *et al.*, 2005; Larson *et al.*, 2003). Viral infection lead to aggravation of iron induced liver disease. Despite iron chelation therapy Hepatic siderosis, portal fibrosis and even cirrhosis may develop (Ragab *et al.*, 2010). Elevated serum ALT levels should alarm the clinician about the onset of hepatitis due to repeated transfusions (Thakerngpoi *et al.*, 1996).The results of our study also show raised serum ALT levels in all four thalassemic groups which ultimately are developing hepatitis.

Superconducting quantum interference device biomagnetometry (SQUID) non-invasively measures a significant correlation between Ferritin iron concentration and individual liver iron concentration. When serum Ferritins are below 2500 µg/ and there is absence of hepatitis the relation between serum Ferritin concentration and liver iron improves (Kaddah *et al.*, 2005; Larson *et al.*, 2003).Our study results also show a significant correlation of serum ALT levels and serum Ferritin levels.

Win *et al.*, 2013 work proved that there was a positive correlation between serum Ferritin and ALT concentrations and the negative correlation between IGF-I concentrations and Ferritin and ALT which indicate that hepatic iron overload impairs the hepatic functions and decreases IGF-I synthesis, even in the absence of hepatitis in these patients.

Development of hepatitis is very common in patients with severe haemosiderosis and hepatic fibrosis that leads to cirrhosis of liver. Patients are also being treated for hepatitis C infection. Thus, early and accurate diagnosis of liver disease followed by prompt intervention may prevent liver disease progression (Telfer *et al.*, 1997).Results of our study also show increase prevalence of hepatitis B and C infections in all the four thalassemic groups along with liver enlargement.

Conclusion

Serum Ferritin concentration is an important determinant of liver enzyme levels, and increased serum Ferritin level is an independent predictor of liver damage in thalassemic patients, so it is useful to identify patients at risk of steatohepatitis and advanced fibrosis. Hepatitis also effects the condition of such patients along with

anemia and low BMI which reduce the ability of thalassemic patients to fight against such infections. In conclusion, available evidence suggests a positive independent association of baseline ALT level with BMI, serum Ferritin and hemoglobin levels. Therefore, elevations of ALT levels in thalassemic patients should be an indication for further clinical evaluation. Careful monitoring should be done at early stage of disease and serum Ferritin levels should also be maintained to prevent further liver damage.

General Discussion

Thalassemic patients are dependent on blood transfusions to maintain the levels of Hemoglobin and packed cell volume in their blood. Transfusion and iron-chelation therapy has prolonged and improved the quality of life in these patients (Borgna-Pignatti *et al.*, 2004). Such a treatment, however, leads to chronic iron overload affecting the endocrine glands (Abdulazahra *et al.*, 2011). In our study we observed that patients suffering from thalassemia major present with endocrine disorders and pubertal delay.

In another study carried out by Al-Rimawi *et al.*, 2005 showed that there was a significant difference in the frequency and regularity of using chelation therapy between pubertal and delayed pubertal groups. Whereas in our study the age of starting chelation therapy was 6-8 months and the patients were on regular blood transfusion and chelation therapy.

Najaf *et al.*, (2008) research revealed that 70% of the males and in 73% of female thalassemic patients of 10–27 years suffered from short stature. While Li *et al.*, (2002) observed short stature in 29.7% of patients. The iron overload leading to endocrinopathies, chronic anemia, zinc and folate deficiencies can lead to short stature. These findings are in accordance to our study results in which we observed reduced Height in all four groups of thalassemic males and females. Therefore, close observation of growth in such individuals can lead to early detection of complication which can be managed to their full extent so, that the individual can achieve their normal adult Height (De Sanctis *et al.*, 1995; Arcasoy *et al.*, 1987).

Under Weight and obesity are assessed in a variety of ways, calculating BMI is one of the most ideal methods to access under Weight, over Weight and obesity in an individual. Under Weight and under-nutrition individuals have decreased energy levels and are vulnerable to develop injury and infection, malfunctioning of multiple endocrine systems and psychological problems (Mahan and Escott, 2000). Patients with thalassemia major are exposed to many growth abnormalities as a outcome of the disease or due to the

adverse effects of chelating therapy which they receive on regular basis as described by Kattamis *et al.*, (1990).

Work done by Ali and Hamdollah, (2004) on thalassemic patients revealed the reduced BMI was more apparent in greater than 10 years of age, which are similar to our study results. Thalassemic males of <13 and ≥13 years and thalassemic females of ≥13 years in our study had reduced BMI (P<0.001) as compared to the control group. The explanation to these results can be that endocrinopathies which appear as a result of iron overload and development of side effects due to prolong use of chelation therapy can be chief contributing factors in development of under Weight thalassemic patients (Ali and Hamdollah, 2004). While considering the prevalence of under Weight (low BMI), in males and females, our study revealed that both <13 and ≥ 13 years thalassemic males suffered from low BMI while on the other hand only ≥13 years thalassemic females developed low BMI. Reason for such a finding requires further studies keeping the pathogenesis of under Weight and low BMI in thalassemic patients into consideration as also explained by Ali and Hamdollah, (2004). Deena *et al.*, (2014) also showed similar results of 18 (30%) patients who had low BMI. The BMI was significantly lower in the patients group as compared to the control individuals of more than 12 years of age. This finding is indicating that low BMI is highly dependent on disease progression and are in accordance with our present findings.

Growth retardation is a common presentation in thalassemic children which may be attributed to their distraction from proper intake of food, loss of appetite from ineffective erythropoiesis, along with the development of anemia. Frequent blood transfusions normally re establishes the normal growth spurt as explained by Viprakasit *et al.*, (2001). However, despite frequent blood transfusions the adolescent growth spurt is often delayed, except if rigorous iron chelation treatment is commenced at an early age in life (Theodoridis *et al.*, 1998). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were over Weight and 6.5% were categorized as obese

(Adil *et al.*, 2012). Although these results are contrary to our study findings where low BMI and reduced Height was detected.

Shalitin *et al.*, (2005) studies revealed that at serum Ferritin levels of 2500 ng/mL there was development of hypogonadism in thalassemic patients and in his studies the serum Ferritin levels were significantly raised as compared to the control groups. While Bronspiegel *et al.*, (1990) discovered that chelation therapy with DFO initiated before puberty could help children to achieve normal sexual maturation as 90% of thalassemic patients in their study received DFO with mean serum Ferritin (1562 ± 445 ng/mL) before the age of 10 years and had normal sexual development as compared with 38% of patients who received chelation therapy after onset of puberty with mean serum Ferritin levels of 4271 ± 1989 ng/mL.

Shalitin *et al.*, (2005) also observed that thalassemic patients receiving effective chelation therapy in prepubertal years still developed short stature with significantly raised serum Ferritin levels. But these finding were contrary to results obtained by De Sanctis *et al.*, (1994) who detected no significant difference in final Height between patients who started chelation therapy during adolescence with high serum Ferritin level and those who started chelation therapy during childhood with low serum Ferritin levels.

Hegazi *et al* ., (2013) observed a significantly low Hb levels and red blood cell count along with significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups. These findings are in accordance with finding of Charles and Linker, (2005); Irshaid and Mansi, (2009) who also reported that Hb levels in thalassemic patients are significantly lower than control. These results are similar to our study results as all thalassemic groups of <13 and ≥13 years had low Hb levels as compared to the control groups.

Hegazi *et al* ., (2013) carried out a study on thalassemic male and female patients of 4-18 years of age, where there was a significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared to control groups. Similarly, Abdulzahra *et al.*, (2011); Patil and Mujawar, (2010); Vahidi, *et al.*, (2003) work also revealed that

iron indices were markedly increased in thalassemic patients, and the mean serum level of Ferritin were also raised as compared to control group. Similarly, in our study high serum Ferritin levels were observed in all four groups of <13 and ≥3 years male and female thalassemic patients as compared to the control groups which was similar to the results reported by Adil *et al.*, 2012, suggesting that increased serum Ferritin levels are related to the endocrinopathies. The raised serum Ferritin levels in thalassemic patients were associated with increased prevalence of development of endocrinopathies along with consequent increase in the serum calcium (Ca), levels, alkaline phosphate and parathyroid hormone levels which was also a similar finding observed by Hussein and Manal in 2013.

In previous study carried out by Hussein and Mohsen (2013), comparison of measured parameters between male and female thalassemic patients of 3-11 years age group were done which showed high Hemoglobin levels in male patients in comparison with female thalassemic patients. The change in Hemoglobin levels can be easily explained by the genetic changes between male and female, males have higher Hemoglobin levels than females. While comparing with our study Hemoglobin levels of thalassemic males and females were significantly low as compared to the control groups. Similarly, in our study serum Ferritin level were high as compared to the control group which was similar to the results reported by Adil *et al.*, (2012), suggesting that increased serum Ferritin levels are related to the endocrinopathies.

Increased serum Ferritin levels were associated with increased incidence of endocrinopathies along with subsequent increase in the serum levels of calcium (Ca), alkaline phosphate and parathyroid hormone levels (Hussein and Mohsen 2013).

Thalassemic children frequently present with growth retardation which may be attributed to their diversion from caloric resources resulting from ineffective erythropoiesis, along with the effects of anemia. Since hyper-transfusion has been shown to frequently restore normal growth rates (Viprakasit *et al.*, 2001). However, the adolescent growth spurt is often delayed, even in children who are hyper-transfused, unless intensive iron chelation therapy is instituted early in life (Theodoridis *et al.*, 1998).

Previous study on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overWeight and 6.5% were categorized as obese (Adil *et al.*, 2012).Whereas, thalassemic patients males and females of all groups included in our study had reduced BMI ($P<0.001$) as compared to the control group.

A high prevalence of endocrine abnormalities in beta thalassemia major patients is reported by Zervas *et al.*, (2002). Relationship between the level of Ferritin and the development of endocrinopathies suggest that serum Ferritin is used as a prognostic marker for survival of these thalassemic patients, prognosis for survival is excellent when serum Ferritin concentration is below 2500ng/ml in thalassemic patients (Costin *et al.*, 1979).Whereas the study results by Zervas *et al.*, (2002) reported absence of such a relation.

During the course of beta thalassemia major, multiple endocrine disorders may develop mainly due to iron overload. Growth retardation and HPG axis dysfunction represent the commonest disorders of the endocrine system (De Sanctis, 2002).

The major breakthrough in the field of reproduction came in 2003, Seminara *et al.*, (2003) showed that GPR54 was crucial for normal puberty. In our study there were increased levels of serum Kisspeptin in thalassemic females of <13 and ≥13 years as compared to the control group. Whereas, high levels of serum Kisspeptin in ≥13 and ≥13 years thalassemic males were observed as compared to the control group, which can be due the reason that high serum levels of Kisspeptin are required during pubertal years.

Work done by Hegazi *et al.*, (2013) revealed that thalssemic males of 4-12 years of age had high levels of serum iron, Ferritin and low levels of FSH ($P<0.05$). Meanwhile, no significant difference was detected between LH levels of thalassemic patients when compared with the control group.

Similarly, Dundar *et al.*, (2007) found that the serum level of FSH in male thalassemic patients were significantly lower as compared to control group. Similarly, in our study

serum FSH levels in thalassemic males and females <13 years and ≥13 year females were higher than the control group. Whereas, serum FSH levels in thalassemic males <13 years and ≥ 13 years were low as compared with the control group. Serum LH levels in less than 13 years thalassemic male patients were higher (P<0.001) than the control group, similarly serum LH levels of thalassemic males ≥13 years were also significantly reduced (P<0.001) than the control group.

Findings of previous work done by Hegazi *et al.,* (2013) showed low serum levels of LH, FSH and Testosterone in thalassemic male of 12-18 years of age as compared to control group. These results were in accordance with studies done by (Anoussakis *et al.,* 2008; Dundar *et al.,* 2007; Vahidi *et al.,* 2003). Which is contrary to the results of our study as thalassemic male of <13 years had high serum Testosterone levels when compared with the control group.

Low levels of serum LH, FSH, progesterone and Estradiol were detected in studies done by Hegazi *et al.*, (2013) on thalassemic females of 12-18 years when compared to control group. Our study had a similar finding as significantly low Estradiol levels were observed in <13years thalassemic females as compared to the control group.

In our study negative correlation of FSH levels with serum Ferritin level (r = -0.511) in <13 years thalassmic females was observed, this result is supported by the studies done by Hegazi *et al.*,(2013) in 4-12 years thalassemic females. The results demonstrated that there was a significant alteration in the activity of gonadotropins (LH and FSH), in the anterior pituitary gland that takes place early in life and affects the function of the gonads at puberty, due to iron overload. Hegazi *et al.,* (2013) found in thalassemic females of 14-18 years age that there existed a negative correlation between serum Ferritin levels and FSH in accordance with Papadimas *et al.,* (1996). These results are contrary to our study results as in ≥13 years thalassemic females there is a non significant negative correlation of FSH with BMI (r=-0.455), serum Ferritin (r=- 0.350) and Hemoglobin levels (r= -0.358).

These results confirmed that there is effect of iron overload on the activity of the pituitary secretion of FSH. Hegazi *et al.*, (2013) revealed a significant elevation of the serum levels of iron and Ferritin and a significant decrease in the mean serum level of FSH ($P<0.05$) in 4-12 years thalassemic males. Our study also showed that FSH had a negative correlation with Hemoglobin in thalassemic males <13 years ($r=-0.479$) and ≥13 years thalassemic males ($r= - 0.346$) and a significant positive correlation with BMI ($r=0.296$) in <13 years thalassemic males.

In our study serum LH also had a negative correlation with Hemoglobin levels ($r = - 0.386$) in <13years thalassemic females whereas, Al-Rimawi *et al.*, (2006) observed that when the gonadotropin levels in the thalassemic delayed puberty patients were compared with the constitutional delayed puberty patients, there were no significant difference in the basal hormonal levels, but the response to GnRHa administration was extremely low in the thalassemic delayed puberty group compared with the response ($P<0.001$) in constitutional delayed puberty group. This indicated a defective gonadotropin reserve in the gonadotropic cells in the thalassemic delayed puberty group compared with normal pituitary function in the constitutional delayed puberty group (Al-Rimawi *et al.*,(2006).

In our study Estradiol in ≥13 years thalassemic females had a positive correlation with BMI ($r= 0.318$) and Hemoglobin levels ($r= 0.286$) These finding are similar to results obtained by Hegazi *et al.*, (2013). Other investigators demonstrated through (magnetic resonance imaging) that pituitary gland atrophy in beta thalassemic patients with hemochromatosis (Soliman *et al.*, 2000) and the signal intensity reduction in the anterior lobe of the pituitary gland correlated with serum Ferritin level and the severity of pituitary dysfunction (Sparacia *et al.*, 2000). Furthermore, even a modest amount of iron deposition within the gland could interfere with its function (De Sanctis, 2002).

Patients with transfusional iron overload begin to develop pituitary iron overload in the first decade of life; however, significant iron deposition were observed beginning in the second decade. Heavy pituitary iron deposition were predictive of hypogonadotropic hypogonadism in these patients (Noetzli *et al.*, 2011).This explains our study results as there is development of pituitary dysfunction more in older age patients than younger

age patients. In the study carried out by Noetzli *et al.*, (2011), the serum levels of Testosterone were significantly lower among male thalassemic patients ≥13 years than control ($P<0.05$) indicating pituitary-gonadal dysfunction. These results are not in accordance with our work as our thalassemic patients of <13 years have significantly high Testosterone levels while ≥ 13 thalassemic males have no significant decrease levels.

When chelation therapy with deferoxamine is used before the age of 3 years it causes marked stunted growth with a clinical and radiologic rickets-like syndrome.The reason can be due to the fact that along with iron overload deferoxamine also chelates other essential minerals. Therefore, after the age of 10 years adequate levels of Hemoglobin are maintained but, many of the thalassemic childerm start developing decelerated growth. Pubertal group of children present with reduced growth spurt with marked deceleration, truncal shortening, most likely due to hypogonadism secondary due to iron overload (De Virgilis *et al.*, 1988; Borgna-Pignattiet et al., 1985).

During the first 10 years of life a Hemoglobin levels above 8.5 g/dl should be maintained because during this period hypoxia due to anemia can be the main factor causing growth retardation. Maintenance of Hemoglobin levels above 10–11 g/dl along with adequate chelation therapy gives the thalassemic childern a chance of normal growth and development (Kattamis and Liakopoulou,1990).In previous study carried out by Hussein and Mohsen 2013, comparison of measured parameters between male and female thalassemic patients of 3-11 years age group were done which showed high Hemoglobin levels in male patients in comparison with female thalassemic patients. The change in Hemoglobin levels can be easily explained by the genetic changes between male and female, males have higher Hemoglobin levels than females (Hussein and Mohsen 2013). While comparing with our study Hemoglobin levels of thalassemic male and female patients were significantly low as compared to the control groups. Similarly, in our study serum Ferritin level were high as compared to the control group which was similar to the results reported by Adil *et al.*, 2012, suggesting that increased serum Ferritin levels are related to the endocrinopathies (Adil *et al.*, 2012). Increased serum Ferritin levels were

associated with increased incidence of endocrinopathies along with subsequent increase in the serum levels of calcium (Ca), alkaline phosphate and parathyroid hormone levels (Adil *et al.*, 2012). Thalassemic children frequently present with growth retardation which may be attributed to their diversion from caloric resources resulting from ineffective erythropoiesis, along with the effects of anemia. Since hyper-transfusion has been shown to frequently restore normal growth rates (Viprakasit *et al.*, 2001). However, the adolescent growth spurt is often delayed, even in children who are hyper-transfused, unless intensive iron chelation therapy is instituted early in life (Theodoridis *et al.*, 1994). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overWeight and 6.5% were categorized as obese (Adil *et al.*, 2012). Whereas, thalassemic patients male and female of all groups included in our study had reduced BMI ($P<0.001$) as compared to the control group. A high prevalence of endocrine abnormalities in beta thalassemia major patients is reported by (Zervas *et al.*,2002). Relationship between the level of Ferritin and the development of endocrinopathies suggest that serum Ferritin is used as a prognostic marker for survival of these thalassemic patients, prognosis for survival is excellent when serum Ferritin concentration is below 2500 ng/ml in thalassemic patients (Costin *et al.*, 1979). Whereas the study results by (Zervas *et al.*, 2002) reported absence of such a relation (Zervas *et al.*,2002). During the course of beta thalassemia major, multiple endocrine disorders may develop mainly due to iron overload. Growth retardation and HPG axis dysfunction represent the commonest disorders of the endocrine system (De-Sanctis *et al.*, 2002). According to Abdulzahra *et al.*, (2011); Pirinççioğlu *et al.*, (2011) thyroid dysfunction is common in thalassemia major, but its prevalence and severity varies. Decreased thyroid levels can lead to growth problems in thalassemic patients as documented by Pirinççioğlu *et al.*, (2011). In study carried out by (Hegazi *et al.*, 2013), thyroid function tests (free T_4, free T_3 and TSH) showed no significant difference between thalassemic patients and controls (Hegazi *et al.*, 2013).

On the contrary, hypothyroidism was detected in different ratios in other studies done by Kurtoglu *et al.*, (2012); De Sanctis *et al.*, (2006) as they found hypothyroidism in 12.8%

of their patients respectively. These results were explained by (Aruratanasirikul *et al.*, 2007) that thyroid dysfunction in thalassemic patients was dependent on many factors like age of studied population, the duration of receiving blood transfusions, the amount of iron overload, the dosage of iron-chelating agent, and the procedure used for evaluation. While in our study serum T_3 levels in <13 years thalassemic females were reduced which are similar to results reported by Karamifar *et al.*, (2003) and Zervas *et al.*, (2002).While ≥13 years thalassemic females had significantly ($P<0.001$) higher levels than the control group.Serum T_4 levels in < 13 years thalassemic male patients were significantly lower ($P<0.001$) than the control group. While TSH levels were significantly lower ($P<0.05$) in thalassemic males of < 13 years of age as compared to the control group. Whereas ≥13 years thalassemic males TSH levels were significantly higher ($P<0.01$) than the control group.

Study carried out by Hegazi *et al.*,(2013), detected a significant positive correlation between age and Ferritin and a significant negative correlation between Ferritin and free T_4.So, thyroid function tests might be affected with progress of age of those patients and increased their serum Ferritin level. Whereas in our study thalassemic female of ≥13 years T_3 had a significant ($P<0.001$) negative correlation with BMI ($r= -0.408$), ($P<0.05$) with Hb levels ($r= -0.329$).While T_3 had a positive correlation ($P<0.05$) with serum Ferritin levels of thalassemic males of < 13 years ($r = 0.523$). Gamberini et al., (2008) observed that serum Ferritin levels of approximately 2,000 ng/mL correlated with hypogonadism while at serum Ferritin of 3,000 ng/mL hypothyroidism, hypoparathyroidism and diabetes mellitus was detected.

Similarly, in our study T_3 has a positive correlation with Kisspeptin levels in thalassemic males of ≥13 years.T_4 had a significant positive correlation with BMI, serum Ferritin and Hb levels in thalassemic females of ≥13 years of age. While T_4 had a positive correlation with Hb levels in < 13 years thalassemic females. Similarly, T_4 has a positive correlation with Hb levels in thalassemic males of ≥ 13 years of age and with Hb levels of <13 years thalassemic male.These findings are in line with studies carried out by Hashemizadeh and Norri, (2012) who found that impaired thyroid function was

associated with iron overload. Also, Irshaid and Mansi, (2009) reported that the rate of thyroid dysfunctions increases steadily with advancing age. Controversially, Abdulzahra *et al.*, (2011); Zervas *et al.*, (2002) stated that no statistically significant correlation was found between serum Ferritin levels and thyroid functions. Results of the work done by Ashraf *et al.*, (2013) have proven that there is increase higher prevalence i.e 35% of hypothyroidism was observed in thalassemic patients by age of 18 years They significantly correlated age of thalassemic patients with T_4 and Ferritin levels. While they also proved a negative correlation of Ferritin level with T_4. Through these findings they proved that patients with thalassemia develop progressive deterioration of thyroid function with time,which is due to iron overload. While in our study thalassemic males of <13 years suffered from hypothyroidism and serum levels of T_4 also showed similar negative correlation with serum Ferritin levels.

Elevated thyroid-stimulating hormone (TSH) level and decreased (low) T_4 are observed in primary hypothyroidism while in secondary or central hypothyroidism there is decreased T_4 and low TSH (Malik *et al.*, 2010). Kurtoglu *et al.*, (2012) work proved that there was higher prevalence of primary rather than secondary hypothyroidism. Filosa *et al.*, (2006) reported that there was increase in hypothyroidism over a period of 12 years. Ashraf *et al.*, (2013) patients also had reduced T_4 levels along with corresponding decrease levels of TSH over a period of 12 years This was indicative of the fact that progressive slow dysfunction of the thyroid gland developed with a degree of pituitary insensitivity to the low T_4 level (central component of hypothyroidism).

Higher incidence of secondary (12%) as compared to primary hypothyroidism in thalassemic patients was an observation made by Hashemi *et al.*, (2012). While there was higher prevalence of primary hypothyroidism in thalassemic patients studied by Malik *et al.*, (2010). Thalassemic male of <13 years in our study developed secondary hypothyroidism while ≥13 years thalassemic males suffered from primary hypothyroidism.This discrepancy in the frequency of pituitary-thyroid axis can be due to difference in the study region, quality of management and treatment protocols (Najafipour *et al.*, 2008).

Whereas, in our study serum GH levels were low in thalassemic male and female of <13 years and the GH levels were high in thalassemic male and females of ≥13 years of age. In our study GH had a positive correlation ($P<0.05$) with Kisspeptin levels in thalassemic male of <13 years. The absence of a pubertal growth spurt during spontaneous or induced puberty has shown required for the attainment of normal final adult Height. Low serum IGF-I and normal GH reserve in short thalassaemic children shows that there is a time when there is relative GH resistance. The rise in IGF-I and changes in growth after giving GH therapy proves that this GH resistance was only partial. In older patients having high serum Ferritin levels present with multiple endocrinopathies, including hypogonadism, hypothyroidism and diabetes mellitus. Better survivals of such individuals is seen after keeping the serum Ferritin below 2000 micrograms/l by regular chelation(Low, 1997).

Beta thalassemia patients are more prone to develop different organ damage due to metabolic dysfunction, the actual mechanism is not clear but anemia and iron overload are most important factors leading to increase mortality and morbidity rate along with lipid peroxidation, oxidative stress and free radical release that cause such condition(Walter *et al.*, 2008).

Frequent blood transfusions normally re establish the normal growth spurt as explained by Viprakasit *et al.*, (2001). However, despite frequent blood transfusions the adolescent growth spurt is often delayed, except if rigorous iron chelation treatment is commensed at an early age in life (Theodoridis *et al.*, 1998). Previous studies on thalassemic patients revealed that average age of 12 ± 8 years occasionally suffered from growth failure as 77.4% of these patients had normal BMI, while 4.8% were overWeight and 6.5% were categorized as obese (Adil *et al.*, 2012). Although these results are contrary to our study findings where low BMI was detected.

Thalassemic patients develop the tendency of reduced or inadequate nutrient intake that leads to undernutrition resulting in stunting of children. In our study Height was

measured in thalassemic groups and all four groups suffered from significantly reduced Height as compared to the control. Similarly, ≥13 years thalassemic female and <13 and ≥13 years thalassemic male patients also had reduced BMI as compared to control groups. These results are consistant with work done by Fuchs *et al.,* (1997) in which thalassemic children had reduced Height and BMI but, changes in Weight, length and body compartments were observed after period of recovery from under nutrition by nutritional support period. Work done by Zamar *et al.,* (2015) also showed that male and female thalassemic patients had reduced Height and BMI, which is consistant with our study results. The reason for such findings is attributed to the fact that childern with thalaseemia suffer from iron deposition in many major organs of the body leading to multiple endocrinopathies, which contribute to reduced Height and low BMI in these children as compared to the healthy children who have strong immune systems which combat such development.

Hegazi *et al* ., (2013) observed a significantly low Hb levels and Red blood cell count along with significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups. These findings are in accordance with finding of Charles and Linker, (2005); Irshaid and Mansi, (2009) who also reported that Hb concentration values in thalassemic patients are significantly lower than controls. These results are similar to our study results as all thalassemic groups of <13 and ≥13 years had low Hb. levels as compared to the control groups.

Hegazi *et al* ., (2013) carried out a study on thalassemic male and female patients of 4-18 years of age, where there was a significant increase in the mean serum levels of iron and Ferritin in thalassemic patients as compared with control groups was observed . Similarly, Abdulzahra *et al.,* (2011); Patil and Mujawar, (2010); Vahidi, *et al.,* (2003) work also revealed that iron indices were markedly increased in thalassemic patients, and the mean serum level of Ferritin were also raised as compared to control group. Similarly, in our study high serum Ferritin level were observed in all four groups of <13 and ≥3 years male and female thalassemic patients as compared to the control groups which was similar to the results reported by (Adil *et al.,* 2012), suggesting that increased serum

Ferritin levels are related to the endocrinopathies. The raised serum Ferritin levels in thalassemic patients were associated with increased prevalence of development of endocrinopathies along with consequent increase in the serum calcium (Ca), levels, alkaline phosphate and parathyroid hormone levels was also a finding observed by Hussein and Manal, (2013).

Alanine aminotransferase (ALT) is an enzyme belonging to the family of transaminases, produced mainly in the liver for catalyzing the transfer of amino groups between L-alanine and glutamate. Hepatic injury and before the appearance of hepatic diseases like jaundice the ALT levels rise (Dufour *et al.*, 2000). Cheema and Dilshad, 2011 found high level of ALT in thalassemic patients receiving multiple blood transfusions. Similarly, we have also found the elevated levels of ALT in the current study, Hence, the results of the present study, are in line with these studies.

High Ferritin levels and the age when transfusions was started are related to abnormal liver function (Kaddah *et al.*, 2005; Larson et al., 2003). Viral infection lead to aggravation of iron induced liver disease. Despite iron chelation therapy Hepatic siderosis, portal fibrosis and even cirrhosis may develop (Ragab *et al.*, 2010). Elevated serum ALT levels should alarm the clinician about the onset of hepatitis due to repeated transfusions (Thakerngpol *et al.*, 1996).The results of our study also show raised serum ALT levels in all four thalassemic groups which ultimately are developing hepatitis.

Superconducting quantum interference device biomagnetometry (SQUID) non-invasively measures a significant correlation between Ferritin iron concentration and individual liver iron concentration. When serum Ferritins are below 2500 µg/ and there is absence of hepatitis the relation between serum Ferritin concentration and liver iron improves (Kaddah *et al.*, 2005; Larson *et al.*, 2003). Our study results also show a significant correlation of serum ALT levels and serum Ferritin levels.

Win *et al.*, 2013 work proved that there was a positive correlation between serum Ferritin and ALT concentrations and the negative correlation between IGF-I concentrations and Ferritin and ALT which indicate that hepatic iron overload impairs the hepatic functions and decreases IGF-I synthesis, even in the absence of hepatitis in these patients.

Development of hepatitis is very common in patients with severe haemosiderosis and hepatic fibrosis that lead to cirrhosis of liver. Patients are being treated for hepatitis C infection. Thus, early and accurate diagnosis of liver disease followed by prompt intervention may prevent liver disease progression (Telfer *et al.*, 1997). Results of our study also show increase prevalence of hepatitis B and C infections in all the four thalassemic groups.

General Conclusion

In beta thalassemic patients growth disturbance or delay is main clinical feature that effects the life and wellbeing of such individuals. Our study has revealed that patients with beta thalassemia suffer from reduced height, BMI which is enhanced in patients having high levels of serum ferritin (ng/mL) and low hemoglobin (gm/dl). The growth retardation seen in these patients with thalassemia major is multifactorial, it can be due to under-nutrition, hypogonadism, hypothyroidism, and other complications of thalassemia such as tissue hypoxia and side effects of chelating therapy with desferrioxamine. So, lifelong care and management of such patients is mandatory which requires significant cost for proper treatment of these patients in all aspects.

Half of the patients with thalassemia major die before reaching an age of 30 years mainly because the conventional iron chelation therapy is too oppressive for full adherence. Patients require an individually made-to-measure treatment plan incorporating new, more tolerable treatment options. By reviewing the transfusion and chelation regimens used for patients we can minimize the iron overload in regularly transfused β-thalassemia patients leading to the normal growth and development along with normal height and BMI.

Our study revealed that the levels of kisspeptin in thalssemic females and males of < 13 years had reduced levels but, ≥ 13 years were raised but, at the same time the levels FSH and LH were significantly deranged. These findings are suggestive that hormone production by hypothalmus was correctly secreting kisspeptin according to the pubertal time frame but, the levels of hormones secreted by anterior pituitary and the gonads were deranged due to damage caused by iron overload at pituitary and gonadal level. Along with disturbed hypothalamic pituitary gonadal axis there was decreased BMI, low hemoglobin and raised serum ferritin levels. These findings are indicating that treatment with double chelation from early life should be considered for better outcomes in thalassemic patients.

Regular blood transfusion followed by iron chelation therapy is just a supportive treatment for the disease of thalassemia which is associated with serious complications. The beneficial effects of regular transfusions on wellbeing of patients with thalassemia have been reported and have been subsequently confirmed by further studies. But, during this supportive treatment, the magnitude of the body iron burden is the principal determinant of clinical outcome for the prime goal i.e of iron-chelating therapy in patients with thalassemia major is to control body iron. The optimal body iron should be reduced both to prevent the adverse effects from the iron-chelating agent to prevent the risk of complications from iron overload. With stable transfusion requirements and in the absence of other confounding factors, the lower the level of body iron desired, increased dose of iron chelator is needed. With effective chelation using DFO, normal growth and sexual maturation can be expected.

Serum Ferritin concentration is an important determinant of liver enzyme levels, and increased serum Ferritin level is an independent predictor of liver damage in thalassemic patients, so it is useful to identify patients at risk of steatohepatitis and advanced fibrosis. Hepatitis also effects the condition of such patients along with anemia and low BMI which reduce the ability of thalassemic patients to fight against such infections. In conclusion, available evidence suggests a positive independent association of baseline ALT level with BMI, serum Ferritin and hemoglobin levels. Therefore, elevations of ALT levels in thalassemic patients should be an indication for further clinical evaluation. Careful monitoring should be done at early stage of disease and serum Ferritin levels should also be maintained to prevent further liver damage.

Annexure – 1

PATIENT CONSENT FORM

TO WHOM IT MAY CONCERN

I_____allow my child history, physical examination and retrieval of blood tests for analysis to be included in the present study. I have been told in detail how the research will be carried out and I can discontinue participation in the research project at any time.

Signature of Parent _____

Signature of Researcher _____

Research Proforma

Patient No. _____

Name: _____

Age: _____

Onset of disease (diagnosis): _____

Family History: _____

General Physical Examination: _____

Height: (cm) _____

Weight (kg) _____

Systemic Examination

- Cardiovascular System

_____

- Gastrointestinal System

_____

- Central Nervous System

_____

- Respiratory System:

_____

Investigation

- Hemoglobin (gm/dL) _____
- Serum Ferritin(ng/ml) _____
- Hb Electrophoresis: _____

Hormone Analysis

- T_3 (ng/mL) _____
- T_4 (µg/dL) _____
- TSH (µIU/mL) _____
- FSH (mIU/mL) _____
- LH (mIU/mL) _____
- Estradiol(pg/mL) _____
- Testosterone (ng/mL) _____
- Growth Hormone (ng/ml) _____
- Kisspeptin (ng/ml) _____

Liver Profile

- Hepatitis B _____
- Hepatitis C _____
- ALT (U/L) _____

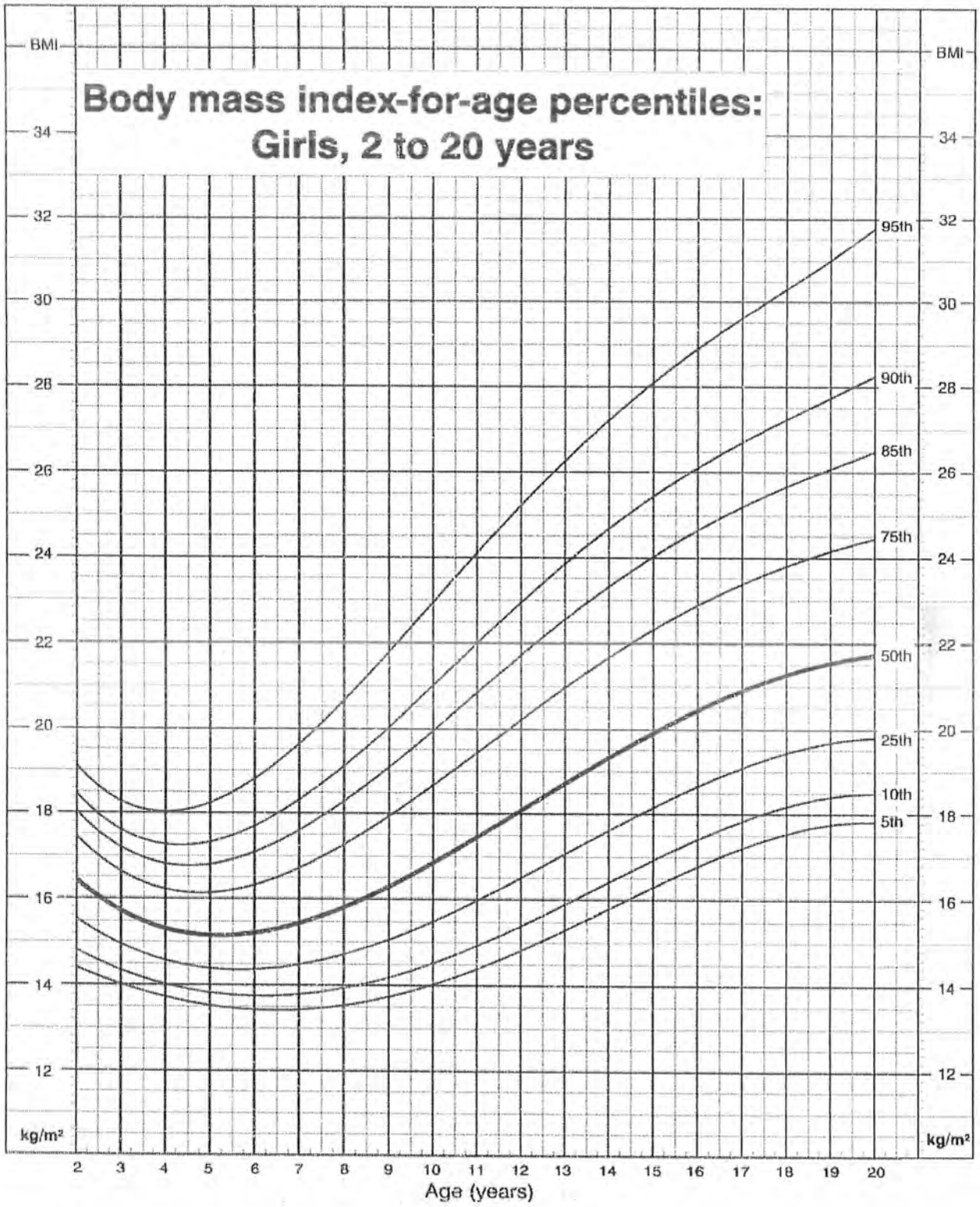
Abdominal Examination

- Enlargement of Liver _____

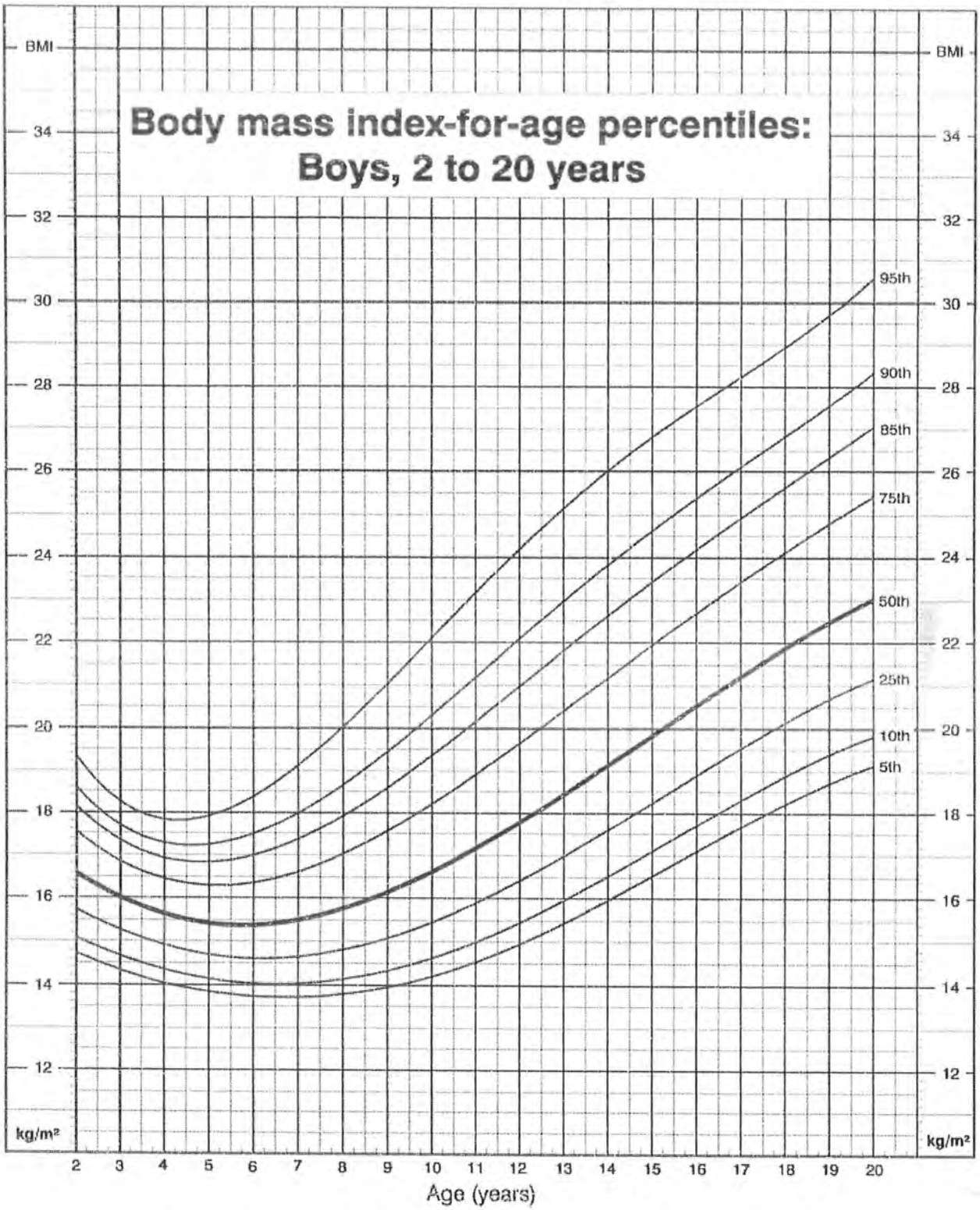
Treatment

- Chelaton Therapy _____
- Regularity of Treatment: _____

Growth Chart



Growth Chart



Psychological Distress and Coping Strategies among Parents of Beta-Thalassemia Major Patients

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Abstract. β-Thalassemia major is a disorder characterized by defective production of hemoglobin and excessive destruction of red blood cells. The usual treatment consists of periodic blood transfusions that can cause iron overload within tissues. Parents of thalassemic patients not only have concerns regarding their children's goal, expectation and standard of life but, also the impact of diagnosis and treatment on family stability and family dynamics. The present study focuses on psychological well being of parents of thalassemic patients.

Key Words: β-Thalassemia major, Puberty, Psychological distress

Introduction

β-Thalassemia major is a disorder characterized by defective production of hemoglobin and excessive destruction of red blood cells. Hemoglobin (Hb) is formed of four protein subunits, two α and two β. Genetic mutations in the gene encoding for the β subunits of the protein, result in reduced or totally absent synthesis of the globin β-chains, leading to the formation of abnormal hemoglobin or even to the absence of β hemoglobin. This defect causes an abnormal development of red blood cells and ultimately anemia, which is the characteristic symptom of the thalassemia. The disease is prevalent among Mediterranean people; the highest frequency is found in the Greek islands, in Italy and in Asia, where the highest concentration of people carrying the genetic mutations underlying thalassemia is found in the Maldives (Borgna-Pignatti, 2004).

The usual treatment consists of periodic blood transfusions that can cause iron overload within tissues. Children on hypertransfusion regimes will maintain normal growth up to puberty. Serum ferritin gives an estimate of the total body iron; levels higher than 2500 mg/l over a period of 15 years are considered a risk factor for cardiac disease (Borgna-Pignatti, 2004). The concept of health is described by WHO as "a state of complete physical, mental, and social well-being, not

merely the absence of disease ...." Like any other chronic disease, for Beta-thalassemia, there is clinical burden on the whole family which includes psychological and social consequences which ultimately affects the well being of the patient. So, it is imperative to study the psychological factors which incorporate, add to distress of the family alongside other factors. Previous studies focused on these points have shown improvement in the quality of life of such patients and their ability to integrate well into their society(Porter and Davis, 2002).

Parents of thalassemic patients not only have concerns regarding their children's goal, expectation and standard of life but, also the impact of diagnosis and treatment on family stability and family dynamics. The disease related concerns of parents are regarding the appearance of their child, bone deformities, short stature, poor self-image, frequent hospital visits for transfusion, delayed or absent sexual development and impaired fertility and other associated complications such as heart disease, bone disease, diabetes, infections etc (Luigi et al., 2009). On parent's perspective it is a frightening and worrisome experience in which they have to cope up with the psychosocial aspects of thalassemia along with their regular visits to the thalassemic centers for blood tests and blood transfusion with iron chelation therapy and their determination to fulfill the treatment. Parents of β-thalassemic patients undergo a significant psychological impact, causing emotional burden, hopelessness, and difficulty with social integration. They experience negative thoughts about their life, guilt, increased anxiety and low self-esteem. They have severe psychosocial problems due to their inability to cope up with painful situation which leads to worseninnig of relationship amongst family members, increased marginalization and isolation. The present study focuses on psychological well being of parents of thalassemic patients. The parents of thalassemic patients elaborate a painful perception of the disease and show impairment in domains involved in physical health, psychological health, quality of life. Poor quality of life (QOL) of parents can be explained by a sense of guilt for having generated a child with a genetically determined disease.

Material and Methods

This prospective, cross sectional study was carried out on Parents of Beta-Thalassemia Major Patients attending thalassemia centre in Rawalpindi, from May 2010 to April 2011. Forty parents

(17 Fathers, 23 mothers) were included into the study. After obtaining informed consent, parents coming for regular treatment were asked to complete the questionnaire in the clinic.

Study Measures

The psychological distress was assessed using the Parental Stress Scale (PSS) and General Health Questionnaire (GHQ) and coping strategies were assessed using COPE Inventory.

Parental Stress Scale

The Parental Stress scale (PSS) is a 19-item self-report measure of parental stress which was developed by Berry & Jones (1995). Parents were asked to agree or disagree with items in terms of their typical relationship with their child and to rate each item on a five-point scale: strongly disagree (1), disagree (2), undecided (3), agree (4), and strongly agree (5). The scores on the scale range between 18-90. Higher scores on the scale indicate greater stress and low score indicate lesser stress. The Parental Stress Scale demonstrated satisfactory levels of internal reliability (.83), and test-retest reliability (.81).

The General Health Questionnaire (GHQ-12)

The General Health Questionnaire (GHQ-12) was used to measure psychological distress. It was originally developed by Goldberg in 1970 (3) and is a widely used tool in primary care to screen for psychological distress and psychiatric morbidity. Its use as screening instrument is well established (4) and is validated in Pakistan (5). Each question has 4 possible responses, i.e. less than usual, no more than usual, rather more than usual, or much more than usual. Cut off point for high scoring was set at a positive response (much more than usual) to at-least 3 of the 12 items.

Brief COPE

The Brief COPE, originally developed by Carver (1997) and translated into Urdu by Akhtar (2005), was used to identify the coping strategies used by parents. Brief COPE is a brief form of COPE Inventory (Carver et al., 1989) consisting of 28 items, categorized into 14 subscales (Self distraction, Active coping, Denial, Substance abuse, Use of emotional support, Use of instrumental support, Behavioral disengagement, Venting, Positive reframing, Planning, Humor, Acceptance, Religion, Self blame). Items are arranged in a 4-point Likert format (1= Never, 2=

Very less, 3= Sometimes and 4= A lot). The items are summed for each subsection separately to get a total score on all 14 categories. The high score on each subscale indicate more use of that particular coping strategy and low score indicate less use of that coping strategy. The respondents' demographic and clinical characteristics were also recorded on history taking proforma. Data was analyzed through SPSS-14. Descriptive statistics were used to describe the data. Independent Samples t-test was used to compare scores between different groups. P-value < 0.05 was considered as significant.

Results

A total of 40 parents, 17 fathers & 23 mothers were included in the study. Age of the parents ranged from 30-50 years. All the parents (40/40) were found to have severe parental stress. Psychological distress was observed in 27 (67.5%) parents as shown in figure 1. Different coping strategies were employed by parents. Most commonly used coping strategies were Active coping (97.5%), Planning (95%), Acceptance (92.5%), Religion (92.5%), self-blame (92.5), Use of instrumental support (90%), Positive reframing (87.5%), and Self-Distraction (82.5%). Others include Use of emotional support (73%), Venting (70%), Behavioral disengagement (62.5%) and Denial (60%). Least used coping strategies include humor (15%), and substance use (7.5%). Details are shown in table1. Significant gender differences were observed on GHQ. Mothers were found to have more distress than fathers. No differences were observed on parental Stress Scale (PSS). Significant differences were found on coping strategies of Denial ($p<0.01$) and Behavioral disengagement ($p<0.05$). Both coping strategies were more prevalent in fathers. Details are shown in table-1 & 2.

Discussion

Thalassemic children are individuals who suffer from a severe chronic hemolytic anemia requiring transfusions as treatment for their survival. This chronic illness is a source of stress for the parents and the rest of the family. Such situation results in the existence of various types of emotional reactions and behavioral patterns in the family, which affects the relationships of family members amongst each other and with their surroundings. Chronic illness of a child

effects the parents at: cognitive levels, emotional level and their daily routine (Monastero et al., 2000).

In the present study psychological distress and coping strategies among parents of β-thalassemic patients showed that all the parents of these thalassemic patients experienced severe parental stress however, psychological distress was reported by 27 (67.5%) parents (Figure-1). Beta thalassaemia is an unending illness that can lead to excessive psychological burden to the patients and their families. Many studies done by Rao P, Pradhan PV have reported that the frequency of psychopathological disorders is higher in parents of children with chronic and disabling diseases (thalassemia) as compared to the normal population (Economouet al., 2006). It is reported by Deepika Shaligram, that fifty seven percent of the caregivers had psychiatric problems and Quality of Life was affected in 50%. Another study revealed that parents of thalassemic patients experienced higher degrees of distressfulness when compared with parents of normal children (Zafeiriou et al., 2006). This frequency of psychological distress leading to parental stress is higher due to the multiple problems which parents have to face while going through the rigorous and painful treatment procedures of thalassemia. They have to face many concerns like the psychosocial adjustment of the child, financial problems, provision of treatment, travelling and other social problems.

An additional goal of the present study was to evaluate the coping strategies of parents. For this purpose COPE Inventory was used which has fourteen types of coping strategies. Different coping strategies were employed by parents. Most frequently used coping strategies were Active coping (97.5%), Planning (95%), Acceptance (92.5%), Religion (92.5%), self-blame (92.5), Use of instrumental support (90%), Positive reframing (87.5%), and Self-Distraction (82.5%). Others include Use of emotional support (73%), Venting (70%), Behavioral disengagement (62.5%) and Denial (60%). Least used coping strategies were humor (15%), and substance use (7.5%). Significant differences were found on coping strategies of Denial ($p<0.01$) and Behavioral disengagement ($p<0.05$). Both coping strategies were more prevalent in fathers. As fathers were found to have difficulty in accepting their child's disease and denied it completely. This behavior made the fathers to be overprotected about their child which inturn effected the psychosocial development of the child. Coping strategy of denial was associated with the guilt of the parents, and any negative remarks from the environment played a significant role to enhance such

behavior. Studies carried out by Dr. Grattan have shown that patients who show behavioral disengage are not realizing their loss and are not making any efforts to improve their condition. Such behavior of fathers hamper the treatment of their children (Beratis, 1993). The awareness of the illness has been suggested as a possible factor influencing both the compliance with treatment and the quality of life of Parents. A previous study done by Aydinok Y found that awareness of the illness by the thalassemic children and their family made them more compliant with the therapy, as they were provided with the psychological support they needed to treat their depression, obsession, paranoia, and hostility(Aydinoket al., 2007).

Conclusion:

Thalassemic children are individuals who suffer from a severe chronic hemolytic anemia requiring transfusions as treatment for their survival. This chronic illness is a source of stress for the parents and the rest of the family. A psychosocial support aimed at reducing emotional distress of the parents, and strengthening their coping strategies for a better integration in daily life, is therefore necessary.

Recommendations

A psychosocial support aimed at reducing emotional distress of the parents, and strengthening their coping strategies for a better integration in daily life, is therefore necessary.

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Table-1: Mean, SD and t-value of Parents (Fathers and Mothers) of Thalassemic Patients on the total scores of GHQ and PSS (N = 40)

Scales	Gender (Fathers) (n = 17)		Gender (Mothers) (n = 23)		t
	M	*SD*	*M*	*SD*	
GHQ)	12.47	4.571	16.57	6.381	-2.250*
PSS	66.24	6.350	65.74	11.017	.166

*df = 38 , *p < .05*

Table-2: Mean, SD and t-value of Parents (Fathers and Mothers) of Thalassemic Patients on the subscales of Brief COPE (N = 40)

Subscales	Gender (Fathers) (n = 17)		Gender (Mothers) (n = 23)		t
	M	*SD*	*M*	*SD*	
Self-distraction	6.06	1.435	5.13	1.866	1.710
Active Coping	7.59	.712	7.39	1.270	.575
Denial	5.29	.849	4.17	1.557	2.6818**
Substance use	2.47	1.375	21.45	2.22	.718
Use of emotional support	5.41	1.278	4.57	2.107	1.467
Use of instrumental support	6.35	1.618	5.96	1.942	.684
Behavioral disengagement	3.65	2.120	4.96	1.894	-2.055*
Venting	4.65	1.656	4.78	1.682	-.237
Positive-reframing	6.47	1.908	6.87	1.817	-.672
Planning	7.12	1.576	7.22	1.380	-.213
Humor	2.82	1.629	2.43	1.472	.789
Acceptance	6.00	1.803	6.43	1.376	-.866
Religion	7.41	1.583	7.30	1.521	.217
Self-blame	6.41	1.326	5.70	1.636	1.480

*df = 38 , **p < .01, *p < .05, p=n.s*

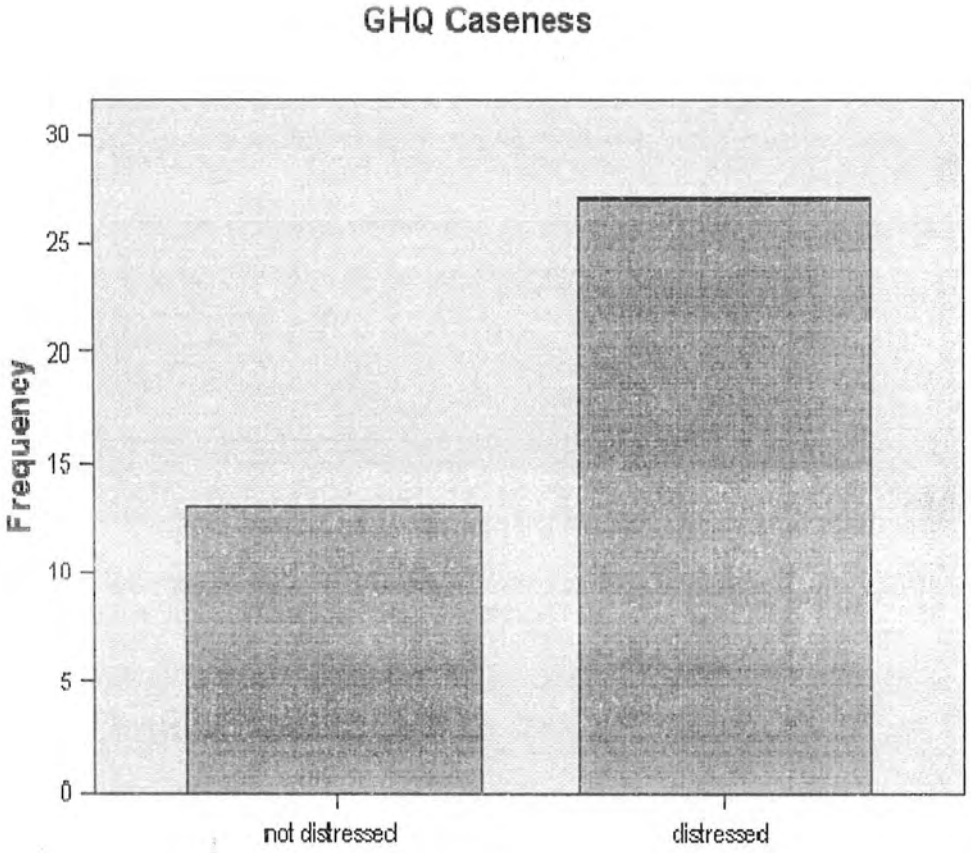

Figure 1: Graph for GHQ Distressed\Not distressed

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