Hematological Analysis of Anemic Severity in Beta Thalassemia

THESIS SUBMITTED TO THE
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Declaration of Authenticity

I, the undersigned, Shadman Shakib Barno, declare that the research work embodying the results reported in this thesis entitled “Hematological Analysis of Anemic Severity in Beta Thalassemia” is my original work, gathered and utilized especially to fulfill the purposes and objectives of this study, and has not been previously submitted to any other institution for a higher degree or diploma. It is further declared that this work has been carried out under joint supervision of Dr. Md Kaiissar Mannoor, Senior Scientist and Head, Institute for Developing Science and Health Initiatives (ideSHi), and Ms. Romana Siddique, Senior Lecturer, Department of Mathematics and Natural Sciences (MNS), BRAC University. I also declare that the publications cited in this work have been personally consulted.

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Abstract

Beta thalassemia is an inherited blood disorder that affects synthesis of the beta chain in the production of haemogoblin, leading to the production of deficient RBCs (red blood cells) that cannot effectively transport oxygen. It is relatively widespread in parts of Asia, and Bangladesh is among the countries where this disorder is prevalent. Beta thalassemia patients require frequent transfusions of healthy blood throughout life in order to mitigate the symptoms, and can suffer from iron overload complications as a result. Transfusions of improperly selected blood can also worsen the condition by encouraging the patient's immune system to attack transplanted healthy RBCs, increasing the amount of transfusions a patient will need throughout life. Erythropoietin (EPO) acts as an erythropoietic protein. Higher-than-normal levels may indicate anemia and in severe cases of anemia, EPO levels in the blood may be a thousand times higher than normal. Serum ferritin is an useful monitoring tool for iron overload in thalassemia major. This study focused on comparing the serum erythropoietin and ferritin levels between Thalassemia patients grouped by the severity of their disease based on transfusion interval. Beta-thalassemia major or E/beta-thalassemia diagnosed patients were enrolled from Bangladesh Thalassemia Samity and Hospital for this study. Peripheral blood specimen was collected from study participants before blood transfusion. Complete Blood Count (CBC) analysis including hemoglobin, RBC, platelet, and reticulocyte counts as well as MCV, MCH and RDW was performed. Serum was separated from the blood specimen and serum erythropoietin and ferritin levels measured using ELISA. Statistical analysis of the obtained results revealed that the EPO level in severe group (irrespective of whether it is β-thalassemia major or E-beta thalassemia) patients was approximately 600 times higher than the control group whereas the less sever group has a concentration as high as 200 times than the healthy control group with a statistically significant difference ($P<0.0001$ and $P<0.005$, respectively). The data showed that serum EPO levels increased in all thalassemia patients despite repeated transfusion. A reason of this high EPO level can be resistance to endogenous EPO. Pro-inflammatory cytokines antagonize the action of EPO by exerting an inhibitory effect on erythroid progenitor cells and by disrupting iron metabolism. EPO resistance might also be caused by inflammation, which has a negative effect on EPO receptors. This study elucidates that the high EPO concentration is linked to anemic severity rather than type of thalassemic condition. Whether the endogenous EPO resistance is a contributing factor to this high magnitude of EPO needs further investigation.

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Introduction

Chapter 1

1.1 Background

Inherited hemoglobinopathies were originally disorders of tropical and sub-tropical areas but now are common worldwide due to migration [1]. Among these hemoglobinopathies, beta thalassemia is one of the most common disorders. Global annual incidence of beta-thalassemia is at one in 100,000 [2]. World Health Organization (WHO) estimates that at least 6.5% of the world populations are carriers of different inherited disorders of hemoglobin [3]. Another WHO report estimates that 3% are carriers of beta-thalassemia and 4% are carriers of Hb E in Bangladesh which accounts which accounts 5.1 million of Beta thalassemia carriers and 6.8 million Hb E beta thalassemia carriers in the whole populations of Bangladesh. In Bangladesh, more than 7000 children are born with thalassemia each year [4]. This inherited autosomal recessive disorder occurs due to a mutation in *HBB* gene. Due to mutation, *HBB* gene expression hampered which leads to a decreased beta-globin chain synthesis which results in the underproduction of HbA. Underproduction of HbA as well as HBB proteins leads to microcytic anemia which requires blood transfusions for treatment [5]. The most severe case of beta thalassemia is beta thalassemia major.

Beta thalassemia is the most frequent type of thalassemia which can be classified further into three subtypes; beta thalassemia major, beta thalassemia intermediate and beta thalassemia minor [6]. Individuals with beta thalassemia major inherit two mutant beta globin alleles (β^0); hence, synthesis of beta chain is completely diminished with a consequence of the development of fatal anemia in early childhood if untreated. Intermediate beta thalassemic individuals carry mutation in one or both of the beta globin genes whereas beta thalassemic trait bears mutation in only one of the two beta globin alleles [7].

Beta globin (hemoglobin subunit beta, HBB) is a globin protein, which along with alpha globin (HBA), makes up the most common form of hemoglobin in adult humans, the HbA. Over 97% of the total RBC Hb is HbA. It consists of two alpha chains and two beta chains Beta thalassemia is characterized by less to no beta globin chain production. This specific type of blood disease results in excessive destruction of red blood cells which in result

leads to anemia [1]. In vertebrates, hemoglobin is the iron-containing oxygen-transport protein that is found in red blood cells which carries oxygen from the lungs to the rest of the body and then brings the carbon dioxide back to the lungs to be dispensed [8]. Individuals with thalassemia produce less health red blood cells which are not enough to help body in carrying oxygen. Individuals with beta thalassemia major usually present within the first two years of life with severe anemia, requiring regular blood transfusions. Majority of thalassemic childs are born in countries with limited resources where priority tends to be given to fight against high rates of infant and child mortality from infection diseases and malnutrition [1]. Findings in untreated or poorly transfused individuals with thalassemia major, as seen in some developing countries, are growth retardation, pallor, jaundice, poor musculature, splenomegaly, development of masses from extramedullary hematopoiesis and skeletal changes that result from expansion of the bone marrow. Regular transfusion therapy leads to iron overload which is related to complications including endocrine complication, failure of sexual maturation, diabetes mellitus, insufficiency of the parathyroid, thyroid, pituitary, and less commonly, adrenal glands, dilated myocardiopathy, liver fibrosis and cirrhosis [9].

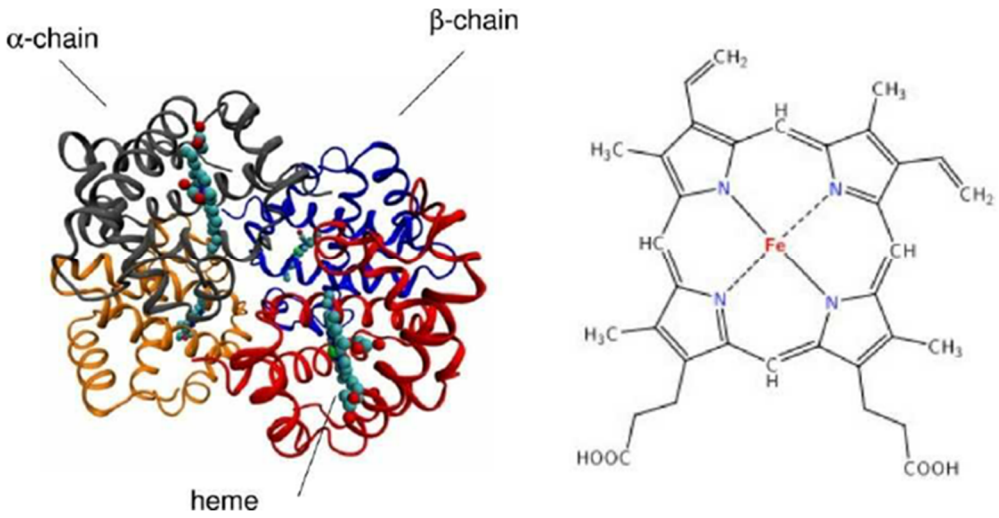

Figure-1.1: The quaternary structure of hemoglobin and its oxygen carrier heme

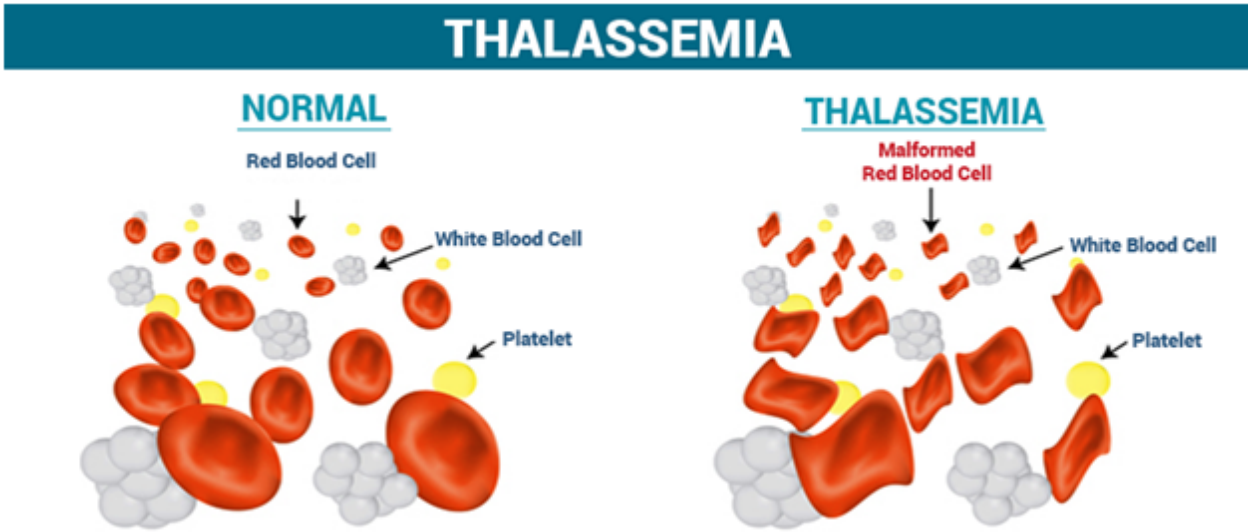

Figure 1.2: Malformed RBC in thalassemia patient compared to normal. Figure adopted from https://byjus.com/biology/thalassemia/

Knowledge about the distribution of carrier status and prenatal diagnosis will provide efficient way of thalassemia management. Previous carrier screening studies in countries with limited resources were proven successful in raising awareness in the local population [10]. This study will end up determining common mutations, present in the Bangladeshi thalassemic patients and association of them with clinical parameters and blood transfusion intervals.

1.2 Epidemiology of beta thalassemia

Geographically the thalassemia belt includes the Mediterranean passing through West and Central Asian countries like Turkey, Iran, Afghanistan onto Pakistan & India and passes on to the South East Asian countries like Indonesia, Burma and Thailand, Vietnam and Cambodia. This makes it most common in African, Greek, Italian, Middle Eastern and Southern Asian populations [11]. Thalassemia affects approximately 4.4 of every 10,000 live births throughout the world. It causes males and females to inherit the relevant gene mutations equally because it follows an autosomal pattern of inheritance with no preference for gender. The widespread of two types of thalassemia is given below [12]:

Alpha thalassemia around the world:

- America: 0-5% of the population has a thalassemia trait, up to 40% may be a genetic carrier
- Eastern Mediterranean: 0-2% of the population has a thalassemia trait, up to 60% may be a genetic carrier
- Europe: 1-2% of the population has a thalassemia trait, up to 12% may be a genetic carrier
- Southeast Asia: 1-30% of the population has a thalassemia trait, up to 40% may be a genetic carrier
- Sub-Saharan Africa: 0% of the population has a thalassemia trait, up to 50% may be a genetic carrier
- Western Pacific: 0% of the population has a thalassemia trait, up to 60% may be a genetic carrier

Beta thalassemia around the world:

- Americas: 0-3% of population is affected by a gene mutation
- Eastern Mediterranean: 2-18% of population is affected a gene mutation
- Europe: 0-19% of population is affected a gene mutation
- Southeast Asia: 0-11% of population is affected a gene mutation
- Sub-Saharan Africa: 0-12% of population is affected a gene mutation
- Western Pacific: 0-13% of population is affected a gene mutation

1.3 Inheritance pattern of beta thalassemia

When the two β- and the four α-globin genes that produce normal adult hemoglobin (HbA, $\alpha_2\beta_2$) work or function normally, the individual is not a carrier and does not have a hemoglobin disorder [2]. Thalassemia major and thalassemia intermedia are inherited in an autosomal recessive pattern, which means both copies of the HBB gene in each cells have mutations. The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene, but they typically do not show signs and symptoms of

the condition [13]. Sometimes, however, people with only one HBB gene mutation in each cell develop mild anemia. These mildly affected people are said to have thalassemia minor.

In a small percentage of families, the *HBB* gene mutation is inherited in an autosomal dominant manner. In these cases, one copy of the altered gene in each cell is sufficient to cause the signs and symptoms of beta thalassemia (OMIM: BETA-THALASSEMIA, DOMINANT INCLUSION BODY TYPE).There are two patterns for beta thalassemia. These are:

Pattern A

When one of the parents carries a non-functional β-globin gene, i.e. when he/she is a β-thalassaemia carrier and the other parent carries 2 functional β-globin genes, then each child born to these parents (i.e. at every pregnancy) has a one-in-two (50%) chance of inheriting the non-functional β-globin gene from the carrier parent as shown below.

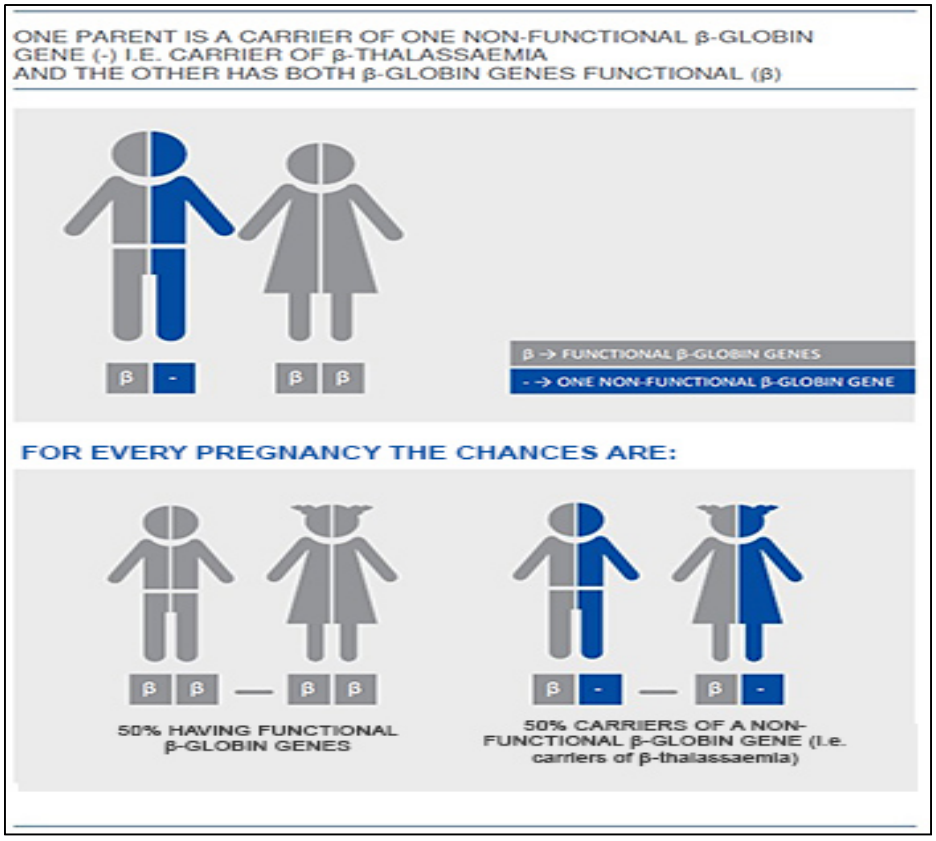

Figure 1.3: Inheritance pattern of beta thalassemia carrier/trait (Figure reprinted from https://www.thalassaemia.org.cy/)

Pattern B

Partners who both carry a non-functional β-globin gene are referred to as an "at-risk" couple and at each pregnancy, the risks involved are as follows:

- a one-in-four (25%) chance that the child will inherit two non-functional β-globin genes (- -), one from the mother and one from the father, and therefore have β-thalassemia major/intermedia, the full-blown disease, also known as Mediterranean Anemia or Cooley's Anemia or homozygous for β-thalassemia
- a one-in-two (50%) chance that the child will be a carrier of β-thalassemia
- a one-in-four (25%) chance that the child will have completely functional β-globin genes as seen below

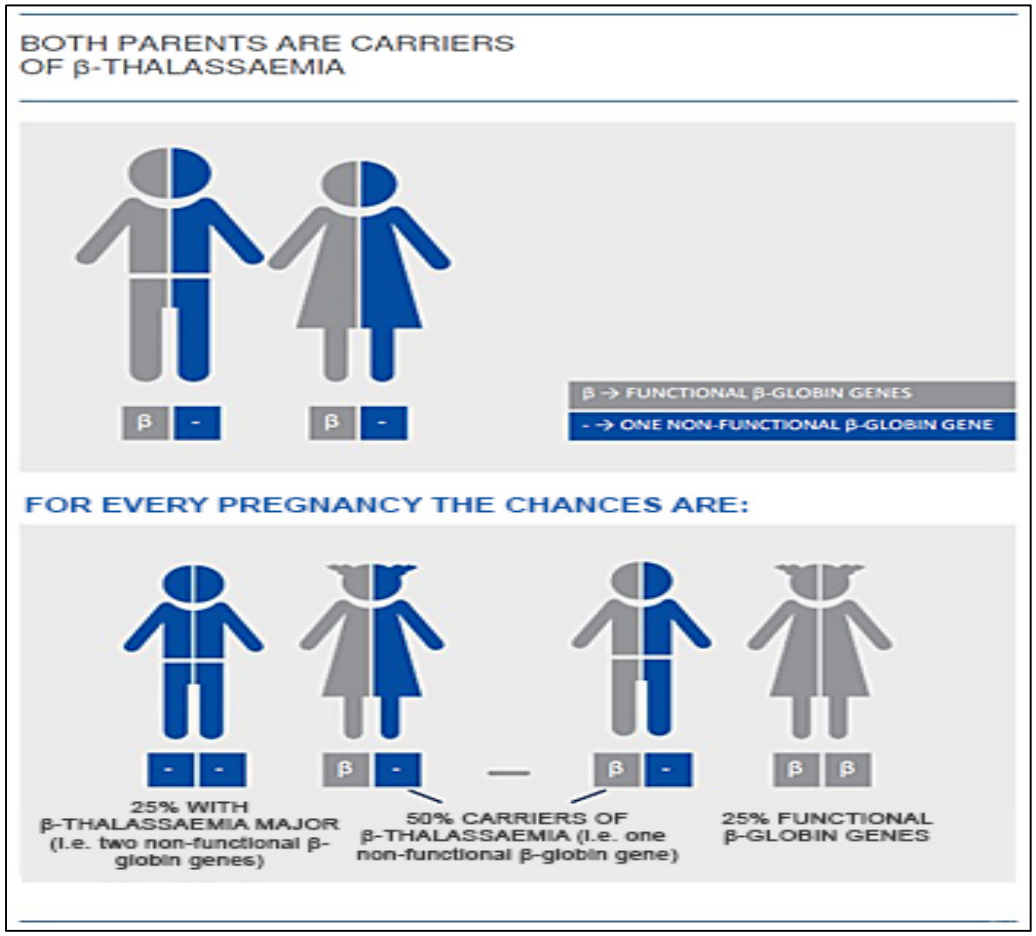

Figure 1.4: Inheritance pattern of beta thalassemia major. (Figure reprinted from https://www.thalassaemia.org.cy)

1.4 Prevalence of beta thalassemia

The β-thalassemia syndromes are much more diverse than the α-thalassemia syndromes due to the diversity of the mutations that produce the defects in the β-globin gene [14].The frequency of β-thalassemia varies widely, depending on the ethnic population. The disease is reported most commonly in Mediterranean, African, and Southeast Asian populations. The highest carrier frequency is reported in Cyprus (14%), Sardinia (10.3%), and Southeast Asia [15]. Population migration and intermarriage between different ethnicities have introduced thalassemia in almost every country of the world, including Northern Europe, where thalassemia was previously absent. About 1.5% of the global populations (80–90 million) are β-thalassemia carriers, with about 60,000 symptomatic individuals born annually. Incidence of symptomatic individuals is estimated at 1 in 100,000 worldwide and 1 in 10,000 in Europe [16]. It is the most common chronic hemolytic anemia in Egypt (85.1%), and its carrier rate has been estimated at 9–10.2% from an examination of 1000 normal random subjects from different geographic areas of the country [17]. β-Thalassemia/HbE disease is the most common form of β-thalassemia in many Asian countries. In Thailand, approximately 3,000 children are born with this condition each year and there are some 100,000 patients in the population. It accounts for over 50% of cases of severe β-thalassemia in Indonesia and Bangladesh and is also very common in Vietnam, Cambodia, Laos, and Malaysia. It also occurs frequently in the eastern side of Indian subcontinent, including Sri Lanka and Maldives [18].

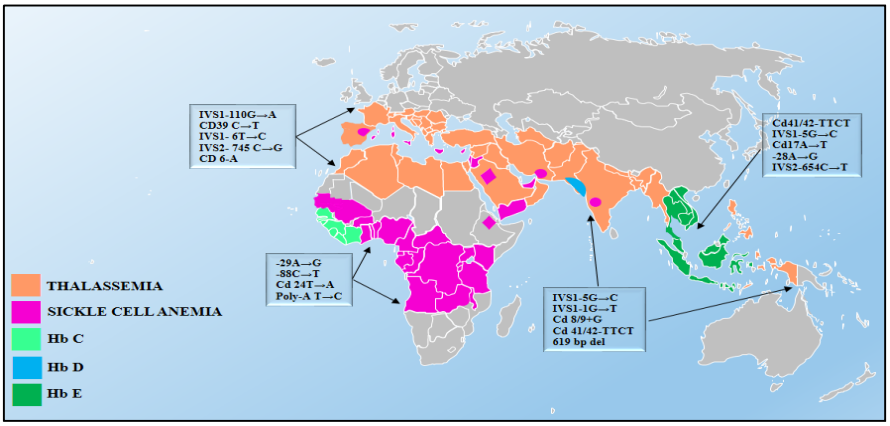

Figure 1.5: Thalassemia distribution and most common β-thalassemia mutations in different countries (Nadia Maria Sposi 2015).

1.5 Classifications of beta thalassemia

There are different types of beta thalassemas. These are given below (Tamer Hassan et al., 2016).

1. β-Thalassemia
    - Thalassemia major
    - Thalassemia intermediate
    - Thalassemia minor
2. β-Thalassemia with associated Hb anomalies
    - HbC/β-thalassemia
    - HbE/β-thalassemia
    - HbS/β-thalassemia
3. Hereditary persistence of fetal Hb and β-thalassemia
4. Autosomal dominant forms of β-thalassemia
5. β-Thalassemia associated with other manifestations
    - β-Thalassemia-trichothiodystrophy
    - X-linked thrombocytopenia with thalassemia

1.6 Pathophysiology of beta-thalassemia

β thalassemia occurs when there is a quantitative reduction of β globin chains that are usually structurally normal [1]. They are caused by mutations that nearly all affect the β globin locus and are extremely heterogeneous. Almost every possible defect affecting gene expression at transcription or post-transcriptional level, including translation, has been identified in β thalassemia [19]. These genetic defects lead to a variable reduction in β globin output ranging from a minimal deficit (mild β+ thalassemia alleles) to complete absence (β° thalassemia).

In β thalassemia, the synthesis of normal α globin chains from the unaffected α globin gene continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α globin. The free α globin chains are not able to form viable tetramers and instead precipitate in the red cell precursors in the bone marrow forming inclusion bodies.

This α chain inclusions can be demonstrated by both light and electron microscopy in the erythroid precursors in the bone marrow as well as in the peripheral red cells following splenectomy. They are responsible for the extensive intramedullary destruction of the erythroid precursors and hence the ineffective erythropoiesis that underlies all β thalassemias [20].

Anemia in β thalassemia thus results from a combination of ineffective erythropoiesis, peripheral hemolysis, and an overall reduction in hemoglobin synthesis. The severity of disease in β thalassemia correlates well with the degree of imbalance between α and non-α globin chains and the size of the free α chain pool. Thus, factors that reduce the degree of chain imbalance and the magnitude of α chain excess in the red cell precursors will have an impact on the phenotype.

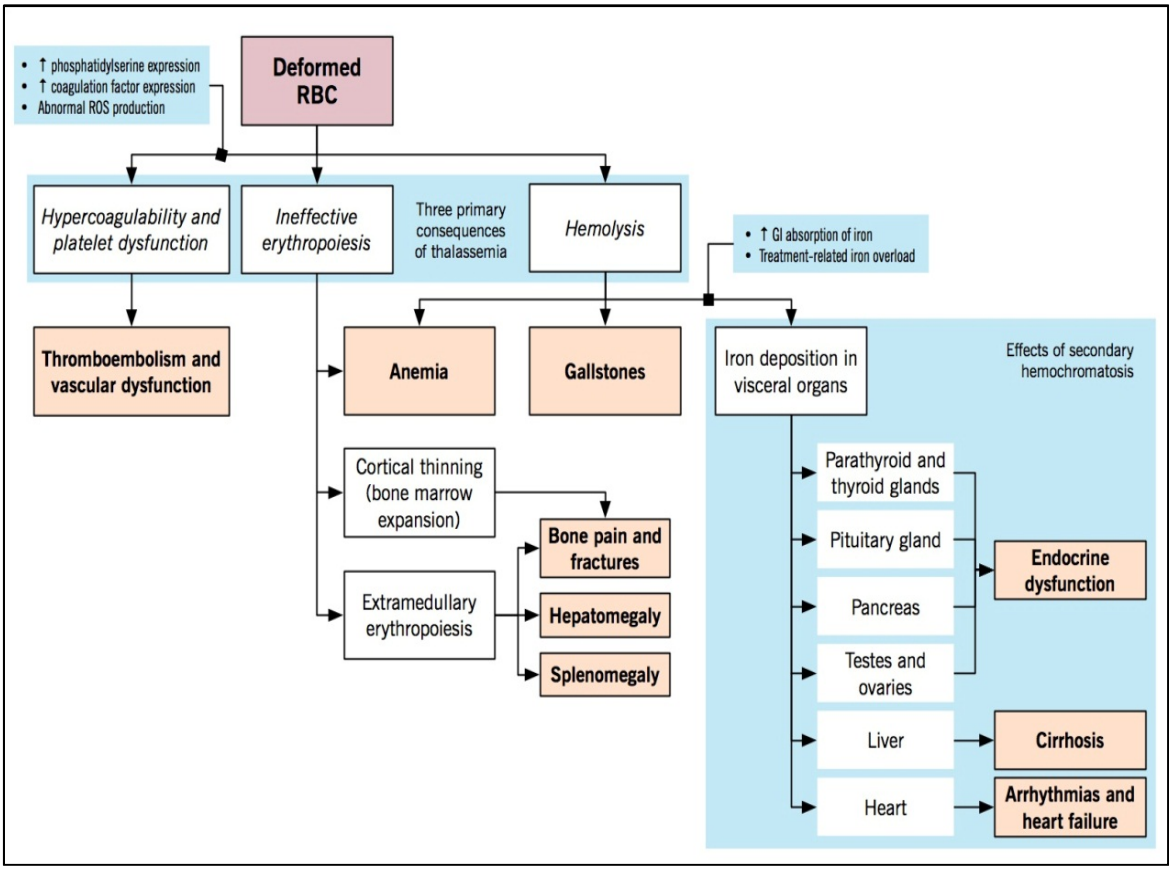

Figure 1.6: Pathophysiology and clinical features of beta thalassemia. (Figure reprinted from http://www.pathophys.org/thalassemia/)

1.7 Iron overload in beta thalassemia

Repeated transfusions represent the major cause of iron overload in thalassemia major. Each unit of blood represents 200-250 mg of iron. Considering that total body iron stores are approximately 4 grams, and that normal daily iron losses are of the order of 1-2 mg (with a very limited capacity for the body to regulate these losses), one can understand that, when a given individual needs for instance one unit of blood every 2 weeks, body iron overload develops rapidly[21]. Thalassemia causes iron to accumulate in the body. There are two main ways in which patients with thalassemia absorb iron: from the diet, and from transfused blood. If this excess iron is not removed, it can cause damage to important organs such as the liver and heart. Thalassemia patients must therefore use special drugs called chelators, which remove iron from the body. In thalassemia major, the body attempts to compensate for the patient's severe anemia by absorbing significantly more iron from the gut than normal (2-5g/year compared to 0.0015g/year in healthy individuals), in order to make more red blood cells. How much more iron is absorbed depends on the severity of the anemia. Other factors may also play a part in determining the amount of iron absorbed by the gut. For example, the presence of vitamin C increases the amount of iron absorbed, while tea and some cereals lead to a decrease. The main source of iron overload in patients receiving transfusions however is blood transfused. In fact, the amount of iron the patient absorbs through blood transfusions is far greater than that absorbed from the diet through the gut. It is therefore important that patients on regular blood transfusions, use iron chelators that bind with iron and remove it from the system [22]. The clinical symptoms of iron overload generally appear around the age of 10, although evidence of the toxic effects of iron has been found in the liver of much younger children. Heart disease is one of the most frequent causes of death in thalassemia major -has also been reported within 10 years of the start of a transfusion regime, although heart failure does not usually occur until after 15 years or more.

Table-1.1: Effect of excess iron deposition in the body [22]

Consequences of excess iron deposition	
Heart	Biventricular failure Arrhythmia
Pituitary	Hypogonadotrophic-hypogonadism Osteoporosis
Endocrine gland	Diabetes Hypothyroid Hypoparathyroid
Liver	Fibrosis Cirrosis

1.8 Ineffective erythropoiesis in beta thalassemia

Erythropoiesis is the process of producing erythrocytes which is stimulated by decreased O_2 in circulation. Erythropoietin is a glycoprotein cytokine secreted by the kidney in response to cellular hypoxia that stimulates proliferation and differentiation of red cell precursors, which activates increased erythropoiesis in the hemopoietic tissues, ultimately producing erythrocytes [23]. Erythropoietin (EPO) has been shown to exert its effects by binding to the erythropoietin receptor (EpoR). EPO binds to the erythropoietin receptor on the red cell progenitor surface and activates a JAK2signalling cascade. This initiates the STAT5, PIK3 and Ras MAPK pathways. This results in differentiation, survival and proliferation of the erythroid cell [24]. Erythropoietin levels in blood are quite low in the absence of anemia, at around 10 mU/mL. However, in hypoxic stress, EPO production may increase up to 1000-fold, reaching 10 000 mU/mL of blood. A feedback loop involving erythropoietin helps regulate the process of erythropoiesis so that, in non-disease states, the production of red blood cells is equal to the destruction of red blood cells and the red blood cell number is sufficient to sustain adequate tissue oxygen levels but not so high as to cause sludging, thrombosis, or stroke. Recent studies have also shown that the

peptide hormone hepcidin may play a role in the regulation of hemoglobin production, and thus affect erythropoiesis. The liver produces hepcidin. Hepcidin controls iron absorption in the gastrointestinal tract and iron release from reticuloendothelial tissue. Iron must be released from macrophages in the bone marrow to be incorporated into the heme group of hemoglobin in erythrocytes [25,26]. Hepcidin, a peptide hormone which is mainly synthesized in the liver, was discovered in 2000. It reduces extracellular iron in the body by several mechanisms:

1) It lowers dietary iron absorption by reducing iron transport across gut mucosal cells (enterocytes);
2) It reduces iron exit from macrophages, the main site of iron storage; and
3) It reduces iron exit from the liver.

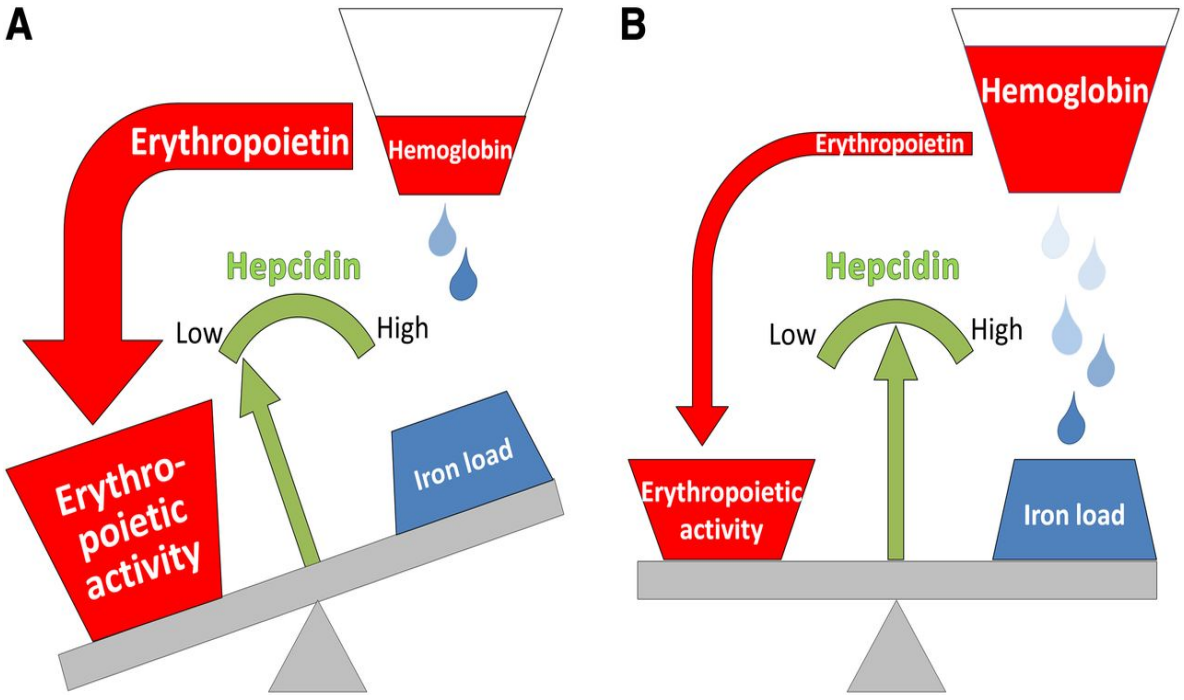

Figure 1.7: Hepcidin regulation in β-thalassemia major. Hepcidin production is modulated by suppressive effects of erythropoiesis and stimulatory effects of iron overload [28].

In all three instances this is accomplished by reducing the transmembrane iron transporter ferroportin. Hepcidin regulates systemic iron status by controlling dietary iron absorption and iron release from macrophages. Hepcidin synthesis is suppressed by erythropoiesis but is upregulated by iron accumulation and inflammation. Normally, these signals are balanced to provide an appropriate level of iron to meet erythropoietic demand, but in thalassemia, excessive erythropoiesis suppresses hepcidin, increasing iron absorption. Iron from transfusions compounds iron loading. Hepcidin concentrations are suppressed in β-thalassemia [27].

Pasricha et al clearly demonstrated that even in β-thalassemia major patients, who are highly iron overloaded, serum hepcidin levels are lower than would be expected because of the exuberant erythropoiesis. The reduction of erythropoietic activity by erythrocyte transfusions partially relieved the suppression of hepcidin [28]. Before transfusion, exuberant erythropoietic activity suppresses hepcidin through an as yet poorly defined mechanism. Lower hepcidin would be expected to result in increased dietary iron loading. After transfusion, ineffective erythropoiesis is alleviated, resulting in hepcidin de-repression. The effect of iron loading becomes apparent chronically rather than immediately after transfusion. Hepcidin measurements should help determine how well ineffective erythropoiesis is managed in β-thalassemia patients (figure 1.7)

1.9 Serum ferritin status in beta thalassemia

Serum ferritin is a useful monitoring tool for iron overload in thalassemia major. The pathophysiology is attributed mainly to increased iron absorption from the gastrointestinal (GI) tract and the occasional transfusions. Ferritin has been reported to increase with age in nontransfused thalassemia patients especially when the iron overload is excessive. Splenectomy has been also reported to influence ferritin level. A higher ferritin level in a splenectomized individual as compared to an individual with intact spleen has been described. The mechanism for higher iron overload in splenectomized patients is postulated by the fact that the intact spleen may be a reservoir of excess iron and possibly has an excess scavenging effect on iron-free fractions, including non–transferrin-bound iron [29].

1.10 Aims and objectives of the study

- Observing relationship between severity of thalassemia based on transfusion interval and frequency of incidence of EBT and BTM
- Comparing serum erythropoietin level between healthy controls and thalassemic patients based on disease severity
- Comparing erythropoietin levels in EBT and BTM patients in the less and more severe groups
- Finding correlation between disease severity and age of first transfusion
- Determining and comparing ferritin level in thalassemia patients.

Methods and Materials

Chapter 2

2.1 Laboratory analysis and Working Place

All the experiments including hematological and immunological tests were performed at the biosafety level-2 laboratory facility of the Institute for developing science and health initiatives (ideSHi), Mohakhali, Dhaka-1212. All the specimens were collected from Bangladesh Thalassemia Samity and Hospital.

2.2 Duration of the study

The study was conducted for 6 months (January, 2018- July, 2018).

2.3 Study site, Study population and Ethical approval

Beta-thalassemia major or E/beta-thalassemia diagnosed patients were enrolled from Bangladesh Thalassemia Samity and Hospital for this study. Written informed consent forms were obtained from the adult patients. In case of children written informed consents were taken from their legal guardians. All the patients were blood transfusion dependent and after standard clinical evaluation, information about the age of first transfusion, transfusion interval, splenectomy status etc. were also taken using a structured questionnaire. Ethical approval for this project was taken from the Bangladesh Medical Research Council (BMRC).

2.4 Specimen collection

Approximately 5 ml of peripheral blood specimen was collected from study participants before blood transfusion. Blood specimens were collected via standard venipuncture method and a portion of the blood were dispensed into a red top BD Vacutainer® tube for serum collection (Becton Dickinson, Franklin Lakes, NJ, USA). The remaining blood was placed in a vaccutainer containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA). The vacutainers containing specimens were immediately kept in a cool box (2 -8˚C) and transported to ideSHi laboratory for analysis. Serum and

plasma specimens were separated from the blood and for long time preservation of the serum/plasma specimens the blood specimens were stored in freezers (-70 ˚C) at the 'Institute for developing Science and Health initiatives (ideSHi)'.

2.5 Serum separation:

Whole blood was kept into a serum collection tube (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to coagulate at room temperature (20 - 25˚C). Serum separation was done by centrifugation of the vacutainer tube at 760 x g for 15 minutes at room temperature within 30 minutes of specimen collection. Specimens were stored at -70 ˚C freezer for quantitation of Erythropoietin using Quantikine® IVD® Human Erythropoietin ELISA kit (R&D Systems Inc., USA).

Figure 2.1: Serum separation using centrifuge from serum collection tube**.**

2.6 Complete Blood Count (CBC) analysis:

Complete Blood Count (CBC) analysis including hemoglobin, RBC, platelet, and reticulocyte counts as well as MCV, MCH and RDW was done according to the manufacturer's instructions using Sysmex kx-21 system Haematology analyzer (Sysmex Corporation, Kobe, Japan) automated hematology analyzer from EDTA containing blood. Among these hematological parameters, blood hemoglobin level was used for this study.

Figure 2.2: Complete Blood Count (CBC) analysis using Sysmex automated hematology analyzer.

2.7 Quantification of serum erythropoietin level

Serum erythropoietin level of patients were measured using Quantikine® IVD® ELISA human erythropoietin immunoassay kit (R&D Systems, Inc., USA) according to the manufacturer's instructions. Briefly, wash buffer (1X) and substrate solution was prepared before starting of the assay. To prepare 2500 mL of wash buffer (1X), 100 mL of concentrated wash buffer (25X) was added into deionized or distilled water. Substrate solution was prepared by mixing equal volume of color reagent A and reagent B within 15 minutes of use. All reagents and specimens were brought to room temperature before use and 100 µL of erythropoietin assay diluent into each well for the microplate strips. The plate frame was tapped for approximately 1 minute after addition of 100 µL of standard, control, or specimen into each well to mix the well contents. The plate was covered with the adhesive strip and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker at 500 rpm. The content of the each well was thoroughly aspirated and 200 µL of erythropoietin conjugate was added into each well. The plate was covered with a new adhesive strip and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker. The content of the each well was aspirated and washed with 400 µL wash buffer using a squirt bottle. After the last wash, the remaining wash buffer was removed by decanting and the plate was dried by blotting it against clean paper towels. The plate was incubated at room temperature for 20 – 25 minutes after addition of freshly prepared substrate solution into each well. After incubation, 100 µL of stop solution was added into each well to stop the reaction. Optical density (O.D.) of each well was measured at 450 nm and 600 nm using EON ELISA reader (BioTek, USA). The readings at 600 nm were subtracted from the readings at 450 nm to correct for optical imperfections of the plate. A standard curve was generated by plotting the absorbance (O.D.) for each standard on the

y axis against the concentration on the x axis. Serum erythropoietin concentrations were measured from the standard curve and expressed as mIU/mL.

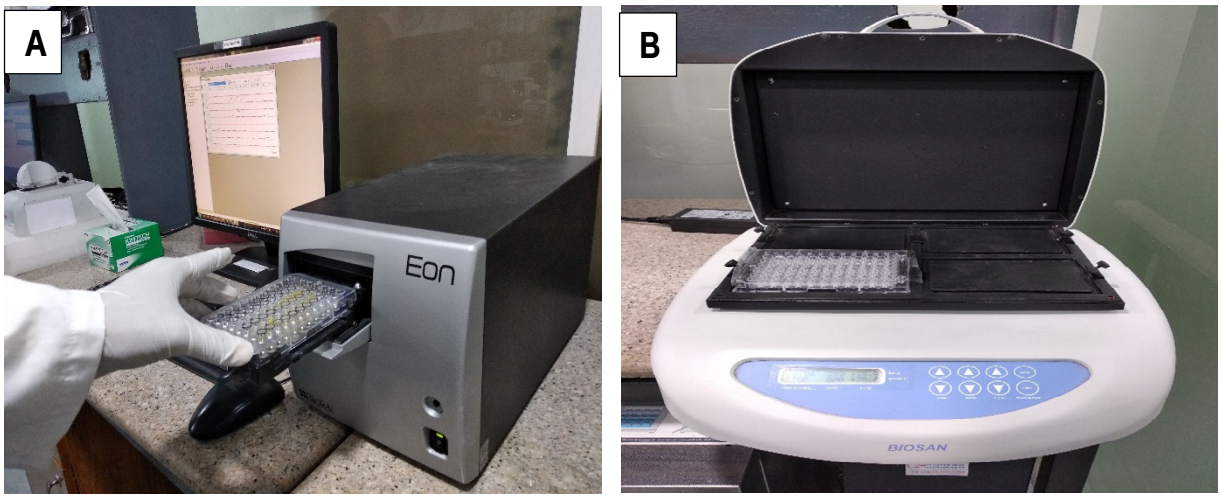

Figure 2.3: Instruments used for quantification of serum erythropoietin immunoassay. (A) EON ELISA reader (BioTek, USA). (B) BioSan (Thermo Fisher Scientific, USA) orbital microplate shaker.

2.8 Quantification of serum serum ferritin level

Quantification of serum ferritin level was done using AccuBind ELISA microwells ferritin test (Monobind Inc., Lake Forest, CA, USA) kit according to manufacturer's instructions. Briefly, working wash buffer was prepared by diluting the concentrated wash buffer in 1000 ml distilled water and working substrate solution was prepared by mixing solution A and solution B. 25µl of the appropriate serum reference, control or specimen into the assigned well of microtiter plate and 100µl of the ferritin biotin reagent was added into the well. The microplate was swirled for 20-30 seconds to mix the reagents and the plate was incubated for 30 minutes at room temperature. After incubation the contents of the microplate was discarded by decanting. The plate was dried using

absorbent paper and the plate was washed with 350µl of wash buffer for 3 times. The plate was incubated for 30 minutes at room temperature after addition of 100µl of the ferritin enzyme conjugate to each well. After incubation, the contents of the microplate was discarded by decanting and washed with 300µl of wash buffer for 3 times. After addition of 100µl of working substrate solution to all wells, the plate was incubated for 15 minutes. Stop solution (50µl) was added to each well and mixed gently for 15-20 seconds. The absorbance in each well was measured at 450nm and the reference wavelength was 630nm to minimize well imperfections using EON ELISA reader (BioTek, USA). Standard curve was prepared by plotting the absorbance for each duplicate serum reference versus the corresponding ferritin concentration in ng/ml. The concentration of the unknown sample was determined from the standard curve and expressed in ng/ml.

Results

Chapter 3

3.1 Study Subject:

A total of 52 Thalassemia patients between 4- 45 years were enrolled in the study. Of them 48% (27/52) were female and 52% (25/52) were male. All patients were confirmed thalassemic by hemoglobin electrophoresis. The demographic information of the patients are tabulated below.

Table 3.1: Demographic data of study subjects

Characteristics		Patients (N=52)
Median age (yrs)		15.5
Gender	Male	25 (52%)
	Female	27 (48%)
Average spleen size		6.7 cm
Average age of first transfusion		4.78 years
No. of patients taking chelation therapy		48

3.2 Grouping of severity of Thalassemia:

Based on differences in transfusion interval, patients were classified into two severity groups. The more severe group with a transfusion interval between 0-30 days and the less severe group with transfusion interval above 30 days

3.3 Prevalence of Thalassemia:

Among the 52 patients enrolled, 13 suffered from Beta Thalassemia major and 34 suffered from E beta Thalassemia across both the severity groups. In the more severe group (0-30 days transfusion interval), 20 patients suffered from E beta Thalassemia, while 13 patients had Beta Thalassemia Major. However, the more severe group only had 14 patients with E beta Thalassemia and no cases of Beta Thalassemia Major. A chi square test was performed and no relationship was found between severity of thalassemia based on transfusion interval and frequency of incidence of EBT and BTM, X^2 (4, N = 47) = 7.62, p =.10.

Table 3.2: Contingency table for distribution of EBT and BTM in severity groups.

	More severe group (0-30 days TI)	Less severe group (>30 days)	Total
EBT	13	0	13
BTM	20	14	34
Total	33	14	47

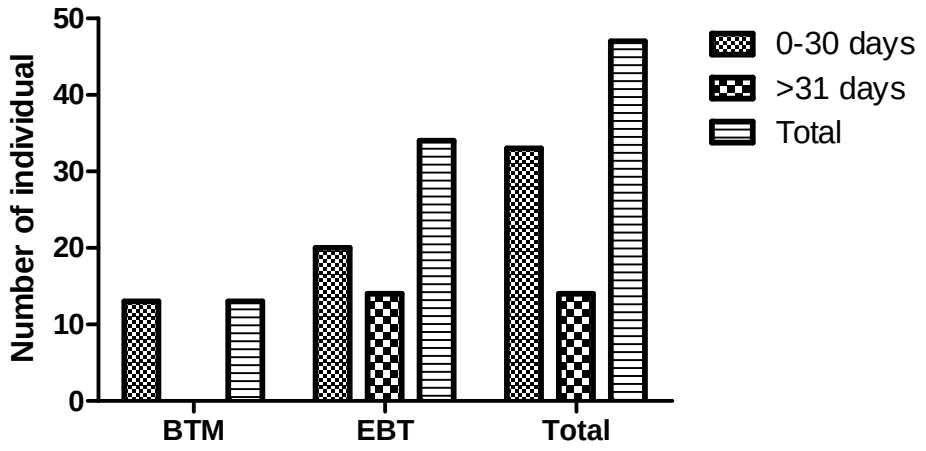

Figure 3.1: Chi-square test of EBT and BTM

3.4 Erythropoietin:

The serum erythropoietin was estimated for 14 normal healthy controls where the EPO mean was 4.84 mIU/ml. The mean of serum erythropoietin in patients within the more severe group with transfusion interval 0-30 days was 617.3 mIU/ml, while for patients above 30 days of transfusion interval the mean was 191.7 mIU/ml. It was observed that the patients in both severity groups with transfusion interval between 0-30 days and >3o days had higher significantly higher EPO levels compared with the control group ($P<0.001$) and ($P=0.02$) respectively.

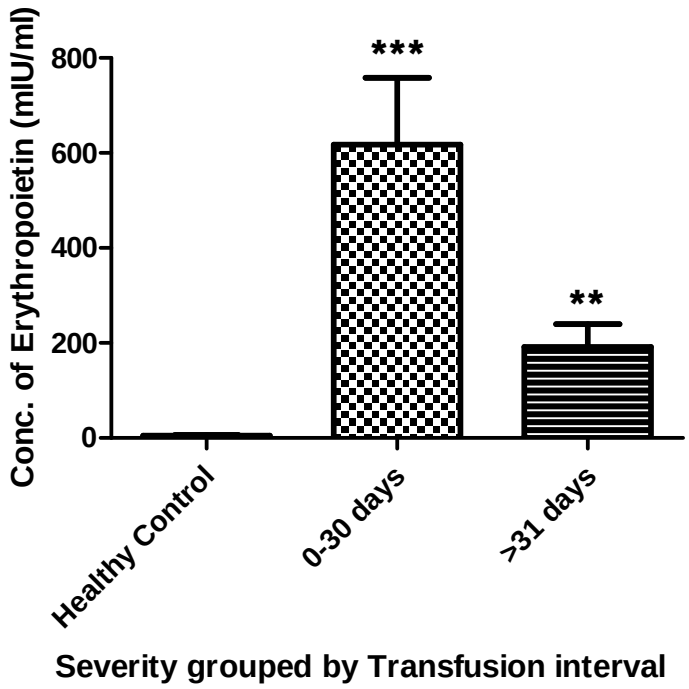

Figure 3.2: Comparison of Erythropoietin concentration of healthy control vs severity groups 0-30d transfusion interval and >30 days transfusion interval. Statistical difference compared to healthy controls: ***, $P<0.0001$; **, $P<0.005$

3.4.1 Comparison of Erythropoietin levels in EBT patients across the two severity groups:

Another analysis compared EPO concentrations in E beta Thalassemia patients in the two severity groups. The mean concentration of serum erythropoietin in EBT patients in the more severe group with 0-30 days transfusion interval was 730 mIU/ml, while for EBT patients in the less severe group with >30 days transfusion interval the mean was 191.7 mIU/ml. The concentrations of EPO was significantly higher in EBT patients from the more severe group (0-30 days transfusion interval) than the less severe group (>31 days) ($P<0.05$).

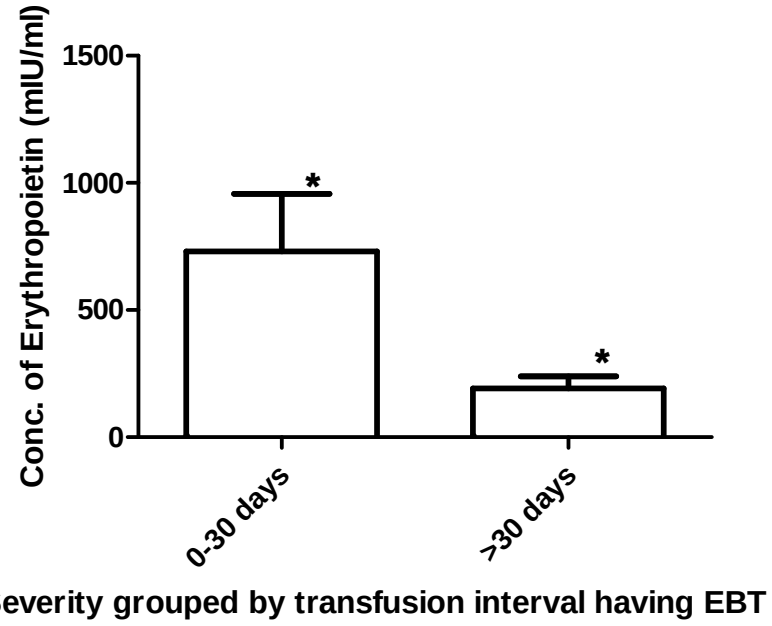

Figure 3.3: Comparison of Erythropoietin concentration of severity groups 0-30d transfusion interval vs 31-547d suffering from EBT

3.4.2. Comparison of Erythropoietin levels in EBT and BTM patients in the more severe group (0-30 days transfusion interval):

There was no significant difference in erythropoietin levels between EBT and BTM patients in the more severe group (0-30 days transfusion interval) (P=0.33).

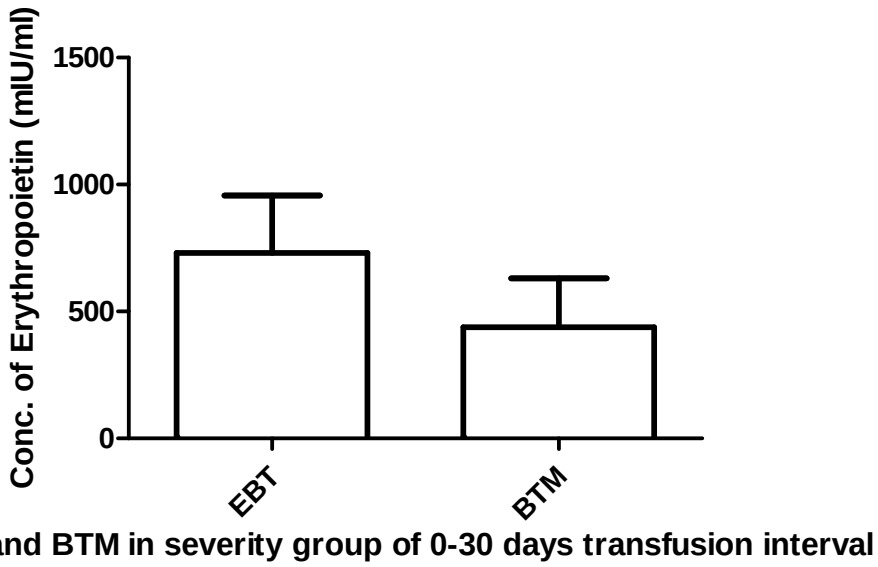

Figure 3.4: Comparison of Erythropoietin concentration of EBT and BTM in severity group of 0-30 days transfusion interval.

3.5. Correlation between disease severity and age of first transfusion:

For patients in the more severe group with 0-30 days transfusion interval, age of first transfusion was significantly earlier when compared to age of first transfusion for patients within the less severe group with >30 days transfusion interval ($P<0.05$). This translates to the fact that patients requiring frequent blood transfusion started taking transfusion at an earlier age that patients who requires transfusion at much longer intervals.

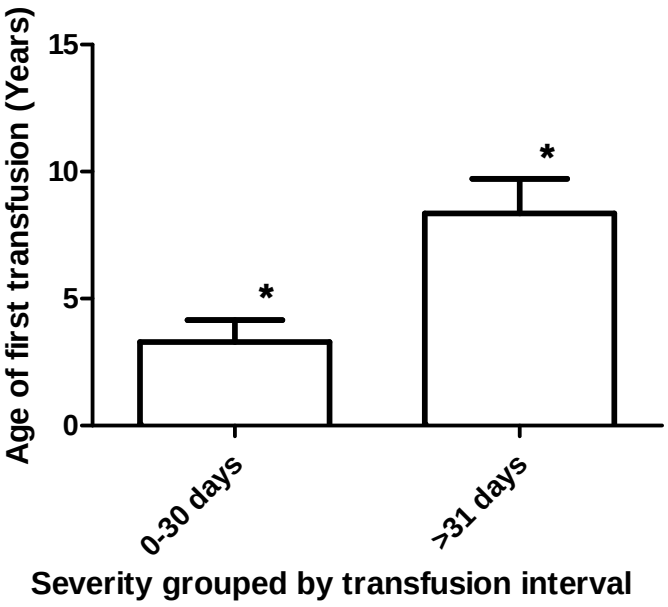

Figure 3.5: Comparison of age of first transfusion between the severity groups of transfusion interval 0-30 days and >31 days

3.6 Ferritin:

We also compared the level of serum ferritin across the two severity groups. No significant differences were found in ferritin levels based on severity (P=0.2)

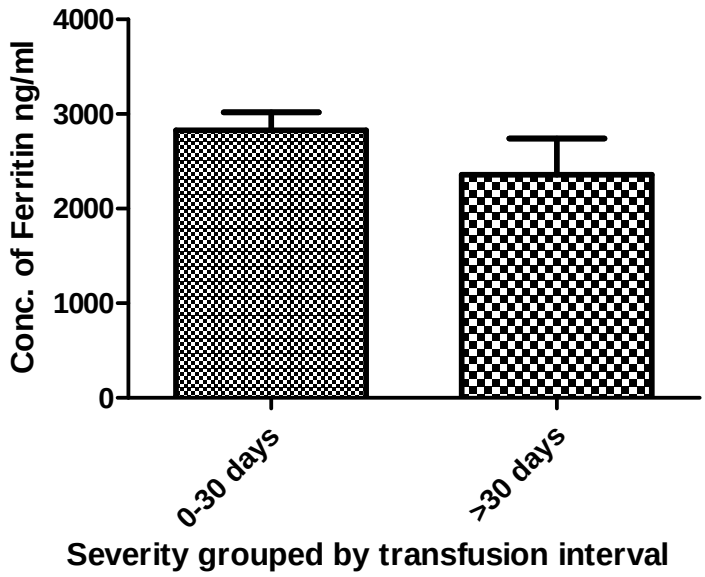

Figure 3.6: Comparison of Ferritin concentration of severity groups 0-30 days transfusion interval vs 31-547 days

Discussion

Chapter 4

Discussion:

β-thalassemia and hemoglobin E-beta thalassemia are highly prevalent autosomal recessive disorders characterized by a reduced or absent expression of the β globin gene and co-inheritance of a β-thalassaemia allele from one parent and the structural variant hemoglobin E from the other, respectively [30]. β-thalassemia is now regarded as the most common inherited single gene disorder in the world including Bangladesh. Like β-thalassemia, Hb E is also supposed to be highly prevalent in Bangladesh. For both of these disorders, the affected individuals suffer from life-threatening anemia and require regular treatment and management of the complications. Though considered one of the major cause of morbidity and mortality worldwide, there is still no universally available cure for thalassemia major [31]. Regular blood transfusions greatly contribute to the quality and length of life of patients with thalassemia major, and have been a central aspect of the treatment of thalassemia. Studies have shown that regular transfusion therapy with safe and appropriately processed blood, combined with regular and effective iron chelation tremendously increase patients' survival and quality of life [32].

Erythropoietin (EPO) acts as an erythropoietic protein; it is produced in the kidneys by peritubular cells and can also be produced in the liver [33]. A deficiency in tissue oxygen levels increases the activity of hypoxia inducible factor 2α, which binds to hypoxia responsive elements located in the enhancer region of the *EPO* gene in order to activate transcription [34]. In the bone marrow, EPO acts synergistically with stem cell factor, granulocyte macrophage colony stimulating factor, interleukin (IL) 3, IL4, IL9 and insulin like growth factor 1 on erythroid progenitor cells to prevent their programmed cell death, thereby stimulating proliferation and maturation of erythroid progenitors through the normoblast stage into reticulocytes and mature erythrocytes [35-38]. The normal range for EPO levels can vary from 3.7 to 36 IU/L. Higher-than-normal levels may mean anemia and in severe cases of anemia, EPO levels in the blood may be a thousand times higher than normal [39]. In this study, the EPO level in severe group (irrespective of whether it is β-thalassemia major or E-beta thalassemia) patients was approximately 600 times higher than the control group whereas the less sever group has a concentration as high as 200 times than the healthy control group with a statistically significant difference ($P<0.0001$ and $P<0.005$,

respectively). The definition of severe (transfusion interval <30 days) and less severe (transfusion interval is more than 30 days) group explains the difference between two groups as the high anemic condition explains the high concentration of EPO. This data is further supported by other findings of this study. When the EPO concentration of severe (transfusion interval within 30 days) and less severe group (transfusion interval more than 30 days) of EBT patients were compared, the similar pattern was found where the concentration of EPO was almost 3.5 times higher in severe group compare to less sever one with a significant P value ($P< 0.05$). Moreover, upon comparison between EBT and BTM group, no statistical significant difference was found which indicates anemic condition might be the underlying cause of such high concentration of anemia. In this study, the blood samples were collected from the patients before they went blood transfusion. Usually the patients come for blood transfusion when hemoglobin concentration is less than the normal range. Some other independent researcher groups also reported high level of EPO in thalassemia major group compare to minor and healthy controls [40, 41]. These data showed that serum EPO levels increased in all thalassemia patients despite repeated transfusion. Multiple transfusion may modulate the response of serum EPO to the degree of anemia, resulting in increased EPO levels. In addition, Nişli et al. did not found any difference in hemoglobin (Hb) level between BTM major and minor group although the EPO concentration was higher in major group. They concluded that some other factors might be contributing to the metabolic adaptation to low oxygen concentration or improvement of the tissue oxygenation are as effective as the Hb concentration in EPO production. It is also suggestive of the fact that some amount of tissue hypoxia cannot be prevented in spite of polytransfusion regimens in BTM patients. Moreover, Serum EPO levels of BTM patients were not found to be age related or correlated with the mean pretransfusional Hb levels. In the BTM patients, the serum EPO concentration was not consistently correlated with clinical signs of etythropoietic activity. This may be indicative of personal differences with respect to the sensitivities of erythroid precursors to the increasing EPO levels in BTM patients. However, similar report on EBT patients are rare. This study has found high level of EPO concentration in both BTM and EBT patients in severe group which concludes, anemic condition is a major factor controlling EPO level in these patients although other factors were not investigated in this study and need to study further.

Another reason of this high EPO level can be resistance to endogenous EPO. EPO has been successfully used for over a decade to treat anemia in patients with chronic kidney disease [42].

Up to 10% of patients receiving EPO are hyporesponsive to therapy and require large doses of the agent. Several mechanisms could explain resistance to endogenous and exogenous EPO. Proinflammatory cytokines antagonize the action of EPO by exerting an inhibitory effect on erythroid progenitor cells and by disrupting iron metabolism. EPO resistance might also be caused by inflammation, which has a negative effect on EPO receptors. Resistant to endogenous EPO has not been reported for thalassemia patients and in requires investigation in case of thalassemia patients in order to find the possible mechanism of increased high level of EPO among severe thalassemia patients. Interestingly, it has been reported that long-term L-carnitine administration reduces erythropoietin resistance in chronic hemodialysis patients with thalassemia minor [43] and another study in Bangladesh on thalassemia patients found decreased level of L-carnitine [44]. The efficacy of L-carnitine administration in improving the quality of life of these patients need further studies with considering the effect of L-carnitine on EPO resistance in thalassemia patients.

Repeated transfusions represent the major cause of iron overload in thalassemia major. Each unit of blood represents 200-250 mg of iron [45]. Considering that total body iron stores are approximately 4 grams, and that normal daily iron losses are of the order of 1-2 mg (with a very limited capacity for the body to regulate these losses), one can understand that, when a given individual needs for instance one unit of blood every 2 weeks, body iron overload develops rapidly [46]. Thalassemia causes iron to accumulate in the body. Ferritin is the major iron storage protein of the body. Ferritin levels can be used to indirectly measure the iron levels in the body. Ferritin has the shape of a hollow sphere that permits the entry of a variable amount of iron for storage. If a ferritin test shows higher than normal levels, it could indicate that you have a condition that causes your body to store too much iron. Although many studies reported high levels of serum ferritin in thalassemia patients, in this study no difference was found between severe and less severe group of thalassemia patients in ferritin concentration [45-47]. One possible explanation of this variation from other studies that 48 of 52 enrolled patients are on chelation therapy (deferoxamine).

This study found that the high EPO concentration is linked to anemic severity rather than type of thalassemic condition. Whether the endogenous EPO resistance is a contributing factor to this high magnitude of EPO needs further investigation.

References

Chapter 5

References:

1. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ 2001; 79: 704-12 pmid: 11545326.
2. Galanello, Renzo; Origa, Raffaella (21 May 2010). "Beta-thalassemia". Orphanet J Rare Dis. Orphanet Journal of Rare Diseases. 5: 11. doi:10.1186/1750-1172-5-11. PMC 2893117Freely accessible. PMID 20492708
3. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86:480–7
4. Khan, W. A. (2017). Thalassemia in Bangladesh. National conference on diagnosis of genetic disorders in Bangladesh: Present situation and Future directions, 10.
5. Nagababu, E., Fabry, M. E., Nagel, R. L., & Rifkind, J. M. (2008). Heme degradation and oxidative stress in murine models for hemoglobinopathies: thalassemia, sickle cell disease and hemoglobin C disease. Blood Cells, Molecules, and Diseases, 41(1), 60-66.
6. Safizadeh, H., & Farahmandinia, Z. (2012). Quality of life in patients with thalassemia major and intermedia in kerman-iran (IR). Mediterranean journal of hematology and infectious diseases, 4(1).
7. Talsania, S., Talsania, N., & Nayak, H. (2011). A cross sectional study of thalassemia in ahmedabad city, Gujarat.(Hospital based). Jourlib Journal/Iapsmgc ISSN, 48-51.
8. Palit, S., Bhuiyan, R. H., Aklima, J., Emran, T. B., & Dash, R. (2012). A study of the prevalence of thalassemia and its correlation with liver function test in different age and sex group in the Chittagong district of Bangladesh. Journal of Basic and Clinical Pharmacy, 3(4), 352–357. http://doi.org/10.4103/0976-0105.105339
9. Borgna-Pignatti C, Vergine G, Lombardo T, Cappellini MD, Cianciulli P, Maggio A, Renda D, Lai ME, Mandas A, Forni G, Piga A, Bisconte MG. Hepatocellular carcinoma in the thalassemia syndromes. Br J Haematol. 2004;124:114–117. doi: 10.1046/j.1365-2141.2003.04732.x.
10. Xu, X.M., Y.Q. Zhou, G.X. Luo, C. Liao and M. Zhou et al., 2004. The prevalence and spectrum of α and β thalassaemia in Guangdong Province: Implications for the future health burden and population screening. J. Clin. Pathol., 7: 517-522.
11. http://www.observerbd.com/details.php?id=135091
12. https://www.news-medical.net/health/Thalassemia-Prevalence.aspx

13. Pandamooz, S., AKHAVAN, N. H., Tabaripour, R., AHMADI, F. M., HADIPOUR, A., AGHAJANPOUR, M., & MOSTAFAZADEH, A. (2011). POSTER PRESENTATIONS: P-61: ISOLATION OF NON-ADHERENT MULTIPOTENT HUMAN DERMAL FIBROBLASTS.
14. Tamer Hassan, Mohamed Badr, Usama El Safy, Mervat Hesham, Laila Sherief and Marwa Zakaria (2016). β-Thalassemia: Genotypes and Phenotypes, Epidemiology of Communicable and Non-Communicable Diseases - Attributes of Lifestyle and Nature on Humankind, Dr. Fyson Kasenga (Ed.), InTech, DOI: 10.5772/64644.
15. Flint J, Harding RM, Boyce AJ, Clegg JB. The population genetics of the haemoglobinopathies. Bailliere's Clin Hematol. 1998;11(1):1–51. DOI: 10.1016/S0950-3536(98)80069-3
16. Vichinsky EP. Changing patterns of thalassemia worldwide. Ann N Y Acad Sci. 2005;1054(1):18–24. DOI: 10.1196/annals.1345.003
17. El-Beshlawy A, Kaddah N, Rageb L, Hussein I, Mouktar G, Moustafa A, Elraouf E, Hassaballa N, T Gaafar and El-Sendiony H. Thalassemia prevalence and status in Egypt. Pediatric Res. 1999;45(S5):760. DOI: 10.1203/00006450-199905010-00132
18. Fucharoen S, Winichagoon P. Haemoglobinopathies in Southeast Asia. The Indian Journal of Medical Research. 2011;134(4):498-506.
19. Forget, B. G. (2001). Molecular mechanisms of b thalassemia. Steinberg, MH.; Forget, BG.; Higgs, DR, 252-276.
20. Thein, S. L. (2005). Genetic modifiers of beta-thalassemia. haematologica, 90(5), 649-660.
21. www.ukts.org/pdfs/hepatitis/hcvpositionpaperfinal.pdf
22. https://www.beximcopharma.com/images/stories/doctors/newsletterpdf/Dec_2005.pdf
23. Sherwood, L, Klansman, H, Yancey, P: Animal Physiology, Brooks/Cole, Cengage Learning, 2005.
24. *Thomson, Angus W.; Lotze, Michael T. (2003-04-22).* The Cytokine Handbook, Two-Volume Set. *Gulf Professional Publishing.* ISBN 9780080518794
25. Koury, M.J. "Erythroferrone: A Missing Link in Iron Regulation". The Hematologist. American Society of Hematology. Retrieved 26 August 2015.

26. Jump up^ Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T (Jul 2014). "Identification of erythroferrone as an erythroid regulator of iron metabolism". Nature Genetics. 46(7): 678–84. doi:10.1038/ng.2996. PMC 4104984. PMID 24880340
27. Jones, E., Pasricha, S. R., Allen, A., Evans, P., Fisher, C. A., Wray, K., ... & Sturges, P. (2014). Hepcidin is suppressed by erythropoiesis in hemoglobin E β-thalassemia and β-thalassemia trait. Blood, blood-2014.
28. Nemeth, E. (2013). Hepcidin and β-thalassemia major. Blood, 122(1), 3-4.
29. Shah, R., Trehan, A., Das, R., & Marwaha, R. K. (2014). Serum ferritin in thalassemia intermedia. Indian Journal of Hematology and Blood Transfusion, 30(4), 281-285.
30. Marengo-Rowe AJ. The thalassemias and related disorders. InBaylor university medical center proceedings 2007 Jan 1 (Vol. 20, No. 1, pp. 27-31). Taylor & Francis.
31. dos Santos DG, Zidova Z, Mikhael M, Hamdi A, Horvathova M, Ponka P. Pathophysiology and Treatment of Beta-Thalassemia: Investigations of Heme Oxygenase 1 and Its Inhibitors.
32. Cohen A, Gayer R, Mizanin J. Long-term effect of splenectomy on transfusion requirements in thalassemia major. Am J Hematol. 1989 Apr;30(4):254–6.
33. Fisher JW *et al.* (1996) Erythropoietin production by interstitial cells of hypoxic monkey kidneys. *Br J Haematol* 95: 27–32
34. Warnecke C *et al.* (2004) Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. *FASEB J* 18: 1462–1464
35. Scortegagna M *et al.* (2005) HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. *Blood* 105: 3133–3140
36. Imagawa S *et al.* (1997) Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* 89: 1430–1439
37. La Ferla K *et al.* (2002) Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF-kappaB. *FASEB J* 16: 1811–1813

38. Dumitriu B *et al.* (2006) Sox6 cell-autonomously stimulates erythroid cell survival, proliferation, and terminal maturation and is thereby an important enhancer of definitive erythropoiesis during mouse development. *Blood* 108: 1198–1207
39. https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=167&contentid=erythropoietin_blood
40. Dore F, Bonfigli S, Gaviano E, Pardini S, Cianciulli P, Papa G, Longinotti M. Serum erythropoietin levels in thalassemia intermedia. Annals of hematology. 1993 Oct 1;67(4):183-6.
41. Nişli G, Kavakli K, Aydinok Y, Oztop S, Cetingül N. Serum erythropoietin levels in patients with beta thalassemia major and intermedia. Pediatric hematology and oncology. 1997 Jan 1;14(2):161-7.
42. Van der Putten K, Braam B, Jie KE, Gaillard CA. Mechanisms of disease: erythropoietin resistance in patients with both heart and kidney failure. Nature Reviews Nephrology. 2008 Jan;4(1):47.
43. Di Iorio BR, Guastaferro P, Cillo N, Cucciniello E, Bellizzi V. Long-term L-carnitine administration reduces erythropoietin resistance in chronic hemodialysis patients with thalassemia minor. Drug target insights. 2007 Jan;2:117739280700200001.
44. Sarker SK, Islam MT, Bhuyan GS, Sultana N, Begum MN, Al Mahmud-Un-Nabi M, Howladar MA, Dipta TF, Muraduzzaman AK, Qadri SK, Shirin T. Impaired acylcarnitine profile in transfusion-dependent beta-thalassemia major patients in Bangladesh. Journal of Advanced Research. 2018 Jul 1;12:55-66.
45. Pootrakul P, Vongsmasa V, La-Ongpanich P, Wasi P. Serum ferritin levels in thalassemias and the effect of splenectomy. Acta haematologica. 1981;66(4):244-50.
46. Shah R, Trehan A, Das R, Marwaha RK. Serum ferritin in thalassemia intermedia. Indian Journal of Hematology and Blood Transfusion. 2014 Dec 1;30(4):281-5.
47. Qureshi TZ, Anwar M, Ahmed S, Khan DA, Saleem M. Serum ferritin levels in carriers of β-thalassaemia trait. Acta haematologica. 1995;94(1):7-9.